De novo expression of transfected Sirtuin 3 enhances susceptibility of human MCF-7 breast cancer cells to hyperoxia treatment

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Abstract

Sirtuin 3 (Sirt3) has a promising role in cancer tumorigenesis and treatment, but there have been controversies about its role as oncogene or tumor suppressor in different types of cancer. Changes in its expression are associated with the excessive production of reactive oxygen species (ROS), thus contributing to mitochondrial dysfunction and age-related pathologies. Hyperoxic treatment (i.e. generator of ROS) was shown to support some tumorigenic properties, but finally suppresses growth of certain mammary carcinoma cells. Due to strikingly reduced Sirt3 level in many breast cancer cell lines, we aimed at deciphering the effect of *de novo* Sirt3 expression upon hyperoxic treatment in the human MCF-7 breast cancer cells. *De novo* expression of Sirt3 decreased metabolic activity and cellular growth of MCF-7 cells, reduced expression of pro-angiogenic and epithelial mesenchymal transition genes, induced metabolic switch from glycolysis to oxidative phosphorylation, and decreased abundance of senescent cells. These effects were enhanced upon hyperoxic treatment: induction of DNA damage and upregulation of p53, with increase of ROS levels followed by mitochondrial and antioxidant dysfunction, resulted in additional reduction of metabolic activity and inhibition of cellular growth and survival. The mitigation of tumorigenic properties and enhancement of the susceptibility of the MCF-7 breast cancer cells to the hyperoxic treatment upon *de novo* Sirt3 expression, indicates that these factors, individually and in combination, should be further explored *in vitro* and particularly *in vivo*, as an adjuvant tumor therapy in breast cancer malignancies.

Keywords:

sirtuin 3, MCF-7, hyperoxia, reactive oxygen species (ROS), mitochondrial function

Introduction

Sirtuin 3 (Sirt3) is a major mitochondrial NAD^+-dependent deacetylase that plays critical role in activation of mitochondrial proteins involved in energy metabolism, ATP production and mitochondrial biogenesis (1). Longer, enzymatically inactive form of Sirt3 (44 kDa) is imported into mitochondria by the N-terminal mitochondrial localization sequence (MLS). Following the import, the MLS is proteolytically cleaved, resulting in catalytically active, short form of Sirt3 (28 kDa) which is able to deacetylate target mitochondrial proteins (2). Sirt3 is the only member of sirtuin family that is linked with prolonged lifespan. Therefore, the control of enzymes involved in energy metabolism by Sirt3 is in accordance with the overall protection against pathological conditions and aging itself (3). However, there have been controversies about the role of Sirt3 in different types of cancer (4).

Cancer cells exhibit prominent genetic, metabolic and bioenergetic differences when compared to normal cells: high rate of glycolysis, lactate production along with the absence of respiration, despite the presence of oxygen thus characterizing the phenomenon known as Warburg effect (5). Because of rapid proliferation, cancer cells quickly exhaust nutrients and oxygen supply, making tumor microenvironment hypoxic. Under hypoxia, tumor's surroundings become acidic as a result of increased lactate production and excretion, thus contributing to the malignant phenotype of the cancer (6). However, although tumor cells often live in hypoxic conditions due to metabolic and structural abnormalities, their angiogenesis, growth and survival depend upon the sufficient supply of oxygen and nutrients. Therefore, one would expect that enhanced oxygenation (hyperoxia) would exert a tumor-promoting effect. On the other hand, several reports demonstrate growth suppression of mammary carcinoma cells upon hyperoxic treatment (7), thus pointing out the therapeutic effect of hyperoxia on tumor growth and proliferation.

The excessive production of reactive oxygen species (ROS) leads to oxidative stress, a crucial event that contributes to mitochondrial dysfunction and age-related pathologies (8). It has been

63 shown that irregular ROS production or scavenging is associated with changes in Sirt3
64 expression (9). Recent data report the association between Sirt3 levels and cancer progression,
65 with either oncogenic or tumor-suppressive role of Sirt3 in cancer (4, 10). Several studies
66 described a prosurvival role of Sirt3 in certain cancer cell lines, thus supporting its oncogenic
67 role in cancer, although the exact mechanism by which Sirt3 exerts its action is not fully
68 understood. On the other hand, Sirt3 is recognized as a fidelity protein that repairs cellular
69 molecules, and it is involved in metabolic reprogramming of some breast cancer cell lines
70 towards oxidative phosphorylation (OXPHOS) (11), and as such may be considered as a tumor
71 suppressor gene. Therefore, the loss of function of Sirt3 may contribute to a more aggressive
72 phenotype in some types of cancers (1).

73 Although hyperoxic treatment is clinically important for treatment of hypoxia, it is known that
74 exposure to the increased oxygen concentrations causes impaired energy metabolism that
75 results in dysfunction of normal cells (12). Since hypoxia is a hallmark of various tumors, we
76 hypothesized that hyperoxia would have negative impact on cancer cells, thus providing
77 therapeutic strategy against their tumorigenic properties. In addition, considering tumor
78 suppressive role of Sirt3 in cancerogenesis, we hypothesized that Sirt3 expression would also
79 negatively impact the cancerous cells, especially in combination with hyperoxic treatment.

80 Breast cancer is the most frequent cancer among women, and ranks as the fifth cause of cancer
81 death worldwide (13). Sirtuin family of genes shows differential expressions in breast cancer
82 cells, which is often different from the expression in the breast cancer tissues (14). Since breast
83 cancer cells have strikingly reduced Sirt3 level, and even 20% of them have almost no
84 detectable Sirt3 protein (15), we wanted to examine the effect of *de novo* Sirt3 expression upon
85 hyperoxic treatment in human MCF-7 breast cancer cells model. This setting of *de novo*
86 expression is advantageous because it is the only changing factor in the control and transfected

cells under the same treatment, thus providing a model which avoids any possible phenotypic distinctions in the cells caused by other factors.

Therefore, in this study we have developed human MCF-7 breast cancer cells transfectants expressing the Sirt3 protein in order to test its potential in affecting the response of these cells upon the hyperoxic treatment, i.e. to decipher whether it sensitizes or makes these cancer cells more resistant to oxidative stress.

Materials and methods

Cell lines, plasmids and transfection

MCF-7 cell line, obtained from the ECACC (Cat.No. 86012803, Public Health England), was grown in Dulbecco's Modified Eagle Medium with high glucose (DMEM, Sigma Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS, Invitrogen/Life Technologies, Carlsbad, CA, USA), 2mM L-glutamine (Sigma Aldrich, St. Louis, MO, USA), 1% nonessential amino acids and antibiotic/antimycotic solution GA-1000 (Lonza, USA), at 37°C with 5% CO_2 in a humidified atmosphere. pcDNA3.1+ plasmid with Flag-tagged Sirt3 (Cat.No. 13814), purchased from Addgene, USA (16), was amplified in *E.coli* strain dH5α and purified with PureYield^{TM} Plasmid Midiprep System (Promega, USA) according to the manufacturer's protocol. pcDNA3.1+ (given by courtesy of dr.sc. Grbeša) was used as a control plasmid. The MCF-7 cells were transfected with the Flag-tagged Sirt3 or empty pcDNA3.1+ plasmid using Lipofectamine 2000 (ThermoFisher Scientific, USA), according to the manufacturer's protocol. Briefly, 1.5 x 10^5 cells were seeded on 24-well plate in growth medium and 24 h later transfected and incubated for additional 24 h. The cells were then split into selective growth medium containing 200 µg/ml G418 antibiotic (Sigma Aldrich, St. Louis, MO, USA). Colonies of stable Sirt3 transfectants were picked, expanded in the growth media containing G418 antibiotic, and cryogenically stored at −80°C.

Normoxic and hyperoxic conditions

Cells were exposed to hyperoxia (95% O_2) for 44 h in a gas-tight modular incubator chamber (StemCell^{TM} Technology Inc., Canada) at 37°C in order to establish hyperoxia-treated groups, while the normoxic groups were exposed to normal growth conditions.

Immunofluorescence and confocal microscopy

Immunofluorescence analysis was performed as described previously (17). Briefly, cells were labelled with 200 nM MitoTracker Deep Red (ThermoFisher Scientific, USA), fixed with 2% paraformaldehyde, incubated with anti-Sirt3 primary polyclonal antibody (dilution 1:100, Santa Cruz, USA), followed by FITC-labeled Goat anti-Mouse IgG secondary antibody (dilution 1:100, Proteintech, USA). DAPI (4,6-diamidino-2-fenilindol, Sigma Aldrich, St. Louis, MO, USA) was used for nuclear staining. Confocal imaging was performed by sequential scanning using Leica TCS SP8 X laser scanning microscope, equipped with a HC PL APO CS2 63×/1.40 oil immersion objective and a white light laser (Leica Microsystems, Germany). The excitation wavelengths and emission detection ranges used were 405 nm and 420-477 nm for DAPI, 490 nm and 500-600 nm for FITC, 644 nm and 665-780 nm for MitoTracker Deep Red.

RNA isolation, reverse transcription and qPCR analysis

Total RNA was isolated from 10^6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Relative gene expression of *sirt3, sirt1*, superoxide dismutase 2 (*sod2*), catalase (*cat*), mitochondrial NADH dehydrogenase subunit 1 (*mtND1*), vascular endothelial growth factor A (*vegfa*), vascular endothelial growth factor receptor 1 (*vegfr1*), *vimentin* and *slug* were quantified by reverse transcription of total RNA and real time quantitative PCR (qPCR) analysis as described previously (18). Assays and primers used in this study are listed in Table 1. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method and presented as the fold-change in gene expression normalized to endogenous reference gene (β-actin) and relative to the control. All reactions were carried out in triplicate.

SDS-PAGE and western blot analysis

For western blot analysis, proteins were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Protein pellets were dissolved in 1% SDS in the presence of protease inhibitors (Roche, USA). Total cellular proteins (20 μg per lane) were resolved by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, CA, USA). Membranes were blocked in 5% nonfat dry milk in TN buffer (50 mM TRIS, 150 mM NaCl, pH 7.4) for 1 h at 37°C and incubated overnight at 4°C with primary polyclonal or monoclonal antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences Inc., USA) for 1 h at 37°C. Primary and secondary antibodies used in this study are listed in Table 2. To confirm equal loading and normalize the bands, AmidoBlack (Sigma Aldrich, St. Louis, MO, USA) was used. The chemiluminescence signals were detected and analyzed with the Alliance 4.7 Imaging System (UVITEC, Cambridge, UK).

MTT, growth curves, and CFU assays

For the MTT assay, $7x10^3$ cells were seeded in 96-well plate and 24 h later treated with hyperoxia. After that, growth medium was removed and 1x MTT was added and cells were incubated for 4 h in the growth conditions, followed by addition of dimethyl sulfoxide and 20 min incubation with gentle mixing. Absorbance was measured at λ=570 nm on ELISA microplate reader (LabSystem Multiskan MS, Artisan Technology group, USA). The same assay was used for determination of cellular growth after seven days of growing in high-glucose medium in normoxic conditions. For the CFU assay, $2x10^3$ cells were seeded in 5 cm Petri dishes and 24 h later treated with hyperoxia. After that, the growth medium was replaced with new one and the cells were incubated for 14 days until the visible colonies were observed. Cells were fixed with methanol for 10 min, followed by 30 min drying at room temperature. Giemsa dye was added and incubated for 30 min, washed by reH_2O and cells were dried. Colonies were

counted and the cell viability was determined in comparison with the control cells from normoxia.

Flow cytometry

Flow cytometry was performed using a Becton-Dickinson FACSCalibur model equipped with a 488 nm argon laser and a 635 nm red diode laser. Cytoplasmic ROS production was measured with 2′,7′-dichlorofluorescein diacetate (H2DCFDA, Sigma Aldrich, St. Louis, MO, USA), mitochondrial superoxide production with MitoSOX Red reagent (ThermoFisher Scientific, USA), variations in the mitochondrial transmembrane potential (ΔΨm) with 3,3′-dihexyloxacarbocyanine iodide (DiOC(6(3))), Enzo Life Science, SAD), and 10-N-*Nonyl acridine orange* (NAO, Invitrogen, Carlsbad, CA, SAD) was used for measurement of mitochondrial mass. In a 6 well plate, $1{,}5x10^5$ cells were seeded and 24 h later treated with hyperoxia. After that, cells were washed with PBS and incubated with specific dye prepared in the growth medium (40 nM DiOC(6(3))), 50 nM NAO, 5 μM H2DCFDA and 3 μM MitoSOX Red) for 30 min in the growth conditions. Cells were washed, collected in PBS, and minimum of 10 000 events were acquired. For discrimination of dead cells, propidium iodide (10 μM) was used. The collected data were analyzed using FCS Express software version 3.

Determination of mtDNA/nDNA ratio

Total DNA was isolated from MCF-7 cell culture pellets containing $2\text{-}5x10^5$ cells, using AccuPrepR Genomic DNA Extraction Kit (Bioneer, Inc., USA), following the manufacturer's protocol. DNA samples were fragmented to approximately 1000 bp in size by using Bioruptor® sonication system (Diagenode, Liège, Belgium). Mitochondrial DNA (mtDNA) copy number was determined by qPCR of the mitochondrial *mtND1* gene relative to the nuclear β-actin housekeeping gene (Taqman® Gene Expression Assays, Applied Biosystems, UK; nDNA). The standard mode thermal cycling conditions were used on ABI PRISM Sequence Analyzer 7300 (Applied Biosystems, UK). All experiments were repeated in at least three independent

biological replicates. MtDNA copy number was determined for each sample using mtDNA/nDNA ratio. Average C_T values were calculated from technical replicates for both mtDNA and nDNA. Relative mtDNA content was calculated as described previously (19), using the formula: 2 x 2 (ΔC_T), where ΔC_T equaled the difference between β-actin gene and *mtnd1* gene C_T values (nDNA C_T – mtDNA C_T).

Mitochondrial oxygen consumption

Oxygen consumption by the cells (10^7 cells from each clone in respiration buffer: 20 mM HEPES pH=7.4, 10 mM $MgCl_2$ and 250 mM sucrose dissolved in reH_2O) was determined polarographically by the Clark type electrode (Oxygraph, Hansatech, Norfolk, UK) in an airtight 1.5 ml chamber at 35°C. After measurement of basal state respiration, the complex III inhibitor antimycin A (2,5 μM) was added to inhibit the cellular respiration. Oxygen consumption was calculated in pmol/min/10^6 cells and normalized to normoxic control group.

Senescence-associated beta-galactosidase (SA-β-gal) activity assay

Cells (4×10^5 cells per well) were seeded into 24-well plate and 24 h later treated with hyperoxia. After that, SA-β-gal staining was performed: cells were fixed for 10 min in 1% glutaraldehyde in DMEM, washed twice with PBS and incubated at 37°C with X-gal (1 mg/mL), dissolved and incubated for 16 h in a solution containing 40 mM citric acid/Na phosphate buffer pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM MgCl2. Senescent cells were evaluated using an Olympus IX50 microscope (Olympus, Japan) under a ×20 lens. Cells were photographed and the percentage of SA-β-gal-positive cells was calculated.

Statistical analysis

Statistical analysis of data was performed using R v2.15.3 (CRAN) and RStudio for Windows, v1.1.423 (20). Before all analyses, samples were tested for normality of distribution using Shapiro-Wilk test. Since all experiments data followed normal distribution, parametric tests for

multiple comparisons of the samples were performed: two-way ANOVA was used for all analyses.

Results

Characterization of stable Sirt3 overexpressing MCF-7 clones in normoxia and hyperoxia

Given the fact that breast cancer cell line MCF-7 has reduced expression of Sirt3, which was shown to act as either tumor suppressor or a tumor promotor in different types of cancer (4), we have generated stable Sirt3 overexpressing clones to explore its role in this cell model. Briefly, MCF-7 cells were stably transfected with empty pcDNA3.1+ plasmid or pcDNA3.1+ plasmid with Flag-tagged Sirt3, and were labelled as MCF-7C or MCF-7S3, respectively. Stable expression of catalytically active Sirt3 (28kDa) in MCF-7S3 was determined with qPCR and western blot analysis. qPCR analysis showed 23-fold increase in Sirt3 gene expression level in MCF-7S3 compared to MCF-7C, and 43-fold increase in hyperoxia (Figure 1A). Western blot analysis confirmed that the specific signal for Sirt3 protein was absent from MCF-7C, while MCF-7S3 expressed high levels of Sirt3 equivalent to molecular weight of 28 kDa, as detected by anti-Sirt3 monoclonal antibody, and this expression was additionally increased upon hyperoxia (Figure 1B-C).

De novo Sirt3 expression is enriched upon hyperoxia and colocalizes with mitochondria

Next we wanted to determine the expression and localization of Sirt3 protein in MCF-7C and MCF-7S3 cells. No signal for Sirt3 was detected in MCF-7C, whereas clear signal was observed in MCF-7S3, and was even stronger upon hyperoxic treatment. These results confirm *de novo* expression of Sirt3 protein in MCF-7S3 cells, as expected, in both normoxia and hyperoxia, with stronger signal per cell in hyperoxia-treated cells (Figure 1D-E). Next, we wanted to analyze whether Sirt3 might be localized in mitochondria. The confocal microscopy analysis of Sirt3 (green) and costaining with MitoTracker Deep Red, which specifically dyes mitochondria (red), revealed overlapping of the two signals (yellow) confirming localization of Sirt3 in

mitochondria. Also, we found that hyperoxia induced mitochondrial signal intensity in both clones. These data demonstrate that hyperoxia induced increase in mitochondrial content in both clones, whereas the Sirt3 expression was increased exclusively in clone with *de novo* Sirt3 expression.

De novo Sirt3 expression enhances downregulation of pro-angiogenic and cancer-malignancy-related genes and proteins upon hyperoxic treatment

To assess the mechanism by which Sirt3 and hyperoxia influence tumorigenic characteristics and potential of MCF-7 cells, we surveyed the expression of genes involved in antioxidant protection (*sod2*, *cat*), metabolic regulation (*sirt1*), mitochondrial electron transfer (*mtND1*), angiogenesis (*vegfa* and *vegfr1*) and epithelial mesenchymal transition (EMT; *vimentin* and *slug*) (Figure 2A). We found that *de novo* Sirt3 expression in normoxia downregulated pro-angiogenic gene *vegfr1* involved in cell proliferation with having no effect on the expression of *vegfa*. In addition, strong downregulation of EMT markers – *vimentin* and *slug*, compared to control clones, was also observed. Hyperoxia induced pro-angiogenic *vegfa*, *vimentin* and *mtND1*, while having no significant effect on other genes. Interaction of *de novo* expressed Sirt3 and hyperoxia downregulated antioxidant defense genes *sod2* and *cat*, along with *sirt1*. In addition, *slug* and *vegfr1* were decreased as well. These results collectively demonstrate that suppressive effect of Sirt3 on pro-angiogenic and cancer-malignancy-related genes in normoxia is enhanced upon hyperoxic exposure.

Next we wanted to explore if this pattern of regulation was followed by changes in the expression of proteins involved in these processes. Therefore, we tested the expression of proteins involved in glycolysis (lactate dehydrogenase A; LdhA), antioxidant defense (Sod2 and Cat), cellular homeostasis (Sirt1), and mitochondrial biogenesis (peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PGC1α) of MCF-7 cells. LdhA, a marker of aerobic glycolysis, which is considered to be a key enzyme to glycolytic phenotype of tumor

cells, was downregulated in MCF-7S3 cells. Hyperoxia significantly upregulated LdhA expression in MCF-7C compared to their normoxic group, while interaction of Sirt3 and hyperoxia abrogated this effect. Sod2 and Cat showed similar pattern of expression: the expression was reduced in MCF-7S3 cells, additionally reduced in hyperoxia, and almost completely absent in hyperoxic MCF-7S3 cells. Hyperoxia lowered Sirt1 level, regardless of Sirt3 expression. PGC1α expression was markedly increased in MCF-7S3 cells, but reduced in hyperoxic MCF-7C, whereas interaction of hyperoxia and Sirt3 partially rescued its expression. Since hyperoxia is known to induce DNA damage, we wanted to check the expression of a marker of DNA double strand breaks - phosphorylated gamma-H2A histone family, member X (pγH2AX) and a major tumor suppressor, p53. In normoxic MCF-7C cells the pγH2AX was absent, whereas the expression of p53 was weak, but slightly increased upon Sirt3 expression. As expected, hyperoxia induced both pγH2AX and p53, thus showing a clear sign of ROS-induced DNA damage which was even more pronounced upon interaction of Sirt3 and hyperoxia. These results collectively suggest that Sirt3 and hyperoxia may act in favor of the inhibiting the proliferation of cancer cells (Figure 2B-I).

De novo Sirt3 expression and hyperoxia decrease proliferation rate and metabolic activity of MCF-7 cells

Since we found that some of the proliferation and angiogenic factors crucial for tumorigenicity were downregulated upon *de novo* expression of Sirt3, we decided to test whether it plays a role in metabolic activity and proliferative potential (growth curve, clonogenic survival) of MCF-7 cells. The growth curve showed that MCF-7S3 cells grow more slowly than their corresponding controls (Figure 3A). MTT test showed that MCF-7S3 cells had small, but significantly decreased metabolic activity (88% of MCF-7C) (Figure 3B). Hyperoxia additionally decreased metabolic activity in both groups irrespective of Sirt3 expression. The drop in metabolic activity in normoxic MCF-7S3 was accompanied by decreased capacity to produce colonies, as showed

with CFU assay (Figure 3C-D). As expected, hyperoxia caused dramatically decreased capacity to produce colonies in both clones, irrespective of Sirt3 expression. These results collectively demonstrate the suppressive effect of either Sirt3 or hyperoxia alone on proliferation and metabolic activity of MCF-7 breast cancer cells.

De novo Sirt3 expression and hyperoxia increase cytosolic and mitochondrial ROS production

Hyperoxia has a well-known effect of increasing cellular ROS levels. Since Sirt3 is considered as a fidelity protein that particularly regulates production of mitochondrial ROS (mtROS), we tested whether *de novo* expression of Sirt3 would influence mitochondrial or cytosolic ROS production. By measuring cytosolic ROS production with H2DCFDA fluorescent probe, we showed that Sirt3 expression increased cytosolic oxidative stress in normoxia (Figure 4A). Hyperoxia *per se* increased cytosolic ROS in MCF-7C which was followed by additional increase in MCF-7S3. Similar pattern was observed for mtROS, i.e. Sirt3 expression and hyperoxia induced mtROS in both clones (Figure 4B). These results collectively demonstrate that *de novo* Sirt3 expression and hyperoxia increase ROS levels in both cytosolic and mitochondrial compartments.

Hyperoxia-induced alteration of mitochondrial function parameters is associated with de novo Sirt3 expression

It has been shown that Sirt3-increased mtROS have capacity to induce mtDNA damage, leading to potential mitochondrial dysfunction (21). Therefore, we wanted to determine whether *de novo* Sirt3 expression can influence mtDNA level, along with other mitochondrial parameters, such as mitochondrial mass, mitochondrial potential and oxygen consumption. First we examined the ratio of mitochondrial to nuclear DNA in all experimental groups and observed significant increase in mtDNA level in MCF-7S3 cells, which was also retained in hyperoxic conditions (Figure 5A). These results demonstrate that in both normoxic and hyperoxic

conditions Sirt3 overexpression increased relative mtDNA level in respect to nuclear DNA. Although combination of hyperoxia and Sirt3 elevated mitochondrial mass, and hyperoxia substantially increased mitochondrial potential regardless of Sirt3 expression (Figure 5B-C), hyperoxic treatment significantly lowered O_2 consumption in both cell lines (Figure 5D).

De novo Sirt3 expression antagonizes senescence

From the above results it is evident that hyperoxia causes DNA damage that eventually results in cell death. However, we did not observe difference in rate of apoptosis as a result of Sirt3 expression or hyperoxia and Sirt3 (data not shown), thus we conclude that hyperoxia-induced cell death did not occur via apoptosis. Furthermore, the unchanged expressions of poly (ADP-ribose) polymerase 1 (PARP-1) imply that it also did not occur via PARP-1-dependent cell death, parthanatos (data not shown). Therefore, we tested whether hyperoxia-induced DNA damage influenced senescence-associated phenotype in both clones. SA-β-gal activity assay staining showed that MCF-7S3 cells had lower ratio of SA-β-gal positive cells compared to MCF-7C cells (Figure 6A-B), whereas hyperoxia induced increase of SA-β-gal positive cells in both groups. Similar to normoxia, the expression of Sirt3 inhibited hyperoxia-induced cellular senescence by 20% compared to hyperoxia-treated MCF-7C cells. Rotenone was used as a positive control for senescence induction (22).

Discussion

In this study we demonstrated the potential of *de novo* Sirt3 expression to suppress pro-angiogenic and cancer-malignancy-related properties of human MCF-7 breast cancer cells, as well as to enhance their susceptibility to hyperoxic treatment. Sirt3 has bifunctional role, acting as both oncogene and tumor suppressor, depending on the tissue and cancer-type specific metabolic programs (4). Because breast cancer cells have strikingly reduced Sirt3 level, and 20% of them have almost no detectable Sirt3 protein (11), we developed *de novo* Sirt3 expressing transfectants to examine its effect on characteristics of MCF-7 breast cancer cells,

combined with hyperoxic treatment which was earlier shown to suppress the growth of certain mammary carcinoma cells (7).

A number of breast cancer cells, particularly MCF-7, depend on a mixture of glycolysis and OXPHOS to support their proliferation (23). The faster growth rate of control MCF-7 clones in high-glucose medium (Figure 3A) implies the preferential use of glycolysis pathway to facilitate the uptake and incorporation of nutrients into the biomass thus supporting their faster proliferation (24). It seems that Sirt3 induced the metabolic shift from glycolysis to OXPHOS, which involves the PGC1α-induced mitochondrial regulation, therefore causing decrease in fast supply of nutrients usually provided by glycolysis and consequently reduced metabolic activity and clonogenic capacity of MCF-7 cells in normoxic conditions. Besides increasing PGC1α, a master regulator of mitochondrial biogenesis, i.e. oxidative metabolism (25), Sirt3 also reduced expression of LdhA, a marker of glycolysis and proliferating cells, thus confirming previous reports indicating that metabolic reprogramming mediated by Sirt3 contributes to its tumor-suppressive role in human breast cancer cell lines (11).

Sirt3 reduced *vegfr1* and two markers of EMT, vimentin and slug. Since vimentin contributes to cell polarity and motility and regulates the expression of slug to further enhance EMT phenotypes and cancer malignancy (26), the dramatic reduction of these genes suggests that Sirt3 indeed acts as a suppressor of EMT transition and malignant properties of the MCF-7 cells. In addition, Sirt3 reduced senescence of MCF-7 cells. Although senescence is usually activated in a variety of premalignant steps to limit tumor progression (27), in certain contexts it may create a pro-oncogenic tissue environment, thus leading to functional decline of tissues and the rise in cancer incidence (28). Therefore, reduced senescence of MCF-7 cells may also contribute to the role of Sirt3 in inhibiting their tumorigenic properties.Another crucial tumor suppressor, p53, is often mutated or deregulated in many human cancers (29). Since cells without a functional p53 lack the DNA-damage-sensing capability to induce the protective

response (30), its slight increase upon the Sirt3 expression may have beneficial effect on suppression of tumorigenic potential. Taking all these factors in consideration, we conclude that Sirt3 inhibits the tumorigenic properties of MCF-7 cells in normoxia.

Although tumor cells often live in hypoxic conditions due to metabolic and structural abnormalities, their angiogenesis, growth and survival depend upon the sufficient supply of oxygen. The high ROS production and pro-angiogenic gene expression upon hyperoxic treatment at first suggest tumor-promoting effects of hyperoxia, since cells respond to elevated ROS levels by increasing the *vegfa* expression through the activation of signaling pathways usually involved in tumorigenesis (31). Hyperoxia also significantly upregulated the EMT marker vimentin, as well as LdhA, an indicator of lactate generation, which initiates the *vegf* production (32) and contributes to aerobic glycolysis. This is also supported by a recent study showing higher uptake of glucose in the hyperoxia-treated MCF-7 cells (33). However, hyperoxia initiated a number of events indicative of suppressing the tumorigenic properties, e.g. strong activation of p53, induction of senescence and mitochondrial dysfunction, as well as depleted PGC1α, Sirt1 and Sod2 proteins abundance, which may finally be responsible for diminished cell proliferation and survival, as shown recently (12, 37). Moreover, hyperoxia increased mitochondrial potential and ROS production. Since even slight increase in mitochondrial potential may cause significant rise in ROS levels (38), this finding implies that tumor-suppressive effect of hyperoxia may be partially achieved through mitochondrial dysfunction. Excessive ROS are also important for establishing cellular senescence (34), which usually suppresses tumors in response to stress and is highly dependent on functional p53 (30). Although one would expected that cells with a lot of DNA damage would undergo apoptosis pathway, MCF-7 cells rather become senescent than enter the apoptosis, probably due to the lack of functional caspase 3 expression (35). This was also presented in previous studies showing that hyperbaric oxygen treatment lowers proliferation, but does not induce apoptosis

386 in MCF-7 cells (36). Collectively and in agreement with previous studies (39, 36), these results
387 show that hyperoxia inhibits proliferation and survival of cancer cells through the mitochondrial
388 dysfunction and activation of p53 upon DNA damage and induction of senescence.
389 Under hyperoxic conditions, Sirt3 additionally downregulated majority of genes involved in
390 angiogenesis and tumor promotion, as well as EMT-inducing gene slug. Furthermore, increased
391 DNA damage was accompanied with additional increase of ROS levels and consequent
392 mitochondrial dysfunction and depletion of antioxidative enzymes. Sirt3 also antagonized
393 senescence in both conditions, as expected (40).
394 The mitigation of tumorigenic properties and enhancement of the susceptibility of the MCF-7
395 breast cancer cells to the hyperoxic treatment upon the *de novo* Sirt3 expression, observed
396 through their reduced survival, proliferation and mitochondrial function, as well as upregulation
397 of the cells growth suppressors, indicates that both Sirt3 and hyperoxia, especially combined,
398 have a potential to negatively modulate the properties of the cancer cells and that they should
399 be further explored, *in vitro* and particularly *in vivo*, as an adjuvant tumor therapy in breast
400 cancer malignancies.

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407 **Disclosure statement**

408 The authors report no conflict of interest.

409

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Tables

Table 1. *Assays and primers used for real time quantitative PCR analyses*

Gene	Assay ID[a]/primers[b]	Product size (bp)
β-actin	Hs01060665_g1	63
mtND1	Hs02596873_s1	143
sod2	Hs01553554_m1	69
cat	Hs00156308_m1	68
sirt1	Hs01009005_m1	94
sirt3	Hs00953477_m1	83
vegfa	Hs00900058_m1	81
vegfr1	Hs01052936_m1	72
vimentin	5′-CGTGATGCTGAGAAGTTTCGTTGA -3′ 5′-CCAAACTTTTCCTCCCTGAACC-3	142
slug	5′-GAGGAGAAAATGCCTTTG -3′ 5′-ATGAGCAATCTGGCTGCT -3′	119

[a] Taqman® Applied Biosystems, UK

[b] Power SYBR™ Green PCR Master Mix, AppliedBiosystems, UK

519 ***Table 2.*** *Primary and secondary antibodies used for Western blot analyses*

Primary antibody	**Dilution**	**Manufacturer**
anti-Sirt3	1:400	Cell Signaling Technology, USA
anti-LdhA	1:200	Santa Cruz Biotehnology, USA
anti-Sod2	1:2000	Abcam, UK
anti-Sirt1	1:300	Santa Cruz Biotehnology, USA
anti-PGC1α	1:1000	Novus Biologicals, USA
anti-Cat	1:500	Abcam, UK
anti-gammaH2AX	1:2000	Abcam, UK
anti-p53 (DO-1)	1:2000	Santa Cruz Biotehnology, USA
Secondary antibody		
anti- rabbit IgG HRP linked	1:4000	GE Healthcare, UK
anti-mouse IgG HRP linked	1:2000	Santa Cruz Biotehnology, USA

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Figure captions

Figure 1. *Characterization of MCF-7C and MCF-7S3 cells.*

(1A) Real-time qPCR analysis of sirt3 gene expression level in MCF-7C and MCF-7S3 cells in normoxia and hyperoxia. Significant effect of sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$) is observed, as well as the effect of their interaction (p=0.015). The results are presented as fold-change ± S.E., and normalized to control group. The fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. β-actin was used as the endogenous control. Experiments were repeated at least three times and representative data are shown. **(1B)** Densitometry analysis of Sirt3 protein expression level in MCF-7C and MCF-7S3 cells in normoxia and hyperoxia. Data are presented as mean ± S.D. Significant effect of Sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.01$) is observed, as well as the effect of their interaction (p=0.008). **(1C)** Immunoblots of Sirt3 protein expression level. Amidoblack was used as a loading control. The experiments were repeated at least three times and representative blots are shown. **(1D, E)** Sirt3 colocalizes with mitochondria and is enriched upon hyperoxia. Confocal image analysis of MCF-7 clones in normoxia and hyperoxia. Representative confocal image analysis of Sirt3 localization (green) in normoxia and hyperoxia. Mitochondria and nuclei were detected with Mitotracker Deep Red or DAPI, respectively. Overlay image shows complete overlap (Yellow) of two staining patterns: fluorescence from the Sirt3–FITC protein (Green) and fluorescence from the Mitotracker Deep Red stained mitochondria (Red).

Figure 2. *De novo Sirt3 expression enhances downregulation of pro-angiogenic and cancer-malignancy-related genes and proteins upon hyperoxia*

(2A) Heatmap of differential gene expression as determined using real-time quantitative PCR method. The fold-change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and β-actin as the endogenous control. Color of the squares on the heatmap corresponds to the mean value of the log fold-change from three biological and three technical replicates. β-actin was used for

normalization**.** Significant effect of sirt3 was found for *vegfr1* – fold-change: -2.0, $p<0.05$; *vimentin* – fold-change: -4.76, $p<0.001$; *slug* – fold-change: -2.70, $p<0.05$. Significant effect of hyperoxia was found for: *vegfa* – fold-change: +2.0, $p<0.05$; *vimentin* – fold-change: +2.17, $p<0.01$; *mtND1* – fold-change: +2.0, $p<0.05$. Significant effect of interaction of sirt3 and hyperoxia was found for: *vegfr1* – fold-change: -2.43, $p<0.05$; *slug* – fold-change: -3.84, $p<0.01$; *sirt1* – fold-change: -2.38, $p<0.05$; *sod2* – fold-change: -4.34, $p<0.001$; *cat* – fold-change: -2.94, $p<0.01$. **(2B)** Protein expression levels of Sirt1, PGC1α, Cat, p53, LdhA, Sod2 and pγH2AX. Amidoblack was used as a loading control. The experiments were repeated three times and representative immunoblots are shown**. (2C-I)** Graphical analysis of immunoblots of protein expression. **(2C)** For Sirt1 there was significant effect of sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$). **(2D)** For PGC1α there was significant effect of sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$), as well as of their interaction ($p<0.001$). **(2E)** For Cat there was significant effect of sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$), as well as of their interaction ($p<0.001$). **(2F)** For p53 there was significant effect of sirt3 **(**$^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$). **(2G)** For LdhA there was significant effect of sirt3 **(**$^{a}p<0.001$), hyperoxia ($^{b}p<0.05$) and their interaction ($p=0.015$). **(2H)** For Sod2 there was significant effect of sirt3 **(**$^{a}p<0.01$) and hyperoxia ($^{b}p<0.001$). **(2I)** For γH2Ax there was significant effect of sirt3 $^{a}p<0.001$, hyperoxia ($^{b}p<0.001$) and their interaction ($p<0.001$).

Figure 3. *De novo Sirt3 expression and hyperoxia result in decrease of metabolic activity and survival.*

(3A) Growth curves of MCF-7C and MCF-7S3 clones in high-glucose medium in normoxia. The representative experiment is shown. Results are presented as mean ± S.D. ***$p<0.001$, MCF-7C vs. MCF-7S3. **(3B)** Percentage of metabolic activity of MCF-7S3 cells in normoxia and hyperoxia compared to MCF-7C cells. Significant effect of Sirt3 **(**$^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$) on metabolic activity is observed. Results are shown as mean ± S.D. **(3C)** Graphical

display of number of colonies (CFU assay) relative to normoxic MCF-7C. Significant effect of Sirt3 ($^{a}p<0.005$) and hyperoxia ($^{b}p<0.001$) on cellular clonogenic capacity is observed. The experiments were repeated at least three times. **(3D)** Representative plates stained with crystal violet.

Figure 4. *De novo Sirt3 expression and hyperoxia increase cytosolic and mitochondrial ROS production.*

(4A) Cytosolic ROS levels were examined by H2DCFDA staining. Results are presented as mean ± S.D. The experiments were performed at least three times and representative data are shown. Significant effect of Sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$), and their interaction on cytosolic ROS production is observed ($p<0.01$). **(4B)** mtROS levels were measured with MitoSOX Red. Mean fluorescence intensity was compared to normoxic control clone. Significant effect of Sirt3 ($^{a}p<0.001$) and hyperoxia ($^{b}p<0.001$), and their interaction on mitochondrial ROS production is observed ($p=0.01$).

Figure 5. *Hyperoxia-induced alteration in mitochondrial function parameters is associated with de novo Sirt3 expression*

(5A) mtDNA content of MCF-7C and MCF-7S3 in normoxia and hyperoxia. Significant effect of Sirt3 ($^{a}p<0.001$) and the interaction of Sirt3 and hyperoxia ($p<0.05$) on mtDNA content is observed. **(5B)** Significant effect of Sirt3 ($^{a}p<0.05$) and hyperoxia ($^{b}p<0.001$) on mitochondrial potential is observed. **(5C)** Significant effect of Sirt3 ($^{a}p<0.001$), hyperoxia ($^{b}p<0.001$) and their interaction ($p<0.05$) on mitochondrial mass is observed. Mean fluorescence intensity of all experimental groups was compared to normoxic control clone. **(5D)** Significant effect of hyperoxia ($^{b}p<0.001$) on oxygen consumption is observed. Data are presented as percent of mean ± S.D. The experiments were performed at least three times and representative data are shown.

Figure 6. *De novo Sirt3 expression antagonizes senescence in both normoxia and hyperoxia* **(6A)** Graphical display of the ratio of β-gal positive cells in normoxic and hyperoxic conditions. Data present percentage of positive senescent cells analyzed by β-galactosidase staining. The experiments were repeated three times and representative data are shown. Significant effect of Sirt3 ($^{a}p<0.001$), hyperoxia ($^{b}p<0.001$) and their interaction ($p<0.01$) on senescence-associated β-galactosidase activity is observed. **(6B)** Representative images of cells stained for senescence-associated β-galactosidase activity. Rotenone was used as a positive control for senescence induction.