Endogenous Myoglobin in Breast Cancer Is Hypoxia-inducible by Alternative Transcription and Functions to Impair Mitochondrial Activity

A ROLE IN TUMOR SUPPRESSION?\(^*\)\(^\text{§}\)

Received for publication, February 4, 2011, and in revised form, September 6, 2011 Published, JBC Papers in Press, September 19, 2011, DOI 10.1074/jbc.M111.227553

Glen Kristiansen\(^\text{††},\) Junmin Hu\(^\text{‡‡},\) Daniela Wichmann\(^\text{§},\) Daniel P. Stehl\(^\text{‡},\) Michael Rose\(^\text{‡},\) Josefine Gerhardt\(^\text{**},\) Annette Bohnert\(^\text{**},\) Anette ten Haaf\(^\text{**},\) Holger Moch\(^\text{**},\) James Raleigh\(^\text{**},\) Mahesh A. Varia\(^\text{**},\) Patrick Subarsky\(^\text{††},\) Francesca M. Scandurra\(^\text{**},\) Erich Gnaiger\(^\text{**},\) Eva Gleixner\(^\text{**},\) Anne Bicker\(^\text{**},\) Max Gassmann\(^\text{**},\) Thomas Hankeln\(^\text{**},\) Edgar Dahl\(^\text{**},\) and Thomas A. Gorr^\text{††}

From the \(^4\) Institute of Pathology, University Hospital Bonn, 53127 Bonn, Germany, the \(^5\) Institute of Veterinary Physiology, Vetsuisse Faculty, Zurich Center for Integrative Human Physiology, University of Zurich, 8057 Zurich, Switzerland, the \(^6\) Institute of Physiology, University of Zurich, 8057 Zurich, Switzerland, the \(^7\) Institute of Pathology, University Hospital of the RWTH, 52074 Aachen, Germany, the \(^8\) Institute of Surgical Pathology, University Hospital Zurich, 8091 Zurich, Switzerland, the \(^9\) University of North Carolina School of Medicine, Chapel Hill, North Carolina 19044, the \(^55\) D. Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck, 6020 Innsbruck, Austria, the \(^66\) Institute of Molecular Genetics, Johannes Gutenberg University Mainz, 55099 Mainz, Germany, and the \(^10\) Center for Pediatrics and Adolescent Medicine, University Medical Center Freiburg, 79106 Freiburg, Germany

**Background:** The role of MB in tumors cells is yet unclear.

**Results:** MB is induced by hypoxia in breast cancer cell lines, possibly by an alternative transcription start site. Knockdown of MB in breast cancer cells is functionally relevant and significantly alters cellular respiration.

**Conclusion:** MB might impair mitochondria in hypoxic cancer cells.

**Significance:** MB might have tumor-suppressive functions, not described so far.

Recently, immunohistochemical analysis of myoglobin (MB) in human breast cancer specimens has revealed a surprisingly widespread expression of MB in this nonmuscle context. The positive correlation with hypoxia-inducible factor 2α (HIF-2α) and carbonic anhydrase IX suggested that oxygen regulates myoglobin expression in breast carcinomas. Here, we report that MB mRNA and protein levels are robustly induced by prolonged hypoxia in breast cancer cell lines, in part via HIF-1/2-dependent transactivation. The hypoxia-induced MB mRNA originated from a novel alternative transcription start site 6 kb upstream of the ATG codon. MB regulation in normal and tumor tissue may thus be fundamentally different. Functionally, the knockdown of MB in MDA-MB468 breast cancer cells resulted in an unexpected increase of O2 uptake and elevated activities of mitochondrial enzymes during hypoxia. Silencing of MB transcription attenuated proliferation rates and motility capacities of hypoxic cancer cells and, surprisingly, also fully oxygenated breast cancer cells. Endogenous MB in cancer cells is apparently involved in controlling oxidative cell energy metabolism, contrary to earlier findings on mouse heart, where the targeted disruption of the Mb gene did not effect myocardial energetics and O2 consumption. This control function of MB seemingly impacts mitochondria and influences cell proliferation and motility, but it does so in ways not directly related to the facilitated diffusion or storage of O2. Hypothetically, the mitochondrial-imparing role of MB in hypoxic cancer cells is part of a novel tumor-suppressive function.

Mammalian myoglobin (MB)\(^3\) is a cytoplasmic heme-containing respiratory protein of cardiac myocytes and oxidative type I/Ila skeletal muscle fibers. In striated human muscles, MB occurs at concentrations of ~200–300 μM. Here, the monomeric globin is widely accepted to function as a temporary “store” for oxygen, able to buffer short phases of exercise-induced increases in O2 flux during which it supplies the gas to mitochondria (2). Another more controversially discussed role of MB is the facilitation of O2 diffusion within muscle cells (3, 4). MB knock-out (Mb\(^{-/-}\)) mice exhibited normal development and exercise capacity and showed no signs of compromised cardiac energetics due to multiple systemic compensa-

---

\(^{*}\) This work was supported in part by grants from the Swiss National Science Foundation (to M. G.), a grant from the Faculty of Medicine, RWTH Aachen (START network) (to E. D.), and by contributions from K-Regio Project MitoCom Tyrol (to E. G.). Parts of this paper have been presented at the International Congress of Respiratory Science, August 9–13, 2009, Bad Honnef, Germany.

\(^{††}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

\(^{1}\) To whom correspondence should be addressed: Institute of Pathology, University Hospital Bonn (UKB), Sigmund-Freud-Str. 25, 53127 Bonn, Germany. Tel.: 49-228-287-15375; Fax: 49-228-287-15030; E-mail: glen.kristiansen@ukb.uni-bonn.de.

\(^{2}\) Both authors should be considered senior authors.

\(^{3}\) The abbreviations used are: MB, myoglobin; DCIS, ductal carcinoma in situ; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ERα, estrogen receptor α; HIF, hypoxia-inducible factor; LoF, loss-of-function; ROX, residual O2 consumption; ETS, electron transfer capacity; HRE, hypoxia-response element; scr, scrambled; si, silencing; KD, knockdown; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; CAIX, carbonic anhydrase IX.
Myoglobin in Breast Cancer, Expression and Function

The fact that these compensations (i.e. increases in capillarity, coronary flow, coronary reserve, and hematocrit) acted in concert to reduce the diffusion path length for \( \text{O}_2 \) between capillaries and the mitochondria strongly argued for the importance of Mb with regard to the delivery of \( \text{O}_2 \). Follow-up studies stressed the additional relevance of functional Mb in maintaining nitric oxide (NO) homeostasis in muscle through either scavenging (6) or producing the NO molecule (7). Beyond transporting/storing diatomic gaseous ligands (\( \text{O}_2, \text{NO} \)), the physiological facets of MB might also include synthesis of peroxides (8), scavenging of reactive \( \text{O}_2 \) species (9), and binding of fatty acids (10, 11). However, the oxygenation-support function of MB in myocytes is widely accepted and further underscored by the \( \text{O}_2 \) responsiveness of the Mb1a-expression function discussed in this study (30). In this study, we focus on transcriptional implications in this setting (30). In this study, we focus on the mechanistic details of human MB expression in malignant tumors of human subjects, including liver, brain, and gills. More than that, the carp generates not one but two Mbs from distinct genes (\( \text{Mb}-1 \) and \( \text{Mb}-2 \)) of which one (\( \text{Mb}-1 \)) is robustly induced as transcript and protein in all nonmuscle tissues upon exposure of the fish to day-long \( \text{O}_2 \) deprivation. Both proteins also occur in nonmuscle tissues of the closely related and equally hypoxia-tolerant goldfish (\( \text{Carassius auratus} \)) (21). MB is also present in smooth muscles of birds (i.e. the gizzard (22)) and humans (i.e. rectal sphincter (23)). In addition, low levels of MB are variably detected in different malignant tumors of human subjects, including breast cancer (24–28), but the physiological function of endogenous MB in cancer cells remains a mystery.

We recently conducted the first comprehensive analysis of MB expression in a large and representative cohort of human breast cancer (29). Subsequently, we documented other globins, namely hemoglobin and cytoglobin, to also be expressed in human breast cancer and have begun to unravel their functional implications in this setting (30). In this study, we focus on the mechanistic details of human MB expression in malignant breast carcinomas and cancer cell lines. CarcinoMa-expressed MB was found strongly associated with known marker genes of hypoxia. Cultured human breast cancer cells, but not healthy breast epithelial cells, robustly induce the expression of MB mRNA and protein when challenged by prolonged hypoxia. This induction by hypoxia requires, to a large extent, transactivation by HIF-1 and -2. Transcription of the MB gene in breast cancer cells is launched via a novel alternative start site 6 kb upstream of the translation initiation codon. In line with the hypoxic activation of the gene, the alternative start site is flanked by a functional HRE. RNA interference-mediated silencing of MB function suggested that the hemoprotein is involved in regulating respiratory, proliferative, and motility activities in oxygenated and hypoxic breast cancer cells. Accordingly, MB in breast cancer might aid in adjusting the proliferative and oxidative capacities of the cells during prolonged hypoxia. In addition, MB promotes the growth of fully oxygenated breast cancer cells, perhaps through controlling fatty acid homeostasis and lipogenesis (29).

**Experimental Procedures**

**Cell Lines and Hypoxia Exposures**

Human mammary epithelial cell line MCF12A and the breast cancerous cell lines MDA-MB231, MDA-MB468, and MCF7 were obtained from ATCC (Manassas, VA) and cultured as recommended. Culture atmospheres included the following: normoxia = ventilated room air in water-saturated 5% \( \text{CO}_2 \) atmosphere = 141.6 mm Hg or 18.6% \( \text{O}_2 \) (sea level, 37 °C); hypoxia = water-saturated 1% \( \text{O}_2, 5\% \text{CO}_2/balance \text{N}_2 \) atmosphere at 37 °C. We used Hera cell 240 incubators (MultiTemp Scientific AG, Kloten, Switzerland), an IG150 incubator (Jouan, Unterhaching), a CB 53 CO\(_2\) incubator (Binder), a polymer Coy glove box (Coy Laboratory Products Inc., Grass Lake, MI), or Invivo 400 hypoxia workstations (Ruskin Technology Ltd., Leeds, UK) for hypoxic exposures.

**Quantitative Real Time Reverse Transcription-PCR**

Quantitative PCR experiments used the ABI Prism 7500 Fast SDS (Applied Biosystems, Darmstadt, Germany) and primers given in Table 1 (see Ref. 31 for details). Mean (± S.D.) expression levels of standard (NM_005368, \( \text{M} \text{B-S} \)) and alternative (NM_203377, \( \text{M} \text{B-A} \)) MB transcripts were calculated by the standard curve approach measuring \( C_t \) values for hypoxic and normoxic samples (\( n = 2 \) experiments, triplicate assays). Differences in relative abundance between \( \text{M} \text{B-S} \) and \( \text{M} \text{B-A} \) transcripts in normoxic MDA-MB468 cells were calculated using the standard curve method and a standard plasmid construct harboring both amplicons, \( \text{M} \text{B-S} \) and \( \text{M} \text{B-A} \) (\( n = 4 \) experiments, duplicate assays).

**Western Blots**

Following normoxic exposure for 72 h and hypoxic (1% \( \text{O}_2 \)) exposure for 4, 8, 24, 48, and 72 h, MDA-MB468 and MCF7 were harvested with a lysis buffer containing 0.1% Nonidet P-40, 400 mM NaCl, 1 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0), and protease inhibitors. Protein extracts were electrophoresed on a 15% SDS-polyacrylamide gel. Primary antibodies (Abs) included the following: (a) monoclonal Ab (mAb) mouse anti-myoglobin (catalog no. 113-0533, clone Z001, Zytomed Systems GMBH, Berlin, Germany; dilution, 1:1000); (b) anti-
Light signal quantification was measured in a Glomax 96-well reporter assays were performed at 72 h post-hypoxia exposure. To investigate the influence of human GAPDH (GenBank accession number NM_002039) as sense 5’-GCACCATGAGATTTCCCATCATCCAGG-3’ and antisense 5’-AACTACAGAGAGTTCTCCATCT-3’ sequences; (b) nucleotides 1010–1030 of human HIF-1α mRNA (GenBank accession number NM_001430) as sense 5’-CAGCAUCUUUAGAUAGCAGGudTdT-3’ and antisense 5’-ACUGCUUAAAGAUGCUGudTdT-3’ sequences. Both siRNAs and the SiCONTROL nontargeting pool 2 of scrambled siRNAs were purchased from Dharmacon Research Inc. (Lausanne, Switzerland). 6-Well plate Oligofectamine transfections (Invitrogen) of siRNAs used final concentrations of 200 nm of either HIF-1α or -2α single siRNA and 100 nm each of HIF-1α and -2α siRNA in combined transfections. Targeting MB—Short hairpin RNAs (shRNA) were used for targeting human MB mRNA (GenBank accession number NM_203377) at nucleotides 284–304 (construct 83), 483–503 (construct 84), 340–360 (construct 85), and 415–435 (construct 86). Inserted into the mammalian expression vector pLKO.1-puro, these four shRNA constructs were purchased as bacterial glycerol stock from Sigma. Stable shRNA MB knockdown MDA-MB468 cells were established by overnight calcium phosphate transfection followed by selection and maintenance in 0.75 μg/ml puromycin containing medium. For transient knockdown, MDA-MB468 cells were Lipofectamine-transfected (Invitrogen) with 20 (i.e. for knockdown 0–72 h) or 100 pmol (i.e. for knockdown 0–144 h) siRNA6 and -8 or all-Stars negative control scrambled RNA (Qiagen). Knockdown efficiency was confirmed on protein and/or RNA level using Western blot and real time quantitative PCR, respectively, as above.

High Resolution Respirometry

Oxygen kinetics of trypsinized, suspended, and heavily stirred MDA-MB468 cells, both of MB control and knockdown make up, were measured at ~1 × 106/ml densities in twin-chamber Oroboros® Oxygraph-2k high resolution respirometers as summarized previously (34–36). Each chamber was filled with cells suspended in 2 ml of culture medium (glucose-free DMEM + 10% FBS + penicillin/streptomycin + 0.75 μg/ml puromycin). To measure routine respiration, the medium was initially free of glucose. Later, 25 mM glucose were added to measure the extent of its inhibition on respiration. To control oxygen levels and record sequentially replicated aerobic-anoxic transitions of respiratory activity of MDA-MB468 cells, injections of 40 μM H2O2 triggered the release of con-
Myoglobin in Breast Cancer, Expression and Function

trolled amounts of oxygen because the medium also contained 280 IU/ml catalase. Catalase dismutates rapidly hydrogen peroxide into oxygen and water such that the small amounts of added hydrogen peroxide do not induce any oxidative stress (37). For the recording of maximum oxygen flow as a measure of mitochondrial electron transfer capacity (ETS), oxidative phosphorylation was uncoupled with increasing doses (4.0–5.5 μM) of the protonophore carbonyl cyanide-4-(trifluromethoxy)phenylhydrazone (FCCP). Finally, mitochondria were poisoned through injection of 0.5 μM myxothiazol to obtain the residual O2 consumption (ROX). The oxygen partial pressure supporting half-maximal respiratory flux, \( P_{50}(O_2) \), was calculated from aerobic-anoxic transitions in the routine state of respiration (with and without glucose in the medium), fitting a hyperbolic function between respiration \((R)\) and oxygen pressure \((pO_2)\) using the Hill equation,

\[
R = R_{\text{max}} \cdot \frac{pO_2}{(pO_2 + p_{50})}
\]

(Eq. 1)

In Vitro Proliferation, Mitochondrial Activity and Migration Assays

Proliferation rates of wild type (WT) and selected stable MB-control or MB-knockdown (KD) MDA-MB468 clones was determined over a 96-h time course in normoxic and hypoxic atmospheres by direct counting of viable cells (trypan blue exclusion assay). In addition, we used the dimethylthiazolidi-phenyltetrazolium bromide (MTT) assay to infer viability from the colorimetric measurement of mitochondrial dehydrogenase activity in MDA-MB468 cells transfected with MB-silencing siRNA or scrambled (scr) control RNA oligonucleotides as a function of four different O2 concentrations (normoxic atmosphere, 5, 1, and 0.2% O2) over a 144-h time course.

In vitro motility of MDA-MB468 cells was assessed in transiently siRNA-transfected cells by performing a monolayer scratch wound assay at normoxic and hypoxic tensions (38). The wound closure dynamics was determined as percentage of remaining wound size by evaluation of the acellular gap width in relation to the initial wound width at three different sites for each wound in each picture (data as mean relative wound size ± S.D. of triplicate measurements/clone).

Statistical Analysis

Expression analysis (mRNA, protein), siRNA treatment, MTT assays, and respirometry results were analyzed using the package STATA 10.0 (StataTM 10.0; StataCorp., College Station, TX). Mean MB or CAIX mRNA and MB protein induction levels along a hypoxia time course (4, 8, 24, 48, 72 h, 1% O2) were compared with basal abundance in normoxia controls (72 h). Also, MB induction was compared for corresponding time points between siControl versus siHIF-1α or siHIF-2α or the combined siHIF-1α/2α treatments. Mean O2 consumption rates or \( P_{50} \) measurements were compared pairwise between pooled MB control and knockdown (KD) samples. Statistical significance was calculated by the following: (i) unpaired Student’s \( t \) tests or one-way analysis of variance when normality of data population and variance equality among samples was ascertained (e.g. MB mRNA induction; MTT assay, scr versus si activity at same O2 and time point; respirometry: \( R(-G) \), \( R(G) \), ROX O2 consumption rates, all \( P_{50} \) data) or (ii) Welch-approximated \( t \) tests allowing unequal variances (e.g. MB protein induction; siRNA 72-h hypoxia time point comparison; ETS consumption rate). \( p \) values < 0.05 were considered significant.

RESULTS

We recently described that MB in breast cancer is positively and significantly correlated with estrogen (ERα), progesterone receptor, hypoxia-inducible factor-(HIF)-2α, and carbonic anhydrase IX (CAIX, Ref. 29). The typical expression profile of a MB-positive case is shown in Fig. 1 as follows: strong expression of MB and ERα, moderate but clearly discernible HIF-2α and CAIX expression, and weak expression or absence of HIF-1α and GLUT1. The positive correlation of MB with markers of tissue hypoxia (CAIX and HIF-2α) implied a possible control of MB expression by O2. To clarify if MB expression also correlates with hypoxic tissue areas in vivo, we reanalyzed 137 cases of ductal carcinoma in situ (DCIS). DCIS represents an interesting in vivo model for hypoxia research because this tumor has no intraductal vasculature. Thus, ambient oxygen can only be supplied by diffusion across the outer basal membrane. This forms a radial O2 diffusion gradient (normoxic rim; hypoxic to anoxic center) along with a central necrotic area (Fig. 2). Expression of the hypoxia-driven GLUT1 protein closely followed this gradient in our cohort of DCIS and showed a typical zonal distribution in 65% of cases (Fig. 2A). However, with regard to MB, only in 27% of MB-positive DCIS cases a hypoxia-like gradient with stronger staining in the peri-necrotic region was found (Fig. 2A). The majority of cases (73%) showed a diffuse MB immunoreactivity (Fig. 2B). This indicates that hypoxia may up-regulate MB in vivo, although in the majority of cases stimuli other than low \( pO_2 \) are driving the induction of MB. A similar conclusion was reached when we
assessed the possible co-localization between MB and hypoxic tumor areas as indicated by the hypoxia marker pimonidazole (supplemental Fig. S1).

We subsequently analyzed the responsiveness of the MB gene toward hypoxia (1% O2) by quantitative PCR in the benign breast cell line MCF12A and three breast cancer cell lines (MDA-MB231, MDA-MB468, and MCF7). Although hypoxia did not affect the transcription of MB in benign MCF12A cells (supplemental Fig. S2), normalized steady state levels of MB mRNA increased 3–4-fold in hypoxic MDA-MB231 (data not shown), MDA-MB468, and MCF7 cells (Fig. 3A). For all cases, a significant activation of the MB gene required at least 24 or 48 h of hypoxia treatment. Proper hypoxic responsiveness of MCF7 cells was confirmed through assessment of transcription of the highly hypoxia-inducible CAIX transcript (Fig. 3A).

Of all examined breast cancer cell lines, MDA-MB468 cells contained the most abundant levels of MB transcripts under basal conditions (29). Superior abundance of MB in MDA-MB468 cells was also seen at a protein level (i.e. MDA-MB468, 65 ng of protein/10^6 cells (29); other breast cancer cell lines contain 24–32 ng of MB/10^6 cells (28)). Exceeding hypoxia (1% O2) over 48 h (Fig. 3B) in MDA-MB-468 (left panel) and MCF7 cells (right panel) induced MB protein expression between 3.4- and 5.1-fold. The hypoxic response was transient when centered around HIF-1α (MCF7) or persistent (up to 72 h, 1% O2) when HIF-2α acted as the predominant regulator (MDA-MB468) (Fig. 3B).

Next, the role of HIF in the hypoxic induction of MB was examined using HIF-1α and HIF-2α siRNA oligonucleotides. The siRNA assay focused on scrambled control and HIF-1α (≈ siHIF-1α), -2α (≈ siHIF-2α), and combined knockdown (KD) effects (≈ siHIF-1α/2α) after 52–96 h of exposure to 1% O2 (Fig. 3C). Levels of MB steadily increased during siControl transfections to reach a maximal ~7-fold induction at 72 h of hypoxia (Fig. 3C). This peak induction was partially reduced upon HIF-1α or -2α single siRNA treatment. Yet the combination of both siRNAs yielded an ~20% residual amount of either HIF factor, which significantly attenuated the MB induction to ~1.7-fold at 72 h of hypoxia. In conclusion, this finding implies that both HIF-1 and HIF-2 participate in transactivating the MB gene by hypoxia.

To follow-up on this hypoxic transactivation of the MB gene by HIF-1/2, we compared the human MB gene locus to expressed sequence tags and detected the presence of several alternatively spliced MB transcripts in addition to the published mRNA (39). These transcript variants contain different non-coding 5′-untranslated regions (5′-UTRs) and hence are transcribed from different MB promoters (Fig. 4A). Comparison of expressed sequence tag numbers revealed that mRNA NM_005368 (NCBI-AceView Hsa MB variant B (40)) is the dominant “standard” transcript (MB-S) in muscle tissues of skeletal and heart. Transcript NM_203377 (NCBI-AceView Hsa MB variant D) was found encoded by the majority of cancer-associated expressed sequence tag reads. This mRNA (designated “MB-A” for “alternative”) thus represented a candidate for analyzing its expression in breast cancer cells (Fig. 4A). Calculating the ratio of MB-S versus MB-A mRNA expression levels in normoxic MDA-MB468 breast cancer cells revealed a clear preference for MB-A, which was roughly 300-fold more abundantly expressed than MB-S (Fig. 4B). In MDA-MB468 cells subjected to either normoxia or 1% O2 for 72 h, the steady state levels of MB-S were unaltered. On the contrary, the amount of MB-A transcripts increased 2.2-fold in hypoxic compared with normoxic cells. Using the rVISTA tool, we inspected the genomic regions of human and mouse MB/Mb genes for the presence of HIF-binding HREs, as characterized by the conserved consensus motif 5′-RCGTG-3′ (41). We detected one putative HRE at ~2.7 kb upstream of exon 1 (Fig. 4A), which consists of two inverted HIF-1-binding sites at an interval of 6 bp contained within a conserved stretch of 53 bp. This candidate MB-HRE has 92% sequence similarity to an upstream promoter region from the human heat shock protein HSPB1 gene (Fig. 4C).

To investigate the functionality of the candidate HRE, Dual-Luciferase reporter assays were performed. MDA-MB468 cells were transfected with pGL3 promoter constructs that included a 376-bp section around the candidate HRE. 72 h post-hypoxia exposure, normalized luciferase activity was quantified in cell
batches raised either under normoxic or hypoxic conditions (Fig. 4D). During hypoxia, the HRE-encoding construct showed an enhancer activity, which increased the amount of generated transcript by 43%, compared with the pGL3 promoter vector as a control. Under normoxia, no enhancer activity of the construct was observed.

To unravel functional properties of endogenous MB in breast cancer cells, we generated stable MB knockdown clones of MDA-MB468 cells using four different short hairpin (sh) RNA constructs (83–86). Applying Fick’s law of diffusion, we aimed to see if the shRNA-mediated loss-of-function (LoF) of MB would yield a steeper oxygen diffusion gradient reflected by a higher \( p_{50}(O_2) \) of cell respiration at identical oxygen flow. O2 consumption kinetics were investigated by high resolution respirometry using two control clones (boxed, 84#5 and 84#14) and two knockdown (KD) clones (boxed, 83#4 and 84#31) (Fig. 5A). O2 flow/s and million cells (red tracing) was recorded as a function of O2 concentration (blue tracing) across four cellular activity states (Fig. 5B) as follows: (i) routine respiration without glucose (=R(-G)); (ii) routine respiration with 25 mM glucose (=R(G)); (iii) maximal respiration through uncoupling of oxidative phosphorylation by FCCP (=ETS); (iv) residual O2 consumption after poisoning of mitochondria with myxothiazol (ROX). \( R(-G) \) and \( R(G) \) states indicate oxygen consumption at higher versus lower fluxes, respectively (Crabtree effect). To our surprise, all mitochondrial and residual O2 fluxes were significantly increased in MB KD cells compared with control cells (Fig. 5C). In contrast, mean cellular cytochrome c oxidase \( p_{50}(O_2) \) values (i.e. \( p_{50}(O_2) \) of respiration) under \( R(-G) \) and \( R(G) \) conditions were statistically indistinguishable in control versus KD cell comparisons for either glucose-deficient or -proficient respiration (Fig. 5D). Differences in \( p_{50} \) values from high versus low oxygen fluxes were also nonsignificant.

We also investigated cellular proliferation during a 0-48-96-h time course in wild type (WT) and stable MB-CON and MB-KD MDA transfectants under both normoxic (continuous lines) and hypoxic (1% \( O_2 \), hatched lines) atmospheres by employing trypsin blue-exclusion assays (Fig. 6A). MB LoF in stable knockdown transfectants yielded a significant diminishment of viable cells over the 96-h course, relative to WT cells or control transfectants, in both normoxic and hypoxic conditions.

Next, we assessed MDA-MB468 viabilities at normoxic and hypoxic \( pO_2 \) following siRNA-based transient silencing of MB expression to generate LoF phenotypes. This time we employed MTT assays during which mitochondrial dehydrogenases reduce a yellow tetrazolium substrate into a purple formazan compound as proxy for the oxidative capacity of living cells. Prior to the assay, cells were transfected with scrambled (scr) or MB-silencing siRNA (si) oligonucleotides. MTT reduction was followed over a 0-48-96-144 h time course as a function of four different \( O_2 \) concentrations: normoxia and 5, 1, or 0.2% \( O_2 \). Control RT-PCR analyses of MB mRNA expression ensured the MB knockdown effect to last over the entire 144-h course (Fig. 6B, gel picture below graph). As expected, MTT conversion increased, because of continued cell and mitochondrial proliferation, linearly and with equivalent slopes over time in oxygenated cells (normoxia, 5% \( O_2 \)), suggesting respiration of MDA-MB468 cells to operate in an oxyregulated fashion between 20 and 5% \( O_2 \). Compared with that, MTT conversion rose far less at 1% \( O_2 \) or was even ~2-fold inhibited during the time course at 0.2% \( O_2 \). Of note, pairwise comparisons of scr (continuous lines with filled symbols) versus MB-silencing treatments (hatched lines with open symbols) were, at the same \( O_2 \) conditions and time points, statistically indistinguishable in high oxygen (normoxia, 5% \( O_2 \)) while revealing a borderline significant benefit of the MB knockdown on the activity of the MTT-converting mitochondrial enzymes in hypoxic cells (see scr/si \( p \) values at 0.2% \( O_2 \) course; Fig. 6B). 144 h si/si ratios of MTT reduction rose steadily from near equality at 5% \( O_2 \) to a +18% (1% \( O_2 \)) and, eventually, +46% (0.2% \( O_2 \)) benefit in MB-silenced cells facing hypoxia (Fig. 6B).

Finally, we considered whether MB expression in breast cancer cells also influences the migration of normoxic or hypoxic cells on the basis of a scratch assay. As can be seen in Fig. 6, C and D, control cells (i.e. wild type MDA cells, either untreated or treated with scrambled RNA) were able to reconstitute the gap notably faster than MB KD cells treated with siRNA 6 or 8. This remarkable retardation of the in vitro motility in cells with repressed MB expression established, especially after prolonged (48 h + 72 h) cultivation, a good agreement between loss of MB and impaired cellular migration potential in high and low \( pO_2 \).

**DISCUSSION**

To date, the existence of regional hypoxia in most solid tumors (for review see Refs. 42–45), including breast cancer (46), has been clearly established. Tumor hypoxia often associates with therapy resistance and poor prognosis. In accordance with Flonta et al. (28), we demonstrate de novo expression and hypoxic responsiveness of human MB mRNA and protein in human breast cancer cell lines. In contrast to nontransformed epithelial cells, malignant breast cancer cells induced both MB mRNA and protein 3–7-fold in response to prolonged exposure to 1% \( O_2 \). Peak induction of the MB protein in chronically

**FIGURE 3. MB expression in hypoxic breast cancer cells.** A, MB (left) and CAIX (right) mRNA expression during indicated times of hypoxia (1% \( O_2 \)) in MCF7 cells (normalized expression, MB/L28, CAIX/L28; mean ± S.D., n = 3). Maximal mRNA abundance at 72 h of hypoxia was set to 1. Hypoxic MB/L28 or CAIX/L28 mRNA expressions (black bars) show significantly elevated values (\( p < 0.05, <0.01, <0.001 \)) relative to a basal steady state at 72 h normoxia (white bar) are indicated by 1–3 asterisks, respectively. B, protein expression of (top-bottom) HIF-1a, HIF-2a, MB, and \( \beta \)-actin in MDA-MB468 (left) and MCF7 (right) cells during indicated times of normoxia and hypoxia (1% \( O_2 \)), respectively. Lower bar graphs show the densitometric summary of mean fold inductions (± S.D., n = 3) for normalized MB protein expression (i.e. MB/\( \beta \)-actin) during the hypoxia time course relative to the proteins normoxic levels (= 1) for MDA-MB468 (left) and MCF7 (right) cells. Hypoxic inductions (black bars) showing significantly elevated values (\( p < 0.05 \)) relative to basal MB levels at 72 h of normoxia (white bars) are indicated by single asterisks. C, transfection of MDA-MB468 cells with scrambled RNA (siControl) or HIF-1a and HIF-2a transcript-targeting siRNAs, Representative Western blots show (top-bottom) HIF-1a, HIF-2a, MB, and \( \beta \)-actin protein abundance for 96 h of normoxia versus 52, 56, 72, and 96 h of hypoxia (1% \( O_2 \)). The protein loading control is shown in the middle. Bottom graph, corresponding MB protein fold inductions (mean ± S.D., n = 3) during indicated hypoxia times are plotted relative to 96 h normoxia (set to 1). Relative to the siControl value at a given time point, siHIF-1a, siHIF-2a, and siHIF-1a/2a values of MB content were nonsignificantly (ns) or significantly different (*, \( p < 0.05 \)).
hypoxic MDA-MB468 cells further required the involvement of HIF-1 and HIF-2 protein. We further noted that the standard TATA box promoter of both human and mouse MB/Mb genes (39, 47), which drives the expression in striated muscle cells, lacks candidate HREs (17). Consequently, we found expression of the standard MB transcript (NM_005368; MB-S) to not be affected by O₂. However, a noncanonical MB mRNA (NM_203377; MB-A), transcribed from a novel promoter proximal to the 5’-UTR upstream exon, greatly exceeded in MDA-MB468 cells the copy numbers of the MB-S mRNA, suggesting that MB-A, tentatively, can be regarded as a cancer-specific transcript. Discovery of the O₂ responsiveness of MB-A, in conjunction with the found HIF-1/2-driven transactivation of the MB gene during hypoxia, was backed by the presence of a can-
didate HRE at 2.7 kb upstream of exon 1 of the MB gene. The motif consists of two inverted HIF-1-binding sites at an interval of 6 bp, embedded in a conserved stretch of 53 bp. This MB-HRE has 92% sequence similarity to an upstream promoter region from the HSP27 human heat shock protein encoding gene HSPB1, which was reported to house a functional and HIF-1 binding HRE (48). Our preliminary analysis indicates that this MB-HRE features an enhancer activity of 43% upon exposure to hypoxia. Based on these findings, we assume that breast cancer cells induce MB in response to longer periods of low oxygen via an alternative and perhaps tumor-specific promoter whose enhanced activity might be also dependent on the binding of HIF-1/2 to the HRE located 2.7 kb upstream of the genuine ATG. The statistically significant positive correlation of MB with HIF-2α and CAIX in breast carcinomas further points toward mechanistic networking of these factors under hypoxia. However, our in vivo data also provide evidence that MB can be expressed by breast epithelia irrespective of hypoxia (29) and that even severe hypoxia in pimonidazole-stained carcinoma does not necessarily trigger MB induction (supplemental Fig. S1).

MB has multiple known or alleged functions in muscle tissue, including short term O₂ storage and buffering, facilitating O₂ diffusion, scavenging of NO and reactive oxygen species and also the reverse (peroxidase activity, NO production), and the binding plus transport of fatty acids (2–4, 6–11, 49). To assess whether MB also confers O₂-buffering or facilitates O₂ diffusion in cancer cells, we used high resolution respirometry to measure O₂ consumption kinetics in MB expressing control

**FIGURE 5.** High resolution respirometry of control and MB knockdown MDA-MB468 cells. A, MB and β-actin Western blot of 12 MDA-MB-468 shRNA clones, derived from shRNA constructs 83, 84, and 85, together with positive (pos.) MB control extractions from a human skeletal muscle biopsy. MB control (84#5, 85#14) and knockdown (83#4, 84#31) clones used for respirometry are boxed. B, original MDA-MB-468 cell respirometry shown as O₂ flows and million cells (red tracing) as a function of O₂ concentration (blue tracing) across four cellular activity states as follows: routine respiration without/with glucose (R(-G), R(G)); maximal respiration through uncoupling of oxidative phosphorylation by FCCP (ETS); residual O₂ consumption after poisoning of mitochondria with myxothiazol (ROX) (see under “Experimental Procedures” for more details). C, O₂ consumption rates of MB control (con; dark gray column) and MB knockdown clones (kd; light gray + white column) for the R(-G), R(G), ETS, and ROX states. D, oxygen kinetic p50(O₂) data of respiration in MB control (black + dark gray column) and MB KD clones (light gray + white column) for R(-G) and R(G) states. Mean p50(O₂) values are highlighted within the respective column.
and KD MDA-MB468 cells, expecting the latter to develop higher intracellular oxygen gradients (reflected by a higher $p_{50}(O_2)$ of cell respiration at identical oxygen flow) than controls. Because facilitated diffusion of oxygen can only occur when MB is partially desaturated with oxygen, the above prediction of Fick’s law of diffusion was carefully assessed with $p_{50}(O_2) \geq 1.1$ to 0 kPa (= 8.3 to 0 mm Hg; see blue tracing of $[O_2]$ in Fig. 5B) and note development of controlled amounts of oxygen by H$_2$O$_2$ injections into catalase-containing medium to generate replicate aerobic-anoxic transitions and thus a more accurate determination of $p_{50}(O_2)$ of cell respiration (37). With the 37 °C half-saturation ($Y_{50}$) of MB occurring at $p_{O_2}$ of 2.4 mm Hg (50), a transition from 1.1 to 0 kPa corresponds to a fractional O$_2$ saturation of MB ranging from ~78% to complete deoxygenation (see under “Experimental Procedures” for details). Although our respirometric conditions were well suited to trigger MB into O$_2$ unloading and to potentially detect any facilitated O$_2$ diffusion, it needs to be stressed that $p_{50}(O_2)$ measures of respiration of small cells and isolated mitochondria usually are below 0.1 kPa (51). Thus, only a small scope for intracellular oxygen gradients exists (51). Within these physical constraints, this study was unable to provide any evidence for O$_2$ gradients that are differentially influenced by the presence or absence of MB protein. In fact, the $p_{50}(O_2)$ of MB-CON and -KD MDA-MB468 cells was found to lie in a similar range (0.08 kPa). These results do not suggest a functional role of MB in oxygen transport in small, suspended cells in vitro. However, they do not exclude the possibility of larger oxygen gradients developing in the solid tumor and a potential contribution of MB to facilitated oxygen diffusion under in vivo conditions. Furthermore, routine respiration in MDA-MB468 cells was activated ~55% (i.e. R(G)) and ~40% (i.e. R(G)), respectively, of the mitochondrial capacity for electron transfer (ETS), which is comparable with various primary cultured cells such as human umbilical vein endothelial cells and fibroblasts (34, 52, 53). Mitochondrial oxygen kinetics and coupling control in malignant MDA-MB468 cells is therefore not indicative of a mitochondrial deficiency frequently considered as a specific feature (Warburg effect) of cancer cells. In addition to these respirometry data from O$_2$-limiting hypoxia, we also noticed the intensified O$_2$ uptake rate across all four physiological activity states considered (R(-G), R(G), ETS, ROX) by MDA knockdown cells during mild hypoxia. As a hemoprotein, MB can effectively interact with the gaseous...
Myoglobin in Breast Cancer, Expression and Function

nitric oxide (NO). MB knock-out mouse models (1, 5) have been instrumental to elicit the critical role of MB in maintaining NO homeostasis in muscle tissue. Whether MB expressed in neoplasms exerts similar controls remains to be seen. At this point, we can only speculate that the respiratory activation and enhancement of substrate turnover by MTT-converting mitochondrial dehydrogenases, both noted for hypoxic MDA cells in response to MB LoF, might result from the capacity of the oxygenated fraction of MB (i.e. MBO2) to scavenge NO (i.e. NO + MBO2 → metMB + NO3), a key stimulus of mitochondrial biogenesis (54). Alternatively, the presence of MB may also exert some oxidative stress (i.e. bound O2 is released as reactive oxygen species) particularly during stages of hypoxia, and in that way act to inhibit mitochondrial activity. Our results on oxygen kinetics, however, suggest that MB does not play a detectable role in regulating levels of NO that inhibit oxidative phosphorylation (OXPHOS) in suspended breast cancer cells. Using inducible NOS-transfected HEK293 cells to achieve regulated intracellular NO production, however, demonstrated that nanomolar concentrations of NO could trigger an increase of low pO2 (i.e. <0.1 kPa) values by 2 orders of magnitude, which underpinned the tight coupling between NO homeostasis and O2 consumption kinetics in this cell model (55).

So far only two studies have analyzed the potential roles of myoglobin in nonmuscle contexts by employing artificial expression systems. Nitta et al. (56) induced MB expression in hepatocytes by an adenoviral gene transfer, which were henceforth significantly more resistant to hypoxia. Galluzzo et al. (57) were the first to introduce abundant levels of mouse Mb into the human tumor cell line A549 (lung carcinoma) by lentiviral gene transfer. Experimental tumors expressing ectopic Mb displayed reduced or no hypoxia, minimal HIF-1α levels, lower vessel density along with a more differentiated cancer cell phenotype, and largely suppressed local and distal metastatic spreading. The authors correlated these beneficial outcomes of Mb overexpression primarily with the reduction of tumor hypoxia (57). Although these findings appear to match our in vivo observation from patients, where patients with higher MB levels show a better prognosis (29), both situations are quite different. First, the low quantities of endogenous MB measured by us in normoxic MDA-MB468 breast cancer cells (~65 ng of MB protein/10⁶ cells) cannot confer meaningful O2 storage/buffering capacity. Second, our respirometry data failed to provide evidence for a functional role of MB in the transport of oxygen in vitro. Finally, at least in hypoxic breast cancer cells, MB has an interfering (i.e. not promoting) impact on O2 uptake and oxidative energy metabolism, which suggests a tumor-suppressive function of MB in deoxygenated malignancies. These fundamental discrepancies require, in our opinion, a cautionary note when overexpression-based molecular evidence is directly interpreted in terms of endogenous protein functions.

In summary, MB abundance in breast cancer cells is regulated not only by estrogen signaling and possibly fatty acid levels or growth factors but also by hypoxia (29). The intensified respiration and mitochondrial enzyme activities in hypoxic cancer cells in response to a silencing of MB expression might either result from mitochondrial compensation(s) (e.g. increase in mitochondrial density/size because of NO overabundance) or, perhaps, reflect a higher level of oxidative stress exerted by the presence of this hemoprotein. The fact that MB LoF yields declining proliferation or motility rates even in fully oxygenated cells suggests MB in cancer cells to occupy rather unconventional functions that are not directly related to the binding and transport of O2. By acting as a putative shuttle for fatty acids, MB could possibly support active lipogenesis and cellular growth even at times when the O2 supply is nonlimiting and the protein is in its fully O2-saturated state (i.e. MBO2). Beyond these functional aspects, the regulation in normal and tumor tissue might also be fundamentally different, as the novel description of a tumor-specific MB transcript suggests. Together, these findings further broaden our view on the role of nonmuscle MB that may have fundamental implications for our conception of the biology of solid tumors.

Acknowledgments—The excellent technical assistance of Martina Storz, Silvia Behnke, and Sonja von Serenyi is gratefully acknowledged. We thank Dr. Peter Uciechowski (RWTH Aachen, Germany) for giving us access to the hypoxia workstation.

REFERENCES

1. Gödecke, A., Flögel, U., Zanger, K., Ding, Z., Hirchenhain, J., Decking, U. K., and Schrader, J. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 10495–10500
2. Ordway, G. A., and Garry, D. J. (2004) J. Exp. Biol. 207, 3441–3446
3. Wittenberg, J. B. (1970) Physiol. Rev. 50, 559–636
4. Jürgens, K. D., Peters, T., and Gros, G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3832–3833
5. Garry, D. J., Ordway, G. A., Lorenz, J. N., Radford, N. B., Chin, E. R., Grange, R. W., Bassel-Duby, R., and Williams, R. S. (1998) Nature 395, 905–908
6. Flögel, U., Merz, M. W., Gödecke, A., Decking, U. K., and Schrader, J. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 735–740
7. Bendgen-Cotta, U. B., Merx, M. W., Shiva, S., Schmitz, J., Becher, S., Klare, J. P., Steinhoff, H. J., Gödecke, A., Schrader, J., Gladwin, M. T., Kelm, M., and Rassaf, T. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 10256–10261
8. Kan, K. K., Mondal, M. S., Padhy, L., and Mitra, S. (1998) Eur. J. Biochem. 257, 547–555
9. Flögel, U., Gödecke, A., Klotz, L. O., and Schrader, J. (2004) FASEB J. 18, 1156–1158
10. Sirram, R., Kreutzer, U., Shih, L., and Jue, T. (2008) FEBS Lett. 582, 3643–3649

11. Tomita, A., Kreutzer, U., Adachi, S., Koshihara, S. Y., and Jue, T. (2010) J. Exp. Biol. 213, 2748–2754
12. Reynafarje, B. (1962) J. Appl. Physiol. 17, 301–305
13. Vogt, M., Punstchart, A., Geiser, J., Zuleger, C., Billerter, R., and Hoppeler, H. (2001) J. Appl. Physiol. 91, 173–182
14. Wittenberg, B. A. (2009) Am. J. Physiol. Cell Physiol. 296, C390–C392
15. Ameln, H., Gustafsson, T., Sundberg, C. J., Okamoto, K., Jansson, E., Poelinger, L., and Makino, Y. (2005) FASEB J. 19, 1009–1011
16. Chang, H., Shyu, K. G., Wang, B. W., and Kuan, P. (2003) Clin. Sci. 105, 447–456
17. Wystub, S., Ebner, B., Fuchs, C., Weich, B., Hankeln, T., and Poellinger, L. (2004) FASEB J. 18, 1009–1011
18. Poellinger, L., and Makino, Y. (2005) FASEB J. 19, 1009–1011
19. Roesner, A., Mitzi, S. A., Hankeln, T., and Burmester, T. (2008) FESB J. 275, 10256–10261
20. Fordel, E., Geuens, E., Dewilde, S., De Coen, W., and Moens, L. (2004) FASEB J. 18, 301–305
Myoglobin in Breast Cancer, Expression and Function

3633–3643

22. Enoki, Y., Morimoto, T., Nakatani, A., Sakata, S., Ogba, Y., Kohzuki, H., and Shimizu, S. (1988) Adv. Exp. Med. Biol. 222, 709–716

23. Qiu, Y., Sutton, L., and Riggs, A. F. (1998) J. Biol. Chem. 273, 23426–23432

24. Eusebi, V., Bondi, A., and Rosai, J. (1984) Am. J. Surg. Pathol. 8, 51–55

25. Smith, T. W., and Davidson, R. I. (1984) Cancer 54, 323–332

26. Iseki, M., Tsuda, N., Kishikawa, M., Shimada, O., Hayashi, T., Kawahara, K., and Tomita, M. (1990) Am. J. Surg. Pathol. 14, 395–398

27. Zhang, P. J., Goldblum, J. R., Pawel, B. R., Fisher, C., Pasha, T. L., and Barr, F. G. (2003) Mod. Pathol. 16, 229–235

28. Flonta, S. E., Arena, S., Pisacane, A., Michieli, P., and Bardelli, A. (2009) Am. J. Pathol. 175, 201–206

29. Kristiansen, G., Rose, M., Geisler, C., Fritzsche, F. R., Gerhardt, J., Lu¨ke, C., Ladhoff, A. M., Knüehl, R., Dietel, M., Moch, H., Varga, Z., Theurillat, J. P., Fabrizius, A., Laufs, T., Bauer, T., Koslowski, M., Horn, S., Burmester, T., Hankeln, T., and Kristiansen, G. (2011) Acta Physiologica 202, 563–581

30. Gorr, T. A., Wichmann, D., Pilarsky, C., Theurillat, J. P., Fabrizius, A., Laufs, T., Bauer, T., Koslowski, M., Horn, S., Burmester, T., Hankeln, T., and Kristiansen, G. (2011) Acta Physiologica 202, 563–581

31. Wirthner, R., Wrann, S., Balamurugan, K., Wenger, R. H., and Stiehl, D. P. (2008) Carcinogenesis 29, 2306–2316

32. Loots, G. G., and Ovcharenko, I. (2004) Nucleic Acids Res. 32, W217–W221

33. Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, R., Meinhardt, T., Prüss, M., Reuter, I., and Schacherer, F. (2000) Nucleic Acids Res. 28, 316–319

34. Steinlechner-Maran, R., Eberl, T., Kunc, M., Margreiter, R., and Gnaiger, E. (1996) Am. J. Physiol. 271, C2053–C2061

35. Gnaiger, E., Steinlechner-Maran, R., Méndez, G., Eberl, T., and Margreiter, R. (1995) J. Bioenerg. Biomembr. 27, 583–596

36. Gnaiger, E. (2001) Respir. Physiol. 128, 277–297

37. Pesta, D., and Gnaiger, E. (2011) in Mitochondrial Bioenergetics: Methods and Protocols (Palmeira, C., and Moreno, A., eds) Humana Press, New York

38. Rosman, D. S., Phukan, S., Huang, C. C., and Pasche, B. (2008) Cancer Res. 68, 1319–1328

39. Weller, P., Jeffreys, A. J., Wilson, V., and Blanchetot, A. (1984) EMBO J. 3, 439–446

40. Thierry-Mieg, D., and Thierry-Mieg, J. (2006) Genome Biol. 7, Suppl. 1, S12, 1–14

41. Wenger, R. H., Stehl, D. P., and Camenisch, G. (2005) Sci. STKE 2005, re12

42. Vaupel, P., Schlenker, K., Knoop, C., and Höckel, M. (1991) Cancer Res. 51, 3316–3322

43. Thews, O., Koenig, R., Kelleher, D. K., Kutzner, J., and Vaupel, P. (1998) Br. J. Cancer 78, 752–756

44. Brown, J. M. (1999) Cancer Res. 59, 5863–5870

45. Brown, J. M., and Wilson, W. R. (2004) Nat. Rev. Cancer 4, 437–447

46. Arcasoy, M. O., Amin, K., Karayal, A. F., Chou, S. C., Raleigh, J. A., Varia, M. A., and Haroon, Z. A. (2002) Lab. Invest. 82, 911–918

47. Blanchetot, A., Price, M., and Jeffreys, A. J. (1986) Eur. J. Biochem. 159, 469–474

48. Whitlock, N. A., Agarwal, N., Ma, J. X., and Crosson, C. E. (2005) Invest. Ophthalmol. Vis. Sci. 46, 1092–1098

49. Wittenberg, B. A., Wittenberg, J. B., and Caldwell, P. R. (1975) J. Biol. Chem. 250, 9038–9043

50. Schenkenman, K. A. (2001) Am. J. Physiol. Heart Circ. Physiol. 281, H2463–H2472

51. Scandurra, F. M., and Gnaiger, E. (2010) Adv. Exp. Med. Biol. 662, 7–25

52. Hutter, E., Renner, K., Pfister, G., Stöckl, P., Jansen-Dürr, P., and Gnaiger, E. (2004) Biochem. J. 380, 919–928

53. Smolková, K., Bellance, N., Scandurra, F., Génot, E., Gnaiger, E., Pécitá-Hlavatá, L., Jezek, P., and Rossignol, R. (2010) J. Bioenerg. Biomembr. 42, 55–67

54. Nisoli, E., Clementi, E., Carruba, M. O., and Moncada, S. (2007) Circ. Res. 100, 795–806

55. Aguierre, E., Rodríguez-Juárez, F., Bellelli, A., Gnaiger, E., and Cadenas, S. (2010) Biochim. Biophys. Acta 1797, 557–565

56. Nitta, T., Xundi, X., Hatano, E., Yamamoto, N., Uehara, T., Yoshida, M., Harada, N., Honda, K., Tanaka, A., Sosnowski, D., Chance, B., and Yamaoka, Y. (2003) J. Surg. Res. 110, 322–331

57. Galluzzo, M., Pennacchietti, S., Rosano, S., Comoglio, P. M., and Michieli, P. (2009) J. Clin. Invest. 119, 865–875