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Nrf2-heme oxygenase-1 axis in mucoepidermoid carcinoma of the lung: Antitumoral effects associated with down-regulation of matrix metalloproteinases

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Lung mucoepidermoid carcinoma (MEC) is a very poorly characterized rare subtype of non-small-cell lung cancer (NSCLC) associated with more favorable prognoses than other forms of intrathoracic malignancies. We have previously identified that heme oxygenase-1 (HO-1, encoded by HMOX1) inhibits MEC tumor growth and modulates the transcriptome of microRNAs. Here we investigate the role of a major upstream regulator of HO-1 and a master regulator of cellular antioxidant responses, transcription factor Nrf2, in MEC biology. Nrf2 overexpression in the NCI-H292 MEC cell line mimicked the phenotype of HO-1 overexpressing cells, leading to inhibition of cell proliferation and migration and down-regulation of oncogenic miR-378. HMOX1 silencing identified HO-1 as a major mediator of Nrf2 action. Nrf2- and HO-1 overexpressing cells exhibited strongly diminished expression of multiple matrix metalloproteinases and inflammatory cytokine interleukin-1 β , which was confirmed in an NCI-HO-1 xenograft model. Overexpression of HO-1 altered not only human MMP levels in tumor cells but also murine MMP levels within tumor microenvironment and metastatic niche. This could possibly contribute to decreased metastasis to the lungs and inhibitory effects of HO-1 on MEC tumor growth. Our profound transcriptome analysis and molecular characterization of the mucoepidermoid lung carcinoma helps to understand the specific clinical presentations of these tumors, emphasizing a unique antitumoral role of the Nrf2-HO-1 axis.

Keywords: Mucoepidermoid carcinoma, Non-small cell lung cancer, Nrf2, Nuclear factor (erythroid-derived 2)-like 2 Heme oxygenase-1, Matrix metalloproteinases, MMP-1

1. Introduction

Nrf2 (nuclear factor erythroid 2-related factor 2, encoded by *NFE2L2*) is a redox-sensitive transcription factor driving major molecular responses to protect cells against oxidative and electrophilic stress. Under physiological conditions, Nrf2 is sequestered within the cytoplasm while bound to its negative regulator Keap-1 (Kelch-like erythroid-derived cap-n-collar homology-associated protein-1), which serves as an adapter for cullin-3-dependent E3 ubiquitin ligase directing Nrf2 for proteasomal degradation. Upon stress, the Nrf2-Keap-1 complex is disrupted through modifications of key sensory cysteine residues of Keap-1 by reactive oxygen species or electrophiles [1]. In the nucleus, Nrf2

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dimerizes with small Maf protein and binds to the antioxidant response element (ARE) or electrophile response element (EpRE) to transactivate transcription of a broad array of cytoprotective genes, including phase 2 detoxifying enzymes and antioxidant genes such as NAD(P)H:quinone oxidoreductase-1 (NQO-1), glutathione S-transferase (GST), and heme oxygenase-1 (HO-1), an inducible isoform of the enzyme that degrades pro-oxidant heme into biliverdin, ferrous iron, and carbon monoxide.

Nrf2 plays a dual role in tumor growth and its action is largely context-dependent. On one hand, regarding tumor initiation, Nrf2 deficiency has been associated with increased susceptibility to carcinogenesis in multiple models and thus the transcription factor is a central target for chemoprevention [2,3]. On the other, aberrant Nrf2 activation enhances tumorigenic potential, not only through its cytoprotective effects, but also by actively promoting cancer cell proliferation, angiogenesis, and metastasis [4]. Similar oncogenic properties have been attributed to the principal Nrf2 target protein HO-1 in many tumor types [5]. In lung cancers, the neoplasms causing the largest number of cancer-related deaths worldwide, Nrf2 gain-of-function and/or Keap1 loss-of-function mutations often occur [6]. Accordingly, high intratumoral levels of Nrf2 and HO-1 have been associated with poor clinical outcomes in most common types of lung tumors [7,8]. Nevertheless, we have recently identified a particular subtype of non-small-cell lung carcinoma (NSCLC) where HO-1 acts rather as a tumor suppressor, inhibiting cancer cell proliferation, migration, tumor growth, and angiogenesis [9].

Mucoepidermoid carcinoma (MEC) of the lung is a very rare form of intrathoracic malignancy originating from the submucosal bronchial glands and affecting approximately 0.2% of lung cancer patients [10]. Interestingly, this subtype is associated with more favorable prognoses than other NSCLCs, as the MEC tumors usually have lower grades and are diagnosed at lower clinical stages, allowing more effective surgical procedures [11]. Yet very little is known about the microenvironment and molecular characteristics of this subtype. Besides our recent profiling of microRNAs in the NCI-H292 MEC cell line overexpressing *HMOX1* [9], there is one clinical study showing that low-grade pulmonary MECs are characterized by significantly attenuated expression of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 in comparison to typical lung cancer [12]. MMPs are proteolytic enzymes degrading extracellular matrix components, which play an important role in tumor growth and progression [13]. Accordingly, we found that the expression levels of MMP-2 were associated with tumorigenic potential in NCI-H292 xenografts [9], indicating that the regulation of MMPs may be of importance for the growth of MEC.

Here we investigate the molecular consequences of activation of the Nrf2-HO-1 axis in a model of human mucoepidermoid carcinoma of the lung. We show its atypical antitumoral actions in this subtype of NSCLC and identify the down-regulation of MMPs as one of the potential mechanisms contributing to the phenotype.

2. Materials and methods

2.1. Ethics statement

All experiments were carried out in accordance with good animal practice and were approved by the I Local Ethical Committee for Animal Research at the Jagiellonian University, Krakow.

2.2. Cell culture

The human NSCLC cell lines NCI-H292 (mucoepidermoid carcinoma, purchased from ATCC), A549 (adenocarcinoma, purchased from ATCC), and NCI-H460 (large cell carcinoma, purchased from

ATCC) were cultured in RPMI 1640 (PAA), and SK-MES-1 (squamous cell carcinoma, purchased from ATCC) was cultured in MEM (Gibco), each supplemented with 10% fetal bovine serum (PAA) and penicillin (100 U/mL)/streptomycin (10 µg/mL) (Sigma) (pen/strep) under standard culture conditions: 37 °C, 5% CO₂, 95% humidity. NCI-H292 cell lines overexpressing *NFE2L2* or *HMOX1* were developed earlier in our laboratory as described in [14] and [9], respectively.

2.3. Measurement of Nrf2 transcriptional activity

Cells were transfected with ARE-Luc plasmid using Lipofectamine2000 (Invitrogen) according to the vendor's protocol, and Luc activity was measured as described earlier [15].

2.4. Measurement of cell proliferation and migration in vitro

Cell proliferation was determined using BrdU incorporation assay (cell proliferation ELISA, Roche) according to the manufacturer's protocol. A scratch assay for measurement of cell migration was performed as in [14].

2.5. Assay of ALDH activity

Cells were collected during trypsinization and stained using an ALDEFLUOR Kit (StemCell Technologies) according to the vendor's protocol. Flow cytometric analysis was performed on BD LSR II (the cytometry platform of the CBM UPR4301, CNRS, Orléans, France).

2.6. Quantitative RT-PCR

RNA was isolated using QIAzol (Qiagen) reagent according to the manufacturer's instructions and reverse-transcribed into cDNA using a RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas) and nCodeVilo (Invitrogen) for analysis of miRNA. Real-time PCR was performed using QuantiTect SYBR Green (Qiagen) or SYBR Green JumpStart Taq Ready Mix (Sigma) on a Light Cycler 480 II (Roche) or a StepOne Plus (Applied Biosystems) platform. Gene expression was calculated according to delta Ct or delta delta Ct methods with EF2 and U6 as reference genes for mRNA and miRNA analysis, respectively.

2.7. siRNA transfection

Cells were seeded in 24-well plates at low confluency (30–50%) and transfected with 50 nM chemically modified siRNA targeted at HO-1 (Stealth RNAi *HMOX1* siRNA, Invitrogen) or control siRNA (Stealth RNAi Negative Control siRNA, Invitrogen) using 1 µl/well of Lipofectamine2000 transfection reagent (Invitrogen) according to the protocol of the supplier.

2.8. Microarray gene expression profiling

NCI-H292 cells, control and overexpressing *HMOX1*, were cultured for 24 h in serum-deprived medium after reaching confluence. Then the cells were washed with PBS and lysed in QIAzol Lysis Reagent (Qiagen) and RNA was isolated by a modified Chomczynski method following the manufacturer's recommendations. The RNA was quantified using a NanoDrop-1000 spectrophotometer and quality was monitored with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA was hybridized to Agilent Whole Human Genome Oligo Microarrays (G4112F) and changes of expression of at least 1.5-fold ($p < 0.05$) were considered significant. GoMiner software was used for segregation of the genes into ontology groups. Datasets are available

through GEO (accession number GSE22030).

2.9. Protein expression analysis

Western blot for Nrf2 and HO-1 was performed as described earlier [16].

ELISA for IL-1 β (R&D Systems) was performed on cell culture media according to the vendor's protocol.

2.10. MMPs activity measurement

Total activity of matrix metalloproteinases was estimated using a SensoLyte 520 Generic MMP Assay Kit (AnaSpec), according to the vendor's instructions.

2.11. In vivo tumor growth

Xenografting of NCI-ctrl and NCI-HO-1 cells has been described earlier [9]. Briefly, 8-week-old NOD SCID mice (males) were injected subcutaneously with 5×10^6 NCI-H292 control or *HMOX1* overexpressing cells in 100 μ l of PBS, and animals were sacrificed 7 to 8 weeks after xenografting and the serum, tumor, and lung samples were collected for further analysis.

2.12. Immunofluorescent staining

For immunofluorescent staining for PCNA (proliferation) samples were incubated overnight at 4 $^{\circ}$ C with anti-human PCNA antibodies (Dako, dilution 1:200) and secondary Alexa 488-conjugated antibodies (Invitrogen, dilution 1:200). Slides were mounted in a Vectashield medium (Vector Labs) containing DAPI dye. Samples were photographed using a Nikon Eclipse TS100 microscope equipped with a Nikon Digital Sight DS-5 M camera. Images were analyzed with GSA Image Analyzer software.

2.13. Screening for inflammatory cytokines in blood

Concentration of human cytokines in mouse serum was evaluated with BD Cytometric Bead Array (Becton Dickinson) according to the manufacturer's protocol. The results were validated with appropriate ELISA assays.

2.14. Statistical analysis

Results are presented as mean \pm SEM. Unpaired two-tailed Student's *t*-tests were used to assess whether the means of two groups differed significantly. For comparison of multiple groups, one-way ANOVA analysis with Bonferroni's post-test was employed.

3. Results

3.1. Establishment of NCI-H292 cell line with stable overexpression of Nrf2

To investigate the action of Nrf2 in lung mucoepidermoid carcinoma cells, NCI-H292 cell line was engineered to overexpress the transcription factor. Human *NFE2L2* cDNA was stably integrated into NSCLC cells by means of retroviral infection and selection of transduced cells. The resulting NCI-Nrf2 cell line exhibited elevated Nrf2 expression and activity, as evidenced using western blotting and ARE-Luc reporter construct (Fig. 1A, 1B). Increased Nrf2 activity was further confirmed by the expression of Nrf2 target genes, including NAD(P)H dehydrogenase, quinone 1 (NQO-1), glutamate-cysteinyl ligase catalytic domain (GCLC), and glutathione S-transferase (GST), which were uniformly up-regulated in NCI-Nrf2 cells (Fig. 1C). Accordingly, NCI-Nrf2 cells exhibited enhanced resistance to oxidative stress (data not shown). The levels of HO-1 mRNA and protein were also significantly elevated (Figs. 1A, 1C). On the other hand, Nrf2 regulatory protein Keap-1

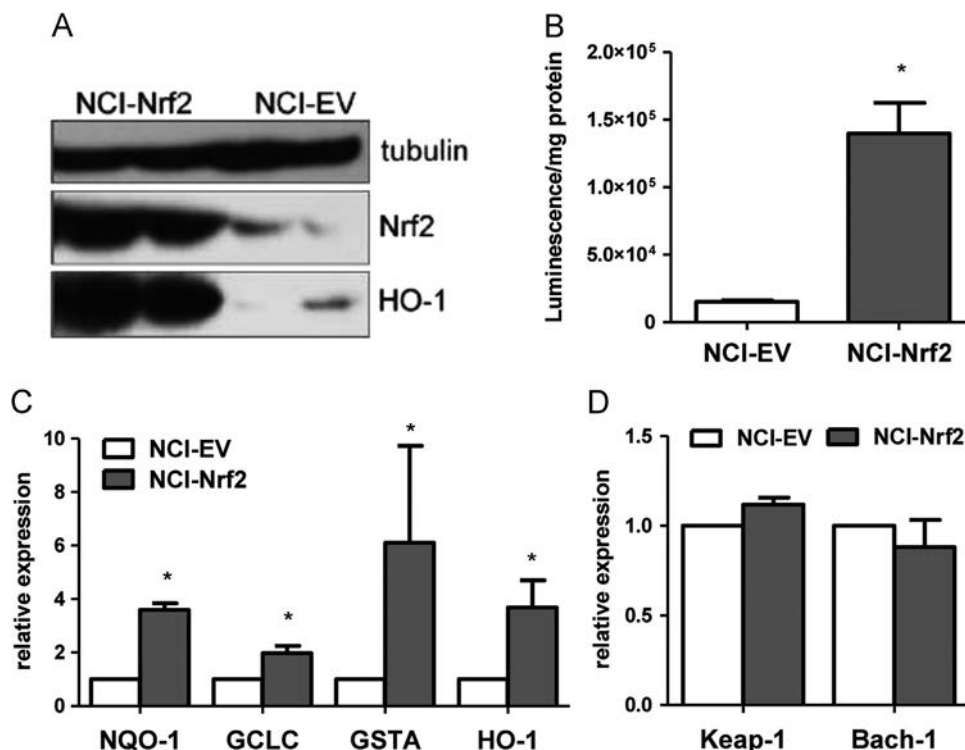


Fig. 1. Validation of NCI-Nrf2 cell line. (A) Western blot analysis of Nrf2 and HO-1 protein expression (representative experiment of 3). (B) Nrf2 transcriptional activity determined by ARE-Luc assay ($n=2$). (C-D) qPCR analysis of mRNA expression of Nrf2 target genes (C) and negative regulators (D) ($n=4$).

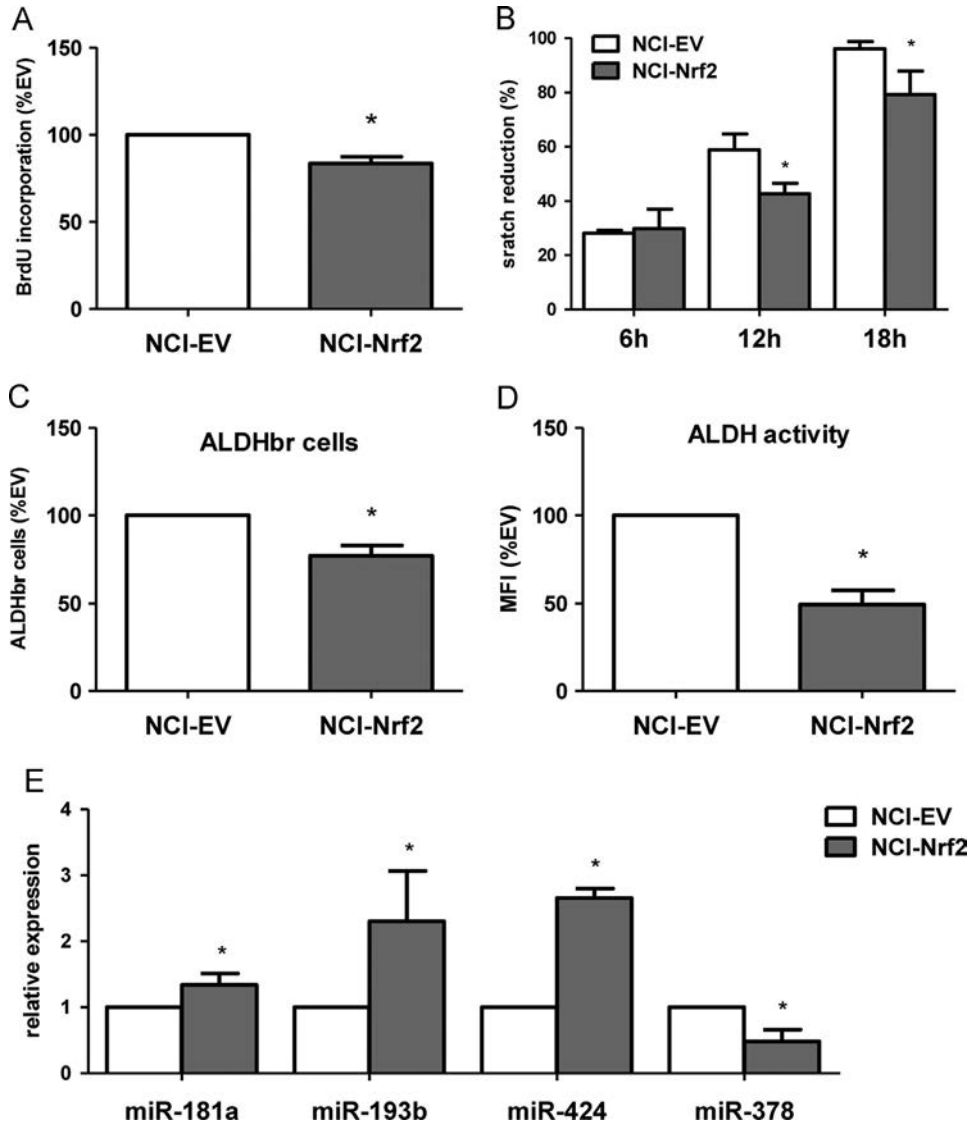


Fig. 2. Basal phenotypic characterization of NCI-H292 cells overexpressing Nrf2. (A) Effect of Nrf2 overexpression on NCI-H292 cell proliferation ($n=3$). (B) Effect of Nrf2 overexpression on NCI-H292 cell migration ($n=3$). (C–D) Effect of Nrf2 overexpression on distribution (C, ALDHbr-bright cells) and activity (D, MFI—mean fluorescence intensity) of ALDH marker of cancer stem-like cells ($n=3$). (E) Effect of Nrf2 overexpression on tumorigenesis-associated miRNA expression ($n=4$). * $p < 0.05$ NCI-Nrf2 vs. NCI-EV.

and Bach-1, which compete for binding ARE sequences, were unaffected (Fig. 1D), indicating that Nrf2 overexpression did not lead to a compensatory regulation of negative modulators of its activity.

3.2. Nrf2 overexpression alters the phenotype of NCI-H292 cells *in vitro* in a manner similar to HMOX1 overexpression

First we investigated the effect of Nrf2 overexpression on basic characteristics of the NCI-H292 cell line. NCI-Nrf2 cells exhibited attenuated proliferation and migration *in vitro* (Fig. 2A, 2B). These effects were similar to the phenotype of the NCI-H292 cell line overexpressing HMOX1 [9]. Moreover, activity of ALDH, an established marker of cancer stem-like cells in non-small-cell lung carcinoma, was also diminished in Nrf2-overexpressing cells (Figs. 2C, 2D). As we have previously established that the attenuation of tumorigenic potential of HMOX1-overexpressing NCI-H292 cells could be associated with an altered expression of microRNAs [9], we next measured the abundance of selected previously identified miRNAs in our model. Tumor suppressor microRNAs miR-181a, miR-193b, and miR-424 were significantly up-regulated in Nrf2-overexpressing cells, while oncogenic miR-378

was down-regulated (Fig. 2E), again resembling the profile of the NCI-HO-1 cell line [9]. Altogether, these data suggested that HO-1 could be an important mediator of Nrf2 action in our model of lung mucoepidermoid carcinoma.

3.3. HMOX1 knockdown reverses the major effects of Nrf2 overexpression in NCI-H292 cells

The involvement of HO-1 in the action of Nrf2 in the NCI-H292 line was determined by knocking down its expression by siRNA transfection. Targeted down-regulation of HMOX1 in NCI-Nrf2 cells to the basal level of control NCI-EV cells (Fig. 3A) resulted in enhanced cell proliferation and an increased migration rate (Figs. 3B, 3C), partially and completely reversing the effects of Nrf2 overexpression, respectively. Moreover, HMOX1 knockdown in Nrf2-overexpressing cells led to up-regulation of miR-378 (Fig. 3D), which plays an important role in NCI-292 tumorigenesis [9]. miR-181a and miR-424 were unaffected (Figs. S1A, S1B), while miR-193b was up-regulated in both the control and the NCI-Nrf2 cells (Fig. S1C) treated with siHO1, which suggests an unknown potential mechanism of HO-1 influence in regulation of this

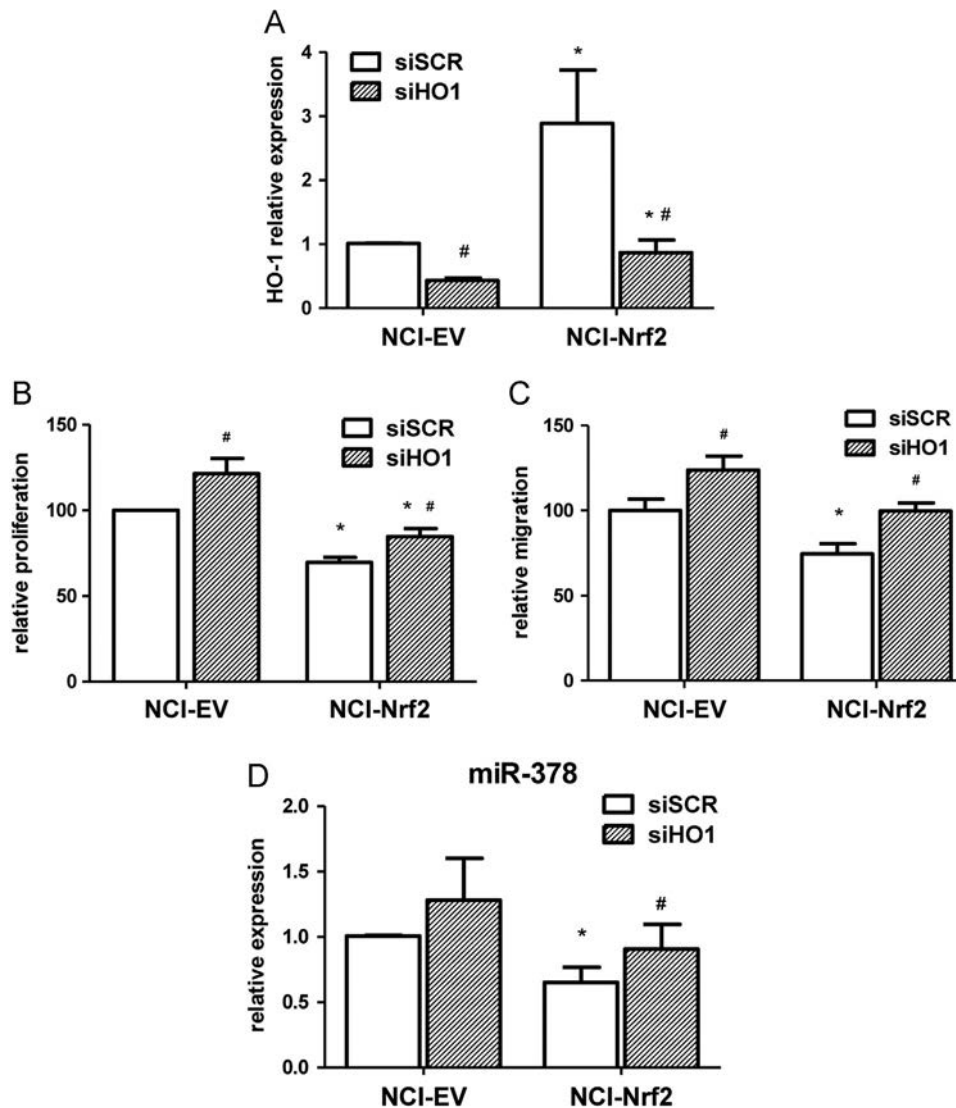


Fig. 3. Involvement of HO-1 in the effects of Nrf2 overexpression in NCI-H292 cell line. (A) Validation of *HMOX1* knockdown in NCI-H292 cells. Cells were transfected with 50 nM siRNA for 48 h, after which gene expression was measured by real-time PCR ($n=3$). (B) Effect of *HMOX1* silencing on cell proliferation ($n=3$). (C) Effect of *HMOX1* silencing on cell migration. Cells were transfected with siRNA for 72 h and allowed to close the wound in monolayer for 24 h ($n=3$). (D) Effect of *HMOX1* silencing on miR-378 expression ($n=3$). * $p < 0.05$ NCI-EV vs. NCI-Nrf2, # $p < 0.05$ siSCR vs. siHO1.

microRNA.

The reversal of Nrf2-overexpression-related attenuation of cell proliferation, migration, and miR-378 expression in NCI-H292 cells by *HMOX1* knockdown indicates that HO-1 is an important downstream mediator of action of Nrf2 in this type of NSCLC.

3.4. Attenuation of tumorigenic potential of NCI-H292 cells overexpressing *HMOX1* is associated with modulation of the mRNA transcriptome

Since HO-1 emerged as the key player in the antioxidant response of NCI-H292 NSCLC cells, we next focused on the mechanisms underlying the antitumor effects of HO-1 in this cell type. We chose to use the model of *HMOX1* overexpression by stable plasmid transfection developed earlier in our laboratory [9], as it closely resembled the moderate HO-1 up-regulation observed in NCI-Nrf2 cells. We have already shown diminished tumor growth of the NCI-HO-1 cell line *in vivo* [9]. Post mortem analyses confirmed the attenuation of tumorigenic potential of NSCLC cells exerted by the enzyme: NCI-HO-1 tumor cell proliferation and metastasis to lungs were severely inhibited (Fig. 4A, B).

To better understand the molecular changes involved in the effects of *HMOX1* overexpression in our model of lung mucoepithelial carcinoma, we performed microarray analysis of mRNA transcriptome of control and NCI-HO-1 cells cultured *in vitro*. We detected a variety of significant alterations in expression levels of genes regulating cell cycle, apoptosis, angiogenesis, cytokine production, and tumor metastasis, which are presented in a heat map (Fig. 4C) and in Supplementary Table 1. We found that expression of cyclins D1 and D2 (*CCND1* and *CCND2*) was down-regulated in *HMOX1* overexpressing cells (over 3.5-fold), while the genes known to inhibit cell proliferation, such as growth arrest-specific 1 (*GAS1*) and cyclin-dependent kinase inhibitor 1C (*CDKN1C*), were very strongly up-regulated (46- and 7-fold, respectively). The analysis demonstrated inhibition of the expression of inflammatory cytokines such as IL-1 α (12-fold), IL-1 β (17-fold), IL-8 (over 200-fold), and proangiogenic growth factor VEGF-A (almost 2-fold) in cells overexpressing *HMOX1*. Proapoptotic caspase-9 (*CASP9*) and *RUNX3* were induced and the mRNA level of *MDM2*, a negative regulator of p53, was diminished. Cells with elevated expression of *HMOX1* also displayed a lower level of urokinase plasminogen activator (*PLAU*, 6-fold) and matrix metalloproteinase-1 (*MMP1*, over 20-fold),

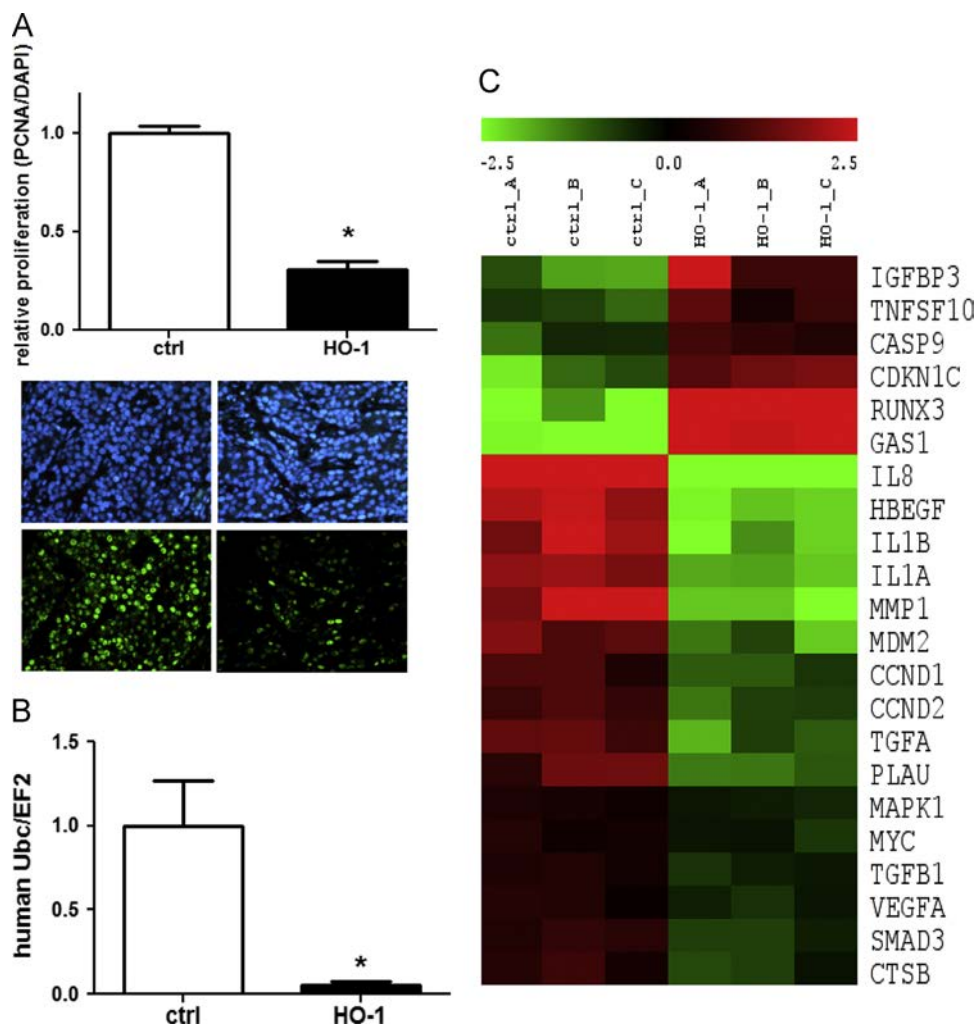


Fig. 4. Effect of moderate *HMOX1* overexpression on tumorigenic potential and transcription profile of NCI-H292 cells. (A) Effect of *HMOX1* overexpression on proliferation *in vivo* in NCI-H292 xenografts from NOD-SCID mice 7 weeks after implantation: immunofluorescent staining of tumor sections for PCNA (green, blue - DAPI) ($n=15$ for control; $n=18$ for HO-1). (B) Effect of *HMOX1* overexpression on metastatic potential of NCI-H292 cells *in vivo*: real-time PCR analysis of expression of human ubiquitin c (Ubc) gene in lungs of xenograft-bearing mice 7 weeks after implantation ($n=15$ for control; $n=18$ for HO-1). * $p < 0.05$ NCI-HO-1 vs. NCI-ctrl. C. Results of microarray analysis of gene expression in control and HO-1-overexpressing NCI-H292 cells *in vitro*.

which could contribute to the decreased metastatic potential of tumor cells.

3.5. *Nrf2*-*HO-1* axis regulates matrix metalloproteinases in NCI-H292 lung carcinoma

Out of the multiple genes regulated by *HMOX1* overexpression in NCI-H292 cells identified by microarray analysis, our attention was drawn to MMP-1, which has a well-established role as a negative prognostic factor in human lung cancer [17] and was potentially down-regulated in NCI-HO-1 cells (Fig. 5A). Real-time PCR analysis additionally revealed inhibition of expression of MMP-9 and MMP-12 in *HMOX1*-overexpressing cells (Fig. 5A), which was corroborated by a significant attenuation of total MMP activity in cell culture media (Fig. 5B), suggesting that HO-1 could be an important regulator of MMPs. The striking down-regulation of MMP-1 and the regulation of MMP-9 was retained in NCI-HO-1 tumors *in vivo*, where diminished expression of MMP-13 and up-regulation of tissue inhibitors of the MMPs (TIMPs) TIMP-3 and TIMP-4 were also identified (Figs. 5C, 5D), which, altogether, could have contributed to the inhibition of xenograft growth and metastasis. Interestingly, we observed a similarly strong attenuation of MMP-1 expression in *Nrf2*-overexpressing cells (Fig. 5E). It was

accompanied by a down-regulation of several other MMPs, including MMP-3, MMP-12, and MMP-13, which, together with MMP-1, form a gene cluster on chromosome 11q22.3, suggesting that there may be a common regulatory mechanism partially related to HO-1. Indeed, siRNA-mediated silencing of *HMOX1* in NCI-*Nrf2* cells reversed the down-regulation of MMP-3 and MMP-12 (Fig. 6B, 6D) and led to the partial restoration of MMP-1 (Fig. 6A). Additionally, a striking induction of MMP-1 and MMP-3 as well as an up-regulation of MMP-12 and MMP-9 was observed in the control cell line with *HMOX1* knockdown (Figs. 6A–6D), where the expression level of the enzyme was lowest (Fig. 3A), further validating HO-1 as a strong modulator of MMP expression in MEC. Involvement of HO-1 in the regulation of expression of MMP-1 was identified also in NCI-H460 large cell carcinoma cells, while *HMOX1* silencing had no effect on MMP-1 mRNA levels in A549 lung adenocarcinoma and SK-MES1 squamous cell carcinoma cell lines (Fig. 6E), showing high specificity of HO-1 action for the NSCLC subtype.

3.6. *Nrf2* and *HO-1* modulate expression of inflammatory cytokines in NCI-H292 cells

A recent report by Petrella et al. described interleukin 1 β as a key factor involved in regulation of expression of MMP-1 and

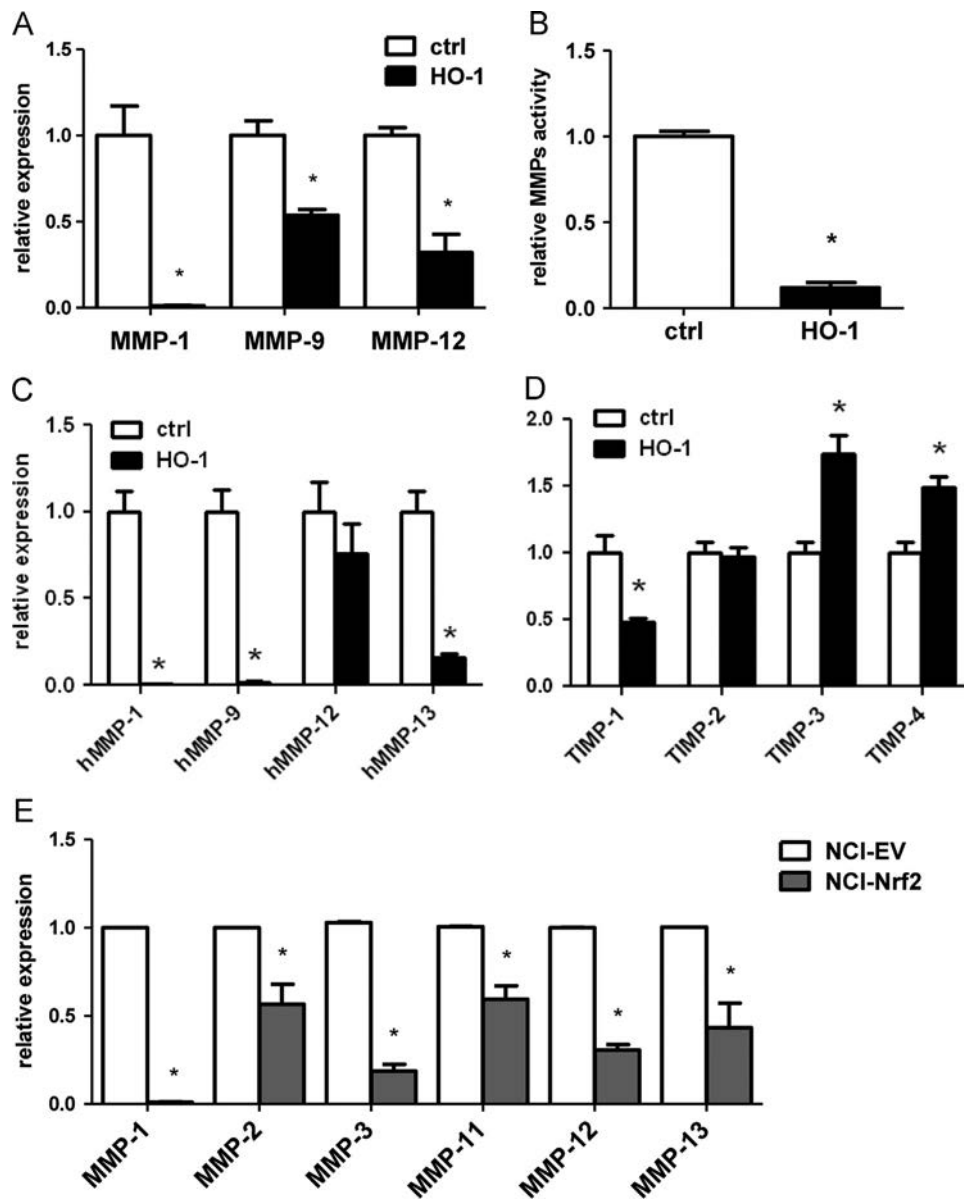


Fig. 5. Effect of HO-1 and Nrf2 on expression of matrix metalloproteinases in NCI-H292 cells. (A) qPCR analysis of MMPs expression in control and *HMOX1*-overexpressing NCI-H292 cells *in vitro* ($n=4$). (B) Measurement of global MMPs activity (MMP-1, -2, -3, -7, -8, -9, -12, -13) in cell culture media of NCI-control and NCI-HO-1 cells ($n=4$). (C–D) Real-time PCR analysis of expression of human MMPs (C) and its inhibitors (D) *in vivo* in tumor tissue from NCI-ctrl and NCI-HO-1 xenografts ($n=15$ for control; $n=18$ for HO-1). (E) qPCR analysis of MMPs expression in control and Nrf2-overexpressing NCI-H292 cells *in vitro* ($n=4$). * $p < 0.05$ NCI-EV/ctrl vs. NCI-transgene.

MMP-3 in A549 lung adenocarcinoma cells [18]. Our microarray analysis indicated that this cytokine was strongly down-regulated in *HMOX1*-overexpressing mucoepidermoid carcinoma cells, which was confirmed by measurement of factor production in cell culture media (Fig. 7A). Also, Nrf2-overexpressing cells exhibited diminished expression of IL-1 β , suggesting a possible association with HO-1 (Fig. 7B). Yet silencing of *HMOX1* in NCI-Nrf2 cells did not alter IL-1 β expression (Fig. 7B), showing that different pathways might be involved in HO-1-dependent regulation of the MMPs in Nrf2-overexpressing cells. On the other hand, a twofold up-regulation of IL-1 β in control cells treated with siRNA against *HMOX1* was observed, which could have contributed to the corresponding induction of MMP-1, -3, -9, and -12. (Figs. 6A–6D). The involvement of HO-1 in the regulation of IL-1 β , however, was specific for MEC cells, as *HMOX1* silencing in cell lines of other NSCLC subtypes did not significantly change IL-1 β expression (Fig. 7C).

Importantly, the attenuation of expression of IL-1 β by HO-1 in

MEC cells was confirmed in xenografts (Fig. 7D). Significant down-regulation of interleukin-6 (IL-6) and tumor necrosis factor α (TNF α) was also observed *in vivo* in NCI-HO-1 tumors (Fig. 7D), consistent with the known anti-inflammatory actions of the HO-1 enzyme in other cell types. Decreased levels of human IL-6 were noticed in murine serum (Fig. 7E), while human IL-1 β and TNF α levels were undetectable. The altered microenvironment of *HMOX1*-overexpressing tumors could have affected stromal cells and metastatic niches, as we observed lower expression of murine MMP-9 in tumors and lungs of NCI-HO-1 xenograft-bearing mice (Fig. 7F).

4. Discussion

The salient finding of the present study is that, contrary to what has been demonstrated for the most common types of NSCLC [19,20], in mucoepidermoid carcinoma the activation of

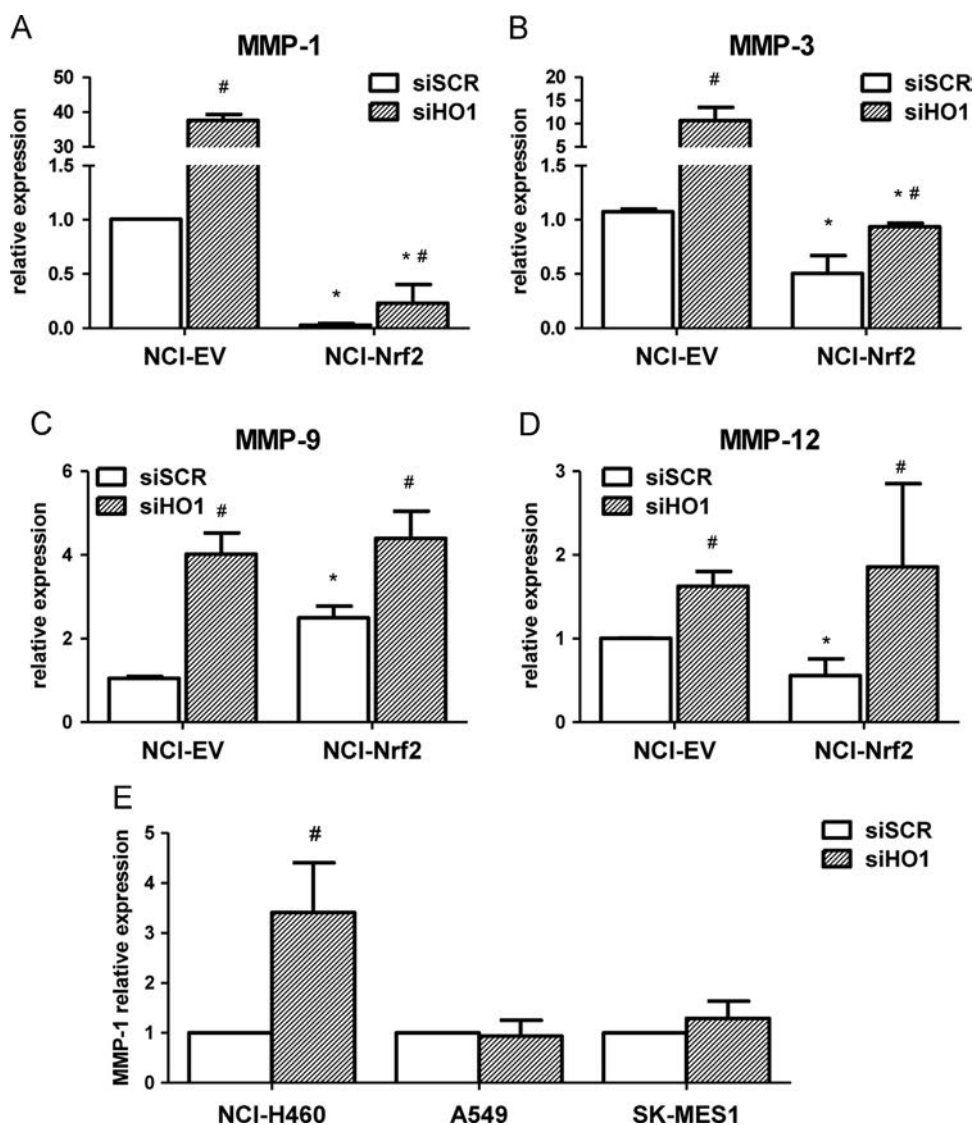


Fig. 6. Involvement of HO-1 in the regulation of MMP expression in NSCLC cells. (A–D) Control and Nrf2-overexpressing NCI-H292 cells were transfected with 50 nM siRNA for 48 h when gene expression was measured by real-time PCR ($n=3$). (E) Effect of *HMOX1* silencing on MMP-1 expression in NSCLC cell lines ($n=4$). * $p < 0.05$ NCI-EV vs. NCI-Nrf2, # $p < 0.05$ siSCR vs. siHO1.

transcription factor Nrf2 exerts antitumoral effects, attenuating tumor cell proliferation, migration, and expression of matrix metalloproteinases. Its actions are largely attributable to the principal ARE-regulated gene heme oxygenase-1, implying that the Nrf2-HO-1 axis could be one of the major molecular pathways contributing to the relatively mild clinical presentations of lung MECs.

Restoration of physiological levels of reactive oxygen species from oncogenic ones by antioxidant actions of Nrf2 target proteins has been associated with suppressed tumor growth in models of glioma, melanoma, and pancreatic cancer [21–23]. Nevertheless, since the treatment of the NCI-H292 cell line with an antioxidant, N-acetylcysteine, resulted in enhanced tumor cell proliferation (data not shown), the effects of Nrf2 overexpression in our model of MEC could rather be redox-independent. Our data suggest that microRNAs may be involved in modulation of MEC cell proliferation, as Nrf2-overexpressing cells exhibited enhanced expression of the tumor suppressor miRNAs miR-193b, miR-181a, and miR-424 and a decreased level of oncogenic miR-378, which we and others have previously shown to play a protumorigenic role in lung NSCLC [9,24]. Down-regulation of miR-378 and attenuation of cell proliferation and migration in Nrf2-overexpressing MEC cells was reversed by *HMOX1* knockdown, establishing this Nrf2 target

gene as a central mediator of its action. This finding could be explained by recent data of Biswas et al. showing that a nuclear form of HO-1 interacts with Nrf2 and stabilizes it, thereby reinforcing its action [25]. Not surprisingly, a key role of HO-1 in mediating the effects of Nrf2 has been also reported for human oral squamous cell carcinoma cells in a model of mollugin-induced growth inhibition and apoptosis [26] and in human breast cancer cells in induction of p53 by 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 [27]. The latter is in accordance with our recent observation of increased p53 levels in NCI-H292 cells overexpressing *HMOX1* [9], additionally corroborated here by microarray data showing a strong decrease in the number of transcripts for Mdm2 protein, which is known to be a negative regulator of p53 protein stability. We have already demonstrated [9] that inhibition of MEC cell proliferation and migration by HO-1 is replicated by treatment of cells with carbon monoxide.

Transcriptome analysis allowed us to reveal additional molecular pathways potentially involved in attenuation of tumorigenic potential of MEC cells by HO-1, including up-regulation of the known negative regulators of NSCLC growth GAS-1 [28] and p57 (CDKN1C) [29], down-regulation of c-myc protooncogene [30], and decreased expression of cyclin D1, which is overexpressed in 25–

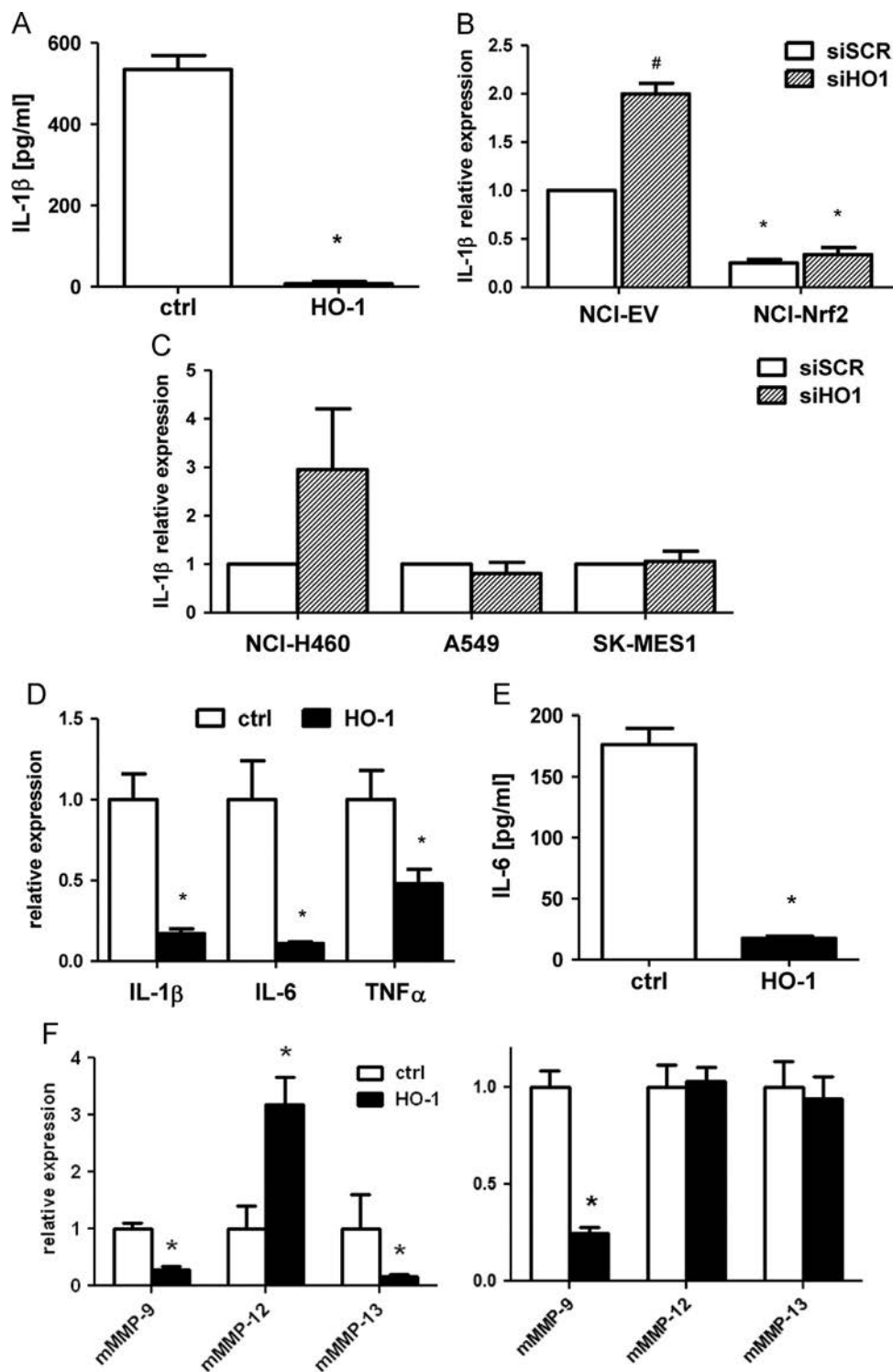


Fig. 7. Involvement of HO-1 in regulation of inflammatory cytokines in NSCLC cells. (A) Measurement of IL-1 β production by NCI-H292 cells *in vitro* by ELISA ($n=4$). (B) Effect of *HMOX1* silencing on IL-1 β expression in NCI-H292 cells ($n=3$). (C) Effect of *HMOX1* silencing on IL-1 β expression in NSCLC cell lines ($n=4$). (D) Real-time PCR analysis of level of expression of human cytokines in NCI-H292 xenografts ($n=15$ for control; $n=18$ for HO-1). (E) Quantification of human IL-6 in serum of tumor-bearing mice ($n=15$ for control; $n=18$ for HO-1). (F) Real-time PCR analysis of expression of murine MMPs in tumors (left) and lungs (right) of tumor-bearing mice. * $p < 0.05$ NCI-EV vs. NCI-transgene, # $p < 0.05$ siSCR vs. siHO1.

47% of clinical cases of NSCLC, correlating with bad prognosis [31].

We have previously shown, using another *HMOX1*-over-expressing NCI-H292 cell line, that inhibition of MEC tumor growth *in vivo* is associated with decreased vascularization and oxygenation of cancer tissue [9]. Here we demonstrate that xenografting of the NCI-HO-1 cell line to NOD-SCID mice reveals

significant attenuation of cancer cell metastatic potential. It coincides with down-regulation of matrix metalloproteinases including MMP-9, an enzyme down-regulated in low-grade lung MEC specimens [12] that has been already reported to be negatively regulated by HO-1 in breast cancer cells [32,33]. Nevertheless, MMP-9 was not inhibited by Nrf2 overexpression, while the

general profile of MMP expression of NCI-Nrf2 cells not only mimicked that of NCI-HO-1 cells, but also displayed attenuated mRNA levels of additional enzymes. This broad inhibitory effect could be attributed to a general modulation of cellular signaling by altered redox status, as it has already been demonstrated that in prostate cancer cells a more reducing intracellular environment is associated with down-regulation of MMPs and decreased cell invasiveness [34], and we have previously reported that *HMOX1*-overexpressing cells exhibit reduced levels of intracellular reactive oxygen species [9]. However, putatively redox-independent actions of Nrf2 target proteins may also be of importance, as exemplified in our study by the increased levels of MMPs in Nrf2-overexpressing cells following HO-1 knockdown. The potential mechanism of direct regulation of MMP expression by HO-1 requires further study. It may involve heme cleavage products such as CO, biliverdin, and bilirubin, which have been previously reported to attenuate activity of ERK1/2 kinases, diminishing nuclear translocation of the transcription factors AP-1 and NF- κ B, and thus inhibiting transcription of MMPs [35,36]. Our findings of the key role of HO-1 in modulating the expression of MMPs in NCI-H292 are further corroborated by a uniform up-regulation of all the examined MMPs in control cells treated with siRNA against HO-1, which exhibited the lowest expression of HO-1 among the cell lines under study. Given the relatively low basal expression of HO-1 in NCI-H292 cells as compared with other NSCLC models [9], this suggests that even a minimal level of HO-1 induction in MECs would be sufficient for suppression of expression of numerous matrix-degrading enzymes, possibly contributing to the relatively mild clinical phenotypes of these tumors.

The strongest effect of *HMOX1* knockdown was observed for MMP-1 and MMP-3, the two proteins that have been reported to be regulated by interleukin-1 β in A549 lung adenocarcinoma [18]. Indeed, silencing of *HMOX1* in NCI-EV cells caused a significant up-regulation of IL-1 β , while it had no effect on the inhibition of IL-1 β expression in Nrf2-overexpressing cells, suggesting that the modulation of expression of the inflammatory cytokine could be involved in the action of Nrf2-HO-1 axis in regulating MMPs. Carbon monoxide, identified by our previous study as the major mediator of HO-1 action in the MEC cells [9], is known to attenuate production [37] and signaling [38] of interleukin-1.

Consistent with the above, *HMOX1*-overexpressing cells produced very low amounts of IL-1 β *in vitro*. NCI-HO-1 xenografts in NOD-SCID mice exhibited inhibited expression of IL-1 β , TNF α , and IL-6, the last of which could be also detected at decreased levels in the blood of tumor-bearing animals. This anti-inflammatory effect coincided with down-regulation of murine MMP-9 and MMP-13 in the tumors and mMMP-9 in the lungs of xenografted animals, showing that the attenuation of the metastatic potential of MEC cells by HO-1 may be also attributed to the modulation of the tumor microenvironment and metastatic niche.

Taken together, we show the unique molecular consequences of activation of Nrf2-HO-1 axis in lung mucoepidermoid carcinoma that are associated with tumor-suppressive phenotype involving strong down-regulation of matrix metalloproteinases, which may explain clinical features of this rare subtype of non-small-cell lung cancer.

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