

## Mesothelioma and Hypoxia: Modulation of the Inflammation-Related Phenotype and Identification of Prognostic Markers

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### Abstract

**Objective:** Malignant Mesothelioma is an aggressive tumor occurring in the context of chronic inflammation and characterized by hypoxic areas.

This study explores how hypoxia impacts on the pro-inflammatory phenotype of MSTO-211H and MPP89 mesothelioma cells and investigates the role of HIF-1 alpha and NF-kB in this process. The prognostic values of two molecules upregulated by oxygen deprivation, HIF-3 alpha and CXCR4, is also analyzed.

**Methods:** Hypoxic condition was obtained in a sealed modular incubator chamber flushed with 1% O<sub>2</sub>. mRNA and protein levels were evaluated by Real-Time PCR and Western Blot. Silencing of HIF-1 alpha was achieved by specific shRNA and NF-kB inhibition by parthenolide treatment. HIF-3 alpha and CXCR4 expression in tumor tissues from mesothelioma patients was detected by immunohistochemistry.

**Results:** The hypoxic stimulation of mesothelioma cells induced an early activation of HIF-1 alpha and NF-kB and a later increase of HIF-3 alpha expression. In addition, the upregulation of a set of inflammation-related genes was observed. Silencing of HIF-1 alpha and treatment with parthenolide highlighted that the observed increase in gene expression depends on both HIF-1 alpha and NF-kB transcriptional activity. A correlation between high expression of CXCR4 in human mesothelioma samples and poor survival was also observed and HIF-3 alpha was suggested as a potential new prognostic marker.

**Conclusions:** This study evidences a cross-talk between hypoxia adaptation and pro-inflammatory phenotype in mesothelioma accomplished through the combined transactivation activity of HIFs and NF-kB. Immunohistochemistry analysis of tissue samples confirms CXCR4 and suggests HIF-3 alpha as potential prognostic markers for mesothelioma.

**Keywords:** Mesothelioma; Hypoxia; HIFs; NF-kB; Cross-talk; Inflammation-related genes; Prognostic markers

**Abbreviations:** MM: Malignant Mesothelioma; NF-kB: Nuclear factor-kappa B; HIF: Hypoxia inducible factor; MSTO: MSTO-211H; MPP: MPP89; RAGE: Receptor for advanced glycation end products; P2X7R: P2X purinoreceptor7; VEGF: Vascular epidermal growth factor A; COX2: Cyclooxygenase 2; PTX3: Pentraxin 3; CXCR4: C-X-C chemokine receptor 4; CXCL12: Chemokine (C-X-C motif) ligand 12; HMOX: Heme oxygenase 1; P: Parthenolide; NTshRNA: Non-targeting shRNA

### Introduction

Pleural Malignant Mesothelioma (MM) is one of the most aggressive solid tumors. It has a poor prognosis due to its resistance to chemo and radiotherapy and to the difficulty in administering anticancer drugs into the pleural tissue [1,2]. Even though it is a rare tumor, its incidence is on the rise and approximately 250,000 deaths are estimated to occur in Western Europe over the next 30 years. MM develops in response to asbestos exposure and is characterized by chronic inflammation [3]. Asbestos exposure generates reactive oxygen and nitrogen species and stimulates macrophages and other cell types to produce different cytokines and growth factors. Similar to other cancers, inflammation has been proposed to play a role in MM tumorigenesis [4]. Recent reports have highlighted the ability of tumour cells to synthesize inflammation-related molecules involved

in breast [5], glioblastoma [6], thyroid [7] and prostate [8] cancer progression. In MM, little is known about the inflammation-related molecules produced by tumor cells. In addition to cyclooxygenase 2 (COX2), a strong prognostic factor and a potential therapeutic target [9,10], C-X-C chemokine receptor 4 (CXCR4) and chemokine (C-X-C motif) ligand 12 (CXCL12) are highly expressed in most MM cell lines and tumor tissues, suggesting their use as biomarkers [11]. Vascular epidermal growth factor A (VEGF) also showed to play a significant role in MM, where a correlation with survival was observed [12].

Like other solid tumors, MM is characterized by areas of hypoxia deriving from poor neoangiogenesis. Hypoxia activates gene

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expression through three isoforms of oxygen inducible transcription factors, HIF-1 alpha, HIF-2 alpha and HIF-3 alpha, and through nuclear factor-kappa B (NF-kB), the master regulator of inflammatory response. Previous studies in different tumors showed that HIF and NF-kB signaling are intricately linked during hypoxic inflammation [13-18]. Moreover, Tafani et al. demonstrated that hypoxia and inflammation are sequentially bridged in tumors [19]. However, the molecular mechanisms by which MM cells adapt to hypoxia are poorly understood and little information is available on the cross-talk between HIF and NF-kB pathways. Based on these considerations, we explored the role of HIF-1 alpha and NF-kB in the inflammation-related gene expressions in hypoxic MM cells in order to clarify their role on MM progression and to identify potential prognostic markers. We used two MM cell lines phenotypically different, MSTO-211H (MSTO) (with fibroblastic-like morphology) and MPP89 (MPP) (with epithelial-like morphology) tumorigenic and not tumorigenic in mice, respectively (unpublished data). We chose a set of inflammation-related genes representative of the main classes of molecules involved in the innate immune response and greatly linked to the progression of many tumors as shown by ours and others' studies. In particular, we analyzed the receptor for advanced glycation end products (RAGE) [20-22] and P2X purinoreceptor7 (P2X7R) [23] that are able to bind endogenous alarmins released by necrotic cells during hypoxic stress; the inducible enzyme COX2 highly expressed during inflammation in many tumors including MM [24,25]; the acute phase protein pentraxin 3 (PTX3) [5,8]; CXCR4, a cytokine receptor associated with increased proliferation, invasion and migration in several tumors and tumor cell lines [26,27]. Moreover, we analyzed VEGF, crucial to the systemic hypoxia response [28] and heme oxygenase 1 (HMOX), an anti-inflammatory enzyme essential in heme catabolism that protects the cells against oxidative injury [29]. HIF-1 alpha silencing and NF-kB inhibition following parthenolide treatment allowed us to gain greater insight on the role of these two master regulators in the modulation of hypoxia induced inflammation-related genes.

## Material and Methods

### Cell lines and culture

The human MM cell lines MSTO and MPP were obtained from the ATCC (Rockville, MD) and from the Genova Institute Culture Collection, respectively. MSTO were cultured as monolayers in flasks using ATCC growth medium with 10% FBS (ATCC) and antibiotics (5000 IU/mL penicillin and 5000 µg/mL streptomycin, GIBCO Life Technologies, Paisley, UK). MPP were maintained in Ham's F10 with 15% FBS, and supplemented with glutamine (2 mM) and antibiotics (GIBCO). Both cell lines were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Hypoxic exposure was achieved by incubating the cells at 37°C in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA, US) flushed with 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub> for 5 min according to the manufacturer's instructions. Parthenolide (P) treatment was carried out at a concentration of 3 µM for the time indicated, preceded by a 4 hour pre-treatment.

### Stable gene silencing

Selective silencing of HIF-1 alpha in MSTO was obtained by small interfering RNAs processed from specifically designed short hairpin RNAs (shRNA) (MISSION products, Sigma-Aldrich, St. Louis, MO). Bacterial cultures harboring sequence verified shRNA lentiviral plasmid vectors cloned into the pLKO.1-puro vectors were amplified from glycerol stocks. The shRNA plasmid DNA was purified (PureLink, Invitrogen Life Technologies, Paisley, UK) and the target cells were

transfected using Lipofectamine™ 2000 (Invitrogen) and selected in presence of 2 µg/ml puromycin (Sigma-Aldrich). Negative control was performed by transfection with non-targeting shRNA.

The effects of selective silencing on inflammatory gene expression in normoxic and hypoxic cells were detected by Real Time PCR (RT-PCR) and Western Blot assay. To reduce the variability of hypoxia response, a mix of three HIF-1 alpha silenced clones (HIF-1α shRNA) and a mix of three non-targeting plasmid vector transfected clones (NT shRNA) were used.

### Nuclear protein extraction and Western Blot analysis

Nuclear cell extracts were prepared by a nuclear extract kit (Active Motif, Carlsbad, CA) in accordance with the manufacturer's instructions. 10-20 µg proteins were loaded onto a 10% SDS polyacrylamide gel and after electrophoresis, were blotted onto nitrocellulose membranes. Primary mouse anti-human HIF-1 alpha (BD Biosciences, San Jose, CA), p65 (Santa Cruz Biotechnology, Santa Cruz, CA), γ-tubulin (Sigma), primary rabbit HIF-2 alpha, phospho-NF-kB p65 (Ser276) (Cell Signaling Technology, Danvers, MA), HIF-3 alpha (Abcam, Cambridge, UK), and the appropriate secondary antibodies (Bio-Rad Laboratories; Hercules, CA) were selected after testing and used in appropriate dilutions. Immuno-complexes were visualized by an enhanced chemiluminescent kit (ECL; Sigma). γ-tubuline was used as loading control. Experiments were repeated at least two times with different cellular extracts.

### RNA isolation and RT-PCR analysis

Total RNA was extracted by Trizol reagent and reverse transcribed using Superscript II Reverse Transcriptase and random primers (Invitrogen). Quantitative RT-PCR was carried out with the ABI Prism 7000 Sequence Detector System, using TaqMan Gene Expression Assay kits (Applied Biosystems Life Technologies, Paisley, UK). PCR amplifications were performed according the manufacturer's default cycling conditions. The mRNA levels of HIF-1 alpha, HIF-2 alpha, HIF-3 alpha and inflammation-related genes were quantified using a standard curve and normalized to the housekeeping gene 18s rRNA. Experiments were repeated at least three times with different cellular preparations.

### Clinical data and tumor specimen acquisition

Tissues from 29 MM specimens (16 epithelioid, 6 sarcomatoid and 7 mixed mesotheliomas) obtained from open biopsies or pleurectomies were collected and fixed in 10% formalin before being embedded in paraffin. All patients were treated at the Second University of Naples between 1980 and 1996. Survival was determined from the date of initial surgery. Follow-up was available for all patients. All patients were treated at least with cytoreductive surgery and 13 patients were then treated with radiotherapy or chemotherapy. The formalin-fixed, paraffin-embedded samples were sectioned at 5 µm and stained with hematoxylin and eosin. The most representative blocks were selected to be cut into new 5 µm-thick sections for immunohistochemical studies.

### Immunohistochemistry

All 29 cases were assessed by immunohistochemistry for CXCR4 and HIF-3 alpha. Sections from each specimen were cut at 5 µm, mounted on glass and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and antibody dilution. Endogenous peroxidase activity was blocked by 5% hydrogen peroxide. The primary antibodies

for CXCR4 (rabbit monoclonal, Novus Biologicals, Europe) and HIF-3 alpha (rabbit polyclonal, Abcam) were applied at room temperature for 1 h at the dilution of 1:100. Subsequently, the sections were immunostained with the streptavidin-biotin system (Dako, Carpinteria, CA), using diaminobenzidine (DAB) as the final chromogen and hematoxylin as the nuclear counterstain. Negative controls for each tissue section were prepared by leaving out the primary antibodies. A suitable positive control was run with each set of slides. All samples were processed under the same conditions.

Two observers (A.B. and B.V.) estimated the staining pattern of CXCR4 and HIF-3 alpha separately. For CXCR4 and HIF-3 alpha quantification, staining intensity of each specimen was analyzed using the following scores: 1 (weak), 2 (medium), and 3 (strong). The level of concordance, expressed as the percentage of agreement between the observers, was 93% (27 of 29 specimens). In the remaining specimens, the score was obtained after collegial revision and agreement.

### Statistical analysis

Student t-test was used to analyze data obtained in Real-Time experiments. Values were expressed as mean  $\pm$  SE. For immunohistochemistry, Fischer's exact test was used to assess the relationship between ordinal data (correlation matrix between immunostaining parameters). The correlation between CXCR4 and HIF-3 alpha was carried out on the original ordinal units. The statistical method used to perform the correlation was the Spearman test. A univariate survival analysis for each prognostic variable on overall survival was estimated according to the Kaplan-Meier method [30]. The terminal event was death attributable to cancer. The statistical significance of the differences in survival distribution among the prognostic groups was evaluated by the log-rank test [31]. The Cox proportional hazards model was applied to the multivariate survival analysis [32]. The prognostic variables on overall survival included: histological types, CXCR4 and HIF-3 alpha expression. P values  $\leq$  0.05 was regarded as statistically significant in two tailed tests. SPSS software (version 10.00, SPSS, Chicago) was used for statistical analysis.

## Results

### HIF-1, HIF-2, and HIF-3 alpha expression profiles in MM cell lines after O<sub>2</sub> deprivation.

mRNA levels and, in particular, nuclear protein translocation of HIFs are a measure of cell response to hypoxic stress. Therefore, the mRNA transcription and the nuclear protein expression profile of HIF-1, HIF-2, and HIF-3 alpha were characterized in MSTO and MPP cells in normoxia and hypoxia at different time points (Figure 1A). HIF-1 and HIF-2 alpha mRNAs were expressed at comparable levels in both normoxic cell lines (data not shown). Conversely, HIF-3 alpha showed a higher basal expression level (about 100 fold) in MPP compared to MSTO (data not shown). In hypoxic conditions, the two cell lines showed similar behavior. In fact, HIF-1 alpha mRNA started decreasing at 8 hours of stimulation and remained down-regulated until 24 hours. Nuclear accumulation of the protein was evident after 1-2 hours, reaching a peak at about 4 hours and then declining to basal level after 12 hours. HIF-2 alpha did not seem significantly involved in the response to hypoxic stress given no variation in mRNA and only a slight nuclear protein up-regulation in MSTO was observed after 1-2 hours stimulation. Finally, hypoxia determined a significant enhancement in HIF-3 alpha mRNA and nuclear protein in both models. In MSTO, mRNA increase started at 8 and peaked at 48 hours of stimulation while in MPP, up-regulation was delayed at 48, and

was maintained up to 96 hours. In both cell lines, HIF-3 alpha nuclear protein accumulation was detectable from 48 and still present at 96 hours of hypoxia. Western Blots densitometry of nuclear HIF-1, HIF-2 and HIF-3 alpha is shown in supplementary data (Figure S1A).

### Hypoxia activates NF-kB signaling in MM cell lines

Nuclear translocation and, especially, phosphorylation of nuclear p65 subunit is a measure of NF-kB activation. NF-kB status was evaluated by Western Blot analysis in time-course experiments of oxygen deprivation (30'-4h) (Figures 1B and S1B). Nuclear P65 accumulation was detected in MSTO (30', 1h) and, to a much lesser extent, in MPP (30'). Hypoxia dependent activation of NF-kB was confirmed by a clear-cut and precocious up-regulation of nuclear phospho P65 (Ser276), the transcriptionally active form of P65. Therefore, in both cell lines NF-kB pathway resulted highly responsive to hypoxia, although MSTO cells showed a more marked and prolonged activation.

### Hypoxic regulation of inflammation-related genes in MM cells

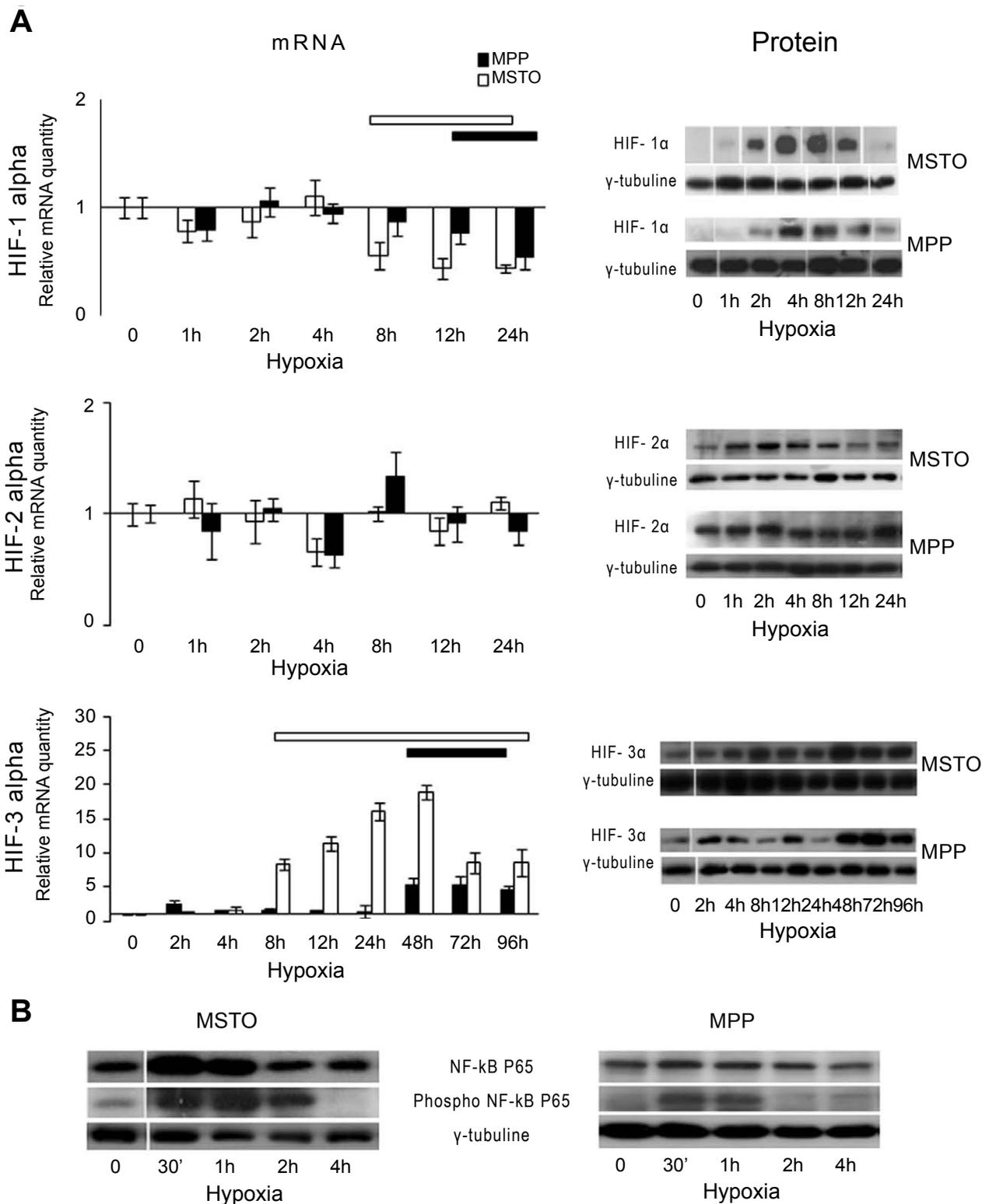
The ability of MM cells to adapt to low oxygen concentrations through the modulation of their pro-inflammatory phenotype was investigated by RT-PCR, in time course experiments under normoxic and hypoxic conditions (Figure 2). Oxygen deprivation up-regulated the expression in a large part of the studied genes with a comparable kinetic in the two cell lines. Except for P2X7R in MSTO, which was induced at 8 hours of hypoxic treatment, transcription of the other molecules started increasing at shorter stimulation times. The highest induction was observed in MSTO for CXCR4 (20 fold) and COX2 (16 fold), and in MPP for CXCR4 and VEGF (3.8 and 3.5 fold, respectively). Indeed, CXCR4 and COX2 were up-regulated at much higher levels in MSTO compared to MPP. HMOX showed a slight but significant increase only in MPP while hypoxia did not have any impact on PTX3 expression in both cell lines.

### HIF-1 alpha knockdown MSTO cells

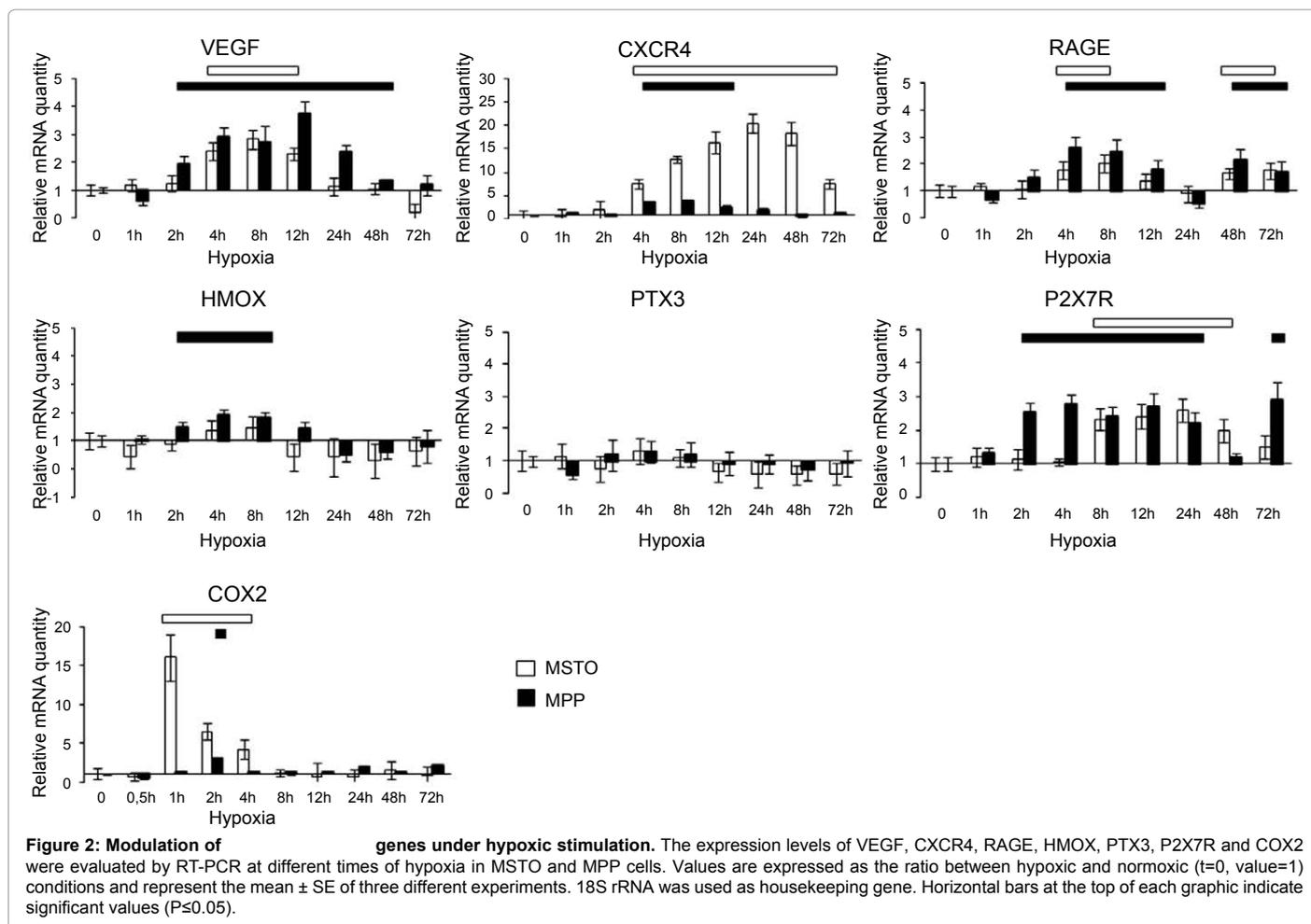
In order to study the reciprocal modulation of HIF-1 alpha and NFkB and to define the role played by the two factors in the transcriptional control of the pro-inflammatory phenotype, HIF-1 alpha knockdown MSTO cells were created. A panel of MSTO cells stably transfected with HIF-1 alpha specific or non-targeting shRNA control vector were analyzed for HIF-1 alpha protein expression at 4 hours of hypoxia (data not shown). The HIF-1 alpha shRNA transfection was effective in HIF-1 alpha gene silencing as shown in Figure 3A depicting the expression of nuclear HIF-1 alpha protein in non-transfected MSTO (wt), in the mix of non-targeting plasmid vector transfected MSTO clones (NT shRNA) and in the mix of HIF-1 alpha silenced MSTO clones (HIF-1 $\alpha$  shRNA) used in the experiments. Characterization of NT shRNA clones for HIF-2 and HIF-3 alpha mRNA and protein levels and for NF-kB status in normoxia and hypoxia did not highlight any significant differences compared to wt cells (data not shown).

### Reciprocal modulation of HIF-1 alpha and NF-kB in normoxic and hypoxic MSTO

The role of NF-kB in HIF-1 alpha expression was investigated in MSTO by evaluating the hypoxic and normoxic levels of HIF-1 alpha mRNA and protein in NT shRNA cells, untreated or treated with the NF-kB inhibitor parthenolide. A significant down-regulation of HIF-1 alpha mRNA, was observed in the presence of the drug, both under normoxic (t=0) and hypoxic conditions (Figure 3B upper panel). Interestingly, this effect did not rebound on the protein level where



**Figure 1: HIF-1, HIF-2, HIF-3 alpha and NF-kB expression in MSTO and MPP cell lines in hypoxic conditions.** (A) On the left side the mRNA expression levels of HIF-1, HIF-2, and HIF-3 alpha at different times of hypoxia were evaluated through RT-PCR. Values are expressed as fold induction with respect to normoxic control (set at 1) and represent the mean  $\pm$  SE of three different experiments. 18S rRNA was used as housekeeping gene. Horizontal bars at the top of each graphic indicate significant values ( $P \leq 0.05$ ). On the right side the nuclear levels of HIF-1, HIF-2, and HIF-3 alpha proteins at different times of hypoxia were analyzed by Western Blot.  $\gamma$ -tubuline was used as loading control. A representative experiment for each gene is shown. (B) The nuclear levels of NF-kB P65 and phospho NF-kB P65 (Ser276) at different times of hypoxia were analyzed by Western Blot in MSTO and MPP.  $\gamma$ -tubuline was used as loading control. A representative experiment is shown for both NF-kB P65 and phospho NF-kB P65 (Ser276).



parthenolide did not seem to affect nuclear HIF-1 alpha protein accumulation at 4 and 8 hours of oxygen deprivation (Figures 3B bottom panel and S2A). The action of HIF-1 alpha on NF-kB activation was highlighted by comparing the normoxic and hypoxic nuclear levels of P65 and phospho (Ser276) P65 in NT shRNA and HIF-1 $\alpha$  shRNA cells. HIF-1 alpha did not appear to be involved in NF-kB nuclear increase in MSTO, since no differences in the basal and hypoxic level of both p65 and phospho p65 were observed between the non silenced and silenced clones at 30 min of hypoxia (Figures 3C and S2B).

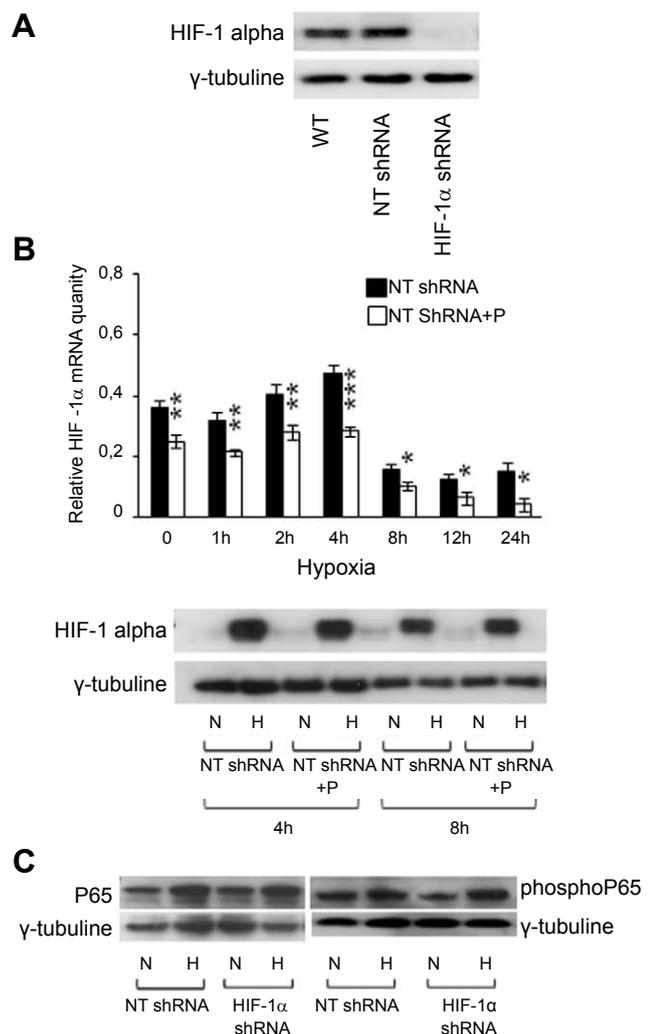
### Role of HIF-1 alpha and NF-kB in HIF-3 alpha modulation

The impact of HIF-1 alpha and NF-kB on HIF-3 alpha was evaluated in MSTO cells.

HIF-1 alpha silencing prevented the hypoxic mRNA induction detected in NT shRNA cells between 8 and 72 hours stimulation (Figure 4A). The analysis of HIF-3 alpha nuclear protein did not highlight a reduction in the nuclear base level in HIF-1 $\alpha$  shRNA cells but confirmed the absence of the hypoxia induced increase observed in NT shRNA cells at 72 hours stimulation (Figures 4B and S3). Therefore, HIF-3 alpha responsiveness to oxygen deprivation in MSTO resulted strictly dependent on HIF-1 alpha activity. Conversely, parthenolide treatment had no effect on HIF-3 alpha mRNA and nuclear protein levels both in NT shRNA and HIF-1 $\alpha$  shRNA cells, independent from oxygen concentration (Figures 4A, 4B and S3), strongly suggesting that NF-kB is not involved in HIF-3 alpha control.

### Role of HIF-1 alpha and NF-kB in the modulation of the pro-inflammatory gene expression

The role of HIF-1 alpha and NF-kB in the modulation of the pro-inflammatory gene expression was investigated in MSTO cells comparing the expression levels of each gene under study in NT shRNA and in HIF-1 $\alpha$  shRNA cells, untreated or treated with parthenolide, in normoxia and under hypoxic conditions, at different time-points (1 - 72 hours) (data not shown). In Figure 5, the results obtained at the time points where hypoxia exerted its maximum increase in transcription, are shown. P2X7R expression was not affected by HIF-1 alpha but strongly down-regulated by parthenolide both in normal and oxygen deprived environment. Therefore, its expression level seems controlled mainly by NF-kB. Conversely, CXCR4 expression was strongly down-regulated in HIF-1 alpha silenced cells compared to NT shRNA cells but was not modified by parthenolide, regardless of oxygen concentration. Consequently, for this gene, HIF-1 alpha control seems to prevail. The level of all the other genes (VEGF, COX2 and RAGE) appeared to be regulated by both HIF-1 alpha and NF-kB. HMOX gene expression underwent a dual and complex modulation. HIF-1 alpha silencing abrogated the hypoxia dependent up-regulation observed in NT shRNA highlighting a role for HIF-1 alpha in its hypoxic increase. On the other hand, parthenolide was able to induce a strong enhancement in HMOX transcription, both in hypoxic and normoxic conditions in HIF-1 alpha silenced and HIF-1 alpha expressing MSTO,



**Figure 3: Reciprocal modulation between HIF-1 alpha and NF-kB.** (A) Nuclear HIF-1 alpha expression in wt MSTO, in a mix of MSTO clones transfected with non-targeting shRNA control vectors (NT shRNA) and in a mix of MSTO clones knockdown for HIF-1 alpha (HIF-1 $\alpha$  shRNA) was detected by Western Blot at 4 hours of hypoxic treatment.  $\gamma$ -tubuline was used as loading control. A representative experiment is shown. (B upper panel) The effect of parthenolide (P) on HIF-1 alpha mRNA expression levels was detected in NT shRNA clones in normoxia and at different times of hypoxia by RT-PCR. Values represent the mean  $\pm$  SE of three different experiments. 18S rRNA was used as housekeeping gene. NT shRNA + P vs NT shRNA: \*P $\leq$ 0.05, \*\*P $\leq$ 0.01, \*\*\*P $\leq$ 0.001. (B bottom panel) The effect of parthenolide (P) on HIF-1 alpha nuclear protein expression was analyzed in NT shRNA clones by Western Blot in normoxia (N) and at 4 and 8 hours of hypoxia (H).  $\gamma$ -tubuline was used as loading control. A representative experiment is shown. (C) The effect of HIF-1 alpha silencing on NF-kB P65 (on the left) and phosphorylated NF-kB P65 (Ser276) (on the right) protein was detected by Western Blot. NT shRNA and HIF-1 $\alpha$  shRNA clones were maintained in normoxic (N) or hypoxic conditions (H) for 30 minutes.  $\gamma$ -tubuline was used as loading control. A representative experiment is shown for both NF-kB P65 and pospho NF-kB P65 (Ser276).

demonstrating a role of NF-kB in a hypoxia-independent inhibition of the gene.

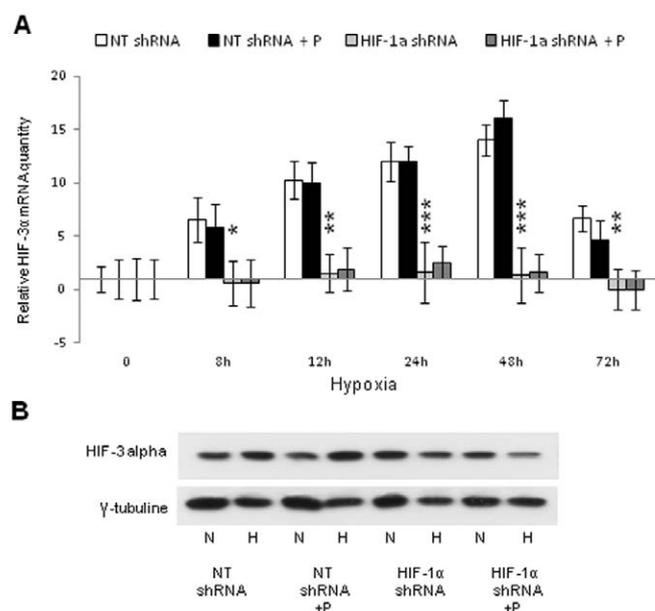
### Expression of CXCR4 and HIF-3 alpha in MM specimens

As suggested by our *in vitro* results, we analyzed the expression of CXCR4 and of HIF-3 alpha in human MM tissues. The table in

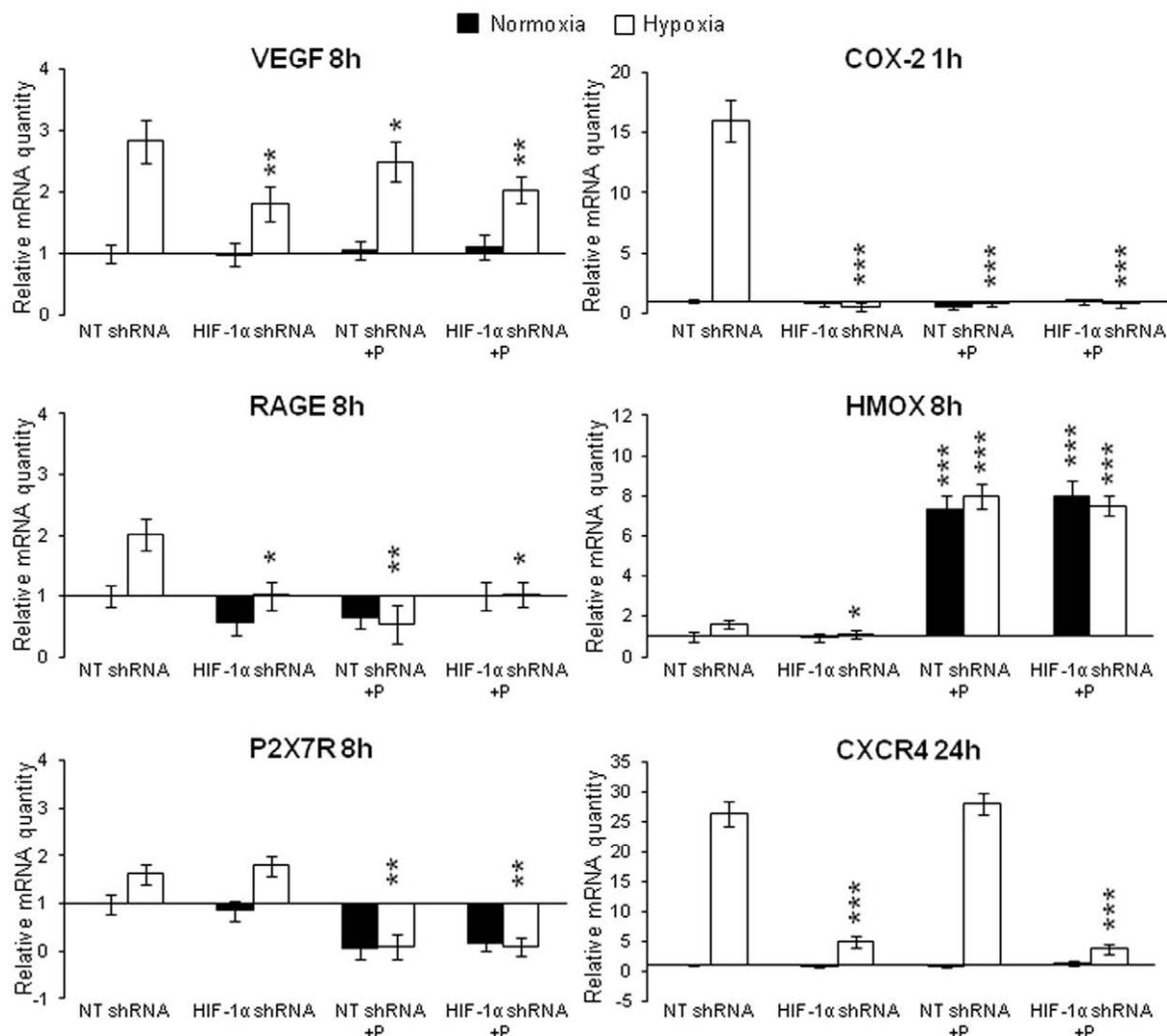
Figure 6 presents summary results from immunohistochemical analysis of 29 MM specimens. Histological tumor specimens contained 16 epithelioid, 6 sarcomatoid and 7 mixed MM. Figure 6 shows typical immunohistochemical stainings for CXCR4 (cytoplasmic) and for HIF-3 alpha (nuclear). By rank correlation matrix, a positive statistically significant correlation was recorded between CXCR4 and HIF-3 alpha expression level (p=0.009) (data not shown). Kaplan-Meier survival plots show a statistically significant association between high levels of both CXCR4 and HIF-3 alpha and poor outcome (p<0.0001 and p=0.032, respectively). Nevertheless, it should be noted that the statistical significance for HIF-3 alpha was borderline and that by a multivariate Cox regression analysis, the only immunohistochemical parameter that had an influence on overall survival was CXCR4 expression (p<0.0001). The histological type did not significantly correlate with immunohistochemical parameters and was not associated with outcome and overall survival (data not shown). Chemotherapy and radiotherapy did not show any impact on overall survival in univariate analysis (data not shown).

### Discussion

In this study, we attempted to further our understanding in adaptation to hypoxia of MM by analyzing the mechanisms underlying inflammation-related gene expression under oxygen deprivation in two cellular models, MSTO and MPP. At the same time, we looked for potential prognostic markers and therapeutic targets among the molecules involved in the response to low oxygen concentration, in particular HIFs and inflammation-related factors, as has already been



**Figure 4: Effect of HIF-1 alpha silencing and NF-kB inhibition on HIF-3 alpha expression.** (A) The mRNA expression levels of HIF-3 alpha at different times of hypoxia were detected by RT-PCR in NT shRNA and in HIF-1 $\alpha$  shRNA clones, in the presence or absence of parthenolide (P). Values are expressed as fold induction with respect to normoxic control (set at 1) and represent the mean  $\pm$  SE of three different experiments. 18S rRNA was used as housekeeping gene. HIF-1 $\alpha$  shRNA vs NT shRNA : \*P $\leq$ 0.05, \*\*P $\leq$ 0.01, \*\*\*P $\leq$ 0.001. (B) Western Blot analysis of HIF-3 alpha nuclear protein was performed in NT shRNA and HIF-1 $\alpha$  shRNA clones in the presence and absence of parthenolide (P) in normoxia (N) and at 72 hours of hypoxia (H).  $\gamma$ -tubuline was used as loading control. One representative experiment is shown.

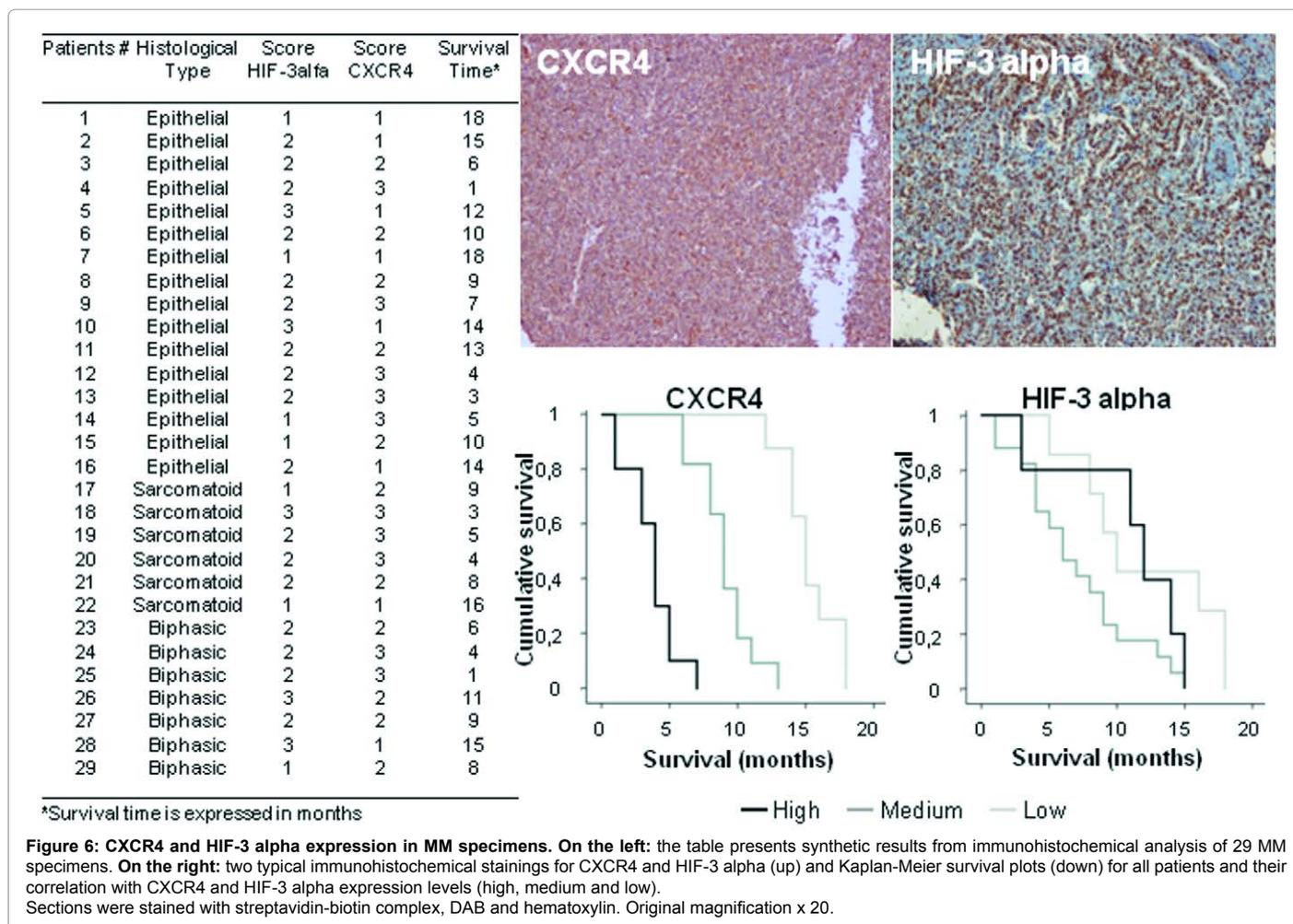


**Figure 5: Role of HIF-1 alpha and NFkB on genes modulation.** The expression levels of VEGF, COX2, RAGE, HMOX, P2X7R and CXCR4 in NT shRNA and in HIF-1α shRNA clones, untreated and treated with parthenolide (P) were detected by RT-PCR in normoxia and at the time points where hypoxia exerted its maximum increase in transcription. Values are expressed as fold induction with respect to normoxic control (set at 1) and represent the mean ± SE of three different experiments. 18S rRNA was used as housekeeping gene. HIF-1α shRNA, NT shRNA +P, HIF-1α shRNA +P vs NT shRNA: \*P≤0.05, \*\*P≤0.01, \*\*\*P≤0.001

done for other tumors [33-35]. As expected, MM cells responded to hypoxic stimulation by HIFs activation. HIF-1 alpha, among the three HIF isoforms, was confirmed to be the main player in hypoxic adaptation in both MSTO and MPP cells, with precocious modifications in transcription and nuclear accumulation comparable to those observed in other tumor cell lines [36]. HIF-2 alpha did not seem to be involved in hypoxic response in MM cell models since a slight accumulation of nuclear protein was detected only in MSTO, whereas mRNA levels remained unchanged in both cell lines. Differently, a delayed HIF-3 alpha mRNA and nuclear protein up-regulation was observed in both cell lines, revealing, for the first time, its role in the MM hypoxic response. HIF-3 alpha is the less known transcriptional factor responsive to hypoxia, it is cell specific and its function is still under debate [37]. The up-regulation of HIF-3 alpha at low oxygen tension resulted clearly reliant on HIF-1 alpha activity, since oxygen deprivation did not increase its expression in HIF-1 alpha silenced MSTO cells. In addition, HIF-3 alpha mRNA and nuclear protein increase peaked

when the expression of the hypoxic pro-inflammatory phenotype was in decline, thus supporting other authors' suggestion that HIF-3 alpha may be a part of a feedback mechanism blunting hypoxic response [38]. Interestingly, the immunohistochemical analysis performed on MM human samples showed an increased expression of HIF-3 alpha in the tumor tissues compared to non tumor and a border-line correlation between HIF-3 alpha expression and survival. This finding allows us to look at HIF-3 alpha as a potential prognostic marker for MM. Knowledge on predictive factors for mesothelioma outcome is poor, thus, the identification of molecular targets is required. Therefore, we plan to validate this attractive hypothesis in a patient's cohort larger than the one reported in this study.

When we analyzed the impact of hypoxia on the pro-inflammatory phenotype of MM cells, we highlighted that oxygen deprivation significantly induced the expression of a selected set of inflammation-related genes. In particular, CXCR4, a gene over-expressed and



associated with increased proliferation, invasion and migration in several tumors [26], showed a remarkable hypoxic up-regulation in both MM cell lines. Both basal and hypoxic expression levels of CXCR4 are higher in MSTO-211H compared to MPP89 cells. Li et al. [11] showed that non tumor mesothelial cell line LP9 do not express CXCR4 while 5 mesothelioma cell lines out of 6 expressed CXCR4 at different levels. Unpublished data from our laboratory showed that MSTO but not MPP are tumorigenic in mice. In addition, our results deriving from the immunohistochemical analysis on MM human samples pointed out, for the first time, a highly significant correlation between CXCR4 expression level and survival. Therefore, we can suggest a link between tumor cell aggressiveness and CXCR4 expression and propose CXCR4, apart from HIF-3 alpha, as a potential prognostic marker for MM. The positive statistically significant correlation recorded between CXCR4 and HIF-3 alpha expression level (data not shown) supports the assumption that these two molecules in association could represent a nucleus of prognostic markers for MM.

As well as HIF-1 alpha, also NF-kB was activated early following hypoxic stress in both MM cell lines, thus confirming what observed in other tumor cells. HIF-1 alpha and NF-kB pathways are intimately associated and a significant cross-talk between them exists on different levels. In fact, a number of stimuli up-regulates the expression of HIF-1 alpha through NF-kB-dependent mechanisms [15,16]. On the other hand, NF-kB was reported to be regulated by HIF-1 alpha in neutrophils

[18]. In different tumors, genes involved in the inflammatory pathway are up-regulated and, in some cases, related to prognosis [39]. Some of them, including inducible NO synthase and COX2, contain functional response elements for both NF-kB and HIF-1 alpha in their promoters, even though the relative contribution of these transcriptional regulators remains controversial [40-43]. We examined the mutual regulation of HIF-1 alpha and NF-kB and their contribution to the modulation of the studied set of inflammation-related genes under hypoxia, by silencing HIF-1 alpha and inhibiting NF-kB. MSTO cells were identified as a suitable model since they showed an NF-kB pathway more responsive to oxygen deprivation and a higher level of CXCR4 and COX2 up-regulation. NF-kB inhibition reduced mRNA HIF-1 alpha levels in both normoxia and hypoxia, confirming the role of NF-kB in basal and stimulated HIF-1 alpha mRNA expression, previously demonstrated for other cellular models [13]. Conversely, HIF-1 alpha silencing did not induce variation in the nuclear translocation of p65 and in the level of phosphorylated Ser276 P65, both in normoxia and hypoxia, strongly suggesting that NF-kB pathway is not dependent on HIF-1 alpha activity in this cell line.

Finally, the hypoxic up-regulation in most of the studied inflammation-related genes, except for CXCR4 and P2X7R, was shown deriving from the combined transactivation activity of both HIF-1 alpha and NF-kB. Therefore, the tuning of the pro-inflammatory phenotype in MSTO, relies on a complex balance between the action of these transcription factors that in turn is strongly influenced by the

environmental changes. The transcriptional modulation of the anti-inflammatory gene HMOX1 appears to be even more complex. HIF-1 alpha silencing reduced the increase of HMOX expression in hypoxia, whereas NF-kB inhibition resulted in a strong up-regulation of this gene in hypoxic and normoxic conditions both in HIF-1 alpha silenced and in HIF-1 alpha expressing MSTO. These results confirm the dual control of this molecule we observed in prostate cells [44], where NF-kB acts as a repressor under normoxia and hypoxia and HIF-1 alpha as an inducer under hypoxia.

## Conclusions

We can summarize our findings as follows:

1. For the first time, a cross-talk between hypoxia adaptation and pro-inflammatory pathway was revealed in MM which was accomplished through the interaction of the transcription factors HIF-1 alpha and NF-kB.

2. The involvement of HIF-3 alpha in MM response to hypoxic stimulation was demonstrated and a possible correlation between its expression in MM tissues and prognosis was suggested.

3. The over-expression of CXCR4 in human MM tissues was confirmed and a statistically significant association between high levels of CXCR4 and poor outcome was highlighted.

## Competing Interests

The authors declare that they have no competing interests.

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