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The effect of tumor microenvironmental stress on malignant mesothelioma

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The effect of tumor microenvironmental stress on malignant mesothelioma

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ABSTRACT

The effect of tumor microenvironmental stress on malignant mesothelioma

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Compared to normal cells, cancer cells survive and acquire more aggressive phenotypes under hostile tumor microenvironment in which oxidative stress and tumor hypoxia are the most pervasive environmental stress. Human malignant mesothelioma (HMM) is asbestos-related aggressive cancer. The importance of tumor microenvironmental stress has been relatively overlooked and rarely studied in HMM progression. In the present study, we investigated the effect of oxidative stress and tumor hypoxia on HMM. Upon the addition of hydrogen peroxide (H_2O_2) to HMM cells, epithelial to mesenchymal transition (EMT) was markedly induced, as evidenced by upregulation of vimentin, *SLUG*, and TWIST1, and

downregulation of E-cadherin. Treatment of HMM cells with H_2O_2 significantly upregulated the expression of stemness genes, such as *OCT4*, *SOX2*, and *NANOG*. Alteration of these genes was mediated via activation of hypoxia-inducible factor 1 alpha (HIF-1α) and transforming growth factor beta 1 (TGF-β1). Immunohistochemically, EMT-promoting protein TWIST1 was solely expressed to the nucleus of sarcomatoid cells in HMM tissues. Hypoxic conditions (2.2% O_2) induced the expression of HIF-1α and HIF-2α in parallel with the upregulation of their target GLUT-1 in HMM cells. HMM cells under hypoxia showed more aggressive phenotypes regarding *in vitro* clonogenicity, apoptosis, drug resistance, and mobility and invasiveness. The enhancement of *in vitro* clonogenicity was mediated by upregulation of HIF-2α, OCT4, and CD44 in hypoxic HMM cells. Meanwhile, the expression of p-Akt and $NOTCH1^{IC}$ was not significantly altered in hypoxic HMM cells. On the other hand, cisplatin rapidly degraded HIF-1α and HIF-2α proteins, and HIFα expression was not detected in HMM cells during hypoxic conditions. Hypoxia-induced upregulation of Bcl-2 was shown to decrease apoptotic potential, and the increased ratio of Bcl-2 to Bax was suggested to mediate hypoxia-induced drug resistance in HMM cells. Hypoxia significantly promoted EMT in HMM cells, as evidenced by downregulation of E-cadherin, upregulation of vimentin, and acquisition of sarcomatoid HMM morphology. Mitochondria within HMM cells were hypothesized to be the potential candidate to overcome drug resistance arising from tumor hypoxia. The viability of HMM cells cultured under

hypoxia (0.1% O_2) was less affected by cisplatin treatment, compared to those cultured under normoxia. Hypoxia significantly inhibited cisplatin-induced apoptosis in HMM cells. HMM cells under hypoxia inhibited cisplatin-induced detrimental effects on $\Delta\Psi_M$, redox status, mitochondrial DNA (mtDNA) integrity, and ultrastructure of mitochondria. Hypoxia exhibited mitochondrial hyperpolarization and inhibited cisplatin-induced mitochondrial depolarization in HMM cells. The mitochondrial hyperpolarization by hypoxia was augmented by the addition of cisplatin. The hyperpolarized phase of mitochondria was not related to ATP production nor reversal of ATP synthase. The mitochondrial depolarization was not due to the opening of mitochondrial permeability transition pore in HMM cells. Hypoxia significantly inhibited cisplatin-induced mitochondrial oxidative stress and consequent damages to mtDNA and mitochondrial ultrastructure. Redox compartmentalization was observed within HMM cells cotreated with cisplatin and hypoxia. Long-term treatment of low dose ethidium bromide significantly depleted mtDNA in HMM cells. The mtDNA-depleted HMM cells showed a significant reduction in cell proliferation, cell viability, $\Delta\Psi_M$, intracellular ATP content, mitochondrial ROS generation, and mitochondrial mass, compared to parental cells. The ρ^0 HMM cells were demonstrated to lose their ability to induce hypoxia-induced drug resistance. Also, ρ^0 HMM cells under hypoxia failed to mitigate cisplatin-induced mitochondrial oxidative stress. Taken together, our results clearly demonstrate that oxidative stress and hypoxia are a critical part of cancer

progression in HMM. A control measure for oxidative stress and tumor hypoxia may be an effective therapeutic strategy to reduce the aggressiveness of cancer cells in patients with HMM. In addition, it can be concluded that mitochondrially encoded ETC subunits are the very core of mitochondria, allowing HMM cells under hypoxia to induce drug resistance. The present study is valuable to provide convincing evidence for the therapeutic potential of mtDNA targeting to overcome drug resistance arising from tumor hypoxia. Data presented in this study will also scientifically contribute to the understanding of molecular mechanisms for cell or organism adaptive response to hypoxic stress encountered during normal and pathophysiological conditions.

Keywords: human malignant mesothelioma, hostile tumor microenvironment, oxidative stress, tumor hypoxia, drug resistance, mitochondria, mtDNA, electron transfer chain system.

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CONTENTS

ABBREVIATIONS

HMM: human malignant mesothelioma

ROS: reactive oxygen species

8-OHdG: 8-hydroxy-2'-deoxyguanosine

HIFα: hypoxia inducible factor alpha

HIF-1α: hypoxia inducible factor 1 alpha

HIF-2α: hypoxia inducible factor 2 alpha

HIF-1/2α: hypoxia inducible factor 1 and 2 alpha

PHD: oxygen-dependent prolyl-hydroxylase

VHL: von Hippel-Lindau

EMT: epithelial to mesenchymal transition

MET: mesenchymal to epithelial transition

ECM: extracellular matrix

CSCs: cancer stem cells

TGF-β1: transforming growth factor beta 1

OCT4: octamer-binding transcription factor 4

RT-PCR: reverse transcription polymerase chain reaction

qPCR: quantitative polymerase chain reaction

Glut-1: glucose transporter 1

Bcl-2: B-cell lymphoma 2

Bax: Bcl-2-associated X protein

Bcl-xL: B-cell lymphoma-extra large

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

MMP: mitochondrial membrane potential, $\Delta\Psi_M$

MPT: mitochondrial permeability transition

mPTP: mitochondrial permeability transition pore

mtDNA: mitochondrial DNA

mtROS: mitochondrial reactive oxygen species

OXPHOS: oxidative phosphorylation

ETC: electron transport chain

TCA: tricarboxylic acid

EtBr: ethidium bromide

MAPK: mitogen-activated protein kinase

P-Akt: phosphorylated Akt

PKC: Protein kinase C

PI3K: phosphoinositide 3-kinase

PTEN: phosphatase and tensin homologue ()

AP-1: activator protein 1

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

DCFDA: 2′ ,7 ′-Dichlorofluorescin diacetate

JC-1: 5,5',6,6'-tetrachloro-1,1',3,3' -tetraethyl-benzimidazolylcarbocyanine chloride

ID_{50}: Half maximal inhibitory dose

CCCP: Carbonyl cyanide m-chlorophenyl hydrazone

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LITERATURE REVIEW

Tumor tissue oxygenation and abnormal tumor vasculature

Molecular oxygen (O_2) is required for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many organic and inorganic reactions (Huang et al. 2006, Brahimi-Horn et al. 2007, Bertout et al. 2008).

Ambient air is 21% O_2 (150 mm Hg) and most mammalian tissues exist at 2% to 9% O_2 (24 to 66 mm Hg; on average 40 mm Hg) (Vaupel et al. 1989, Bertout et al. 2008). Hypoxia is defined as reduced O_2 levels and occurs in a variety of pathological conditions, such as stroke, inflammation, and the growth of the tumor (Bertout et al. 2008). Compared to corresponding normal tissues, tumor tissue oxygenation is poor with the median pO_2 value of less than 10 to 20 mm Hg in tumor tissues of patients (Vaupel et al. 1989, Brahimi-Horn and Pouysségur 2007). Tumor hypoxia is a common feature in human malignancies (Vaupel et al. 1987). Clinical investigations have shown that 50% to 60% of locally advanced solid tumors have hypoxic and ischemic regions (Brown et al. 2004, Bache et al. 2008, Walsh et al. 2014).

Tumor vasculature is poorly disorganized and structurally and functionally abnormal, compared to normal vasculature (Vaupel et al. 1989, Fukumura et al. 2007). Capillaries are frequently fenestrated and discontinuous, and venules are often tortuous, saccular, and dilated (Jain 1988). The arteriovenous anastomoses, a direct shunting of blood vessels from the arterial to the venous side, are commonly found in tumor tissues (Jain 1988,

Vaupel et al. 2001). Consequently, these structural abnormalities contribute to the chaotic tumor microcirculation, such as fluctuation in the bloodstream, intermittent blood perfusion, and even blood flow stasis (Jain 1988, Vaupel et al. 1989, Fukumura and Jain 2007). This perfusion-limited O_2 delivery to tumor cells leads to transient acute hypoxia (Figure 1A) (Vaupel et al. 1989, Fukumura and Jain 2007). Cancer cells that initially access blood-borne oxygen (O_2) from preexisting host vessel start to stimulate neovascularization during rapid tumor expansion (Vaupel et al. 1989). The angiogenesis, however, usually does not overtake uncontrolled, dysregulated proliferation of the cancer cells (Vaupel et al. 1989). Moreover, the diffusion distance of O_2 is as low as approximately 70 to 150 μM from a vessel (Coleman 1988, Vaupel 2004). Thus, tumor cells beyond the diffusion limit become hypoxic or anoxic (Moolgavkar et al. 1981, Vaupel et al. 1989). Chronic hypoxia occurs as the size of tumor cell aggregates reaches the diffusion limit of O_2 from blood vessels (Figure 1B) (Vaupel et al. 1989). Meanwhile, the reduction or the lack of transmural pressure due to interstitial hypertension also aggravates the penetration and delivery of O_2 to tumor cells (Yuan et al. 1994, Fukumura and Jain 2007). The high hydrostatic pressure is a result of no functional lymphatic vessels in tumor tissues (Leu et al. 2000, Padera et al. 2004, Jain et al. 2014)

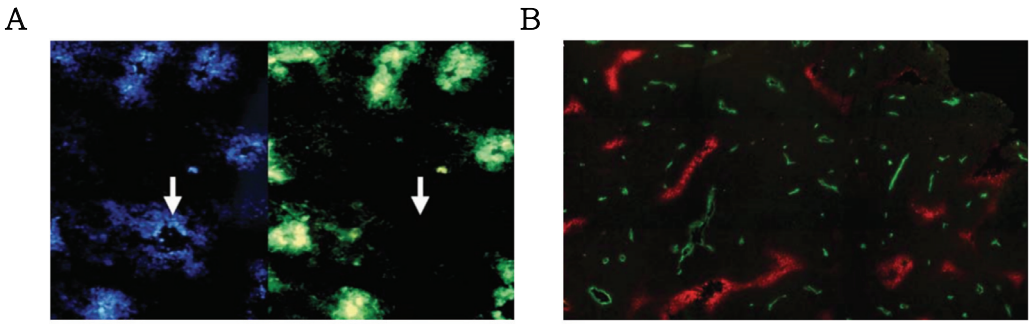


Figure 1. Clinical evidence of acute and chronic hypoxia in human solid tumor. (A) Acute hypoxia. Hoechst 33342 (blue) and DiOC7 (green) should stain the same cells immediately adjacent to the well-perfused vasculature. Staining disparity (arrow) represents heterogeneous chaotic microcirculation and existence of acute hypoxia. A 20-minute period between injection of the first Hoechst 33342 and second DiOC7 dyes. (B) Chronic hypoxia. Red and green colors represent nitroimidazole-positive hypoxic tumor cells and CD31-positive blood vessels, respectively. Human soft tissue sarcoma. Adapted from (Brown 2002).

Tumor hypoxia and cancer progression

Tumor hypoxia is the most pervasive and best characterized of the hostile environmental stresses (Vaupel et al. 1989, Harris 2002). The O_2-depleted areas can arise prior to nutrient deprivation during the very early growth stages of xenografted human tumors (Vaupel et al. 1989). Although a study has revealed that glucose depletion increases metastatic potential of tumor cells (Cuvier et al. 1997), it is still controversial whether glucose starvation plays a critical role in cancer progression *in vivo* (Vaupel 2010). Clinically, glucose concentrations in tumor cells are rarely below 1 mM (Vaupel 1994). Tumor hypoxia is the most independent prognostic factor for predicting tumor behavior, response to treatment, and clinical outcome in advanced disease progression (Höckel et al. 1996). Tumor hypoxia has a negative impact on the effectiveness of curative treatment, leading to local failure and recurrence (Teicher 1994, Kim et al. 2017).

Tumor hypoxia results in cancer progression (Cosse et al. 2008). Accumulating evidence reveals that hypoxia affects most of the tumor cell properties, such as cell proliferation, apoptosis, metabolism, immune responses, genomic instability, vascularization, and invasion and metastasis (Graeber et al. 1996, Höckel et al. 1996, Vaupel et al. 2007, Wigerup et al. 2016). A variety of genes and/or proteins, as well as intracellular signaling pathways, have been reported to mediate tumor cell adaptation to hypoxia, leading to the acquisition of aggressive phenotypes in hypoxic tumor cells (Vaupel 2008). Of the various molecules that sense and respond to hypoxia,

hypoxia-inducible factor (HIF) transcription factor has been extensively recognized the master regulator of hypoxia-induced cellular responses (Semenza 2000, Liu et al. 2012).

The HIF is a heterodimeric complex that consists of O_2-labile α subunit, including HIF-1α and HIF-2α, and an O_2-stable β subunit (Wang et al. 1995). The HIFα subunits are reported to share a high degree of sequence identity and undergo a similar proteolytic regulation in a post-translational manner (Wang et al. 1995, Pugh et al. 2003). Under the reduced O_2 availability, prolines residues 402 and 564 within oxygen-dependent degradation domain of HIFα are hydroxylated by oxygen-dependent prolyl-hydroxylase (PHD) (Ivan et al. 2001, Jaakkola et al. 2001, JEWELL et al. 2001). The hydroxylated HIFα is subjected to binding of von Hippel-Lindau (VHL) tumor, an E3 ligase that ubiquitinates HIFα, which is degraded by 26S proteasomal degradation pathway (Maxwell et al. 1999, Ohh et al. 2000). The β subunit of HIF, also called aryl hydrocarbon receptor nuclear translocator, is constitutively expressed in cells (Wang et al. 1995). HIF-1α is translocated to the nucleus where it dimerizes with HIF-1β, and the HIF-1 α/β complex binds hypoxia-responsive elements in the promoter, 5'-untranslated, or 3'-untranslated regions of its target genes (Semenza 2007). There are hundreds of target genes of HIFα (Loboda et al. 2010, Keith et al. 2012, Liu et al. 2012). The HIF-1α and HIF-2α are reported to transactivate overlapping but distinct set of their target genes (Sowter et al. 2003). Among their targets, genes primarily involved in anaerobic glycolysis,

angiogenesis, proliferation, growth, survival, and apoptosis, are under the control of HIF-1α (Liu et al. 2012). HIF-2α is reported to regulate genes involved in proliferation, stemness, and migration and invasion (Covello et al. 2006, Gordan et al. 2007, Kim et al. 2009).

Tumor cell adaptation to hypoxia has been well-documented as follows. Cancer cells reprogram their energy metabolism by increasing anaerobic glycolysis and decreasing mitochondrial oxidative phosphorylation (Zhang et al. 2007). The metabolic shift is a beneficial trade-off for tumor cell proliferation (Zhang et al. 2007). Firstly, anaerobic glycolysis provides free energy, despite the low-yield, but high-speed ATP production (Pfeiffer et al. 2001, Shestov et al. 2014). Secondly, the glycolysis provides intermediates for biosynthesis of macromolecules, such as pyruvate that is used for lipid or ribose synthesis (Denko 2008). Finally, glycolysis generates less ROS, which contributes to mitigation of oxidative stress in tumor cells (Brand et al. 1997, Zhang et al. 2008).

Tumor cells under hypoxia regulate cell cycle progression by affecting p53 and p21 (Guo et al. 2018). HIF-1α directly represses c-Myc activity, which in turn activates p21 and leads to cell cycle arrest (Koshiji et al. 2004, Gordan et al. 2007). At the same time, the c-Myc activity can be also enhanced by HIF-2α under hypoxia, which is known to progress cell cycle and proliferation (Gordan et al. 2007). Cell cycle arrest has been involved in drug resistance (Tannock 1978, Åmellem et al. 1991, Guo et al. 2018).

Hypoxia is responsible for clonal selection with diminished apoptotic

potential (Graeber et al. 1996). HIF-1α can facilitate the clonal dominance and propagation of the apoptosis-resistant phenotype via tight regulation of pro-and anti-apoptotic proteins (Erler et al. 2004, Sasabe et al. 2005) and activation of key signaling pathways (Mazure et al. 1997, Richard et al. 1999, Zhong et al. 2000, Alvarez-Tejado et al. 2001). In addition to HIF-1α, HIF-2α also plays an important role in aggressive phenotypes of tumor cells, including drug resistance (Holmquist-Mengelbier et al. 2006, Bertout et al. 2009, Rouault-Pierre et al. 2013).

Hypoxia is known to restrain differentiation and maintain the undifferentiated status of tumor cells (Lin et al. 2006, Kim et al. 2009). In a hypoxic niche, some cancer cells are transformed to cancer stem cells (CSCs) with self-renewal capacity (Jordan et al. 2006). HIF-2α plays an important role in the generation and maintenance of the hypoxia-induced stem cell-like phenotype via OCT4 activation (Covello et al. 2006, Heddleston et al. 2009, Qing et al. 2009). The acquisition of stemness by hypoxia is aso associated with cell survival and drug resistance by several mechanisms, such as upregulation of ABC transporter MDR1 (Sakata et al. 1991, Comerford et al. 2002, Liu et al. 2008, Nardinocchi et al. 2009), ABCG2 (Martin et al. 2008), human telomerase (hTERT) (Nishi et al. 2004), and enhanced DNA repair system (Wagemaker 1995, Bao et al. 2006).

During hypoxia, EMT is strongly induced in tumor cells (Jiang et al. 2011). The EMT is defined by the loss of epithelial cell polarity and gain of mesenchymal phenotypes (Yang et al. 2008). The EMT process is

fundamentally associated with loss of cell to cell conjunction, which involves in the enhancement of cell migration, invasion, and metastasis (Yang and Weinberg 2008). Beyond its literal definition, EMT is also significantly related to tumor aggressiveness (Yang and Weinberg 2008). A growing body of evidence has shown that hypoxia and EMT are reciprocally linked to acquisition of stemness of cancer cells and drug resistance (Singh et al. 2010, Jiang et al. 2011). Hypoxia and HIF-1α are reported to stimulate EMT of cancer cells by regulating a variety of EMT-related molecules, such as TGF-β, TNFα, NFκB, and TWIST (Yang et al. 2008, Jiang et al. 2011). HIF-2α can increase the expression of several EMT genes, including SIP1, SNAIL, ZEB1, and vimentin (Kim et al. 2009).

In the long term, hypoxia increases genomic instability (Vaupel et al. 2001, Bristow et al. 2008). Although DNA repair system can be activated by hypoxia in tumor cells (Walker et al. 1994), hypoxia is reported to compromise DNA repair ability of tumor cells by decreasing gene expressions involved in the DNA mismatch repair system, homologous repair, and non-homologous end-joining (Bristow and Hill 2008, Li 2008, Rodríguez-Jiménez et al. 2008). The increased frequency and genomic instability might be harmful for immediate cancer cell survival (Greijer et al. 2004). However, at the same time, hypoxic tumor cells that are genetically unstable are more likely to obtain DNA mutations (Greijer and Van der Wall 2004). The genetic instability is a potential source of the appearance of cancer cells that are more malignant, more aggressive, and less susceptible

to apoptosis, thus rendering them resistant to various therapies (Vaupel et al. 2001, Cosse and Michiels 2008).

Tumor hypoxia can be harmful for cell survival and growth, because rapid onset of O_2 deficiency depletes intracellular ATP, leading to catastrophic loss of intracellular K^+ and necrotic cell death (Boutilier et al. 2000). Chronic and persistent hypoxia can also induce apoptosis via p53, Bcl-2 related pro-apoptotic proteins, c-Jun NH_2-terminal kinases, HIF-1α, and ROS stress (An et al. 1998, Basu et al. 1998, Carmeliet et al. 1998, Kim et al. 2003, Greijer and Van der Wall 2004). Nevertheless, a fraction of tumor cells always adapt and survive under hypoxia, which is the main culprit for the appearance of more aggressive phenotypes (Denko 2008).

ROS, oxidative stress, and cancer progression

ROS encompass a wide range of intermediate oxygen-carrying metabolites with or without unpaired electrons (Policastro et al. 2013). The unpaired electrons or O_2-derived free radical include mainly superoxide anion ($O_2\cdot^-$) and hydroxyl radical ($\cdot OH^-$), while nonradicals mainly include hydrogen peroxide (H_2O_2) (Policastro et al. 2013).

ROS is generated from various cellular sources, including mitochondrial electron transport chain (ETC) complex, NADPH oxidase, cytochrome P450, lipoxygenases, cyclooxygenases, xanthine oxidases, and peroxisomal enzymes (Policastro et al. 2013). Among them, mitochondrial ETC and NADPH oxidase are a major source of ROS production. Within cells, $O_2\cdot^-$ is converted to H_2O_2 spontaneously and/or enzymatically by superoxide dismutase (SODs) (Policastro et al. 2013). The H_2O_2 is finally decomposed into H_2O and O_2 by a variety of antioxidant enzymes, such as catalase, glutathione peroxidase, and peroxiredoxin (Policastro et al. 2013).

Compelling experimental and clinical evidence reveals that tumor cells have underwent persistent oxidative stress, compared to their normal counterparts (Figure 2) (Evans et al. 2004, Bahar et al. 2007, Fruehauf et al. 2007, López-Lázaro 2007, Chang et al. 2008). The increase in basal levels of intracellular ROS is associated with genetic alterations, mitochondrial dysfunction, aberrant metabolism, deregulation of the antioxidant system,

and tumor microenvironments (Cook et al. 2004, Fiaschi et al. 2012). Although excessive ROS accumulation induces oxidative stress and damages to nucleic acids, proteins, and lipids, cancer cells are found to not only sustain their growth, survival, and proliferation but also gain more aggressive phenotypes (Hensley et al. 2000, Evans et al. 2004, Giannoni et al. 2012).

A variety of redox-responsive protein kinases and phosphatase have been reported to play a role in tumor cell growth and proliferation (Schieber et al. 2014). The mode of action of ROS is largely dependent on oxidation-reduction processes that involve oxidation of cysteine residues within proteins (Rhee 2006). Representative examples include Ras-ERK, Ras-JNK, mitogen-activated protein kinase (MAPK), and PI3K/Akt intracellular pathways (Gius et al. 2006, Weinberg et al. 2009, Circu et al. 2010, Zhang et al. 2011).

Oxidative stress promotes more aggressive phenotypes of cancer cells, including apoptosis resistance, stemness, and migration and invasion (Landriscina et al. 2009, Giannoni et al. 2012). For the mechanisms, p53 deficiency (Brown et al. 2001), antioxidant enzymes (Lazo et al. 1998), p-glycoprotein (Ziemann et al. 1999), and PI3K/Akt signaling (Morag et al. 1998) have been implicated. The resistance to oxidative stress is directly linked to development of drug resistance (Yokomizo et al. 1995, Lazo et al. 1998, Landriscina et al. 2009). In the long term, oxidative stress increases

genomic instability and frequency of DNA mutation, which is a critical factor in generation of heterogenous populations of cancer cells, such as cancer cells with decreased sensitivity to drug (Szatrowski et al. 1991).

Oxidative stress is closely associated with EMT (Giannoni et al. 2012). EMT is a critical factor in stemness, drug resistance, and metastasis in cancer cells (Singh and Settleman 2010). A variety of redox-mediated mediators or signaling pathways have been reported to promote EMT (Gius and Spitz 2006, Jiang et al. 2011). Detailed information about the redox-mediated molecular events leading to EMT is well-documented (Giannoni et al. 2012). TGF-β1 is a best-characterized EMT-related molecule (Xu et al. 2009, Giannoni et al. 2012). Exogeneous H_2O_2 activates TGF-β1 signaling via mitochondrial $O_2\cdot^-$ generation in lung cancer cells (Gorowiec et al. 2012, Zhang et al. 2018). The TGF-β1 is an upstream regulator of NOX4 expression that produces $O_2\cdot^-$, which provides breast tumor cells with migratory, invasive, and metastatic properties (Boudreau et al. 2012). During EMT process, TGF-β1 also promotes acquisition of H_2O_2-mediated stemness in breast cancer cells (Karicheva et al. 2016). The oxidative stress-mediated enhancement of tumorigenicity or clonogenicity is mediated by activation of OCT4, SOX2, or Snail (Mahalingaiah et al. 2015, Kim et al. 2017). In addition to EMT and stemness, TGF-β1 is also involved in drug resistance (Karicheva et al. 2016, Tang et al. 2018).

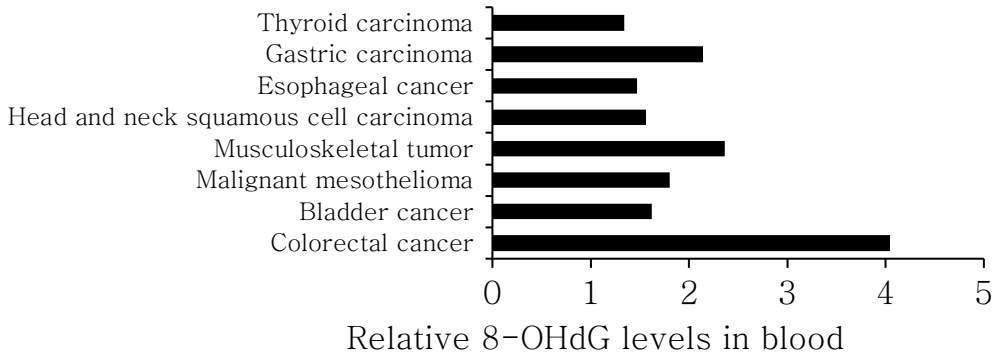


Figure 2. Clinical evidence of ROS stress in human solid tumors. (A) Cancer patients show relative high levels of peripheral blood 8-OHdG, which is normalized to corresponding healthy controls.

Adapted from (Akçay et al. 2003, Amati et al. 2008, Chang et al. 2008, Lagadu et al. 2010, Kumar et al. 2012, Borrego et al. 2013, Tabur et al. 2015, Woraruthai et al. 2018).

Tumor microenvironment, tumor hypoxia, and drug resistance

For the drug resistance, tumor cell intrinsic factors exist, such as enhanced drug efflux or DNA repair system (Gottesman 2002, Gatti et al. 2005). However, numerous extrinsic factors also exist in tumor microenvironment (Morin 2003, Trédan et al. 2007).

The heterogeneous microcirculation, poor perfusion, and interstitial hypertension compromises the extravasation of drugs (Morin 2003). The penetration and distribution of drugs into tumor tissues, especially high-molecular weight agents, is physically impeded by ECM components in tumor microenvironments (Berk et al. 1997, Kuh et al. 1999, Netti et al. 2000, Au et al. 2002, Grantab et al. 2006). A large fraction of tumor cells in solid tumors cannot be exposed to lethal concentrations of drugs, including the cases of doxorubicin, methotrexate, vincristine, vinblastine, cisplatin, and some other drugs (Durand 1986). Acidic tumor microenvironment negatively influences on the activity or transport of drugs, such as in the cases of doxorubicin, mitoxantrone, paclitaxel, vincristine, and vinblastine (Cowan et al. 2001, Mahoney et al. 2003, Trédan et al. 2007). On the other hand, the lack of oxygen itself can reduce the efficacy of drugs, because some drugs, such as bleomycin, etoposide, and doxorubicin, directly utilize O_2 to fully elicit cytotoxicity via free radical generation and oxidative stress (Teicher 1994, Wardman 2001, Shannon et al. 2003).

Despite the arrival of drugs into tumor cells, a fraction of tumor cells is always problematic, because they are transformed to resist hypoxic stress

and refractory to chemotherapy (Kim and Lee 2017). The mechanisms underlying hypoxia-associated drug resistance are highly complex, multifactorial, and different depending on drugs used, cell types, and experimental conditions (Rohwer et al. 2011, Doktorova et al. 2015). Currently, HIFα is considered as a central mediator of hypoxia-associated drug resistance in multiple tumor types (Unruh et al. 2003, Brown et al. 2005, Hao et al. 2008, Liu et al. 2008, Sullivan et al. 2008, Nardinocchi et al. 2009, Sullivan et al. 2009, Daskalow et al. 2010, Sasabe et al. 2010, Deben et al. 2018). Apoptosis inhibition is a major phenotype of drug resistance in hypoxic tumor cells (Rohwer and Cramer 2011), although inhibition of other cell death forms, such as senescence (Sullivan et al. 2008) or necroptosis (Huang et al. 2013), is also involved in hypoxia-induced drug resistance.

Enhanced drug efflux is one of the important mechanisms underlying drug resistance (Chen et al. 2016). It has been demonstrated that HIFα contributes to low intracellular drug concentration via activation of ABC-binding cassette (ABC) transporters, including multidrug resistance 1 (MDR1), ABCG2, and multidrug resistance-associated protein (MRP) (Sakata et al. 1991, Comerford et al. 2002, Liu et al. 2008, Martin et al. 2008, Chen et al. 2009, Chen et al. 2014, Lv et al. 2015).

Dysregulation in many pro- and anti-apoptotic Bcl-2 family members are involved in hypoxia drug resistance in a HIFα-dependent or independent manner, possibly due to inhibition of MPT or mPTP opening (Dong et al.

2004, Cosse and Michiels 2008, Sermeus et al. 2012).

The p53 is a key player to induce apoptosis in response to DNA damages in human cancers (Wang et al. 1996). Hypoxia is a potent selector of p53-deficient apoptosis-defective tumor cells (Teicher 1994, Cavalli et al. 1997), and suppression of p53 induced by hypoxia results in cisplatin resistance (Wang et al. 2006). HIFα is known to antagonize p53-mediated apoptosis in hypoxic tumor cells (Bertout et al. 2009, Rohwer et al. 2010, Sendoel et al. 2010, Nardinocchi et al. 2011). In addition, the cytoprotective effect of hypoxia on cisplatin-induced apoptosis does not occur in p53-mutant cancer cells (Hao et al. 2008). However, the role of p53 in the drug resistance under hypoxic condition is controversial. A recent study suggested that p53 plays a role in low-dose cisplatin-induced drug resistance of hypoxic lung cancer cells (Guo et al. 2018).

Inhibition of DNA damage is a drug resistance phenotype in hypoxic tumor cells (Sullivan and Graham 2009). Previous studies have shown that HIF-1α has a role in the repair of damaged DNA (Walker et al. 1994, Wirthner et al. 2008), and cell cycle arrest or decreased cell division contributes to prevention of initial DNA damage (Valencia-Cervantes et al. 2018). The importance of cell cycle regulation can be noted in hypoxic CSCs (Schoning et al. 2017). The quiescent CSCs in a hypoxic niche are demonstrated to be highly resistant to drugs due to HIFα activation, altered metabolism, enhanced drug efflux, and PI3K/Akt and Wnt/Notch signaling pathway (Liao et al. 2014, Schoning et al. 2017, Uribe et al. 2017, Yan et al. 2018).

Meanwhile, inhibition of DNA damage might be unrelated to DNA repair or cell cycle arrest (Shannon et al. 2003, Wohlkoenig et al. 2011, Cho et al. 2013).

Autophagy is a part of hypoxic adaptive mechanisms as an energy recycler and a metabolic provider of cellular breakdown products to cells for biosynthesis and survival (Zhang et al. 2008). Although it could be controversial whether autophagy confers tumor cells drug resistance under hypoxia, general consensus have indicated that autophagy induced by hypoxia is cytoprotective against drug-induced cytotoxicity (Liu et al. 2010, Hu et al. 2012, Lee et al. 2015, Wu et al. 2015, Chen et al. 2017, Tan et al. 2017, Yang et al. 2018). HIF-1α is a central regulator of autophagy by targeting BNIP3 and BNIP3L that are required for the induction of autophagy (Bellot et al. 2009, Yang et al. 2018).

Mitochondria are a critical mediator of drug resistance, and inhibition of mitochondrial activity or function is correlated with hypoxia-induced drug resistance (Indran et al. 2011). Mounting evidence have revealed that remodeled mitochondrial ETC complexes by hypoxia plays an important role in drug resistance (Wang et al. 2006, Oliva et al. 2010, Oliva et al. 2011, Cho et al. 2013, Okamoto et al. 2017). HIF-1α is reported to modify cytochrome *c* oxidase (COX) IV activity, which contributes to decreased ROS production and protection of tumor cells from oxidative stress in hypoxia (Fukuda et al. 2007, Zhao et al. 2014). HIF-1α also inhibits mitochondrial metabolism by PDK-induced PDH inhibition, which blocks the

conversion of pyruvate into acetyl-coenzyme A, an initial respiratory substrate for TCA cycle within mitochondria (Kim et al. 2006, Lu et al. 2008). Inhibition of mPTP and prevention of intracellular acidification can be also a cause of drug resistance under hypoxia (Brahimi-Horn et al. 2012, Pellegrini et al. 2012, Ferecatu et al. 2018). On the other hand, in some types of cells, mitochondrial inhibition by hypoxia can enhance drug-induced apoptosis (Schwerdt et al. 2005).

Currently, experiments that are designed to overcome the hypoxia-induced drug resistance are largely depended on silencing of HIFα. Although HIFα has been extensively investigated as an important factor to underpin hypoxia-induced drug resistance, it is also true that drug resistance does develop independently of HIF (Doktorova et al. 2015). Previous studies have shown that reactivation of the suppressed mitochondria reverses hypoxia-mediated drug resistance (Lu et al. 2008, Shin et al. 2013). Alternatively, direct perturbations of mitochondrial metabolism or functional integrity appears to be promising to increase drug efficacy of hypoxic cancer cells (Xu et al. 2005, Kumar et al. 2013, Kulikov et al. 2014, Mitani et al. 2014, Xuan et al. 2014). The mitochondrial targeting is beneficial to circumvent the problem of apoptosis resistance upstream of the intrinsic mitochondrial pathway without damaging nuclear DNA (Wisnovsky et al. 2013).

Human malignant mesothelioma and tumor microenvironment

Human malignant mesothelioma (HMM) is a rare but lethal cancer arising from mesothelium lining of pleural space, pericardium, peritoneum, and tunica vaginalis testis and ovarian epithelium (Robinson et al. 2005). More than 80% of HMM cases arise from pleural cavity (Mery et al. 2014, Zhang et al. 2015). Due to the long latency period more than 30 years between exposure and presentation (Peto et al. 1995), no single causal factors sufficiently explain HMM pathogenesis. However, general consensus reveals that occupational or domestic exposure to asbestos is a critical factor for mesothelial carcinogenesis (Wagner et al. 1960, Robinson et al. 2005). The most common types of asbestos fibers related to HMM induction are chrysotile (Suzuki et al. 2002). Asbestos consists of short and long fibers (Goodglick et al. 1990). Both types of fibers are cytotoxic as oxidant in an iron-dependent mechanism (Goodglick and Kane 1990). Asbestos fibers contain iron, which directly generates hydroxyl radicals via the catalysis of the Fenton reaction on the surface in the hemoglobin (Kamp et al. 1992, Maples et al. 1992). Uptake of asbestos fibers by mesothelial cells contributes to further oxidative stress (Dong et al. 1994). Macrophages that fail to uptake long fibers undergo cell death, which is an indirect source of ROS along with inflammatory molecules (Donaldson et al. 2010). The ROS production following the asbestos exposure leads to genomic damages in mesothelial cells (Chew et al. 2015). On the other hand, simian virus 40 (SV40) is another important cofactor to promote HMM development

(Carbone et al. 2000). Other fiber minerals, including erionite, may contribute to HMM development (Attanoos et al. 2018).

Abnormal genetic features of HMM include homozygous deletion of tumor suppressors $p16^{INK4a}$ and $p14^{ARF}$ and dysregulated neurofibromatosis type 2 (NF2) and tumor suppressor BRCA1 associated protein-1 (BAP1) (Papp et al. 2001, de Assis et al. 2014, Yap et al. 2017). Along with these genes, activation of MAPKs, PI3K/Akt/mTOR, and other key signaling pathways are reported to be critically involved in mesothelial tumorigenesis (de Assis et al. 2014, Yap et al. 2017). The role of p53 mutation in mesothelial carcinogenesis remains to be elucidated (Metcalf et al. 1992, Jean et al. 2018).

Over 3,000 patients are newly diagnosed with HMM in North America (Bianchi et al. 2014). The occurrence rate of HMM is anticipated to increase worldwide (Bianchi et al. 2007, Bianchi and Bianchi 2014). The majority of HMM cases show poor prognosis with survival of less than 1 year, which is largely attributable to drug resistance (Fennell et al. 2008, Blomberg et al. 2015). In HMM, apoptosis resistance to drugs occurs due to various mechanisms, including (a) enhanced drug efflux (Soini et al. 2001), (b) upregulation of antioxidant enzymes (Kahlos et al. 2001, Järvinen et al. 2002, Kinnula et al. 2002), (c) existence of tumor-initiating cells (Cortes-Dericks et al. 2014, Pasdar et al. 2015), (d) altered ratio of Bcl-2 family members (O'Kane et al. 2006, Jin et al. 2010), (e) epigenetic impairment of p53 function (Kubo et al. 2011), (f) autophagy induction (Lee et al. 2016), (g)

upregulation of specific molecules, such as heat shock proteins (Roth et al. 2009), Numb (Kang et al. 2013), and insulin-like growth factor 1 receptor (Kai et al. 2009), and (h) signal transduction dysregulation, such as MAPKs, Akt, and PTEN (Kai et al. 2009, Roth et al. 2009, Fischer et al. 2012).

Clinically, the elevated levels of 8-OHdG, a marker of oxidative DNA damage, has been detected from peripheral leukocytes in early and advanced HMM patients (Marczynski et al. 2000, Amati et al. 2008), suggesting the potential involvement of oxidative stress in cancer progression in HMM. Indeed, oxidative stress is demonstrated to play a critical role in HMM cell survival, growth, and proliferation (Chew and Toyokuni 2015, Tanaka et al. 2015). A variety of antioxidant enzymes support adaptation to ROS stress in HMM cells (Kahlos et al. 1998, Kinnula et al. 1998, Kahlos et al. 2001, Kahlos et al. 2001, Kinnula et al. 2002). Various redox-regulated signaling pathways have been reported to expand the initiated transformed HMM cell population (de Assis et al. 2014, Benedetti et al. 2015, Chew and Toyokuni 2015). Adaptation to oxidative stress is also related to chemoresistance in CD44-expressing HMM (Chew et al. 2017).

HMM is extremely heterogeneous with regard to morphology, showing three histological subtypes, including epithelioid type, which constitutes 60% of HMM, sarcomatoid type (20%), and biphasic type (30%) (Law et al. 1982, Attanoos et al. 1997). The morphologic transition from epithelioid to sarcomatoid histologic subtypes is significantly associated with EMT

process and cancer progression in HMM (Schramm et al. 2010, Fassina et al. 2012, Ye et al. 2015). The EMT process is a significant part of the acquisition of aggressive phenotypes of HMM cells, such as proliferation, migration, invasion, and stemness (Casarsa et al. 2011, Fassina et al. 2012). Previous studies have shown that TGF-β1, interleukin-1β, and H_2O_2 promote EMT in human mesothelial cells (Yáñez-Mó et al. 2003, Lee et al. 2007). HMM is demonstrated to undergo continuous oxidative stress (Marczynski et al. 2000, Amati et al. 2008).

Clinical and biological evidence of tumor microenvironment exists in HMM (Miselis et al. 2008, Burt et al. 2011, Wang et al. 2014, Ujiie et al. 2015). Emerging studies have identified tumor hypoxia in HMM tissues *in vivo* (Klabatsa et al. 2006, Ravenna et al. 2014, Francis et al. 2015, Nabavi et al. 2016). HIF-1α is commonly expressed in HMM (Klabatsa et al. 2006). HIF-1α is initially turned out to be not significantly associated with tumor aggressiveness (Klabatsa et al. 2006). Moreover, it is found that hypoxia increases the expression of the tumor suppressor, the estrogen receptor β, with MET induction (Manente et al. 2015). However, a few independent studies using HMM cell lines have next found that 1% to 3% hypoxic conditions can enhance survival (Graziani et al. 2008, Goudarzi et al. 2013, Zonca et al. 2017), drug resistance (Riganti et al. 2008, Giovannetti et al. 2016), and migration and invasion (Goudarzi et al. 2013). Mechanisms underlying the hypoxia-induced aggressive properties of HMM cells are found to relate HIF-1α and its downstream action on Notch1/2, p-

glycoprotein, transglutaminase 2, and MUC1 (Graziani et al. 2008, Riganti et al. 2008, Goudarzi et al. 2013, Goudarzi et al. 2013, Zonca et al. 2017). Inhibition of lactate dehydrogenase A is shown to overcome hypoxia-induced gemcitabine resistance in HMM cells (Giovannetti et al. 2016).

Experiments have been conducted to increase drug efficacy of apoptosis-resistant HMM. Antagonization of anti-apoptotic Bcl-2 family proteins was effective to provoke apoptosis in HMM cells (Hopkins-Donaldson et al. 2003, Cao et al. 2007, Littlejohn et al. 2008, Varin et al. 2010). Induction of mitochondria oxidative stress was found to enhance drug-induced apoptosis in HMM cells and their CSC populations (Tomasetti et al. 2004, Pasdar et al. 2015, Lee et al. 2016, Lee et al. 2016, Lee et al. 2017). More selective perturbations of mitochondrial ETC system or redox status have been proved to overcome drug resistance in HMM (Cunniff et al. 2013, Kovarova et al. 2014).

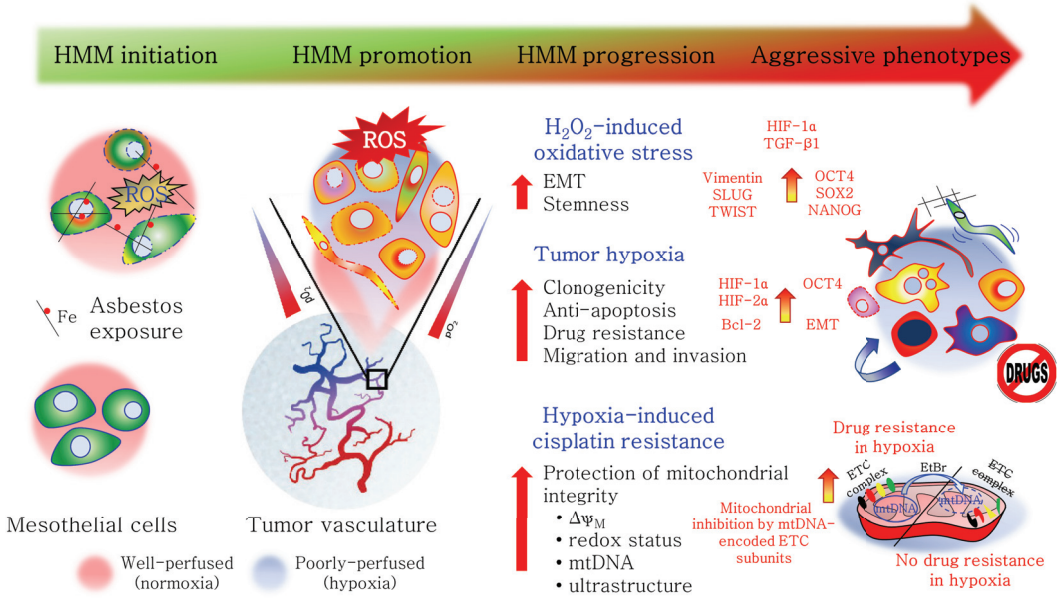
HMM is appreciated as a suitable model for the study of the effect of tumor microenvironmental stress. It is supported by the fact that normal mesothelial cells have a high capacity to cope with upon exposure to hypoxia and oxidative stress, showing phenotypic and biochemical changes, such as EMT (Molinas et al. 2000, Yáñez-Mó et al. 2003, Lee and Ha 2007, Nagai et al. 2013). Moreover, normal tissue oxygenation of pleural and peritoneal mesothelial cells is low compared to other tissues (Wang et al. 2005), and HMM cells undergo additional oxygen deficit (Ravenna et al. 2014, Francis et al. 2015). Currently, a few studies have addressed the

correlation between tumor microenvironment and clinical behaviors of HMM. Moreover, the cause of a poor patient outcome with regard to hypoxia-induced cisplatin resistance remains largely unknown in HMM. Cisplatin is the only established first-line chemotherapy for HMM (van Zandwijk et al. 2013). Time-dependent experiments have shown that nucleus is not a primary target site of cisplatin (Janson 2008).

Summary

Oxidative stress and tumor hypoxia are reciprocally associated with cancer progression (Cook et al. 2004, Fiaschi and Chiarugi 2012). Clinical evidence of prooxidant and hypoxic states exists in HMM *in vivo* (Marczynski et al. 2000, Amati et al. 2008, Francis et al. 2015). However, the effect of hostile environmental stresses is largely unknown with regard to cancer progression in HMM. Herein, we showed the impact of oxidative stress and hypoxia on HMM cell biology and molecular mechanisms underlying acquisition of more aggressive phenotypes. Particularly, the role of mitochondria in the hypoxia-induced drug resistance was studied (Figure 3).

Figure 3. Summary of the effect of tumor microenvironments on HMM. Adapted and modified from (Ramachandran et al. 2015)



CHAPTER I.

HYDROGEN PEROXIDE PROMOTES EPITHELIAL TO MESENCHYMAL TRANSITION AND STEMNESS IN HUMAN MALIGNANT MESOTHELIOMA CELLS

Abstract

ROS are known to promote mesothelial carcinogenesis that is closely associated with asbestos fibers and inflammation. EMT is an important process involved in the progression of tumors, providing cancer cells with aggressiveness. The present study was performed to determine if EMT is induced by H_2O_2 in HMM cells. Cultured HMM cells were treated with H_2O_2, followed by measuring expression levels of EMT-related genes and proteins. Immunohistochemically, TWIST1 expression was confined to sarcomatous cells in HMM tissues, but not in epithelioid cells. Treatment of HMM cells with H_2O_2 promoted EMT process, which was indicated by increased expression levels of vimentin, SLUG and TWIST1, and decreased E-cadherin expression. Expression levels of stemness genes such as OCT4, SOX2 and NANOG were significantly increased by treatment of HMM cells with H_2O_2. Alterations of these genes were mediated via activation of HIF-1α and TGF-β1. Considering that treatment with H_2O_2 results in excessive ROS, the present study suggests that ROS may play a critical role in HMM carcinogenesis by promoting EMT process and enhancing the expression of stemness genes.

Introduction

ROS plays a central role in a variety of cellular processes, such as cell cycle progression, apoptosis, and diabetic complications. Elevated ROS has been found in many types of cancer cells, thus promoting carcinogenesis (Clerkin et al. 2008, Maynard et al. 2008). Recently, it has been proposed that ROS signaling contributes to a phenotypic conversion- so called EMT that is an important process during embryonic development (Kang et al. 2004). EMT also occurs during the progression of epithelial tumors, providing cancer cells with increased metastasizing capability (Tse et al. 2007). Defining molecular features of EMT include down-regulation of epithelial markers like E-cadherin and up-regulation of mesenchymal markers such as vimentin and fibroblast specific protein 1 (Edelman et al. 1983).

EMT program is controlled by various growth and differentiation factors including TGF-β1 and HIF-1α (Xu et al. 2009), mediated through EMT transcription factors such as SNAIL, SLUG, and TWIST1 (Cannito et al. 2008, Yang et al. 2008). HMM cells are overexpressing TGF-β1 and its receptor (Suzuki et al. 2007). Stabilization of HIF-1α transcription complex, caused by intratumoral hypoxia, promotes tumor progression and metastasis via promoting EMT through regulating the expression of TWIST1 (Yang and Wu 2008, Yang et al. 2008). The hypoxic condition recapitulates the HMM microenvironment of the body cavity, and the HIF-1α is commonly expressed in HMM cells but not in normal mesothelial cells (Klabatsa et al.

2006). It is well known that overproduced ROS induced by asbestos promote EMT, producing more strong and resilient cells. Moreover, our results are lined with the previous finding that HIF-1α mediates hypoxia-induced EMT in mesothelial cells (Morishita et al. 2016).

The EMT-related pathways and molecules are also involved in mesothelial carcinogenesis, suggesting the potential role for EMT in the development and progression of HMM (Carbone et al. 2002). A receptor tyrosine kinase, c-Met, which are known to induce EMT are highly expressed in most HMMs, and the CD44, hyaluronic acid receptor, is regulated through the c-Met signaling pathway (Ramos-Nino et al. 2003). E-cadherin expression was detected in 48 % of the epithelioid, 12 % of the mixed, and in only 7 % of the sarcomatoid HMMs, illustrating differential expression patterns between histological subtypes (Abutaily et al. 2003). *Sivertsen et al.* (2006) analyzed the expression pattern of E-, N-, and P-cadherins, MPs and transcriptional regulators of EMT in HMMs (Sivertsen et al. 2006). These published data indicate that EMT may play a significant role in the progression of HMM, however, its biological importance and the detailed mechanisms are not fully characterized.

It has been proposed that carcinogenic effects of asbestos are mediated both by direct interaction of target cells with asbestos fibers and by generation, in response to asbestos, of ROS causing genetic alterations (Huang et al. 2012). The present study was performed to determine whether

the ROS induce EMT in malignant human mesothelioma cell lines and to elucidate the underlying molecular mechanisms.

Materials and Methods

Hydrogen peroxide (H_2O_2) treatment

Seven HMM cell lines with distinct morphologic and genetic properties were selected for the present study. For example, H513 is epithelioid in morphology with mutated p53 gene, whereas MS1 and MSTO-211 are biphasic in morphology with wild type p53 gene. The cell lines were cultured in the RPMI 1640 (Mediatech Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Mediatech Inc.), 10 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 2mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 100U/100ug/ml penicillin/streptomycin (Gibco-Life Technology, Gaithersburg, MD, USA) at 37°C in a humidified atmosphere containing 5% CO_2. In order to explore the potential role of ROS in EMT induction, HMM cells were cultured for 48 hours followed by addition of H_2O_2 (Sigma-Aldrich) to the media. After designated periods of further incubation, the dead and viable cells were collected and processed for Western blot and quantitative real time reverse transcription (RT) PCR assays as described below.

Cytotoxicity assay

To determine the effects of H_2O_2 treatment on the cell proliferation of H513 and MS1 cells, the cell proliferation assay using CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA) was performed according to the

manufacturer's protocol. The 10^3 HMM cells (H513 and MS1) were seeded in a 96-well plate with 100 μl of media, and treated with 0 to 100 μM hydrogen peroxide (H_2O_2) for designated hours. Then, a methanethiosulfonate/phenazine methosulfate solution (20 μl/well) was added and incubated for 1 h at 37°C, 5% CO_2. Absorbance was measured at 490 nm using a microplate reader (Gen5, Epoch, Bio Tek, Winooski, VT, USA). The percentage of growth suppression for each dose was calculated by comparing to the same volume of media-treated control cells.

Quantitative real-time RT-PCR

Total RNAs from control, 10, and 20 μM H_2O_2-treated cells of H513 and MS1 were isolated using Trizol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' recommendations. Reverse transcription of the extracted RNA into cDNA were done using a commercial kit (Takara, cat 6110a). Quantitative real time PCR was performed with QIAGEN SYBR® Green PCR Kit (Cat. No. 204074, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and published methods (Kai et al. 2010). Primers for all genes analyzed were listed in Table 1. Cycle conditions were 95°C for 5 min, and 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Fold increases or decreases in gene expression were determined by quantitation of cDNA from control cells. The GAPDH gene was used as the endogenous control for normalization of initial RNA levels. To determine normalized value, $2^{-(\Delta\Delta CT)}$ values were calculated for treated cells, where the changes

in crossing threshold (ΔCt) = $Ct_{Target\ gene} - Ct_{GAPDH}$, and $\Delta\Delta Ct = \Delta Ct^{control} - \Delta Ct^{treated}$ (Kim et al. 2006).

Table 1. Primer sequences used for quantitative real time RT-PCR

Genes	Direction	Primer sequences (5'->3')
TWIST1	Forward	TCTCGGTCTGGAGGATGGAG
	Reverse	GTTATCCAGCTCCAGAGTCT
SLUG	Forward	GAGCATTTGCAGACAGGTCA
	Reverse	CCTCATGTTTGTGCAGGAGA
β-Catenin	Forward	GCCGGCTATTGTAGAAGCTG
	Reverse	GAGTCCCAAGGAGACCTTCC
E-cadherin	Forward	GATTCTGCTGCTCTTGCTGT
	Reverse	CCTGGTCTTTGTCTGACTCTG
OCT4	Forward	ACATGTGTAAGCTGCGGCC
	Reverse	GTTGTGCATAGTCGCTGCTTG
NANOG	Forward	TTCAGTCTGGACACTGGCTG
	Reverse	CTCGCTGATTAGGCTCCAAC
SOX2	Forward	CGATGCCGACAAGAAAACTT
	Reverse	CAAACTTCCTGCAAAGCTCC
NOTCH1	Forward	GCAGTTGTGCTCCTGAAGAA
	Reverse	CGGGCGGCCAGAAAC
GAPDH	Forward	CTGCACCACCAACTGCTTAG
	Reverse	AGGTCCACCACTGACACGTT

Western blot assay

In order to evaluate expression levels of EMT-related molecules and their inter-relationship in HMM cell lines, Western blot assay were performed according to the published protocol (Kai et al. 2009). Seven HMM cell lines with different phenotypes and genotypes were cultured in 6-cm plates with appropriate cell culture media. The effects of 100 μM H_2O_2 treatment on the gene expression were evaluated in H513, MS1 and MSTO-211H cell lines, as described above. Total cell lysates were obtained using RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing complete protease inhibitors (Roche Applied Science, Indianapolis, IN, USA) and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL, USA). The soluble protein concentrations were determined by the Bradford technique (Bio-Rad Laboratories, Hercules, CA, USA). Primary antibodies include a monoclonal anti-TWIST1 antibody (1:200, Santa Cruz Biotech, Inc., Santa Cruz, CA), a polyclonal anti-E-cadherin antibody (1:1,000, Cell Signaling Technologies), or a mouse monoclonal anti-SNAIL antibody (1:200, Abcam Inc., Cambridge, MA), HIF-1α (1:50, Cell Signaling), TGF-β1 (1:50, Cell Signaling) and vimentin (1:200, Santa Cruz Biotech, Inc.). Antibodies against GAPDH (Santa Cruz Biotech) and tubulin (Santa Cruz Biotech) were used as a loading control. The blot was incubated with 1:1 ratio of SuperSignal West Pico Substrate (Pierce) for 5 min, followed by the exposure to the CL-Exposure film (Pierce) for 1 min to overnight at 4 °C. The intensities of the

specific bands were analyzed using a VersaDoc Imaging System-4000 (Bio-Rad).

Immunohistochemistry

In order to assess the expression of TWIST1 in HMM tissues, immunohistochemical staining was performed on tissue microarray slides purchased from US Biomax Inc. (http://www.biomax.us/tissue-arrays). Individual tissue microarray slide contained 20 HMMs, 2 normal mesothelia, 1 lymph node and 1 tonsil in duplicate. The immunohistochemistry for TWIST1 was carried out according to the protocol routinely performed (Hong et al. 2007) using mouse monoclonal antibody against TWIST1 (Santa Cruz Biotech) at recommended dilutions (1:50). Negative control sections were processed identically, with the exception of omitting the incubation with the primary antibody.

Statistical analysis

All of the *in vitro* experiments described above were performed at least three times and most data were presented as means ± standard deviation (SD). When the variances of the two populations were assumed to be equal using an F test, a two-tailed Student's t-test was used for statistical comparison. When two samples had unequal variances using the F test, Welch's t test was performed for statistical comparison. $P < 0.05$ was accepted as statistical significance.

Results

Cytotoxicity

HMM cells were cultured in the presence of 0, 10, 30, and 100 μM of H_2O_2 for 24-72 hours, followed by assay using CellTiter 96 AQueous One Solution. Treatment of MS1 and H513 cells with 10 and 30 μM H_2O_2 up to 72 hours did not significantly reduce cell viability (Figure 1). Treatment of H513 and MS1 cells with 100 μM H_2O_2 for 48 and 72 hours significantly reduced cell viability by 60 - 90% compared to control cells.

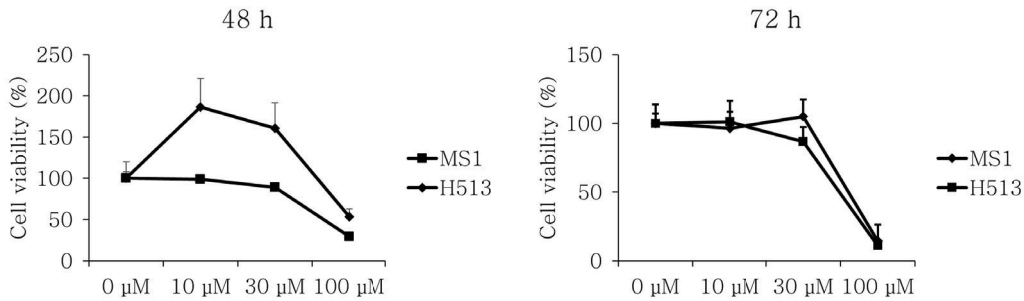


Figure 1. Cell proliferation assay on mesothelial cells treated with H_2O_2. Cells were cultured in reduced serum media (0.5% FBS) containing 0, 10, 30 and 100 μM H_2O_2, followed by cell proliferation assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. In MS1 and H513 cells, treatment with 100 μM H_2O_2 significantly reduced cell proliferation.

Expression profile of EMT-related molecules reflected morphologic phenotypes of HMM cells

Expression levels of TWIST1, SNAIL, E-cadherin and vimentin, and their inter-relationships were assessed in cultured HMM cell lines without chemical treatment. Met5A, H513 cells are classified as epithelioid type with cuboidal shape, and H2373 is sarcomatous type with elongated morphology. MSTO-211H, MS1, H2452, REN cells are classified as biphasic type. On Western blot analysis of the HMM cell lines (Figure 2), TWIST1 expression was detected from high (MSTO-211H) to low (H2452, LRK1A, REN, and H2373), but not detected in Met5A and H513 cells. SNAIL expression was detected in most of the HMM cell lines except LRK1 cell line. E-cadherin expression was only detected in H513 and Met-5A cell lines that had no detectable TWIST1 expression, illustrating clear inverse relationship between the expression levels of E-cadherin and TWIST1. All of the HMM cell lines except H513 express vimentin. Expression profiles of EMT-related genes in H513 and MSTO-211H reflect their own morphologic features.

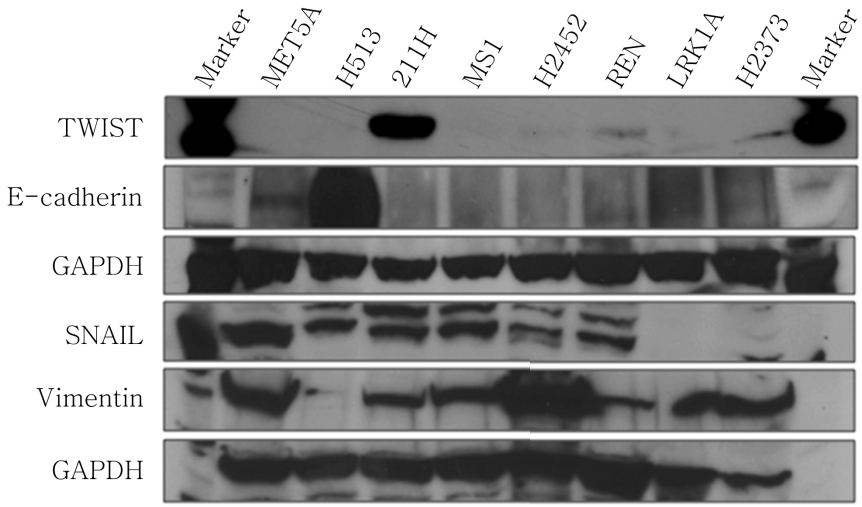


Figure 2. Expression of EMT-related molecules in cultured HMM cell lines determined by Western blot assay. TWIST1 is detected in MSTO-211H, REN and H2373, and E-cadherin is expressed in Met5A, H513 and LRK1A. SNAIL. Vimentin is detected in most of HMM cell lines. Note the inverse relationship between TWIST1 and E-cadherin expression levels in HMM cell lines.

Expression profiles of EMT-related molecules was significantly altered by H_2O_2 treatment

HMM cells (H513 and MSTO-211H) were treated with 100 μM H_2O_2 for designated durations, followed by measurement of the expression levels of EMT-related molecules using Western blot assays (Figure 3). Treatment of MSTO-211H cells with H_2O_2 significantly increased TWIST1 expression. In H513 cells, treatment with H_2O_2 transiently decreased E-cadherin expression. E-cadherin and TWIST1 were not detected in MSTO-211H and H513, respectively. In both H513 and MSTO-211H cells, treatment with 100 μM H_2O_2 significantly increased HIF1α. In MS-1 cells, expression levels pre-TGFβ and HIF-1α were significantly increased after treatment with 100 μM H_2O_2 (Figure 4). Following treatment with H_2O_2, expression level of E-cadherin was decreased, while vimentin was significantly increased (Figure 4).

Following 100 μM H_2O_2 treatment of MS1 and H513, expression levels of multiple genes related to EMT (e.g., SLUG, TWIST1, E-cadherin, β-catenin) and stemness (e.g., OCT4, SOX2, NANOG, NOTCH1) were determined using real time RT-PCR methodology. Overall extent of expression changes for EMT and stemness genes was more dramatic in MS1 cells (Figure 5) than in H513 cells (data not shown). In MS1 cells treatment with H_2O_2 significantly increased the expression levels of SLUG and TWIST1, and decreased E-cadherin expression. In H513 cells, the expression levels of SLUG, TWIST1, and E-cadherin were not significantly affected by H_2O_2

treatment. β-Catenin expression was not affected by treatment with H_2O_2 in both cell lines. For stemness genes, OCT4, SOX2, NANOG and NOTCH1 were evaluated. In MS1 cells, H_2O_2 treatment significantly increased expression levels of OCT4, SOX2, and NANOG, while NOTCH1 was not affected. In H513 cells, H_2O_2 treatment significantly increased the expression levels of OCT4 and NANOG, but SOX2 and NOTCH1 were not affected.

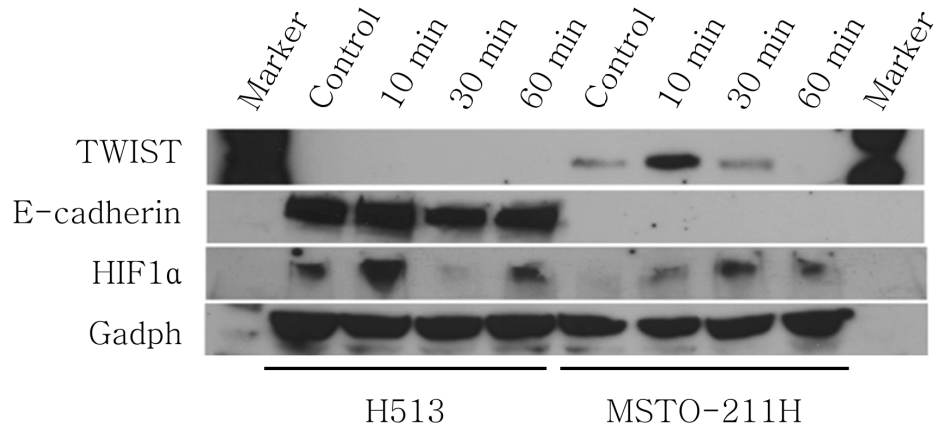


Figure 3. The changes of expression levels of EMT-related genes in HMM cells following treatment with 100 μM H_2O_2 for indicated periods. Lanes 2-4 are H513 and lanes 5-8 are MSTO-211H. Note that TWIST1 expression was significantly increased in MSTO-211H cells at 10 min treatment, while E-cadherin expression was decreased in H513 at 30 min treatment. HIF-1α expression was increased by H_2O_2 treatment in both H513 and MSTO-211H cells.

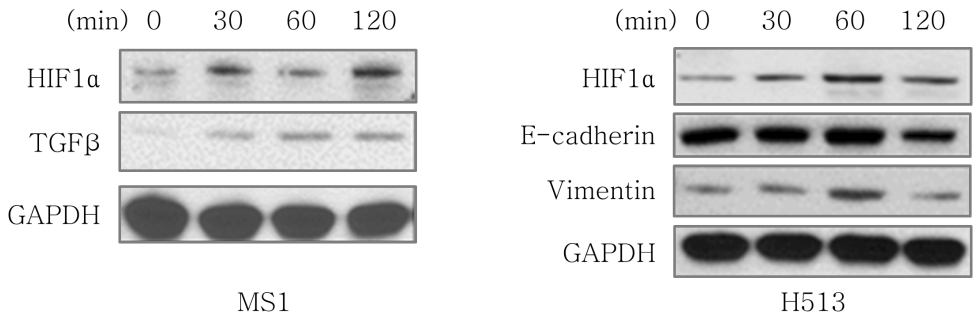


Figure 4. Western blot analysis on the expression levels of signaling molecules induced by H_2O_2. In MS1 cells, expression levels of HIF-1α and TGF-β1 were significantly increased by treatment with 100 μM H_2O_2 for 30 min. Following treatment of H513 cells with 100 μM H_2O_2, expression level of E-cadherin was decreased, while vimentin expression was increased.

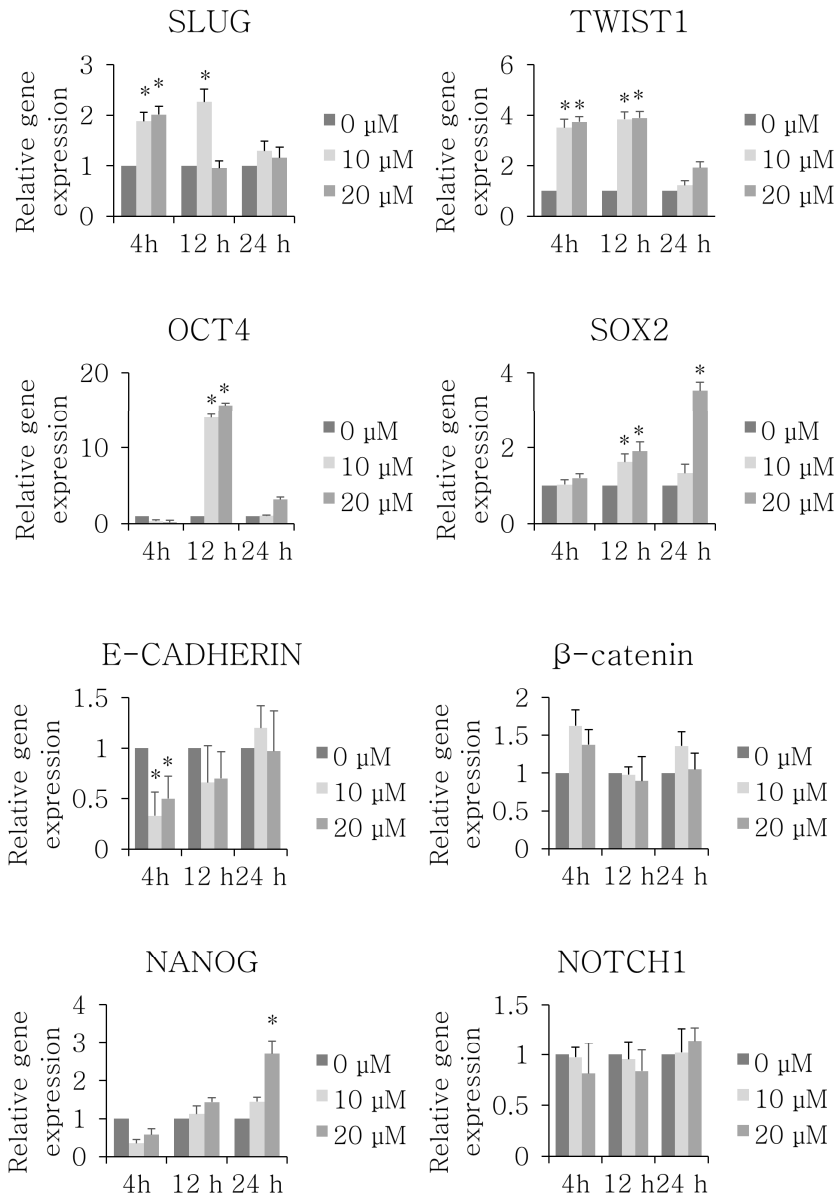


Figure 5. Expression levels of EMT and stemness-related genes measured by real time quantitative RTPCR in HMM cells treated with 0, 10, or 20 μM H_2O_2 for indicated periods. MS1 cells. H_2O_2 treatment significantly enhanced EMT promoting genes such as SLUG, TWIST1, and decreased E-cadherin.

H_2O_2 treatment significantly enhanced expression of stemness genes, such as OCT4, SOX2 and NANOG. The degree of the changes in the gene expression levels induced by H_2O_2 treatment was significant (* indicates $p < 0.05$).

TWIST1 expression in HMM tissues

In cancer tissues, EMT is manifested as spindle-shaped, mesenchymal morphology of cancer cells. TWIST1 was strongly expressed in the nuclei of a subset of mesothelioma cells exhibiting mesenchymal morphology, but not in epithelioid cells (Figure 6). Positive staining for TWIST1 was detected in 6 out of 20 (30%) mesotheliomas which are included in the tissue microarray. Some of the epithelioid mesothelioma cells revealed mild diffuse cytoplasmic staining.

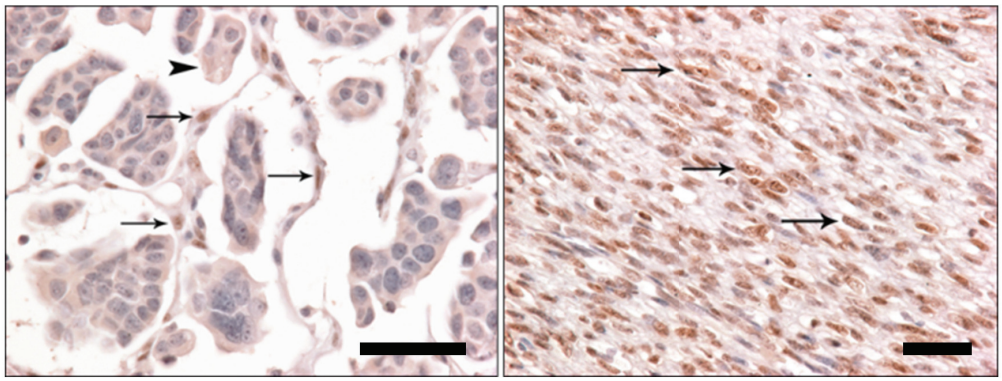


Figure 6. TWIST1 expression detected by immunohistochemical staining of tissue microarray slides containing HMMs. Left panel: Epitheliomatous HMM cells did not show nuclear staining, instead weak diffuse cytoplasmic staining is present (arrowhead). A few spindle cells (arrows) separating the groups of neoplastic epithelioid cells exhibit positive nuclear staining for TWIST1. × 400. Scale Bar = 50 μm, enlarged. Right panel: Most of the sarcomatous mesothelial cells reveal positive staining in their nuclei (arrows). × 200. Scale Bar = 100 μm, enlarged.

Discussion

It has been shown that ROS contribute to the progression of various tumors. However, detailed molecular mechanisms underlying the effect of ROS generated by asbestos fibers on the mesothelial cells remained uncovered. The present study determined the molecular events occurred in the H_2O_2-treated HMM cells. H_2O_2 treatment, a model for excessive ROS production, altered expression levels of EMT-related genes in HMM cells. Additionally, stemness-related genes that may be involved in the survival and aggressiveness of the cancer cells also are significantly increased by treatment with H_2O_2. These data indicated that ROS induced conversion of cellular phenotypes, EMT, resulting in more aggressive and resilient HMM cells.

HMM is invariably lethal tumor arising from the serosal lining cells and is closely associated with exposure to asbestos fibers that induces production of ROS (Ramos-Nino et al. 2006). Although usage of asbestos fibers was banned in the United States and most western European countries in 1970s, the regulation on the asbestos usage has not been forced in many developing countries. Moreover, the asbestos is still common in homes, schools and office buildings that were built prior to the regulation. It is well-known that overproduced ROS from asbestos fibers not only kill mesothelial cells, but also promote mesothelial carcinogenesis. Additionally, previous study showed that ROS induced EMT process in non-malignant mesothelial cells (Lee and Ha 2007). The present study provided additional molecular

evidences involving ROS-induced EMT in malignant mesothelial cells.

Down-regulation of E-cadherin has been widely accepted as a defining molecular feature of EMT (Edelman et al. 1983). Key inducers of EMT are transcription factors that repress E-cadherin expression, such as SNAIL, SLUG, SIP1 and TWIST (Peinado et al. 2007). TWIST1 is a master regulator of EMT and down-regulation of TWIST1 is able to revert EMT, inducing mesenchymal-to-epithelial transition (MET) (Peinado et al. 2007). Our study demonstrated that HMM cell lines differentially expressed TWIST1. TWIST1 expression was detected in HMM cells with biphasic and sarcomatous morphology, but not in H513 with epithelioid morphology. In contrast, E-cadherin expression was observed in H513 but not in biphasic and sarcomatous cells. These expression profile of EMT-related genes clearly reflects the morphologic phenotypes of HMM cells. In support of the notion, the expression of TWIST1 in HMM tissues was confined in the sarcomatous tumor cells. Considering that sarcomatous HMM exhibits poorer prognosis than epithelioid HMM, our data propose that ROS-induced EMT may involve in the progression of HMM carcinogenesis.

Highly aggressive tumor cells should adapt to and survive the hypoxic conditions that enhance ROS generation by increasing HIF-1α activity (Shimojo et al. 2013). Published study shows that HIF-1α is overexpressed in HMM cells, but not in normal mesothelial cells (Klabatsa et al. 2006). In the present study, treatment of HMM cells with H_2O_2 induce the activation of HIF-1α, resulting in EMT. ROS also are known to stimulate TGF-β1,

initiating EMT in a variety of cell types (Kim et al. 2012). Consistent with these published reports, H_2O_2 treatment increased expression of TGF-β1, concurring with induction of the expression of EMT-biomarkers. ROS generated by cells can function as both an upstream signal that triggers p53 activation and a downstream factor that mediates function of p53 (Liu et al. 2008). In our study, MS-1 cells with wild type p53 gene exhibited more dramatic change of the gene expression levels compared to the H513 cells with mutated p53 genes, suggesting the potential role of p53 in the ROS-mediated EMT and stemness.

Through EMT cancer cells acquire drug-resistant, invasive and metastatic properties (Singh and Settleman 2010). As epithelial cells are adherent to each other via E-cadherin, loss of the E-cadherin may be associated with detachment of individual cells from neighboring cells (Cannito et al. 2008), stimulating migration and metastasis. Induction of EMT confers resistance to apoptosis and promotes anchorage-independent growth in epithelial cell lines (Robson et al. 2006, Yang et al. 2006). Depletion of E-cadherin protects mammary cells against anoikis (Geiger et al. 2009). SNAIL represses the cell cycle and enhances resistance to cell death (Vega et al. 2004). TWIST1 promotes intracavitary dissemination of ovarian cancers, which require resistance to anoikis (Terauchi et al. 2007). Taken together, EMT may be a crucial process in the metastasis of HMM cells via intracavitary dissemination. Biological properties of the survived HMM cells following H_2O_2 treatment warrant further studies.

ROS, especially H_2O_2, play an important role in maintaining stemness and differentiating capacity of stem cells. The effect of ROS on the stem cell functions appears to be context and cell type dependent. Lower level of H_2O_2 contributes to maintaining their stemness, whereas a higher level of H_2O_2 promotes differentiation, proliferation and migrations and survival of stem cells (Kobayashi et al. 2012). Recent studies have suggest that cancer stem cells of several tumor types have similar redox patterns to normal stem cells (Kobayashi and Suda 2012). Consistent with the present study, hypoxia enhances the expression of stemness genes such as Sox2 and Oct4 in glioblastoma (McCord et al. 2009). Furthermore, it has been shown that HIF-1α interacts with Notch under hypoxic conditions to maintain a stem cell phenotype and Notch signaling is enhanced by hypoxia (Gustafsson et al. 2005).

In summary, the present study demonstrated that H_2O_2 promoted EMT program, which was mediated through HIF-1α and TGF-β1. Simultaneously, the H_2O_2 increased the expression of stem cell-related genes, suggesting the enhanced potential of survival and proliferation of the HMM cells. These data support the notion that ROS may promote HMM carcinogenesis and progression. The present study provides a background information that may crucial to devise a strategy for preventive or therapeutic interventions of HMM and other ROS-related diseases.

CHAPTER II.

HYPOXIA PROMOTES ACQUISITION OF AGGRESSIVE PHENOTYPES IN HUMAN MALIGNANT MESOTHELIOMA

Abstract

Hypoxia is a hallmark of the solid tumor microenvironment and is associated with poor outcomes in cancer patients. The present study was performed to investigate mechanisms underlying the hypoxia-induced phenotypic changes using HMM cells. Hypoxic conditions were achieved by incubating HMM cells in the air chamber. The effect of hypoxia on phenotype changes in HMM cells were investigated by performing *in vitro* clonogenicity, drug resistance, migration and invasion assays. Signaling pathways and molecules involved in the more aggressive behaviors of HMM cells under hypoxia were investigated. A two-tailed unpaired Student's *t*-test or one-way ANOVA with Bonferroni post-test correction was used in this study. Hypoxic conditions upregulated HIF-1α and HIF-2α in parallel with the upregulation of its target, Glut-1, in HMM cells. *In vitro* clonogenicity of HMM cells was significantly increased in hypoxic conditions, but the proliferation of cells at a high density in hypoxia was lower than that in normoxic conditions. The expression levels of HIF-2α and Oct4 were increased in hypoxic HMM cells. The percentage of cells with high CD44 expression was significantly higher in HMM cells cultured in hypoxia than those cultured in normoxia. Meanwhile, the expression of p-Akt and $NOTCH1^{IC}$ was not significantly altered in hypoxic HMM cells. Hypoxia significantly enhanced the resistance of HMM cells to cisplatin, which occurred through cytoprotection against cisplatin-induced apoptosis. While cisplatin treatment decreased the ratio of Bcl-2 to Bax in normoxic

condition, hypoxia conversely increased the ratio in HMM cells treated with cisplatin. Cisplatin treatment rapidly degraded both HIF-1α and HIF-2α in hypoxic HMM cells. Hypoxia increased the mobility and invasiveness of HMM cells. Epithelial to mesenchymal transition was promoted, which was indicated by the repression of E-cadherin and the concomitant increase of vimentin in HMM cells. The data illustrated that hypoxic conditions augmented the aggressive phenotypes of HMM cells at the biological and molecular levels. The present study provides valuable background information beginning to understand aggressiveness of HMM in tumor microenvironments, suggesting that a control measure for tumor hypoxia may be an effective therapeutic strategy to reduce the aggressiveness of cancer cells in HMM patients.

Introduction

Hypoxia is a common feature of tumor microenvironment (Ruan et al. 2009). There are two types of hypoxia in solid tumors, intermittent hypoxia and chronic hypoxia. Intermittent hypoxia results from abnormal blood flow associated with transient fluctuations in tumor perfusion and the high permeability of tumor vessels with interstitial hypertension (Fukumura and Jain 2007). Chronic hypoxia arises due to the inability of the vascular system to supply the growing tumor mass with adequate amounts of oxygen (Cosse and Michiels 2008). Both types of tumor hypoxia have been reported to be correlated with poor outcomes in many cancer patients (Vaupel and Mayer 2007). Hypoxia enhances cancer cell survival, metastasis, and drug resistance in multiple tumor types (Vaupel and Mayer 2007, Ruan et al. 2009).

One of the adaptive cellular responses to hypoxia is to increase the expression of HIFα, a subunit of the heterodimeric transcription factor HIF (Zhou et al. 2006). In normoxia, the HIFα subunit is hydroxylated by PHD and recognized by an E3 ubiquitin ligase, VHL protein, which proteasomally degrades the HIFα protein (Lee et al. 2004). Under hypoxia, however, HIFα becomes stable and starts to accumulate in cancer cells by blocking the von Hip-mediated ubiquitin-proteasome pathway (Lee et al. 2004). HIFα heterodimerizes with HIF-1β and migrates to the nucleus. The heterodimer recognizes and binds the hypoxia responsive element located in the promotor of hundreds of genes (Lee et al. 2004). Transcriptional activity of

HIF in cancer cells is largely mediated by HIF-1α and HIF-2α (Keith et al. 2012). The function of HIF-1α has been extensively investigated in nearly every stage of tumor progression (Semenza 2010). Recently, growing evidence has suggested that HIF-2α is also a critical mediator of aggressive cancer phenotypes including metastasis and dedifferentiation (Qing and Simon 2009). Although HIF-1α and HIF-2α activate numerous hypoxia-induced genes harboring HIF binding motifs, each HIFα subunit has its own preferential targets. For example, HIF-1α induces genes primarily involved in anaerobic glycolysis, angiogenesis, and apoptosis (Cosse and Michiels 2008, Semenza 2010). On the other hand, HIF-2α regulates genes that promote invasion and stemness (Covello et al. 2006, Qing and Simon 2009). Depending on tumor types or hypoxic duration, the HIFα isoforms are mutually cooperative or exclusive for biological functions and phenotypes (Keith et al. 2012).

Human malignant mesothelioma (HMM) is an aggressive malignancy arising from the mesothelium on the surface of the body cavity (Robinson et al. 2005). Exposure to asbestos fibers increases the risk of HMM, but simian virus 40 may also have a role in HMM tumorigenesis (Robinson et al. 2005). The occurrence rate of HMM is anticipated to increase worldwide (Bianchi and Bianchi 2007). HMM is highly resistant to traditional anticancer drugs (Mujoomdar et al. 2010). Several mechanisms of drug resistance have been proposed in HMM, including drug transporters, anti-apoptosis, and antioxidant defenses (Fennell et al. 2004). Despite the advances in systemic

chemotherapy using an antifolate-platinum regimen have improved clinical outcomes in HMM patients, overall prognosis remains poor with median survival times of 4 to 13 months from the initial diagnosis (Fennell et al. 2008, Blomberg et al. 2015).

The existence of hypoxic cells within a tumor is associated with modulation of the malignant process in many cancers (Zhou et al. 2006, Ruan et al. 2009). Recent studies have revealed that HMM contains hypoxic regions, suggesting a potential link between tumor hypoxia and ineffective therapeutic efficacy (Klabatsa et al. 2006, Francis et al. 2015). However, the mechanism underlying the effect of hypoxia on HMM remains largely unknown. The present study showed that hypoxia promotes aggressive phenotypes of HMM cells. Hypoxia enhances *in vitro* clonogenicity, migration, invasion, and drug resistance through inhibition of apoptosis in HMM cells. Various signaling networks and molecular candidates were suggested for the aggressive biological behaviors of HMM cells, including HIF-1/2α and Oct4 signaling pathways, EMT, and Bcl-2 regulation. Exploiting tumor hypoxia may be an alternative therapeutic strategy to reduce the aggressive behavior of HMM cells.

Materials and Methods

Cell culture and cell lines

MS1 and H513 cell lines were kindly provided by Dr. Jablons (University of California, San Francisco) and Dr. R Kratzke (University of Minnesota), respectively. The cell lines were cultured in RPMI 1640 medium (Mediatech Inc.) containing 10% FBS (Mediatech Inc.), 10 mM of glucose, 10 mM of HEPES (Sigma-Aldrich), 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 1 mM of sodium pyruvate (Sigma-Aldrich), and 100 U/100 μg/mL penicillin/streptomycin (Gibco-Life Technology) at 37°C in a humidified atmosphere containing 5% CO_2. To establish a glucose-starved condition, HMM cells were cultured in DMEM medium (Gibco-Life Technology) supplemented with 0 or 1 mM of D- (+)-glucose (Sigma-Aldrich). The HMM cell lines were determined to be free of mycoplasma contamination by using e-Myco Mycoplasma PCR detection kit (e-Myco, iNtRON Biotechnology, Sungnam, Korea).

Hypoxic condition

Hypoxia was generated by infusing a pre-analyzed air mixture (2.2% O_2/5% CO_2/92.8% N_2) at a flow rate of 5 L/min for 15 minutes into an air chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) with inflow and outflow valves. Hypoxic treatment of cells was achieved by incubating the cells in the air chamber maintained in a humidified environment at 37°C. The culture medium was replaced just before carrying out hypoxic treatment.

Cell proliferation and cytotoxicity assay

Cell proliferation was determined by counting the number of cells and/or measuring cell metabolic activity using MTT dye in each well at defined intervals. Cells were seeded on 6-well plates or 96-well plates at a different density per well. For cell proliferation by manual counting and MTT assay, each group was replicated in five and six separate wells, respectively. The following day, the cells were subjected to normoxia or hypoxia for the indicated periods either without drugs or with varying concentrations of cisplatin (Dong-A Pharm, Seoul, the Republic of Korea). For cytotoxicity assay, each group was replicated in three separate wells. After the treatment, the cells were enumerated using a hemocytometer under an Olympus CK2 microscope (Optical Co., Ltd., Tokyo, Japan). After incubation in MTT dye (5 mg/mL) for 2 h at 37°C, protected from the light, the absorbance values were determined at 570 nm by the microplate reader (Gen5). The absorbance from untreated control cells under each normoxic and hypoxic condition was considered representative of 100% cell viability. All other measurements were expressed as a percentage of the control cell value ± SD.

Clonogenicity assay

To prevent cell to cell contact or the overlapping of too many colonies, 200 cells were chosen to seed on 6-well plates, as previously described

(Franken et al. 2006). Each group was replicated in four separate wells. When attached to the plate, the cells were subjected to incubation for 48 h under normoxia or hypoxia. After the incubation, the culture medium was replaced, and the cells were further incubated at 37°C for 5 days. The cells were fixed with methanol for 5 minutes, stained with Diff-Quik solution (Merck, Darmstadt, Germany), and dried. Groups of more than 50 cells were counted as viable colonies. The colony forming ability was determined by calculating the percentage of surviving cells based on the plating efficiency that is a ratio of the number of colonies to the number of cells seeded (Franken et al. 2006).

Wound healing assay

HMM cells were seeded on 24-well plates at cell densities of 10^5 cells per well in triplicate. When the cells reached 90% confluence, the culture medium was replaced with one containing mitomycin C (Sigma-Aldrich) at a final concentration of 2 μg/mL, followed by further incubation for 2 h. Mitomycin C was used to minimize the proliferative effect of cancer cells, and the concentration used in this study was found to be non-cytotoxic (Jampel 1992). The cell monolayer was manually scratched with a 1,000 μl pipette tip. The cells were subjected to further incubation in normoxia or hypoxia for 48 h. The area of the scratch distance was photographed under a phase contrast microscope using an Olympus CK2 camera (Optical Co.) at 0 and 48 hr of incubation. Cell migration was determined by measuring the

migration distance. The results were normalized to the initial scratch distance and presented as % with normoxic condition set at 100%.

Invasion assay

The 24-transwell plates (8.0 μM pore size with poly-carbonate membrane; Corning Costar, Lowell, MA, USA) covered with 2 mg/ml basement membrane Matrigel matrix (BD Biosciences, Bedford, MA, USA) were used for the invasion assay. Each group was replicated in three separate wells. The coated trans-well was hydrated with culture medium for 2 hr prior to cell seeding. HMM cells were resuspended in serum-free medium and seeded at a cell density of 2.5×10^4 into the upper invasion chamber. Culture medium containing 10% FBS was then added to the lower chamber. The cells were incubated at 37°C for 24 h under normoxia or hypoxia. After a day of incubation, the cells that had invaded the lower surface of the membrane were fixed with methanol for 5 min, stained with Diff-Quik solution (Merck), and quantified by counting five random fields using a phase contrast microscope. Invasion was expressed as the ratio of invading cells incubated under hypoxia compared to the controls in normoxia.

Apoptosis assay

HMM cells were seeded on 60 mm^2 petri dishes with a confluency of 60% density in quadruplicate. On the following day, the cells were incubated in normoxia or hypoxia for 48 h either with or without cisplatin (10 μM). After

the incubation, apoptosis was evaluated using the Annexin V-FITC apoptosis detection kit (Komabiotech, Inc, Seoul, Korea). Briefly, cells were harvested, washed, and incubated in binding buffer containing a saturating concentration of FITC-conjugated Annexin V in the dark for 15 minutes at room temperature. After being washed with a binding buffer, the cells were resuspended and incubated with 500 μl of binding buffer containing 10 μl of propidium iodide (PI) on ice. The cells were immediately analyzed for the fluorescence of FITC and PI using flow cytometry (Becton Dickinson, San Jose, CA, USA). Cells undergoing early and late apoptosis were determined.

Cell cycle analysis

HMM cells were plated on 6-well plates at a confluency of 50% density in triplicate. After a day of incubation, the cells were subjected to normoxia or hypoxia for 24 and 48 hrs. At the indicated time points, cells were harvested and fixed with ice-cold 70% ethanol for 2 hr at -20°C. After being washed with PBS, the cells were incubated with PI/RNase staining buffer (BD Pharmingen, BD Biosciences) for 15 minutes at room temperature. The cells were immediately subjected to analysis using a flow cytometer (BD Biosciences) for the determination of their DNA contents.

Measurement of cell surface CD44 expression

HMM cells were plated on 6-well plates in triplicate. After 48 h of hypoxia incubation, HMM cells resuspended in PBS were incubated with primary

antibody against CD44 (Genetex, CA, USA) at 4°C for 1 h in the dark. Secondary antibody goat anti-rat IgG-PE (Santa Cruz Biotech) for CD44 analysis was incubated at room temperature for 30 min in the dark. After washing with PBS, HMM cells were resuspended in PBS and subjected to flow cytometric analysis for CD44 expression. Unstained cells were used to gate on live cells. After excluding cell debris from the gated populations, a minimum of 10,000 events per condition were collected for the analysis.

Western blot analysis

After washing cells with cold PBS twice, cell lysates were obtained using RIPA lysis buffer containing complete protease inhibitors. The nuclear proteins were extracted by using Cell Fractionation Kit (Abcam) according to manufacturer's instruction. The same amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; subsequently, the protein bands were transferred onto a nitrous membrane by a wet transfer apparatus. After blocking with 5% non-fat milk at room temperature for 60 minutes, the nitrous membrane was placed in 0.1% Tween 20 PBS (T-PBS) containing primary antibodies including HIF-1α (1:1000, Cell Signaling), HIF-2α (1:1000, Cell Signaling), E-cadherin (1:1000, Cell Signaling), Bcl-2 (1:1000, Cell Signaling), Bax (1:1000, Cell Signaling), Bcl-xL (1:1000, Cell Signaling), Oct4 (1:1000, Cell Signaling), $NOTCH1^{IC}$ (1:1000, Santa Cruz Biotech), p-Akt (1:1000, Santa Cruz Biotech), HDAC2 (1:1000, Santa Cruz Biotech), Lamin A/C (1:1000, Santa

Cruz Biotech), and vimentin (1:1000, Cell Signaling) and incubated overnight at 4°C. An antibody against β-actin (1:1000, Cell Signaling) was used as a loading control. After the blot was washed with T-PBS three times for 10 minutes each, peroxidase-labeled secondary anti-rabbit or anti-mouse antibodies (1:2000) were applied to the blot for 90 minutes. The protein levels were detected on CL-Exposure film with the use of enhanced chemiluminescence detection reagents (Advansta, Menlo Park, CA, USA) according to the manufacturer's instructions. Densitometric analysis was performed using the ImageJ program (ImageJ, US National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

Gene expression and quantitative Real-time RT-PCR

Total RNA was isolated from the HMM cell lines using the RNeasy Plus Mini Kit protocol (Qiagen). The quality and quantity of the total RNA were assessed using Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). Total RNA of 500 ng was used to synthesize cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed using the Rotor-Gene SYBR Green RT-PCR Kit (Qiagen). PCR conditions were as follows: 1 cycle at 95°C for 10 min, followed by 45 cycles of 95°C for 10 seconds, and then 60°C for 30 seconds. The expression level of each gene was normalized based on endogenous GAPDH expression. The analysis of relative gene expression was determined according to the $2^{-\Delta\Delta}$Ct

method, as previously described (Kim et al. 2013). The primer sequences used in this study are listed in Table 1.

Table 1. The list of primers used in this study

Target genes	Direction	Primer sequences (5'->3')
Oct4	Forward	GGAGATATGCAAAGCAGAAAC
	Reverse	GAACAAATTCTCCAGGTTGCC
Sox2	Forward	CGATGCCGACAAGAAAACTT
	Reverse	CAAACTTCCTGCAAAGCTCC
Nanog	Forward	TTCAGTCTGGACACTGGCTG
	Reverse	CTCGCTGATTAGGCTCCAAC
Smo	Forward	GAATGAGGTGCAGAACATCAAG
	Reverse	GTCCTCGTACCAGCTCTTG
Gli1	Forward	GAGCCCATCTCTGGGATTC
	Reverse	GTCCAGCTCAGACTTCAGC
Shh	Forward	CAGAGGTGTAAGGACAAGTTG
	Reverse	CGTAGTGCAGAGACTCCTC
β catenin	Forward	GCCGGCTATTGTAGAAGCTG
	Reverse	GAGTCCCAAGGAGACCTTCC
Notch1	Forward	GCAGTTGTGCTCCTGAAGAA
	Reverse	CGGGCGGCCAGAAAC
Bmi1	Forward	CCGGGATTTTTTATCAAGCAG
	Reverse	GTTGTGGCATCAATGAAGTACC
GAPDH	Forward	CTGCACCACCAACTGCTTAG
	Reverse	AGGTCCACCACTGACACGTT

Statistical analysis

All data were presented as the means ± SD. Statistical analyses were done by using Microsoft Excel (Microsoft, Seattle, WA, USA) and SPSS software (IBM, Armonk, NY, USA). P values were calculated by a two-tailed unpaired Student's t-test or one-way ANOVA with Bonferroni post-test correction. The results were confirmed in at least three independent experiments and considered to be statistically significant when P value was less than 0.05.

Results

Experimental induction of hypoxia *in vitro*

Experimental establishment of hypoxia was verified by HIFα induction in HMM cells. Western blot analysis confirmed the upregulation of HIF-1α and the *de novo* synthesis of HIF-2α under hypoxia (Figure 1A). As hypoxia was prolonged, HIF-1/2α target Glut-1 expression was also elevated, suggesting a functional transcriptional activity of HIF-1α in the hypoxic state (Figure 1B). Glucose starvation was used as a positive control for Glut-1 expression.

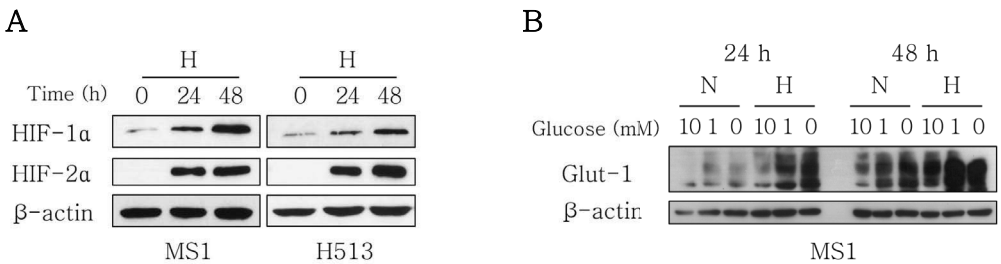


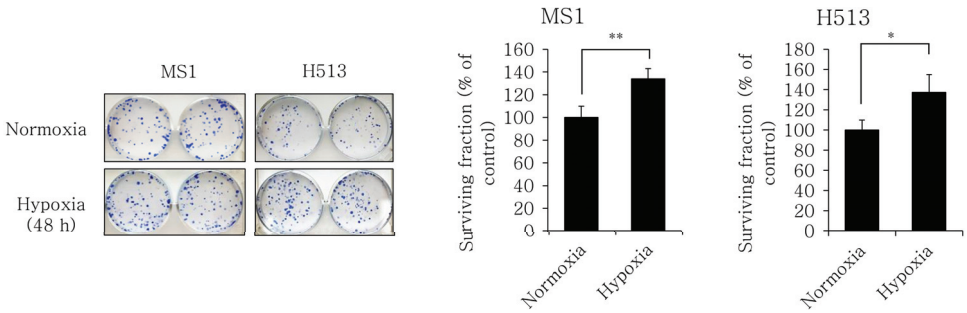
Figure 1. The experimental establishment of tumor hypoxia in HMM cells. (A) Hypoxia markedly increased HIF-1α expression and induced HIF-2α expression *de novo* in HMM cells. (B) A HIF-1/2α target Glut-1 increased in response to hypoxia and glucose starvation in MS1 cells. Abbreviations: N, normoxia; H, hypoxia.

Hypoxia enhanced *in vitro* clonogenicity but reduced proliferation of HMM cells

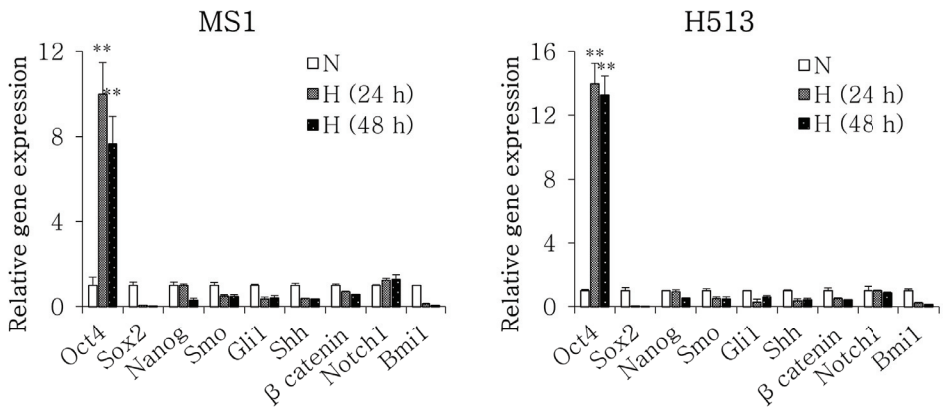
The plating efficiency of the untreated control was approximately 0.6 in HMM cells. Hypoxia significantly increased the surviving fraction by 34% and 37% in MS1 and H513 cells, respectively, compared to that of normoxic cells (Figure 2A). Because the ability of tumor cells to form a single colony is related to the acquisition of stemness properties, the levels of a variety of stemness genes were investigated. Among them, Oct4 gene expression was significantly increased in HMM cells under hypoxia (Figure 2B). The Oct4 protein was also significantly elevated under hypoxia (Figure 2C). We also attempted to determine cell surface markers that correlate with stem cell signatures, and hypoxia was found to significantly increase the percentage of HMM cells with the high CD44 expression, a putative marker of cancer stemness of HMM (Figure 3) (Ghani et al. 2011, Cortes-Dericks et al. 2014). It has been previously reported that HMM cells survive in hypoxic conditions by activating NOTCH1 that subsequently phosphorylates Akt (p-Akt) (Graziani et al. 2008). In the present study, however, the expression of p-Akt and $NOTCH1^{IC}$ was not significantly altered in hypoxic HMM cells (Figure 4). On the other hand, chronic hypoxia did not enhance the proliferative capacity of HMM cells. As the cell density increased, an inhibitory effect of hypoxia on cell growth was detected (Figure 5A). The parallel measurement using MTT dye also confirmed the significant reduction in cell proliferation of HMM cells under hypoxia. The absorbance-

based cell viability was decreased after 48 h of hypoxia from the initial seeding density of 1,000 and 5,000 in MS1 and H513 cells, respectively (Figure 5B). The reduced proliferation under hypoxia was not attributable to the cell cycle arrest at the $G_1/_0$ phase (Figure 5C). The data indicated that hypoxia improved single cell survivability that was mediated through stemness acquisition in HMM cells.

A


MS1
H513
Normoxia
Hypoxia (48 h)
MS1
H513
Surviving fraction (% of control)
Normoxia
Hypoxia
**
*

B


MS1
H513
Relative gene expression
N
H (24 h)
H (48 h)
Oct4
Sox2
Nanog
Smo
Gli1
Shh
β catenin
Notch1
Bmi1

C

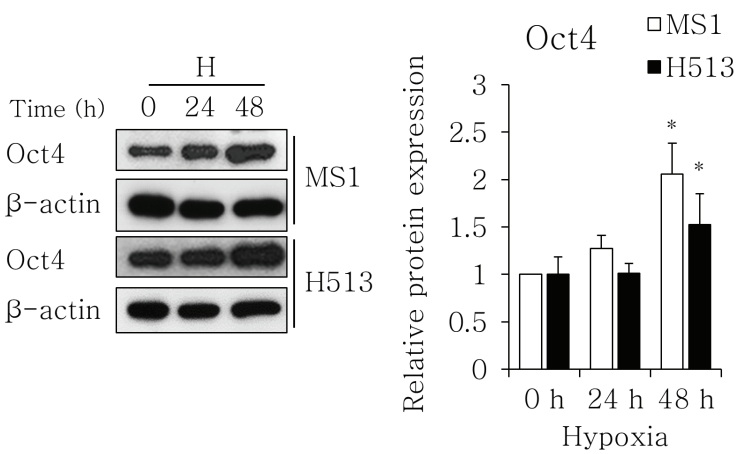

H
Time (h)
0 24 48
Oct4
β-actin
Oct4
β-actin
MS1
H513
Oct4
MS1
H513
Relative protein expression
0 h 24 h 48 h
Hypoxia

Figure 2. The effect of hypoxia on *in vitro* clonogenicity in HMM cells. (A) Hypoxia enhanced the colony forming ability of HMM cells. Representative microscopic examinations are presented. P value was calculated by Student's *t*-test. Hypoxia significantly upregulated the expression of Oct4 at transcriptional (B) and translational (C) levels in HMM cells. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. Abbreviations: N, normoxia; H, hypoxia.

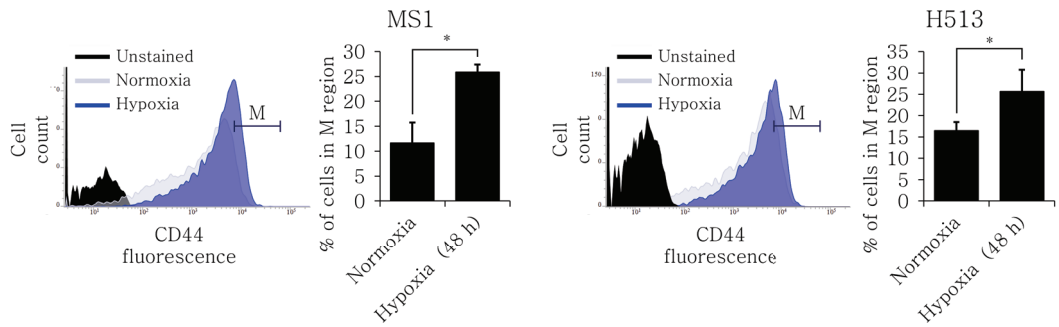


Figure 3. The effect of hypoxia on the abundance of HMM cells with CD44 expression. The percentage of cells with high CD44 expression is significantly higher in HMM cultured in hypoxia than those cultured in normoxia. Representative histogram of CD44 expression is presented. * P value < 0.05, as calculated by Student's *t*-test.

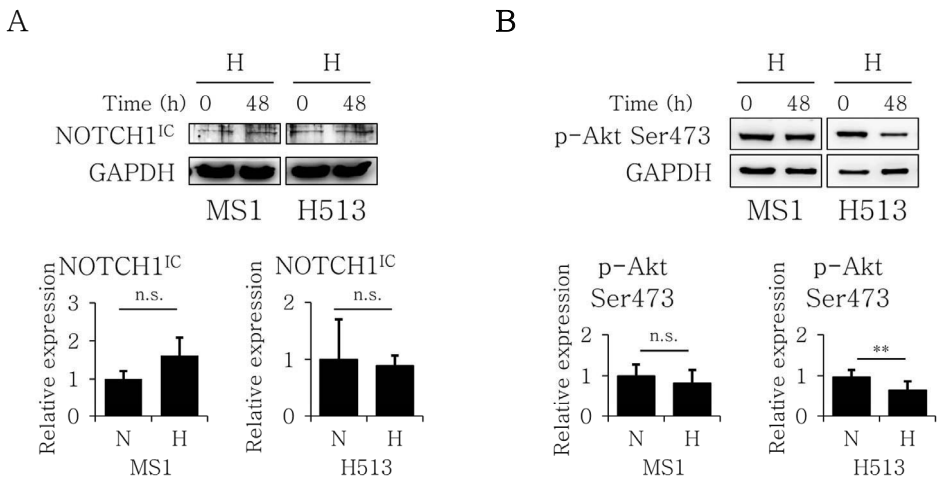
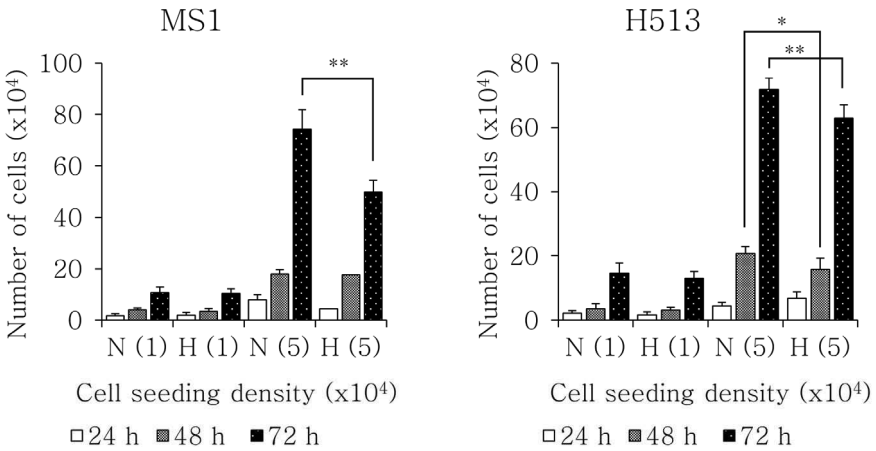
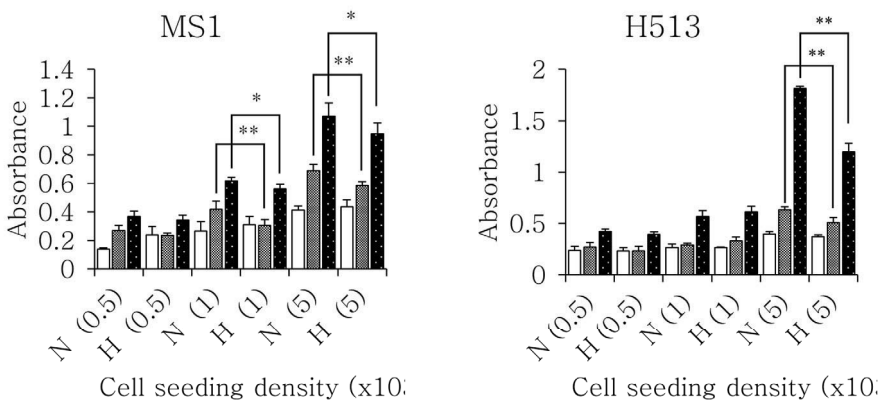


Figure 4. The effect of hypoxia on the expression of NOTCH1^{IC} and p-Akt in HMM cells. (A) The expression of NOTCH1^{IC} is not significantly different between HMM cells under normoxic and hypoxic conditions. (B) Hypoxia does not significantly alter the expression of p-Akt in MS1 cells, but significantly downregulate the p-Akt expression in H513 cells, compared to those in normoxic conditions. ** P value < 0.01, as calculated by Student's *t*-test.

A


MS1
H513
Number of cells (x10⁴)
N (1) H (1) N (5) H (5)
Cell seeding density (x10⁴)
24 h 48 h 72 h

B


MS1
H513
Absorbance
N (0.5) H (0.5) N (1) H (1) N (5) H (5)
Cell seeding density (x10

C

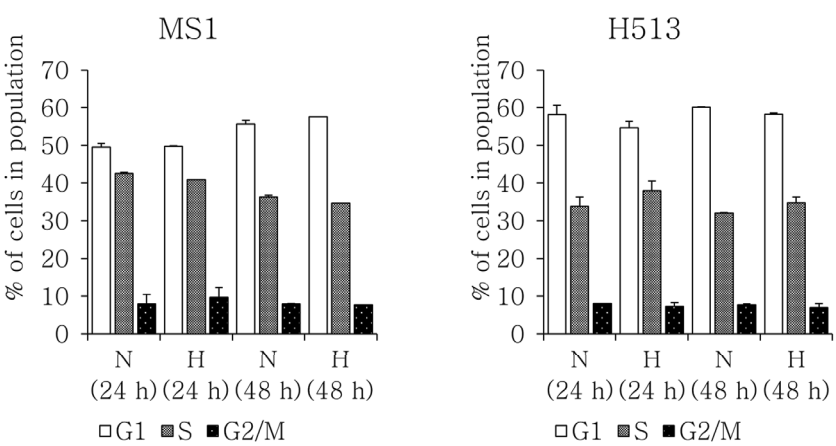

MS1
H513
% of cells in population
N (24 h) H (24 h) N (48 h) H (48 h)
G1 S G2/M

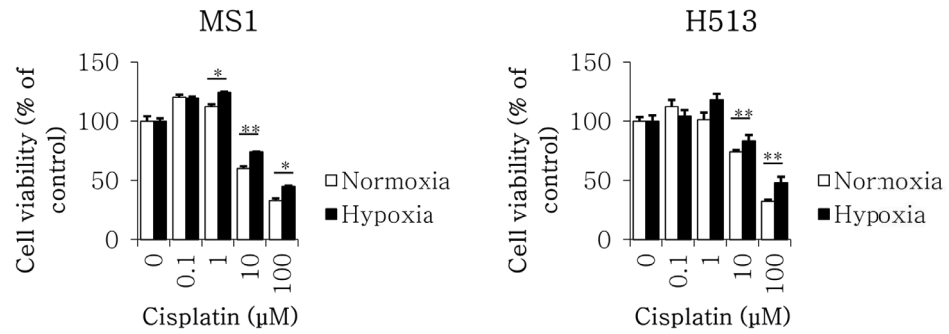
Figure 5. The effect of hypoxia on cell proliferation in HMM cells. Hypoxia significantly decreased proliferation and viability in HMM cells at high cell seeding density. (A) Counting cell numbers. (B) MTT assay. Number of cells initially seeded is presented in parentheses. Cell cycle profiles did not appreciably differ between normoxic and hypoxic HMM cells (C). * P value < 0.05, ** P value < 0.01, as calculated by Student's *t*-test. Abbreviations: N, normoxia; H, hypoxia.

Hypoxia induced drug resistance in HMM cells

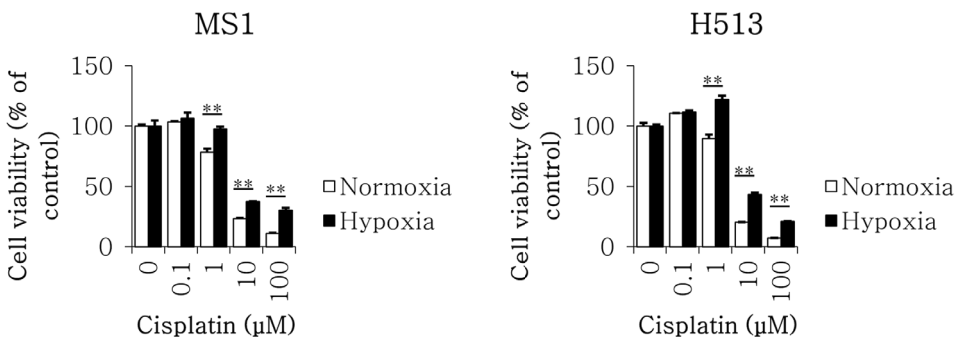
In the normoxic state, cisplatin treatment decreased the cell viability of HMM cells in a dose-dependent manner, but hypoxia significantly reduced the sensitivity of the cells to the drug (Figure 6A and 6B). Among HMM cell lines, MS1 and H513 cells are the most sensitive to cisplatin treatment, and these cell lines displayed the highest response to hypoxia, leading to drug resistance (Figure 6C). Because apoptosis has been used for the evaluation of the chemotherapeutic efficacy of cisplatin (Fennell and Rudd 2004), a bivariate Annexin V/PI analysis was performed. Following cisplatin treatment, a major form of cell death was apoptosis in HMM cells where necrosis occurred less than 3% of total cell death. Compared to the apoptosis of cells exposed to cisplatin in normoxia, hypoxia not only decreased apoptosis in HMM cells without cisplatin treatment but also significantly inhibited cisplatin-induced apoptosis (Figure 7A). The expression levels of representative pro- and anti-apoptotic Bcl-2 family members were determined by immunoblot analysis. As shown in Figure 7B, the Bcl-2 level was increased in HMM cells under the hypoxic state compared to the level in HMM cells under normoxic conditions. However, the level of Bcl-xL remained almost unchanged in hypoxic MS1 cells, and the increase of Bcl-xL was much less in hypoxic H513 cells, compared to that in normoxic H513 cells. A profound elevation of Bax expression was detected in hypoxic MS1 cells, but it remained almost unchanged in H513 cells. Due to the high Bax expression under hypoxia, the ratio of either Bcl-

2 or Bcl-xL to Bax was decreased in MS1 cells. The Bcl-2 to Bax ratio was remained as increase in H513 cells following hypoxia, but the increase in the ratio of Bcl-xL to Bax was not significant. Figure 7C shows expression profiles of the Bcl-2, Bcl-xL, and Bax in HMM cells treated with cisplatin. In the normoxic state, cisplatin reduced the expression of Bcl-2, but hypoxia increased and maintained the Bcl-2 expression in HMM cells treated with cisplatin. With cisplatin treatment, Bcl-xL expression was decreased in HMM cells as hypoxia was prolonged. Densitometric analysis confirmed the upregulation of Bax expression following cisplatin treatment in MS1 cells. The extent of increase in Bax expression was diminished in hypoxic MS1 cells, compared to the level in MS1 cells in normoxia. While the Bcl-2 to Bax ratio decreased in the normoxic state following cisplatin treatment, hypoxia increased the ratio. The Bcl-xL to Bax ratio was lowered in hypoxic HMM cells treated with cisplatin, compared to that of HMM cells treated with cisplatin in normoxia. It has been previously reported that HIFα is a critical regulator of Bcl-2 family members (Cosse and Michiels 2008). However, HIF-1/2α begun to rapidly degrade by cisplatin and were rarely expressed in HMM cells under normoxic and hypoxic conditions (Figure 7D). Taken together, these results indicated that hypoxia promoted the resistance of HMM cells to cisplatin by regulating Bcl-2 family members.

A



B



C

HMM cell lines	ID_{50} for cisplatin (μM)	
	Normoxia	Hypoxia
H513	3.0	13.0
MS1	4.7	12.2
H2373	7.0	13.0
H2461	48.7	98.2
LRK1A	8.3	5.8
REN	11.2	11.6

Figure 6. The effect of hypoxia on drug sensitivity in HMM cells. The sensitivity to cisplatin was decreased in HMM cells under hypoxia for 24 h (A) and 48 h (B), compared to the sensitivity of those in normoxia. (C) The

results of ID_{50} for cisplatin in HMM cell lines. * indicates a significant difference compared with the corresponding normoxic cisplatin-treated control. * P value < 0.05, ** P value < 0.01, as calculated by Student's *t*-test.

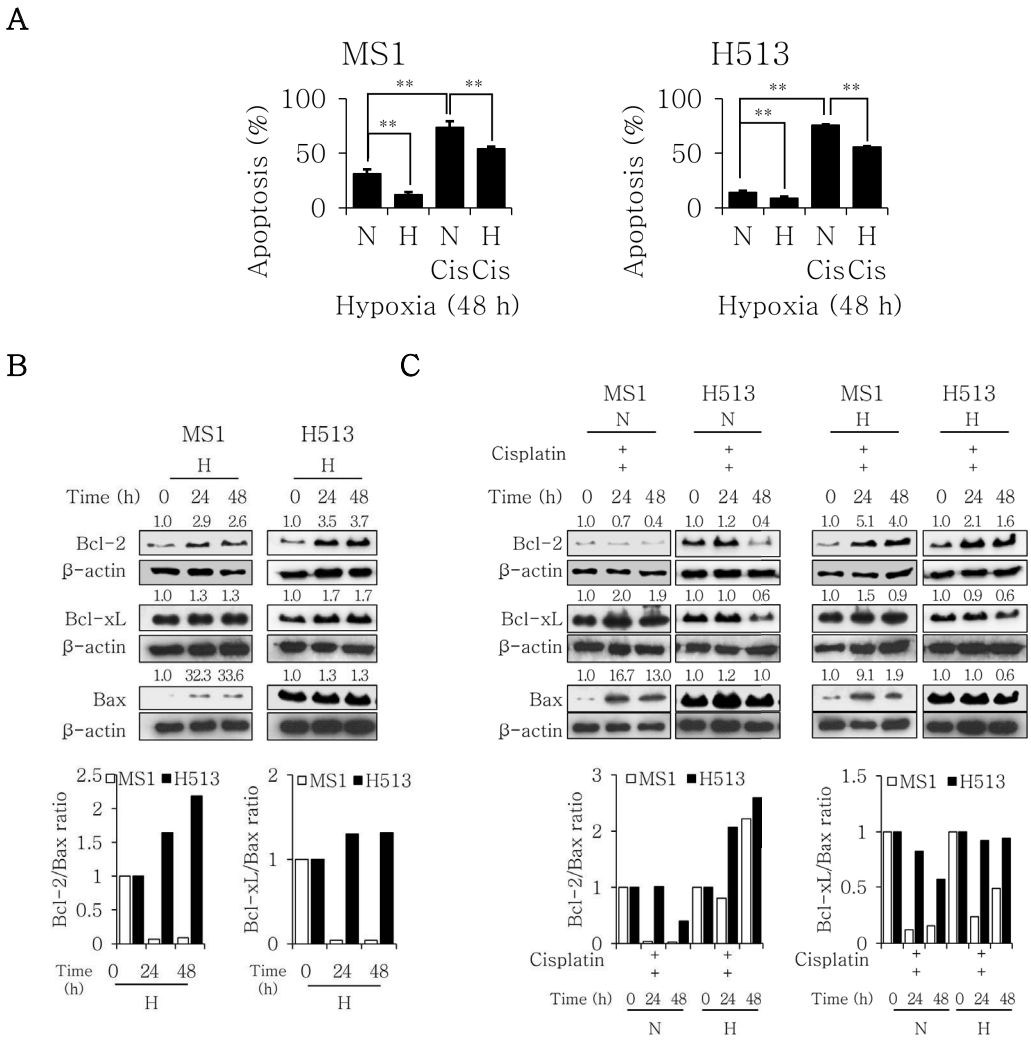

A
MS1
H513
Apoptosis (%)
100
50
0
**
N H N H
Cis Cis
Hypoxia (48 h)
B
MS1
H513
H
Time (h) 0 24 48
Bcl-2
β-actin
Bcl-xL
Bax
1.0 2.9 2.6
1.0 3.5 3.7
1.0 1.3 1.3
1.0 1.7 1.7
1.0 32.3 33.6
1.0 1.3 1.3
Bcl-2/Bax ratio
Bcl-xL/Bax ratio
MS1 H513
Time (h) 0 24 48
H
C
MS1
H513
N
H
Cisplatin
Time (h) 0 24 48
1.0 0.7 0.4
1.0 1.2 0.4
1.0 5.1 4.0
1.0 2.1 1.6
1.0 2.0 1.9
1.0 1.0 0.6
1.0 1.5 0.9
1.0 0.9 0.6
1.0 16.7 13.0
1.0 1.2 1.0
1.0 9.1 1.9
1.0 1.0 0.6
Bcl-2/Bax ratio
Bcl-xL/Bax ratio
N
H
D
4 h
48 h
N
H
Cisplatin
HIF-1α
HIF-2α
Lamin A/C
HDAC2
β-actin

Figure 7. The effect of hypoxia on cisplatin-induced apoptosis in HMM cells. (A) Hypoxia significantly reduced apoptosis either with or without cisplatin in HMM cells. (B) Hypoxia upregulated the expression of anti-apoptotic Bcl-2, whereas Bcl-xL levels remained almost unchanged in HMM cells. Marked increase of pro-apoptotic Bax expression was detected in MS1 cells only. The ratio of Bcl-2 and Bcl-xL to Bax increased in H513 cells. (C) Hypoxia increased Bcl-2 levels and maintained the expression during exposure to cisplatin treatment in HMM cells, compared to the expression level in cells in normoxia. With cisplatin treatment, Bcl-xL expression either tended to decrease or remained unchanged in HMM cells under hypoxia, compared to the level in cells in normoxia. The cisplatin-induced increase in Bax expression was less under hypoxia than under normoxia in MS1 cells. The Bcl-2 to Bax ratio increased in HMM cells under hypoxia following cisplatin. All protein expression was normalized to endogenous β-actin level and is presented as densitometric values at the top of each protein blot. (D) Rapid degradation of nuclear HIF-1/2α is detected during 48 h of hypoxia in HMM cells. * P value < 0.05, as calculated by one-way ANOVA with Bonferroni post-test.

Hypoxia enhanced migration, invasion, and EMT of HMM cells

In the wound healing assay, HMM cells in hypoxia displayed a smaller gap distance than did cells under normoxia (Figure 8A). Under hypoxia, H513 cells showed increased invasiveness (Figure 8B). The H513 cells were round to oval or occasionally polygonal with a small amount of cytoplasm, showing high nucleus to cytosol ratio. The MS1 cells were generally spindle to polygonal (Figure 8C). The HMM cells exposed to hypoxia underwent a morphologic change, showing a neuron-like appearance characterized by pseudopodia protrusions (Figure 7C). To investigate the mechanisms underlying hypoxia-induced cell migration, the expression levels of two representative EMT-related markers, E-cadherin and vimentin, were analyzed. Western blot analysis revealed that hypoxia reduced the expression of E-cadherin and concomitantly increased the expression of vimentin in HMM cells (Figure 8D). Vimentin was upregulated in MS1 cells, but E-cadherin was not detected. It might be due to the infrequent expression of E-cadherin in HMM cell lines or primary tumors with mesenchymal cell phenotype (Kim et al. 2013). These results showed that hypoxia enhances the acquisition of migratory and invasive phenotypes that are associated with EMT process in HMM cells.

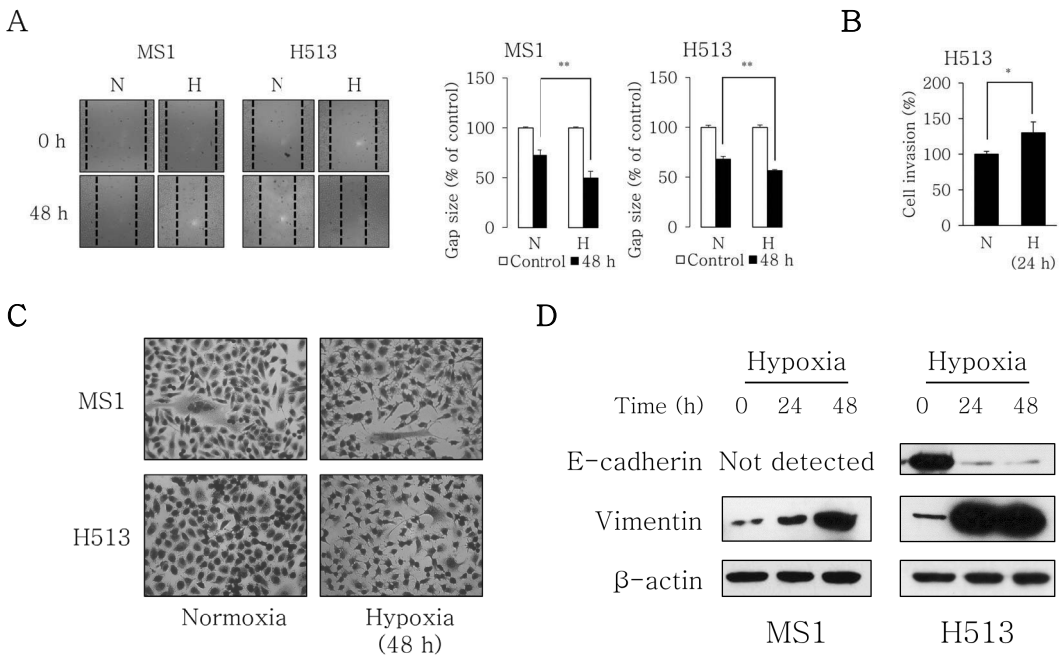


Figure 8. The effect of hypoxia on migration and invasion in HMM cells. (A) Hypoxia significantly increased migration in HMM cells. (B) Hypoxia significantly enhanced invasion in H513 cells. (C) Phase contrast images (400× magnification) of HMM cells cultured for 48 h under normoxia or hypoxia are presented. (D) Hypoxia induced a loss of E-cadherin expression and a gain of vimentin expression in HMM cells. E-cadherin expression was not detected in MS1 cells. * P value < 0.05, as calculated by Student's *t*-test.

Discussion

Tumor cell adaptation to hypoxic condition leads to cancer progression (Zhou et al. 2006, Fukumura and Jain 2007, Ruan et al. 2009). The present study demonstrates that hypoxia causes HMM cells to behave more aggressively at the biological and molecular levels. Hypoxia enhanced *in vitro* clonogenicity, migration, invasion, and drug resistance to cisplatin in HMM cells. Various signaling pathways and molecular targets were associated with the hypoxia-induced aggressive behaviors, including HIF-1/2α, EMT, Oct4, and anti-apoptotic Bcl-2. The data presented in this study emphasize the clinical importance of hypoxia in the biology of HMM. Exploiting molecular signaling pathways affected by the hypoxia may help to overcome the low efficacy of traditional anticancer therapy for HMM patients.

Hypoxia enhanced the *in vitro* colony forming capability of HMM cells. Bcl-2 antagonization reduces *in vitro* clonogenicity through apoptosis induction (Campos et al. 1994, Cao et al. 2008). In this context, the present study showed that the increased Bcl-2 activity likely provided an additional survival advantage to individual HMM cells during colony formation in hypoxia. On the other hand, a published study showed that NOTCH and Akt signaling plays a crucial role in hypoxic cell survival in primary HMM cultures (Graziani et al. 2008). Different cell types and experimental conditions may be responsible for the discordant response to hypoxia with regard to the molecular change seen *ex vivo*. The effect of hypoxia on the

proliferation of HMM cells in culture condition has been reported (Goudarzi et al. 2013). Hypoxia enhances tumor stemness by reprogramming non-stem cancer cells to be cancer stem cells (CSCs) with tumor initiating capacity (Mimeault et al. 2013). The increase of cells with high CD44 expression supports the notion that hypoxia enhanced the stemness of HMM cells (Ghani et al. 2011, Cortes-Dericks et al. 2014). The transformation to CSCs is through the activation of HIFα and stemness-related transcriptional factors, including Oct4, c-Myc, and Notch (Mimeault and Batra 2013). In the present study, the enhanced clonogenicity of HMM cells might be associated with increased Oct4 expression, which was mediated through the transcriptional activity of HIF-2α. The upregulation of Oct4 at mRNA level in hypoxic condition contributes to the formation of more viable colonies with different origins of CSCs (Ma et al. 2011, Yeung et al. 2011, Li et al. 2013). Heddleston *et al.* demonstrated the functional significance of HIF-2α and Oct4 in the maintenance of the CSC state under hypoxia (Heddleston et al. 2009).

The weak correlation between Oct4 mRNA and protein expression may suggest the involvement of post-transcriptional modification of Oct4 and/or another stemness factors in enhancing the clonogenicity of HMM cells under hypoxia (Saxe et al. 2009). CSC factors like c-Myc or Notch are tightly regulated together with Oct4 by competitive cooperation of HIF-1α and HIF-2α in hypoxic tumor cells (Gordan et al. 2007, Hu et al. 2014). Further studies are warranted to elucidate the functional relationship between HIFαs

and stem cell factors including Oct4 in HMM cells. On the other hand, once the HMM cells proliferated in high cell populations after completing colony formation, cell growth was decelerated by hypoxia. This phenomenon may be attributable to the insufficient supply of energy required for cellular proliferation under hypoxia. One of the principal actions of HIF-1α is to shift tumor metabolism from oxidative phosphorylation to anaerobic glycolysis (Semenza 2010). The hypoxic cancer cells consume more glucose by augmenting glucose influx through the upregulation of glucose transporters, such as Glut-1 (Zhou et al. 2006, Ruan et al. 2009). Accordingly, Glut-1 overexpression induced by hypoxia suggested the high energy demand or hypoxia-associated nutrient deprivation of HMM cells in this study. Because cell cycle arrest was not determined in hypoxia in this study, the process of protein synthesis could be inhibited, resulting in the decelerated proliferation of HMM cells, as previously suggested (Goudarzi et al. 2013).

An acquired apoptosis resistance is closely associated with ineffective cancer therapy (Cosse and Michiels 2008). Hypoxia inhibits apoptosis in many cancer types following drug treatment (Rohwer and Cramer 2011). The present study showed that HMM cells acquired a drug-resistant phenotype through inhibition of cisplatin-induced apoptosis under hypoxia. In particular, alteration of Bcl-2 might be responsible for the hypoxia-induced chemoresistance in HMM cells. The Bcl-2 family is a potential blocker of apoptosis and plays a key role in the drug resistance of multiple tumor types (Gross et al. 1999). The anti-apoptosis by Bcl-2 is largely due

to the inhibition of mitochondrial membrane permeabilization, which can also be activated by pro-apoptotic molecules upon various stimuli that initiate apoptosis (Brunelle et al. 2009). Due to its hydrophobic BH3 binding groove, Bcl-2 engages in multiple anti- and pro-apoptotic Bcl-2 protein-protein interactions, i.e., by binding and sequestering the pro-apoptotic Bax (Brunelle and Letai 2009). In this context, the increase of Bcl-2 expression relative to Bax may represent an aspect of hypoxia-induced drug resistance involved in mitochondrial protection upon exposure to cisplatin in HMM cells. In this study, the role of Bcl-xL in promoting drug resistance under hypoxia is unclear. The inability of H513 cells to induce Bax expression in response to external stimuli may be attributable to the p53 mutation. It has been reported that tumor cells with mutant p53 do not induce Bax expression upon apoptotic stimuli (Miyashita et al. 1995). This is consistent with the previous findings that H513 cells failed to induce Bax expression during TNF-related apoptosis-inducing ligand-mediated apoptosis induced by a histone deacetylase inhibitor (Reddy et al. 2007). On the other hand, the present study contradicts the previous finding that drug resistance occurring in hypoxia is a HIF-1α-dependent in HMM cells (Riganti et al. 2008). Our results are supported by a previous finding that HIF-1α is required for aggressive cancer phenotype, including apoptosis resistance, but it is not mandatory for an initial selection of an apoptotic resistance phenotype (Coffey et al. 2005). Although HIF-1α has been extensively investigated as the most important factor in hypoxia-induced drug

resistance, it also holds true that drug resistance does develop independently of the HIF-1α in hypoxic conditions (Doktorova et al. 2015).

Similar to the previous study (Goudarzi et al. 2013), hypoxia enhanced the migratory and invasive properties in HMM cells. Additionally, the present study showed that hypoxia promoted EMT process in HMM cells. In HMM, it was shown that the miR-205-mediated reduction of ZEB1 and ZEB2 increased the expression of E-cadherin, which inhibited migration and invasion (Fassina et al. 2012). The EMT is closely associated with hypoxia and is regulating a more aggressive behavior of cancer cells (Jiang et al. 2011). In HMM carcinogenesis, HIF-1α stabilization promotes EMT process and stemness via an increased expression of TGF-β and stem cell factors (Kim et al. 2013). Moreover, EMT is associated with the emergence of CSCs in HMM cells (Casarsa et al. 2011). Tumor hypoxia is known to recapitulate the HMM microenvironment of the body cavity with HIF-1α expression, and EMT has been potentially implicated in mesothelial carcinogenesis (Klabatsa et al. 2006, Schramm et al. 2010). HIF-1α regulates the expression of a variety of genes involved in EMT-triggering pathways, including TGF-β, Notch, and NF-κB, and EMT-promoting transcription factors, including Twist, Snail, Slug, and, Sip (Jiang et al. 2011). In the same vein, HIF-2α is also implicated in the stimulation of EMT-related factors, such as E-cadherin, LOX, CXCR4, Twist, and Zeb1 (Qing and Simon 2009, Jiang et al. 2011, Keith et al. 2012). On the other hand, a published study showed that hypoxia induced MET through the activation of HIF-2α in

MSTO-211H cells, an HMM cell line (Manente et al. 2016). Cell types and experimental conditions may be responsible for the distinct response to hypoxia with regard to the phenotypic change. Although further studies are required to determine the underlying mechanisms, EMT phenotypes under hypoxia can shed light on understanding how the hypoxic microenvironment governs the malignant progression of HMM.

In conclusion, like other tumors, HMM contains significant areas of hypoxia, but little information is available on the relationship between hypoxia and tumor aggressiveness. The present study illustrated the importance of hypoxia in progression of HMM cells. Understanding about the signaling pathways and molecular mechanisms affected by hypoxia may contribute to the development of therapeutic strategies targeting the microenvironmental influence on HMM biology.

CHAPTER III.

HYPOXIA INDUCES DRUG RESISTANCE THROUGH PRESERVATION OF MITOCHONDRIAL INTEGRITY IN HUMAN MALIGNANT MESOTHELIOMA CELLS

Abstract

The present study was performed to investigate the role of mitochondria in the hypoxia-mediated drug resistance in human malignant mesothelioma (HMM). The viability of HMM cells cultured under hypoxia was less affected by cisplatin treatment compared to those cultured under normoxia. Hypoxia significantly inhibited cisplatin-induced apoptosis in HMM cells. Hypoxia induced mitochondrial hyperpolarization, while cisplatin caused mitochondrial depolarization in HMM cells. The mitochondrial hyperpolarization by hypoxia was augmented by the addition of cisplatin in HMM cells. Mitochondrial depolarization was not related to the opening of mitochondrial permeability transition pore in HMM cells. The generation of mitochondrial hyperpolarization was not related to ATP production or reversal of ATP synthase. Hypoxia significantly inhibited cisplatin-induced mitochondrial ROS stress in HMM cells, while hypoxia enhanced cisplatin-induced ROS stress defined by DCF signals in HMM cells. Amplification efficiency for mitochondrial DNA (mtDNA) revealed that hypoxia significantly decreased cisplatin-induced mtDNA damage in HMM cells. Also, hypoxia inhibited cisplatin-induced degradation of mitochondrial internal structure in HMM cells. Long-term treatment of low dose ethidium bromide depleted mtDNA in HMM cells. The mtDNA-depleted HMM cells showed a significant reduction in cell proliferation, cell viability, mitochondrial membrane potential, intracellular ATP levels, mtROS, and mitochondrial mass. The HMM cells lacking mtDNA were found to lose their

ability to induce drug resistance in hypoxia. Moreover, mtDNA-depleted HMM cells under hypoxia failed to mitigate hypoxia failed to cisplatin-induced mtROS stress. The present study demonstrates that mtDNA-encoded ETC subunits are the core of mitochondria, leading to hypoxia-induced drug resistance in HMM cells. Data in this study may suggest the potential for the therapeutic potential of mtDNA targeting to overcome drug resistance arising from tumor hypoxia.

Introduction

Tumor hypoxia is a hallmark of almost all types of tumor (Hockel et al. 2001). The hypoxic condition is significantly associated with tumor aggressiveness (Vaupel and Mayer 2007). Drug resistance arising from tumor hypoxia is a major obstacle for effective therapy in cancer patients (Cosse and Michiels 2008). The mechanisms underlying the drug resistance in hypoxia are multifactorial, complex, and different depending on cell types, drugs used, and experimental settings (Rohwer and Cramer 2011, Doktorova et al. 2015). Although HIFα has been extensively investigated as an important factor to underpin hypoxia-induced drug resistance, drug resistance can occur independently of HIFα (Doktorova et al. 2015).

Mitochondria are a double-membraned intracellular organelle (Wallace 2012). Mitochondria participate in various physiological functions, including ATP production, apoptosis, ionic homeostasis, reactive oxygen species (ROS) generation, and various intracellular signaling pathways (Wallace 2012). The fact that mitochondria are involved in the regulation of both cell survival and death pathways makes them an attractive target for cancer chemotherapy (Fulda et al. 2010). Direct perturbation in mitochondrial integrity has been suggested to be a promising approach to overcome hypoxia-induced drug resistance (Kumar et al. 2013, Kulikov et al. 2014, Xuan et al. 2014). Mitochondrial have their own genome known as mitochondrial DNA (mtDNA), which encodes 13 polypeptides involved in respiration and oxidative phosphorylation, 2 ribosomal RNAs, and a set of

22 tRNAs that are essential for the protein translation and synthesis in mitochondria (Attardi et al. 1988). The electron transfer chain system is closely associated with modulation of apoptosis (Kwong et al. 2007).

Human malignant mesothelioma (HMM) is an aggressive cancer arising from mesothelium lining of body cavities (Robinson et al. 2005). Occupational exposure to asbestos is closely linked to HMM pathogenesis (Robinson et al. 2005). HMM is highly resistant to traditional anticancer drugs, and cisplatin combined with pemetrexed is the only established first-line chemotherapy (van Zandwijk et al. 2013). Although advances in systemic chemotherapy and diagnostic method have improved clinical outcomes, overall prognosis is still dismal with a mean survival of 9 to 17 months from diagnosis (Blomberg et al. 2015). The major obstacle in a clinical oncology of HMM is an apoptosis resistance to cisplatin (Fennell and Rudd 2004). Published studies have shown that hypoxia is a causal factor for the inefficacy of drugs in HMM, suggesting the potential involvement of mitochondria in drug resistance in hypoxia (Riganti et al. 2008, Giovannetti et al. 2016, Kim et al. 2018). At present, there is no study reporting the role of mitochondria in hypoxia-induced cisplatin resistance in HMM.

In the present study, we scrutinized mitochondrial events affected by hypoxia during cisplatin exposure to HMM cells. We confirmed that hypoxia protects mitochondrial integrity against cisplatin toxicity in HMM cells. In particular, we performed mtDNA depletion, which demonstrated that mtDNA-encoded ETC subunits are a critical part of mitochondria in

hypoxia-induced drug resistance in HMM cells. Data presented in this study are valuable to provide evidence that mitochondrial targeting may be a breakthrough to improve therapeutic efficacy for HMM cells refractory to cisplatin treatment due to tumor hypoxia.

Materials and Methods

Cell culture and reagents

HMM cell lines, MS1 and H513 cells, were maintained at a subconfluent state in RPMI 1640 medium (Mediatech Inc.) with 10% FBS (Mediatech Inc.) and supplements at 37°C in a humidified atmosphere containing 5% CO_2, as previously described (Kim et al. 2018). Cisplatin was purchased from Dong-A pharm. Unless stated otherwise, HMM cells were treated with 10 μM cisplatin. After cisplatin treatment, only attached cells were subjected to experimental analysis. Ethidium bromide (EtBr), uridine, MTT, and Oligomycin were from Sigma-Aldrich. Inhibitor of pan-caspases (zVAD-fmk) was purchased from MBL (Nagoya, Japan). HMM cells were preincubated with zVAD-fmk for 1 h before the addition of cisplatin or hypoxia.

Hypoxic condition

Hypoxic condition (0.1% O_2/5% CO_2/94.9% N_2) was established, as previously described (Kim et al. 2018). HMM cells were incubated in the air chamber and maintained with a humidified environment for 48 h at 37°C.

Flow cytometry analysis

Flow cytometric analysis was performed based on FACS system (Becton Dickinson) equipped with a 488-nm argon laser. Unstained cells were used to gate on live cells. After excluding cell debris from the gated populations, a minimum of 10,000 events per condition were collected for the analysis.

Flowing Software version 2.5.1 (www.flowingsoftware.com) was used to calculate the mean fluorescence intensities of fluorochromes, and the results were represented as an arbitrary unit.

Measurement of cell viability and drug response

The cell viability and drug response were determined by MTT assay, as previously described (Kim et al. 2018). Briefly, HMM cells were subjected to MTT reduction after cisplatin treatment under normoxia or hypoxia. The absorbance values were determined at 570 nm by the Microplate Reader (Gen5). The absorbance from drug-untreated cells under normoxia or hypoxia was normalized to 100%, and all other measurements were expressed as the percentage obtained by comparing the value of control cells with SD.

Apoptosis assay

Apoptosis assay was performed, as previously described (Kim et al. 2018). Briefly, HMM cells were subjected to Annexin V-FITC and PI staining after cisplatin treatment under normoxia or hypoxia. The annexin V or PI positive cells on flow cytometric analysis were considered to be apoptotic and presented as the percentage of apoptotic cells with SD.

Measurement of DNA fragmentation

HMM cells in the log-phase of growth were cultured in normoxia or hypoxia either with or without cisplatin. The cells were harvested and fixed with cold 70% ethanol at -20℃ for 2 h. After washing with PBS, the cells were incubated with 500 μl PI/RNase staining buffer (BD Pharmingen) for 15 min at room temperature. Cell suspensions were analyzed by flow cytometry to determine the cellular DNA contents. Sub G1 phase was considered to be DNA hypoploidy and was presented as the percentage of cells using Modfit LT software (Verity Software House Inc., Topsham, Maine, USA).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP, $\Delta\psi_M$) was evaluated by JC-1 assay (Molecular Probes, Invitrogen) according to manufacturer's instruction. After the exposure to cisplatin or hypoxia, HMM cells were subjected to the staining of JC-1 at a final concentration of 2 μM at 37℃ for 30 min, protected from light. The HMM cells incubated with JC-1 in the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) at 50 mM were used as a positive control for JC-1 assay. HMM cells were harvested, resuspended, and analyzed on a flow cytometer. The MMP was calculated by the ratio of JC-1 aggregates to monomer. The JC-1 ratio less than 1 in CCCP-treated cells was considered to be a dissipation of membrane potential. The JC-1 ratio of untreated control cells under normoxia was considered as 100% MMP, and all other measurements were expressed as

the percentage of the control cell value with SD. For determination of mode of action of F_1F_0 ATP synthase activity, HMM cells were incubated with JC-1 in the presence of oligomycin, an inhibitor of the ATP synthase.

Semi-quantification of intracellular ATP

Cellular ATP level was semi-quantified using ATP-based CellTiter-Glo Luminescent Cell Viability Kit (Promega) according to manufacturer's instruction. Briefly, Cell Titer-Glo reagent was added to HMM cells that were treated with cisplatin under either normoxia or hypoxia. The luminescence values were determined by using luminescent plate reader (Thermo Lab system, Franklin, MA, USA).

Measurement of mitochondrial permeability transition pore opening

Calcein AM fluorescent dye (Molecular Probes) at a final concentration of 2 μM was used to determine mitochondrial permeability transition pore (mPTP) opening. All of procedure in mPTP assay was done according to manufacturer's instruction. After the exposure to cisplatin or hypoxia, HMM cells were harvested and resuspended in pre-warmed Hanks' balanced salt solution (HBSS) containing 2 mmol/L Ca^{2+}. The cells were loaded with calcein AM for 15 min at 37°C in the presence of $CoCl_2$, protected from the light. After washing with HBSS, the cell resuspension was analyzed for calcein fluorescence by flow cytometry. The cells loaded with calcein AM

in the presence of ionomycin were used as a positive control of mPTP opening.

Measurement of mitochondrial mass

Mitotracker Deep Red (Molecular Probes) at the final concentration of 200 nM was used to determine mitochondrial mass. After the exposure to cisplatin or hypoxia, HMM cells were incubated with mitotracker dye at 37°C for 30 min, protected from light. The stained cells were harvested, resuspended, and immediately subjected to flow cytometric analysis.

Measurement of cellular oxidative stress

Cellular oxidative stress was assessed by measuring the fluorescent intensity of ROS with various fluorochromes designed to selectively detect ROS. HMM cells after cisplatin or hypoxia exposure were incubated with 2 μM of cellular hydrogen peroxide H_2O_2 indicator CM-DCFDA (Molecular Probes) for 30 min, 2 μM of mitochondrial superoxide $O_2\cdot^-$ indicator MitoSox Red (Molecular Probes) for 15 min, and 5 μM of mitochondrial hydrogen peroxide H_2O_2 indicator Mito PY1 (Sigma-Aldrich) for 30 min for 30 min at 37 °C, protected from light. After harvest, the cells were suspended in PBS and analyzed by flow cytometry to determine changes in ROS levels.

Measurement of mitochondrial DNA damage

MtDNA damage was measured based on the amplification efficiency for a large fragment mtDNA relative to a short amplicon of mtDNA where various types of DNA lesions slow down or block the progression of DNA polymerase (Mutlu 2012). Total DNA was extracted by using DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instruction. The DNA quality and quantity were determined using Nanodrop 1000 Spectrophotometer (Nanodrop Technologies). The mitochondrial long fragment was amplified by using GoTaq® Long PCR Master Mix kit (Promega) according to manufacturer's instruction. Ten nanograms of genomic DNA were subjected to long PCR reaction (94°C for 2 min 20 s and 19 cycles of 65°C for 9 min and 72°C for 10 min) using the following primer sequences: for 8.9 kb mitochondria fragment, 5'-TCT AAG CCT CCT TAT TCG AGC CGA-3' sense and 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' antisense (Mutlu 2012). After gel electrophoresis, quantification of PCR products was archived with Image J software. To account for the alteration in mtDNA copy number between samples, long PCR amplification values were normalized to the geometric mean by combining levels of COXI, tRNA-Leu (UUA), and short PCR result that is the beginning site of corresponding 8.9 kb mitochondrial genome. The primer sequences for the small fragments used in this study were as follows: 5'-CCC CAC AAA CCC CAT TAC TAA ACC CA-3' sense and 5'-TTT CAT CAT GCG GAG ATG TTG CAT GG-3' antisense (Mutlu 2012). The mtDNA copy number was normalized to amplification of nuclear B2-microglobulin fragment using the

following primer sequences: 5'-TGC TGT CTC CAT GTT TGA TGT ATC T-3' sense and 5'-TCT CTG CTC CCC ACC TCT AAG T-3' antisense (Mutlu 2012). The mitochondrial short fragments were amplified by a quantitative, real-time PCR based on delta delta Ct method (Kim et al. 2018). Ten nanograms of total genomic DNA were subjected to PCR reaction (95°C for 10 min, 45 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 30 s). Each PCR reaction contained 10 µl of SYBR Green Mix, 1 µl of each 10 µM primer, and 7 µl of DNA in RNase free water.

Transmission electron microscopy

Transmission electron microscopy (TEM) examination was performed as previously described (Morris 1965). Briefly, HMM cells were harvested and fixed by Karnovsky's solution for overnight. After washing with 0.05 M sodium carcodylate, the cells were post-fixed by 2% osmium tetroxide for 2 h. The cells were subsequently dehydrated in an ascending alcohol series and finally embedded in Spurr's resin. After complete ultrathin section by EM UC-7 Ultramicrotome (Leica Microsystems, Vienna, Austria), HMM cells were examined by a transmission electron microscope (LIBRA 120, Carl Zeiss, Jena, Germany).

Western blot analysis

Western blot analysis was performed to HMM cells, as previously described without modifications (Kim et al. 2018). Briefly, protein samples were

suspended in sodium dodecyl sulfate loading buffer. After boiling, equal amounts of the proteins were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a nitrous membrane by a wet transfer apparatus. The membranes were probed with primary antibodies using standard techniques. Antibodies against GAPDH β-actin, SOD2, TRX2, PRX3, PINK1, MFN1, and DRP1 were obtained from Santa Cruz Biotechnology (Santa Cruz Biotech); antibody against cleaved Caspase-3 from Cell Signaling Technology (Cell Signaling); antibodies against γ-H2AX and cleaved PARP-1 from Abcam Technology (Abcam); antibody against LC3 from Novus Biologicals (Novus Biologicals, Littleton, CO, USA). The β-actin or GAPDH was used for a loading control. The protein bands were visualized using enhanced chemiluminescence detection reagents (Advansta) on LAS (Las 4000 mini, GE Health Care, USA). The densitometric analysis was performed using the ImageJ program.

Depletion of mitochondrial DNA

Depletion of mtDNA was performed, as previously described (Hashiguchi et al. 2009). HMM cells were cultured in the presence of 50 ng/ml EtBr with 50 μg/ml uridine for more than 4 weeks. The mtDNA-depleted HMM cells, also referred to ρ^0 cells, were maintained under EtBr treatment with uridine throughout the entire experiment. PCR analysis using mtDNA specific primers verified mtDNA depletion of H513 ρ^0 cells, compared to parental cells. The primer sequences used in this study are listed in Table 1. The

mtDNA copy number was normalized to amplification of nuclear B2-microglobulin.

Table 1. Primer sequences used for measurement of mtDNA copy number in this study.

Genes	Direction	Primer sequences (5'->3')
ND1	Forward	AACATACCCATGGCCAACCT
	Reverse	GGCAGGAGTAATCAGAGGTG
ND2	Forward	TAAAACTAGGAATAGCCCCC
	Reverse	TTGAGTAGTAGGAATGCGGT
ND3	Forward	CACAACTCAACGGCTACATA
	Reverse	TTGTAGTCACTCATAGGCCA
ND4	Forward	TCTTCTTCGAAACCACACTT
	Reverse	AAGTACTATTGACCCAGCGA
ND6	Forward	TCCGTGCGAGAATAATGATG
	Reverse	ATAACCTATTCCCCCGAGCA
Cytochrome b	Forward	AGTCCCACCCTCACACGATTC
	Reverse	ACTGGTTGTCCTCCGATTCAGG
COXI	Forward	ACACGAGCATATTTCACCTCCG
	Reverse	GGATTTTGGCGTAGGTTTGGTC
COXII	Forward	ATCAAATCAATTGGCCACCAATGGTA
	Reverse	TTGACCGTAGTATACCCCCGGTC
COXIII	Forward	ACATCCGTATTACTCGCATC
	Reverse	AACCACATCTACAAAATGCC
ATPase 6	Forward	CTCACCAAAGCCCATAAA
	Reverse	AGGCGACAGCGATTTCTA
ATPase 8	Forward	TGCCCCAACTAAATACTACC
	Reverse	ATGAATGAAGCGAACAGATT
tRNA-Leu (UUA)	Forward	CACCCAAGAACAGGGTTTGT
	Reverse	TGGCCATGGGTATGTTGTTA

Statistical analysis

All data are presented as the means ± SD. The bars in the graphs represent at least three biological replicates and representative experimental results were present. P values were calculated by a two-tailed unpaired Student's t-test or one-way ANOVA with Bonferroni post-test correction. Statistical analyses were done by using Microsoft Excel (Microsoft) and SPSS software (IBM). The results were considered to be statistically significant when P value was less than 0.05: * $P < 0.05$ and ** $P < 0.01$.

Results

Hypoxia induces drug resistance through apoptosis inhibition after cisplatin treatment in HMM cells

Hypoxia significantly reduced cell viability of HMM cells (Figure 1A). The viability of HMM cells cultured under hypoxia was significantly less affected by cisplatin at 10 μM, compared to those cultured under normoxia (Figure 1B). We next investigated whether hypoxia affected the apoptosis in HMM cells following cisplatin treatment. Apoptosis analysis showed that hypoxia tended to slightly induce apoptosis, but it did not reach statistical significance (Figure 2A). Cisplatin showed a significant increase in apoptosis, and hypoxia significantly inhibited cisplatin-induced apoptosis in HMM cells (Figure 2A). Parallel measurements of DNA fragmentation also revealed that hypoxia induced a significant reduction in cisplatin-induced sub G1 populations that represent the DNA hypoploidy (Figure 2B). The western blotting analysis also supported that hypoxia protected HMM cells from apoptosis caused by cisplatin. Hypoxia remarkably inhibited the cisplatin-induced formation of caspase 3 and proteolytic fragments of its substrate PARP-1 (Figure 2C). The caspase activation was also confirmed by the treatment of pan-caspase inhibitor, which appreciably prevented apoptosis execution in HMM cells following cisplatin treatment (Figure 2D). Based on these results, it was evident that hypoxia induces drug resistance through inhibition of cisplatin-induced apoptosis.

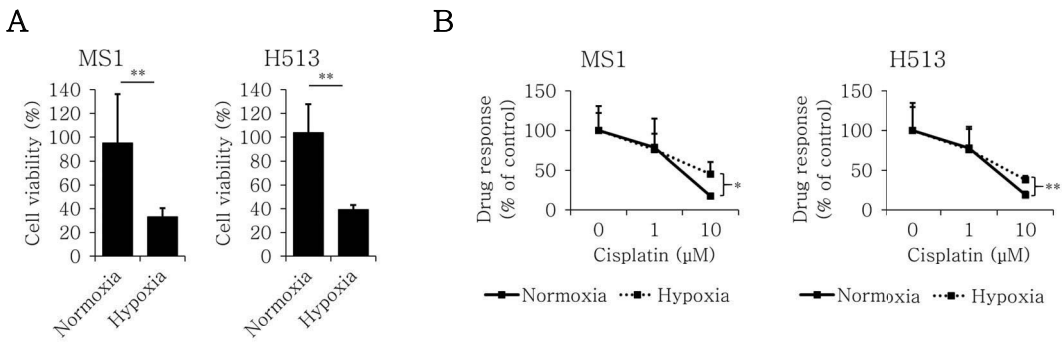


Figure 1. Hypoxia decreases cell viability but reduces the drug sensitivity to cisplatin in HMM cells. (A) HMM cells exposed to hypoxia show a significant reduction in the cell viability. (B) HMM cells under hypoxia significantly decreased the drug sensitivity to cisplatin at 10 μM, compared to cells under normoxia. *P value < 0.05, **P value < 0.01, as calculated by Student's t-test. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

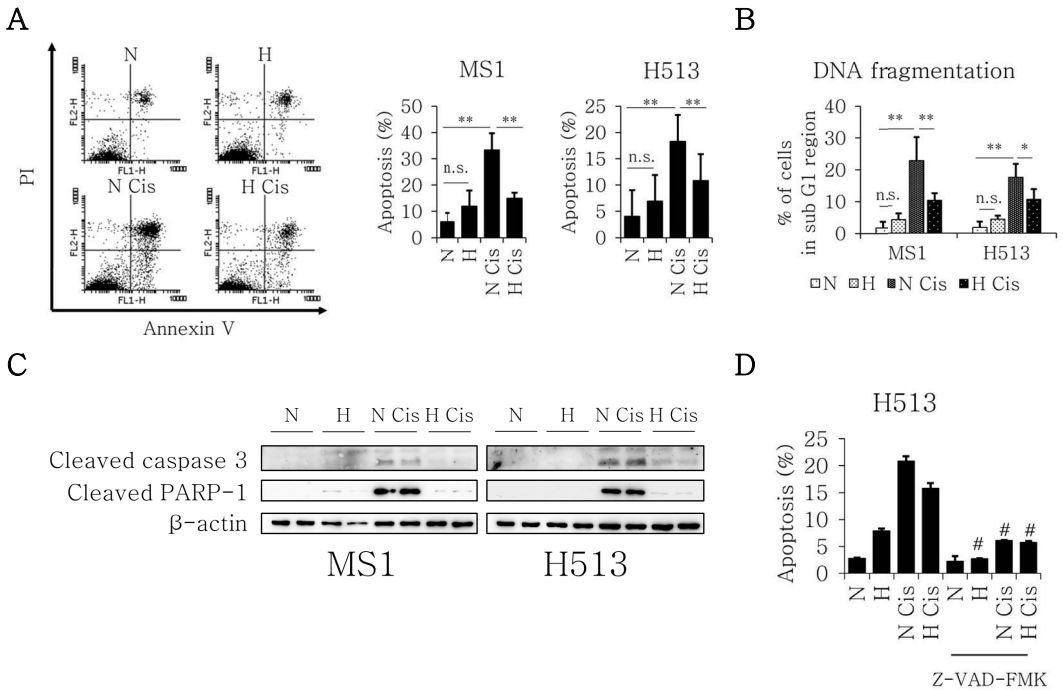


Figure 2. Hypoxia induces apoptosis resistance to cisplatin in HMM cells. (A) Cisplatin induces apoptosis in HMM cells, but hypoxia significantly inhibits the apoptotic induction after the cisplatin treatment. Representative scatter plot for H513 cells is present. (B) Hypoxia reduces the cisplatin-induced increase in sub G1 populations that represent DNA fragmentation in HMM cells undergoing apoptosis. (C) Hypoxia inhibits the cisplatin-induced cleavage of caspase-3 and PARP-1. (D) Treatment of pan-caspase inhibitor Z-VAD-FMK (20 μM) significantly blocks the apoptosis induction in HMM cells. P value was calculated by one-way ANOVA with Bonferroni post-test. *P value < 0.05, **P value < 0.01. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin. # significant different from control groups without treatment. #P value < 0.05.

Hypoxia inhibits cisplatin-induced mitochondrial depolarization and induces mitochondrial hyperpolarization in HMM cells

We investigated mitochondrial membrane potential (MMP, $\Delta\psi_M$) as an integrated appraisal of mitochondrial function in HMM cells. Cisplatin dramatically induced mitochondrial depolarization, as manifested by a decrease of JC-1 red to green fluorescence ratio in HMM cells (Figure 3A). In contrast, hypoxia did not induce the collapse of $\Delta\psi_M$ but exhibited mitochondrial hyperpolarization. Moreover, when HMM cells were co-exposed to cisplatin and hypoxia, the mitochondrial hyperpolarization was further augmented (Figure 3A). These MMP results were not resulted from an increase in mitochondrial mass. The extent of increase in mitochondrial mass was the highest in cisplatin-treated HMM cells in normoxia (Figure 3B). Collectively, these results indicated that hypoxia prevents the disruption of $\Delta\psi_M$ caused by cisplatin and induces hyperpolarization of mitochondrial inner membrane.

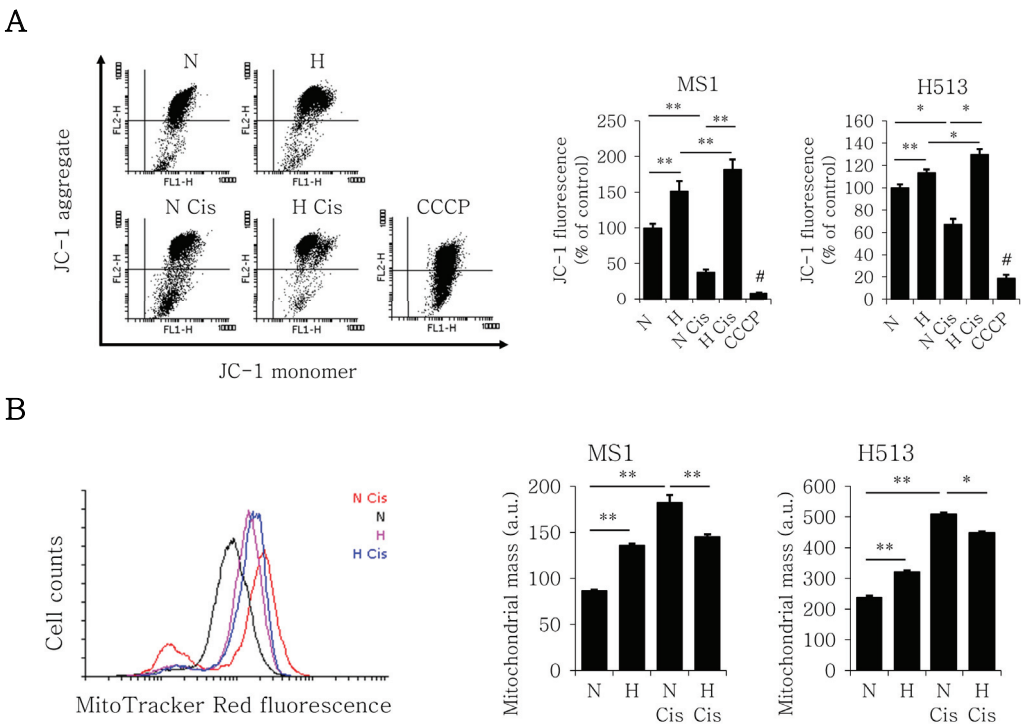


Figure 3. Hypoxia induces mitochondrial hyperpolarization while cisplatin disrupts the membrane potential of mitochondria in HMM cells. (A) Hypoxia prevents cisplatin-induced mitochondrial depolarization. Hypoxia induces mitochondrial hyperpolarization. Hypoxia-induced mitochondrial hyperpolarization is augmented by cisplatin in HMM cells. Representative JC-1 dot plots for MS1 cells are present. (B) The increase of mitochondrial mass is the highest in cisplatin-treated HMM cells. Representative mitotracker histogram for MS1 cells is present. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. # significant different from control groups without treatment. # P value < 0.05. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Hypoxia-induced mitochondrial hyperpolarization is a nonenergetic phenotype of HMM cells to resist cisplatin-induced apoptosis

Mitochondrial membrane potential is known to reflect energetic functionality of mitochondria (Wallace 2012). We investigated whether mitochondrial hyperpolarization is related to ATP production. Semi-quantification of intracellular ATP, however, showed that $\Delta\Psi_M$ did not reflect mitochondrial energy production. It was turned out that cisplatin similarly decreased intracellular ATP amount in HMM cells regardless of oxygenated conditions (Figure 4A). On the other hand, mitochondrial depolarization was not due to opening of mitochondrial permeability transition pore (mPTP), but it appeared to be related to inner membrane integrity of mitochondria (Mancini et al. 2001). In HMM cells, mPTP remained inactivated following treatment of hypoxia or cisplatin where hypoxia significantly decreased the degree of cisplatin-induced mPTP inactivation (Figure 4B). Meanwhile, mitochondrial hyperpolarization can occur due to either inhibition of electron transfer chain (ETC) system or severe ETC dysfunction (Huber et al. 2011, Forkink et al. 2014). In severely damaged cells, F_1F_0 ATP synthase is converted into F_0F_1 ATPase, which hydrolyzes ATP and maintains the $\Delta\Psi_M$ by H^+ efflux from the mitochondrial matrix into intermembrane space. The mode of action of ATP synthase was further investigated in cisplatin-treated hypoxic HMM cells. As a result, co-incubation of JC-1 and oligomycin was found to further increase the $\Delta\Psi_M$ (Figure 4C), confirming H^+ influx into mitochondrial matrix by a forward-mode of ATP synthase

activity. Taken together, these results indicate that hypoxia-induced mitochondrial hyperpolarization is nonenergetic but associated with preservation of inner membrane integrity of mitochondria. Moreover, our results suggest that HMM cells under hypoxia endure cisplatin-induced mitotoxicity via ETC inhibition.

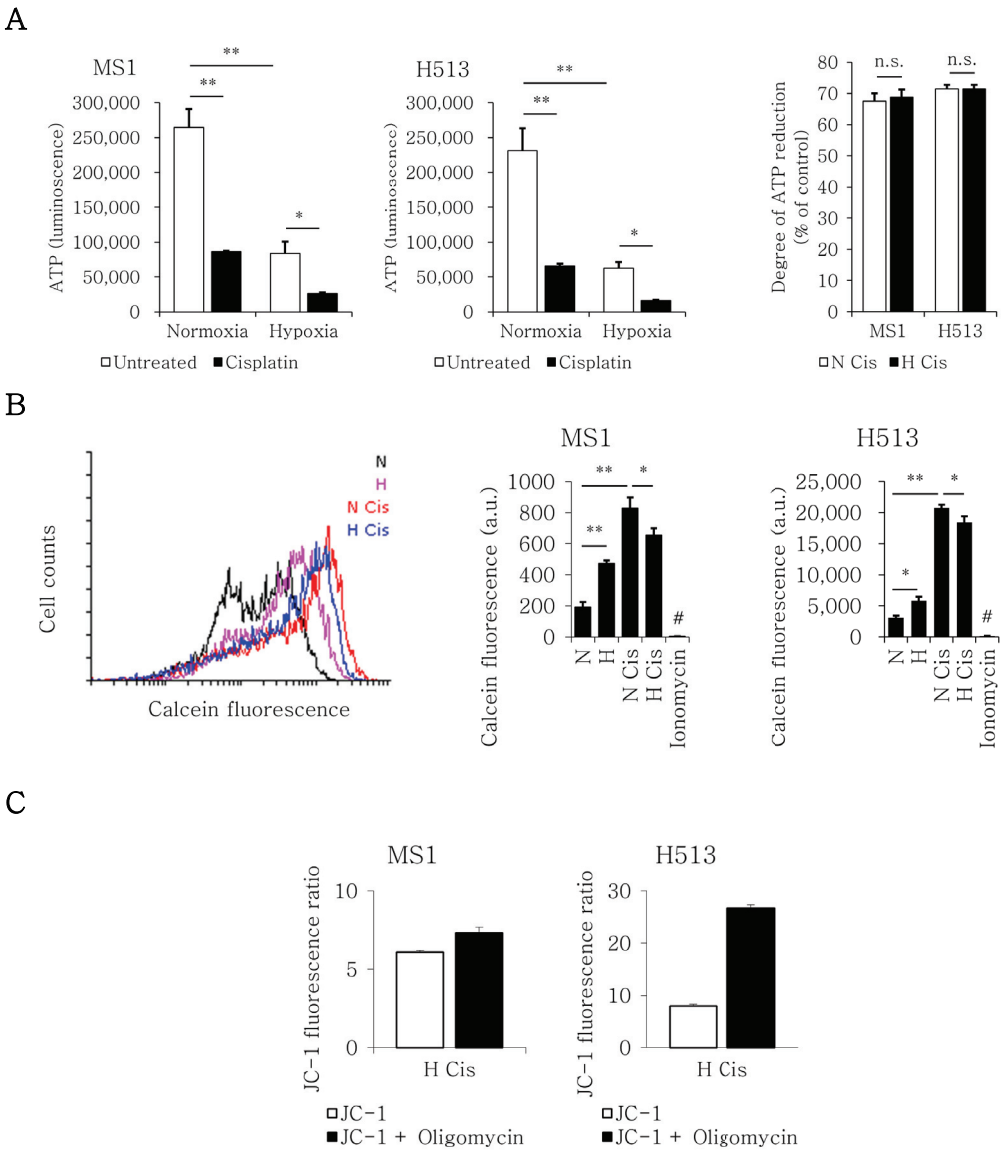


Figure 4. Mitochondrial hyperpolarization is unrelated to mitochondrial energetics, mPTP opening, and reverse mode of action of F_1F_0 ATP synthase in HMM cells. (A) HMM cells exposed to either cisplatin or hypoxia show a significant reduction in intracellular ATP reduction. The degree of cisplatin-induced ATP reduction is not significantly different in HMM cells

under normoxia or hypoxia. (B) The mPTP remained inactivated in HMM cells following cisplatin or hypoxia treatment. Hypoxia attenuates the mPTP inactivation in cisplatin-treated HMM cells. Representative histograms of mPTP results for MS1 cells are present. (C) Oligomycin additionally increases the $\Delta\Psi_M$ in cisplatin-treated hypoxic HMM cells, which confirms the forward mode of F_1F_0 ATP synthase, not the reversal of ATP synthase. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. # significant different from control groups without treatment. # P value < 0.05. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

HMM cells under hypoxia mitigate cisplatin-induced mitochondrial ROS stress

Mitochondrial respiratory chain is a potential source of ROS (Wallace 2012). We hypothesized that mitochondrial ETC inhibition by hypoxia contributes to drug resistance via attenuation of cisplatin-induced oxidative stress in HMM cells. Cytofluorimetric analysis with DCF signals showed that cisplatin or hypoxia significantly induced ROS production (Figure 5A). Unexpectedly, however, hypoxia enhanced the cisplatin-induced oxidative stress in HMM cells (Figure 5A). Because DCF signals are lacking specificity for the detection of mitochondria ROS (Chen et al. 2010, Cardoso et al. 2012), we decided to use mitochondrial-targeted ROS probes. Consistent to our initial hypothesis, hypoxia was found to significantly mitigate cisplatin-induced mitochondrial oxidative stress (Figure 5B and 5C). To investigate whether mitochondrial antioxidant enzymes are involved in the redox homeostasis, western blot analysis was performed. Hypoxia tended to upregulate the expression of mitochondrial antioxidant enzymes, including superoxide dismutase 2 (SOD2), thioredoxin 2 (TRX2), and peroxiredoxin 3 (PRX3) (Figure 5D), although it did not reach statistical significance. However, the expression levels of these proteins were not significantly altered between normoxic and hypoxic HMM cells when treated with cisplatin (Figure 5D). Collectively, these results indicated that hypoxia blocks cisplatin-induced mitochondrial oxidative stress in a possibly non-enzymatic manner in HMM cells.

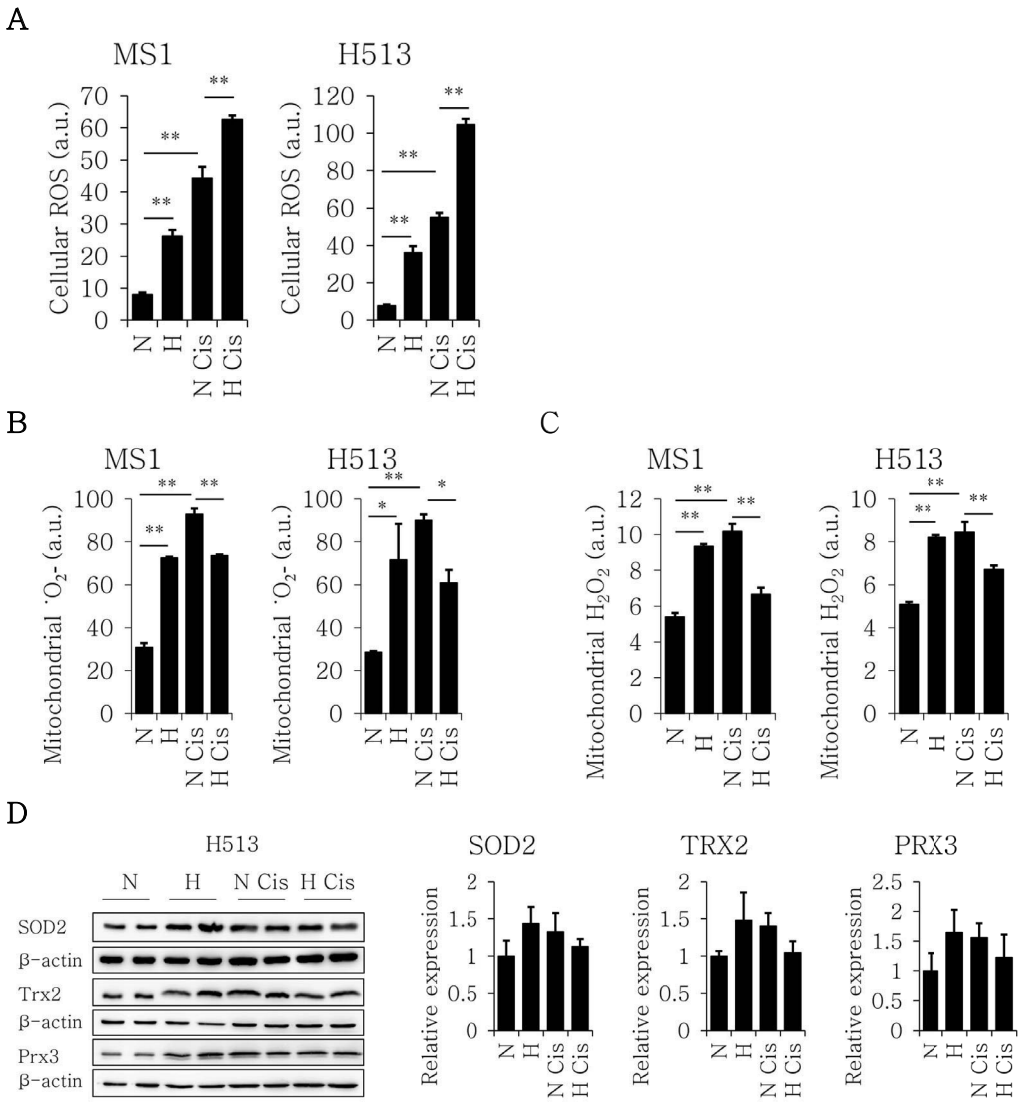


Figure 5. Hypoxia mitigates cisplatin-induced mitochondrial oxidative stress in HMM cells. (A) Cisplatin remarkably increases the ROS levels in HMM cells. Hypoxia enhances the cisplatin-induced ROS accumulation in the cytosolic compartment within HMM cells. In contrast, however, hypoxia significantly inhibits cisplatin-induced accumulation of $O_2 \cdot^-$ (B) and H_2O_2 (C) in mitochondrial compartment within HMM cells. (D) Immunoblot analysis of

mitochondrial redox enzymes. Hypoxia does not upregulate the expression of mitochondrial antioxidant enzymes in cisplatin-treated HMM cells. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Hypoxia protects cisplatin-induced oxidative damages to mtDNA in HMM cells

Mitochondrial oxidative stress results in damages to mitochondrial components, including mtDNA, lipid, or protein (Lenaz 1998). We next investigated the mtDNA damages by measuring mtDNA amplification efficiency in which decreased mtDNA amplification efficiency refers to presence of oxidative mtDNA damages (Mutlu 2012). Analysis of relative mtDNA PCR amplification revealed that cisplatin significantly decreased the mtDNA amplification efficiency in HMM cells, but hypoxia did not affect the amplification efficiency (Figure 6A). However, HMM cells under hypoxia significantly prevented the cisplatin-induced reduction in mtDNA amplification efficiency, compared to those cultured in normoxia (Figure 6A). Figure 6B showed the changes in mtDNA of HMM cells. Cisplatin significantly increased mtDNA content in HMM cells, while hypoxia maintained mtDNA copy number at the basal levels. These results indicated that hypoxia is beneficial for preservation of mtDNA integrity in cisplatin-treated HMM cells.

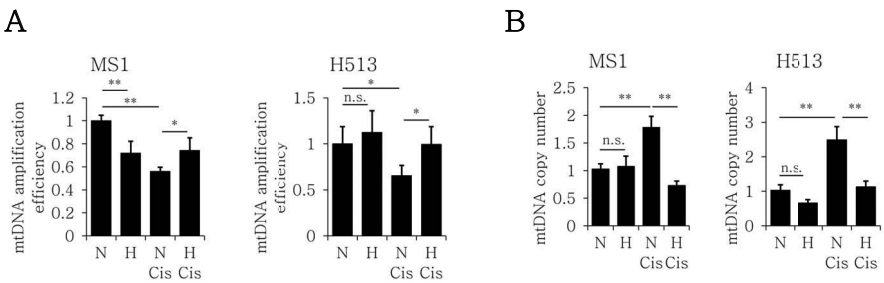


Figure 6. Hypoxia reduces cisplatin-induced mtDNA damage in HMM cells.

(A) Cisplatin significantly induces mtDNA damage in HMM cells. However, hypoxia is found to significantly decrease cisplatin-induced mtDNA damage in HMM cells. (B) Cisplatin significantly increases mtDNA copy numbers in HMM cells. The mtDNA copy number was not significantly altered by hypoxia in HMM cells. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Hypoxia inhibits cisplatin-induced degradation of mitochondrial internal ultrastructure in HMM cells

We also performed to TEM examination in order to investigate whether hypoxia is involved in the preservation of mitochondrial structures in HMM cells. Compared to well-formed cristae of normal mitochondria (Figure 7A), cisplatin caused vesicular structures of mitochondria with a lucent-swelling matrix (Figure 7B). Following cisplatin treatment, mitochondria also contained large electron-lucent space and disorganized few cristae (Figure 7B). Under hypoxia, HMM cells showed almost normal morphologies of mitochondria (Figure 7C). In combination of cisplatin and hypoxia, HMM cells revealed hyper-condensed mitochondria characterized by spherical, bean, or tubular shaped electron-dense matrix (Figure 7D). Moreover, the rarefaction of the matrix and degeneration of internal cristae were rarely observed in cisplatin-treated hypoxic HMM cells. Taken together, these results indicated that hypoxia protects structural integrity of mitochondria in HMM cells following cisplatin treatment.

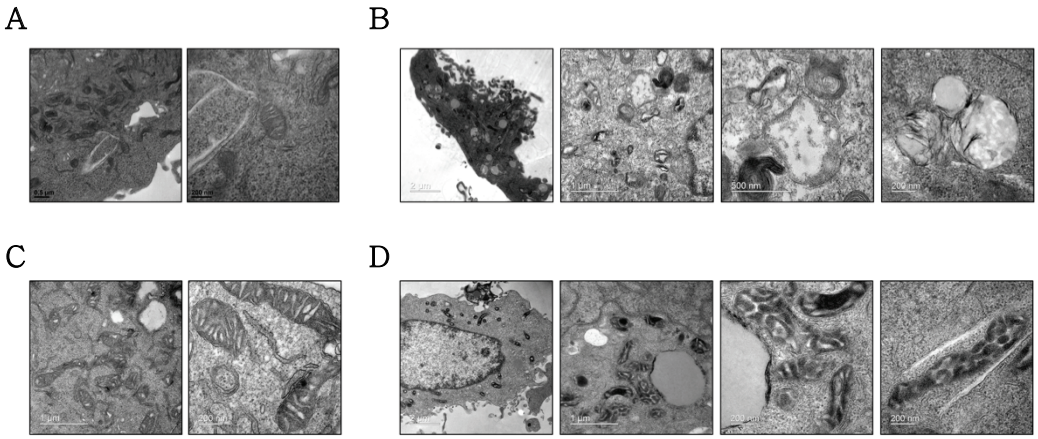


Figure 7. Hypoxia protects internal ultrastructure of mitochondria against cisplatin mitotoxicity in HMM cells. (A) Normal mitochondria with well-formed cristae are present in H513 cells. (B) Abnormal mitochondria with membrane disruption and degraded internal structures are present in H513 cells after cisplatin treatment. (C) A few small electron-lucent spaces were observed in hypoxic mitochondria within H513 cells. (D) Mitochondria display thickening of cristae with spherical, bean, or tubular shaped electron-dense matrix within H513 cells exposed to cisplatin and hypoxia together.

Mitochondrial dynamics is not involved in hypoxia-induced drug resistance in HMM cells

Mitochondrial dynamics, such as fusion, fission, and mitophagy, has been reported to involve drug resistance. Autophagic elimination of damaged mitochondria contributes to prevention of cells from apoptosis (Jangamreddy et al. 2012). Autophagy induction was investigated by the expression of the microtubule-associated protein 1 light chain 3 (LC3)-II. During hypoxia or cisplatin treatment, there was no significant alteration in the expression of LC3-II in MS1 cells (Figure 8A). In H513 cells, however, cisplatin significantly reduced the LC3-II expression, and hypoxia inhibited the cisplatin-induced downregulation of LC3-II (Figure 8A). Meanwhile, the expression levels of PINK1 were decreased by cisplatin or hypoxia treatment (Figure 8B). On the other hand, the expression levels of MFN1 and DRP1 were downregulated by hypoxia or cisplatin treatment (Figure 8C). Hypoxia did not significantly affect the expression of MFN1 and DRP1 in cisplatin-treated HMM cells (Figure 8C). Taken together, these results indicated that mitochondrial dynamics is unlikely to play a critical role in hypoxia drug resistance in HMM cells.

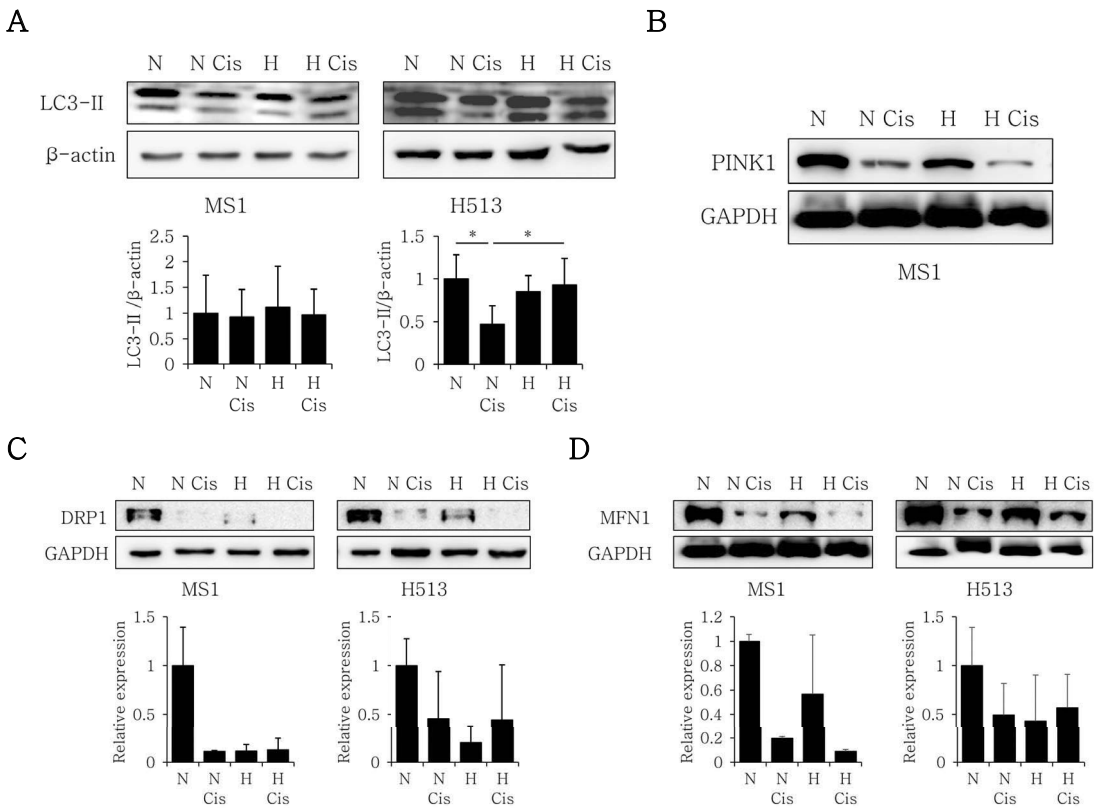


Figure 8. Immunoblot analysis of proteins involved in mitochondrial dynamics and mitophagy in HMM cells. (A) LC3. (B) PINK1. (C) DRP1. (D) MFN1. P value was calculated by one-way ANOVA with Bonferroni post-test. * P value < 0.05. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Mitochondrial DNA depletion overcomes hypoxia-induced drug resistance in HMM cells

Mitochondrial ETC complex is a potential site with regard to ROS production, $\Delta\psi_M$ generation, and apoptosis modulation (Kwong et al. 2007). We hypothesized that mitochondrial ETC complex would be a critical factor in hypoxia drug resistance in HMM cells. Long-term exposure of low-dose EtBr to HMM cells significantly depleted large mtDNA regions (Figure 9A). The ρ^0 HMM cells showed a significant reduction in the cell proliferation (Figure 9B), cell viability (Figure 9C), $\Delta\psi_M$ (Figure 9D), intracellular ATP (Figure 9E), mitochondrial ROS production (Figure 9F), and mitochondrial mass (Figure 9G), compared to parental cells. Finally, we investigated the impact of mtDNA depletion on hypoxia drug resistance in ρ^0 HMM cells. Hypoxia still induced drug resistance in parental HMM cells, but ρ^0 cells failed to develop hypoxia drug resistance (Figure 9H). Moreover, it was found that hypoxia significantly enhances cisplatin-induced apoptosis in ρ^0 H513 ells. Western blot analysis also supported these phenotype changes. The expression levels of caspase 3 and PARP-1 proteolytic fragments were similar between normoxic and hypoxic ρ^0 cells following cisplatin treatment (Figure 9I). With cisplatin treatment, on the other hand, hypoxia significantly decreased the cisplatin-induced upregulation of ɣ-H2AX, a marker of nuclear DNA double-strand breaks, in both parental and ρ^0 HMM cells (Figure 9I). Taken together, these results indicate that mtDNA-encoded

ETC subunits are critical for the development of drug resistance under hypoxia in HMM cells.

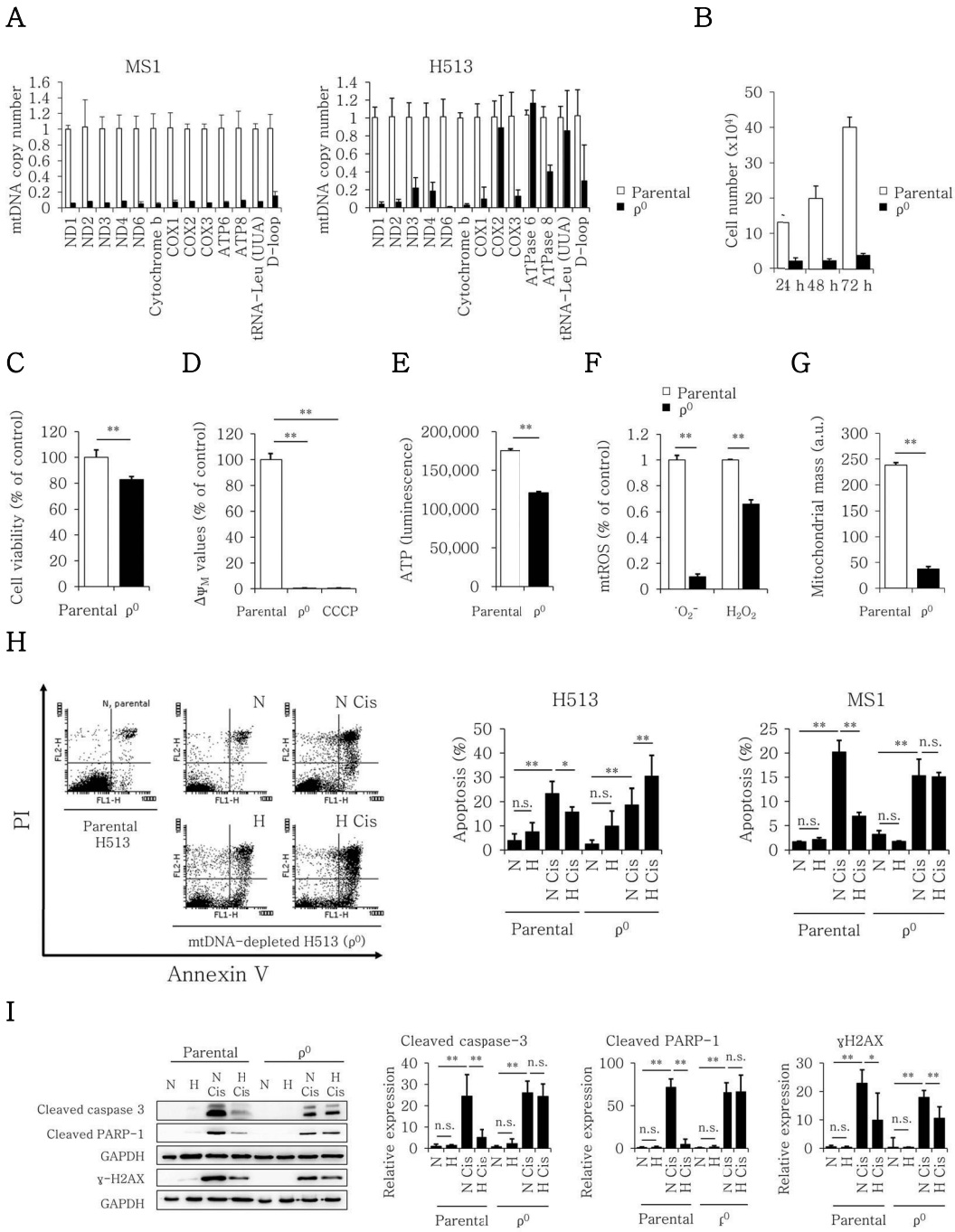


Figure 9. MtDNA depletion abrogates apoptosis resistance to cisplatin in hypoxic HMM cells. (A) Establishment of mtDNA-depleted HMM cells.

Treatment of low concentration of EtBr significantly depletes mtDNA copy numbers in HMM cells. The mtDNA-depleted MS1 cells rarely proliferate (B). The H513 cells lacking mtDNA show a significant reduction in cell viability (C), a $\Delta\Psi_M$ (D), intracellular ATP levels (E), mitochondrial ROS production (F), and mitochondrial mass (G), compared to that of parental cells. (H) The mtDNA depletion abrogates hypoxia-induced apoptosis resistance to cisplatin in HMM cells. While parental HMM cells under hypoxia shows apoptosis resistance to cisplatin, a considerably greater increase in apoptosis was found in cisplatin-treated hypoxic ρ^0 H513 cells. In ρ^0 MS1 cells, hypoxia does not significantly decrease cisplatin-induced apoptosis. (I) Western blot analysis. In ρ^0 H513 cells, hypoxia does not inhibit the cisplatin-induced activation of caspase 3 and PARP-1, compared to parental cells. Also, it is noteworthy that hypoxia shows a similar degree of reduction in nuclear DNA damage after cisplatin treatment in parental and ρ^0 H513 cells. P value was calculated by Student's *t*-test or one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Hypoxia enhances cisplatin-induced mitochondrial ROS stress in mtDNA-depleted HMM cells

We next investigated biochemical alterations of mitochondria that may be related to the diminishment of the hypoxia-induced drug resistance in mtDNA-depleted HMM cells. The ρ^0 HMM cells tended to recover the $\Delta\psi_M$ following exposure to hypoxia or cisplatin, but it still remained completely depolarized, compared to $\Delta\psi_M$ of parental HMM cells (Figure 10A). In ρ^0 HMM cells, intracellular ATP contents continued to decrease by the addition of cisplatin or hypoxia (Figure 10B). The ATP level was approximately 3% of that of parental HMM cells when ρ^0 HMM cells were treated with cisplatin and hypoxia together. In contrast to parental HMM cells (Figure 5C), it was noteworthy that hypoxia did enhance cisplatin-induced mitochondrial oxidative stress in ρ^0 HMM cells (Figure 10C). Taken together, these results finally confirm that hypoxia is closely related to mitochondrial redox control involved in hypoxia-induced drug resistance in HMM cells.

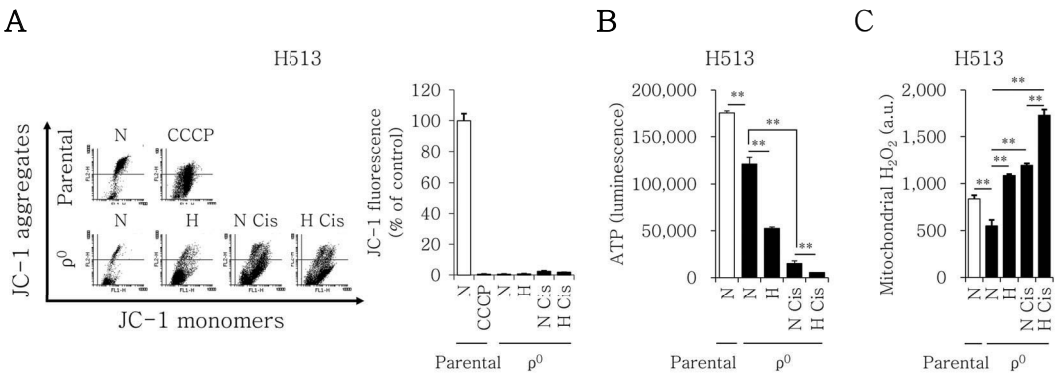


Figure 10. Analysis of biochemical alterations of mitochondria in mtDNA-depleted HMM cells exposed to hypoxia or cisplatin. (A) The mtDNA-depleted HMM cells show complete depolarization of the mitochondrial inner membrane, compared to basal level of $\Delta\Psi_M$ in parental HMM cells. (B) Intracellular ATP levels continue to reduce in ρ^0 HMM cells as an external stimulus is added. Severe ATP depletion is observed in ρ^0 HMM cells cotreated with hypoxia and cisplatin. (C) In ρ^0 HMM cells, mitochondrial H_2O_2 continues to increase as an external stimulus is added. P value was calculated by Student's *t*-test or one-way ANOVA with Bonferroni post-test. * P value < 0.05, ** P value < 0.01. Abbreviations: N, normoxia; H, hypoxia; Cis, cisplatin.

Discussion

Clinical and biological evidence of tumor hypoxia exists in HMM (Francis et al. 2015). Previous studies reported that hypoxia promotes the acquisition of more aggressive behaviors in HMM cells, including drug resistance (Riganti et al. 2008, Giovannetti et al. 2016, Kim et al. 2018). The present study was performed to investigate the role of mitochondria in the hypoxia-induced cisplatin resistance in HMM cells. In the present study, it is demonstrated that hypoxia mitigates cisplatin-induced detrimental effects on mitochondrial integrity in HMM cells. Moreover, mtDNA-encoded ETC subunits are proved the very core of mitochondria, allowing HMM cells under hypoxia to induce apoptosis resistance to cisplatin.

Mitochondrial hyperpolarization is commonly found in various types of cells that show resistance to diverse death stimuli (Zamzami et al. 1995, Liang et al. 1999, Beltrán et al. 2000, Bonnet et al. 2007, Huang et al. 2013). The hyperpolarized mitochondria are also a phenomenological feature of cisplatin-resistant cancer cells (Andrews et al. 1992, Zinkewich-Péotti et al. 1992, Isonishi et al. 2001). Currently, it is not completely understood whether mitochondrial hyperpolarization has a direct role in cell fate decision or are indirectly involved. However, mounting evidence favors pro-survival nature of mitochondrial hyperpolarization. The maintenance of $\Delta\Psi_M$ is important to preserve mitochondrial integrity upon hostile conditions, including hypoxia (Boutilier and St-Pierre 2000, Huang et al. 2013). Mitochondrial hyperpolarization is suggested to protect further mitochondrial damages in cells (Zamzami et al. 1995). Disruption of $\Delta\Psi_M$ is closely related to the killing of cancer cells, including HMM cells (Hopkins-

Donaldson et al. 2003, Stewart IV et al. 2007, Kovarova et al. 2014, Lee and Lee 2016). In the present study, hyperpolarized phase of mitochondria was not related to severe bioenergetic crisis occurring just before a terminal stage of cell death (Huber et al. 2011). Based on these results, mitochondrial hyperpolarization is believed to represent a phenotypic manifestation of hypoxic HMM cells that evade cisplatin-induced apoptosis. Conflicting data, however, have been also presented regarding the biological implication of mitochondrial hyperpolarization in cell survival. Few reports argued that mitochondrial hyperpolarization is an initial, transient phenomenon occurring in dying cells (Gergely Jr et al. 2002, Iijima et al. 2003). In this regard, mitochondrial hyperpolarization might not be a unifying protective mechanism of all types of cells undergoing mitochondrial apoptosis. For these inconsistent results, cell types and variety of conditions, such as duration or intensity of death-inducing signals used, might be involved, which warrants further investigation.

Mitochondrial hyperpolarization appears when mitochondrial respiration is inhibited (Beltrán et al. 2000, Forkink et al. 2014). The inhibition of mitochondria has been correlated with drug resistance in which reduced mtROS generation and oxidative damages are suggested to have a role (Santamaría et al. 2005, Chen et al. 2007, Oliva et al. 2010, Oliva et al. 2011, Cho et al. 2013, Okamoto et al. 2017). Pharmacologic inhibition of mitochondrial respiratory chain also experimentally demonstrated the protective effect of hypoxia against cisplatin-induced apoptosis (Wang et al. 2006). Likewise, reactivation of mitochondria showed increased therapeutic efficacy of cisplatin in hypoxic lung cancer cells (Shin et al. 2013). Thus,

based on these results, it is highly likely that inhibition of mitochondria is a central mechanism of HMM cells under hypoxia to induce drug resistance. The consequence of mitochondrial inhibition would be related to mitigation of cisplatin-induced oxidative stress to mitochondria. The results of mtDNA damage and ultrastructural examination of mitochondria strongly support this notion. The direct correlation between less presence of oxidative mtDNA lesions and drug resistance has been proved (Hirama et al. 2006, Cao et al. 2007). Loss of mitochondrial ultrastructure is interpreted to be a failure of cell adaptation to mitochondrial oxidative toxicity (Mancini et al. 2001, Li et al. 2005, Graves et al. 2012). The observation that mtDNA depletion reversed the pattern of cisplatin-induced mtROS generation also supports the relation between hypoxia and mitochondrial redox control with regard to drug resistance in HMM cells. Meanwhile, a published study argued that hypoxia enhances cisplatin-induced apoptosis in renal collecting cells (Schwerdt et al. 2005). Mitochondria from different cell types and experimental conditions might be responsible for the contrary response to hypoxia.

Considering high stability and membrane permeability of H_2O_2, redox compartmentalization observed in this study is noteworthy. Our finding is quite similar to that of previous study in that mitochondrial ROS starts to excessively accumulate in cytosol following mitochondrial hyperpolarization (Zorov et al. 2006). Although underlying mechanisms have not been elucidated in detail, hypoxia and mitochondria might be involved in active redistribution of intracellular ROS between organelles. Growing body of evidence has shown that hypoxia facilitates the shift of mitochondrial ROS

release from mitochondrial matrix toward intermembrane space and cytosol in tumor cells (Muller et al. 2004, Guzy et al. 2006). Moreover, there is evidence that hyperpolarized mitochondria at least in part participate in non-enzymatic $O_2{\cdot}^-$ dismutation and efflux of the expedited H_2O_2 into the cytosol (Zorov et al. 2006, Afanas' ev 2012, Cardoso et al. 2012, Policastro et al. 2013). In the present study, extramitochondrial ROS alone did not explain cisplatin-induced apoptosis in hypoxic HMM cells. Conceivably, it is plausible that redox status of mitochondria is *bona fide* the most critical for apoptosis determination. Indeed, cytosol and nuclear compartments are better durable to oxidative stress than mitochondria (Kaludercic et al. 2014). On the other hand, our notion is also supported by the fact that either formation of nuclear DNA adduct alone cannot solely explain the execution of apoptosis after cisplatin treatment (Wohlkoenig et al. 2011). For the ROS burst in the cytosol, there might be a noncytotoxic role of ROS at least in our experimental conditions. A study suggested that elevated $\Delta\Psi_M$ triggers signaling transduction that modulates drug sensitivity in cisplatin-resistant cancer cells (Andrews and Albright 1992). Another study also demonstrated that hypoxia augments H_2O_2 signaling in the cytosol while reducing oxidative stress in the mitochondria (Waypa et al. 2010). Indeed, H_2O_2 can be non-apoptotic, contrary to $O_2{\cdot}^-$ (Sawada et al. 2001, Devadas et al. 2002, Afanas' ev 2009). Thus, it might be possible that cytosolic ROS acts as a signaling molecule. Redox processes, such as modification of protein kinases or phosphatases of enzymatic cascades (Rhee 2006) or mitochondrial retrograde signaling pathway (Liu et al. 2006), might be involved in

hypoxia-mediated drug resistance in HMM cells. Further insight into this aspect is left to future work.

The mtDNA depletion was performed to verify the role of mitochondria. The EtBr treatment successfully depleted mtDNA in HMM cells, followed by mitochondrial defects, which were consistent to previous studies using HMM cell lines (Tomasetti et al. 2014, Lim et al. 2015). In the present study, the sensitivity of HMM cells to cisplatin remained unaffected by mtDNA depletion. Consistent to our results, a study reported no alteration in cisplatin sensitivity between parental and mtDNA-depleted HMM cells (Lim et al. 2015). The sensitivity to drugs, including cisplatin, in mtDNA-depleted tumor cells, can be highly variable depending on different cell origins, conditions, and compensatory mechanisms, and the specific reason is unclear (Cavalli et al. 1997, YEN et al. 2005, Yang et al. 2006).

Data presented in this study confirms the requirement of all ETC subunits for hypoxia-induced drug resistance in HMM cells. Previous studies favor this finding. Mammalian ρ^0 cells with defective OXPHOS system cannot cope with hypoxic conditions (Brunelle et al. 2005). Intact mitochondria are required for hypoxia-induced cisplatin resistance in kidney cells (Schwerdt et al. 2005). At present, no study satisfactorily explains our finding, but remodeled ETC complexes have the possibility of the loss of mitochondrial ability to induce drug resistance in hypoxic conditions (Oliva et al. 2010, Oliva et al. 2011). For example, chronic inhibition of complex I and sustained low activity of other complexes underlie mitocondrial inhibition (Forkink et al. 2014). The complex I is particularly critical for adaptation to hypoxia (Calabrese et al. 2013). Likewise, cellular oxygen sensing and hypoxic

adaptation requires complex III (Guzy et al. 2005, Guzy and Schumacker 2006). Analysis of a single mtDNA mutation further strenghens the importance of the presence of the respiratory chain subunits. The mutation of mitochondrial NADH dehydrogenase (ND) 6 gene resulted in abnormality in complex I activity and assembly, which leads to a defective response of glioma cells to hypoxia (DeHaan et al. 2004). The complex I defect arising from non-assembled ND1 subunit was demonstrated to impair apoptosis resistance (Stiburek et al. 2012). The mutation of cytochrome b gene has potential to cause a combined deficiency of complex I and III (Lamantea et al. 2002). Mitochondrial ATP6 and ATP8 subunits are key constituents for complex V whose repression is involved in apoptosis resistance in human carcinomas (Santamaría et al. 2005). The mutation in mitochondrial ATP6 or ATP8 gene results in mtROS generation due to impairments of Fo part of ATP synthase (Baracca et al. 2007). Either generation of trans-mitochondrial cybrid, characterized by the fusion of a ρ^0 cell line with enucleated cells that contain mitochondria with an interest of mtDNA mutation, or mitochondrial genome editing appears to be promising to scrutinize the role of a mtDNA-encoded subunit (Taylor et al. 2005, Jo et al. 2015). On the other hand, it cannot be excluded that rRNAs or tRNAs encoded by mtDNA produced generalized mitochondrial respiratory chain deficiency.

In the present study, mitochondria do not appear to be actively involved in compensatory energy production under severe hypoxia, as previously described (Schwerdt et al. 2005). Published studies have shown that hyperpolarized mitochondria are energetically nonfunctional (Hirama et al.

2006, Marrache et al. 2014). Moreover, $\Delta\Psi_M$ and ATP can change independently (Beltrán et al. 2000, Poppe et al. 2001, Wang et al. 2006). ATP-independent processes, such as substrate availability, ionic homeostasis, protein import, or membrane topology, might be potentially related to mitochondrial inhibition underlying hypoxia-induced drug resistance in HMM cells (Hunter Jr et al. 1956, Young 1973, Skulachev 1988). Of interest, ATP was not a limiting factor or significant consideration for apoptosis despite severe ATP depletion predicted in ρ^0 HMM cells encountered during cisplatin and hypoxia treatment. A minimum level of ATP enough to execute apoptosis in HMM cells seemed to be maintained in our experimental condition.

The biological implication of mPTP inactivation is unclear in this study. According to Fennell *et al.*, the closure of mPTP pore might be a result resulting from disruption of core apoptosis machinery in HMM (Fennell and Rudd 2004). The increase of mitochondrial mass and mtDNA contents following external stimulus is interpreted to be a mitochondrial adaptive response. A previous study reported that during apoptosis proliferation of hypofunctional mitochondria appears in a redox-dependent manner (Mancini et al. 2001). Our preliminary data alluded that mitochondrial dynamics less likely plays a role in hypoxia-induced drug resistance. However, morphology study of mitochondria using fluorescence confocal microscopy is additionally needed to consolidate our conclusion.

Clinically relevant concentration of cisplatin is found to target only mitochondria in head and neck cancer (Cullen et al. 2007). Apoptosis still occurs even in enucleated cells during cisplatin treatment (Fuertes et al.

2003). Moreover, there is a large body of data documenting that mitochondrial targeting breaks through hypoxia-mediated problem of apoptosis resistance in multiple cancer types (Xu et al. 2005, Kumar et al. 2013, Kulikov et al. 2014, Mitani et al. 2014, Xuan et al. 2014). Currently, there is no study to focus on mtDNA depletion aimed at examining the importance of mitochondria in hypoxia and drug resistance. Generation of ρ^0 cell lines has been reported in multiple types of cancer. Thus, our experimental approach is novel, and data will be valuable to provide convincing evidence for the therapeutic potential of mtDNA targeting to overcome drug resistance arising from tumor hypoxia. Mutational study of mtDNA gene will be useful to identify specific function and mode of action of mitochondrially encoded subunits as well as their actual contribution to hypoxia-induced drug resistance. Finally, results presented in this study will contribute to the understanding of molecular mechanisms for cell or organism adaptive response to anaerobic stress encountered during normal and pathophysiological conditions.

GENERAL CONCLUSION

Contrary to normal cells, cancer cells survive in hostile tumor microenvironments characterized by high oxidative stress and O_2 deprivation (Cerutti 1985, Vaupel et al. 1989). The adaptation to hostile conditions plays an important role in cancer progression. In the present study, tumor hypoxia and oxidative stress are demonstrated to be a critical part of cancer progression in HMM. Moreover, mitochondria are the key component responsible for hypoxia-induced drug resistance in HMM cells. Thus, a control measure of oxidative status and hypoxia will be valuable to establish an effective therapeutic strategy to reduce the aggressiveness of HMM. The present study next highlights that mtDNA can be an ideal prospect for therapeutic intervention in advanced cancer. Finally, our data will be precious to understand molecular mechanisms for cell or organism adaptive response to anaerobic stress encountered during normal and pathophysiological conditions.

Chapter I.

1) H_2O_2-induced oxidative stress induces EMT and acquires stemness in HMM cells.

2) H_2O_2-induced oxidative stress upregulates EMT-related signaling molecules HIF-1α and TGF-β1 in HMM cells.

3) HIF-1α and TGF-β1 is an upstream regulator of EMT-related

molecules, including E-cadherin, vimentin, SLUG, and TWIST1, in H_2O_2-treated HMM cells.

4) TWIST1 represents ROS-mediated EMT and cancer progression in HMM *in vivo*.

5) Oxidative stress enhances stemness through upregulation of OCT4, SOX2, and NANOG genes in HMM cells.

Chapter II.

1) Hypoxia is a critical factor in the acquisition of more aggressive behaviors of HMM cells, including *in vitro* clonogenicity, migration, invasion, anti-apoptosis, and drug resistance.

2) HIF-2α enhances OCT4-induced stemness in HMM cells under hypoxia.

3) HMM cells under hypoxia enhance motility and invasiveness through HIF-1/2α and EMT activation.

4) Anti-apoptotic Bcl-2 plays an important role in hypoxia-induced drug resistance in HMM cells.

5) It is unlikely that HIF-1α and HIF-2α have a critical role in hypoxia-induced drug resistance in HMM cells.

Chapter III.

1) Hypoxia induces drug resistance through apoptosis resistance to

cisplatin in HMM cells.

2) Hypoxia is critical for the protection of mitochondrial integrity regarding $\Delta\psi_M$, redox status, mtDNA, and ultrastructure, against cisplatin cytotoxicity in HMM cells.

3) Mitochondrial inhibition sustained by respiratory complex activities is believed to be a central mechanism that allows HMM cells under hypoxia to induce drug resistance.

4) Redox compartmentalization may be a strategy of HMM cells under hypoxia to evade cisplatin-induced apoptosis.

5) Targeting of mtDNA has the potential to overcome hypoxia-induced drug resistance in HMM cells.

Limitations and future directions

A potential limitation of this study is the establishment of hypoxia, because the setting of hypoxia in a cell culture incubator does not perfectly reflect *in vivo* physiological situations, including intermittent hypoxia or reoxygenation (Wenger et al. 2015). Another limitation of our study is that underlying mechanisms have not been addressed in detail. For example, it should be answered how mitochondria govern protein signaling network that elicits the protection schemes of HMM cells in hypoxic conditions against cisplatin cytotoxicity. Additional investigations might be also required to

study whether parameters for mitochondrial function are involved independently or in association with each other. Validation of our findings using both *in vitro* and *in vivo* different types of cancer models will draw more consolidate and general conclusion. Introduction of future technologies designed to precisely delete specific mtDNA region will identify distinct subsets of ETC subunits that play a key role in hypoxia-induced drug resistance. This will substitute the method of chemically-induced large mtDNA depletion, which opens an avenue for experimental manipulation of mitogenome in various normal and pathophysiological conditions.

REFERENCES

Abutaily, A., J. Collins and W. Roche (2003). "Cadherins, catenins and APC in pleural malignant mesothelioma." The Journal of Pathology 201(3): 355-362.

Afanas' ev, I. (2012). "Enhanced Free Radical Status of Cancer Cells Success and Failure of Prooxidant/Antioxidant Treatment." Current Signal Transduction Therapy 7(3): 228-236.

Afanas' ev, I. B. (2009). Signaling mechanisms of oxygen and nitrogen free radicals, CRC Press.

Akçay, T., I. Saygılı, G. Andican and V. Yalçın (2003). "Increased formation of 8-hydroxy-2'-deoxyguanosine in peripheral blood leukocytes in bladder cancer." Urologia Internationalis 71(3): 271-274.

Alvarez-Tejado, M., S. Naranjo-Suárez, C. Jiménez, A. C. Carrera, M. O. Landázuri and L. del Peso (2001). "Hypoxia induces the activation of the phosphatidylinositol 3-Kinase/Akt cell survival pathway in PC12 cells protective role in apoptosis." Journal of Biological Chemistry 276(25): 22368-22374.

Amati, M., M. Tomasetti, M. Scartozzi, L. Mariotti, R. Alleva, E. Pignotti, B. Borghi, M. Valentino, M. Governa and J. Neuzil (2008). "Profiling tumor-associated markers for early detection of malignant mesothelioma: an epidemiologic study." Cancer Epidemiology and Prevention Biomarkers 17(1): 163-170.

Åmellem, Ø. and E. Pettersen (1991). "Cell inactivation and cell cycle inhibition as induced by extreme hypoxia: the possible role of cell cycle arrest as a protection against hypoxia-induced lethal damage." Cell Proliferation 24(2): 127-141.

An, W. G., M. Kanekal, M. C. Simon, E. Maltepe, M. V. Blagosklonny and L. M. Neckers (1998). "Stabilization of wild-type p53 by hypoxia-inducible factor 1α." Nature 392(6674): 405-408.

Andrews, P. A. and K. D. Albright (1992). "Mitochondrial defects in cis-diamminedichloroplatinum (II)-resistant human ovarian carcinoma cells." Cancer Research 52(7): 1895-1901.

Attanoos, R. and A. Gibbs (1997). "Pathology of malignant mesothelioma." Histopathology 30(5): 403-418.

Attanoos, R. L., A. Churg, F. Galateau-Salle, A. R. Gibbs and V. L. Roggli (2018). "Malignant mesothelioma and its non-asbestos causes." Archives of Pathology & Laboratory Medicine 142(6): 753-760.

Attardi, G. and G. Schatz (1988). "Biogenesis of mitochondria." Annual Review of Cell Biology 4(1): 289-331.

Au, J. L.-S., S. H. Jang and M. G. Wientjes (2002). "Clinical aspects of drug delivery to tumors." Journal of Controlled Release 78(1-3): 81-95.

Bache, M., M. Kappler, H. Said, A. Staab and D. Vordermark (2008). "Detection and specific targeting of hypoxic regions within solid tumors: current preclinical and clinical strategies." Current Medicinal Chemistry 15(4): 322-338.

Bahar, G., R. Feinmesser, T. Shpitzer, A. Popovtzer and R. M. Nagler (2007). "Salivary analysis in oral cancer patients: DNA and protein oxidation, reactive nitrogen species, and antioxidant profile." Cancer 109(1): 54-59.

Bao, S., Q. Wu, R. E. McLendon, Y. Hao, Q. Shi, A. B. Hjelmeland, M. W. Dewhirst, D. D. Bigner and J. N. Rich (2006). "Glioma stem cells promote radioresistance by preferential activation of the DNA damage response." Nature 444(7120): 756-760.

Baracca, A., G. Sgarbi, M. Mattiazzi, G. Casalena, E. Pagnotta, M. L. Valentino, M. Moggio, G. Lenaz, V. Carelli and G. Solaini (2007). "Biochemical phenotypes associated with the mitochondrial ATP6 gene mutations at nt8993." Biochimica et Biophysica Acta (BBA)-Bioenergetics 1767(7): 913-919.

Basu, S. and R. Kolesnick (1998). "Stress signals for apoptosis: ceramide and c-Jun kinase." Oncogene 17(25): 3277-3285.

Bellot, G., R. Garcia-Medina, P. Gounon, J. Chiche, D. Roux, J. Pouysségur and N. M. Mazure (2009). "Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains." Molecular and Cellular Biology 29(10): 2570-

2581.

Beltrán, B., A. Mathur, M. R. Duchen, J. D. Erusalimsky and S. Moncada (2000). "The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death." Proceedings of the National Academy of Sciences 97(26): 14602-14607.

Benedetti, S., B. Nuvoli, S. Catalani and R. Galati (2015). "Reactive oxygen species a double-edged sword for mesothelioma." Oncotarget 6(19): 16848-16865.

Berk, D. A., F. Yuan, M. Leunig and R. K. Jain (1997). "Direct in vivo measurement of targeted binding in a human tumor xenograft." Proceedings of the National Academy of Sciences 94(5): 1785-1790.

Bertout, J. A., A. J. Majmundar, J. D. Gordan, J. C. Lam, D. Ditsworth, B. Keith, E. J. Brown, K. L. Nathanson and M. C. Simon (2009). "HIF2α inhibition promotes p53 pathway activity, tumor cell death, and radiation responses." Proceedings of the National Academy of Sciences 106(34): 14391-14396.

Bertout, J. A., S. A. Patel and M. C. Simon (2008). "The impact of O 2 availability on human cancer." Nature Reviews Cancer 8(12): 967-975.

Bianchi, C. and T. Bianchi (2007). "Malignant mesothelioma: global incidence and relationship with asbestos." Industrial Health 45(3): 379-387.

Bianchi, C. and T. Bianchi (2014). "Global mesothelioma epidemic: trend and features." Indian Journal of Occupational and Environmental Medicine 18(2): 82-88.

Blomberg, C., J. Nilsson, G. Holgersson, P. Edlund, M. Bergqvist, L. Adwall, S. Ekman, D. Brattström and S. Bergström (2015). "Randomized trials of systemic medically-treated malignant mesothelioma: a systematic review." Anticancer Research 35(5): 2493-2501.

Bonnet, S., S. L. Archer, J. Allalunis-Turner, A. Haromy, C. Beaulieu, R. Thompson, C. T. Lee, G. D. Lopaschuk, L. Puttagunta and S. Bonnet (2007). "A mitochondria-K+ channel axis is suppressed in cancer

and its normalization promotes apoptosis and inhibits cancer growth." Cancer Cell 11(1): 37-51.

Borrego, S., A. Vazquez, F. Dasí, C. Cerdá, A. Iradi, C. Tormos, J. M. Sánchez, L. Bagán, J. Boix and C. Zaragoza (2013). "Oxidative stress and DNA damage in human gastric carcinoma: 8-Oxo-7'8-dihydro-2'-deoxyguanosine (8-oxo-dG) as a possible tumor marker." International Journal of Molecular Sciences 14(2): 3467-3486.

Boudreau, H. E., B. W. Casterline, B. Rada, A. Korzeniowska and T. L. Leto (2012). "Nox4 involvement in TGF-beta and SMAD3-driven induction of the epithelial-to-mesenchymal transition and migration of breast epithelial cells." Free Radical Biology and Medicine 53(7): 1489-1499.

Boutilier, R. and J. St-Pierre (2000). "Surviving hypoxia without really dying." Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 126(4): 481-490.

Brahimi-Horn, M. C., D. Ben-Hail, M. Ilie, P. Gounon, M. Rouleau, V. Hofman, J. Doyen, B. Mari, V. Shoshan-Barmatz and P. Hofman (2012). "Expression of a truncated active form of VDAC1 in lung cancer associates with hypoxic cell survival and correlates with progression to chemotherapy resistance." Cancer Research 72(8): 22140-22150.

Brahimi-Horn, M. C. and J. Pouysségur (2007). "Oxygen, a source of life and stress." FEBS Letters 581(19): 3582-3591.

Brand, K. A. and U. Hermfisse (1997). "Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species." The FASEB Journal 11(5): 388-395.

Bristow, R. G. and R. P. Hill (2008). "Hypoxia and metabolism: hypoxia, DNA repair and genetic instability." Nature Reviews Cancer 8(3): 180-192.

Brown, J. M. (2002). "Tumor microenvironment and the response to anticancer therapy." Cancer Biology & Therapy 1(5): 453-458.

Brown, J. M. and W. R. Wilson (2004). "Exploiting tumour hypoxia in cancer treatment." Nature Reviews Cancer 4(6): 437-447.

Brown, L. M., R. L. Cowen, C. Debray, A. Eustace, J. T. Erler, F. C. Sheppard, C. A. Parker, I. J. Stratford and K. J. Williams (2005). "Reversing hypoxic cell chemoresistance in vitro using genetic and small molecule approaches targeting hypoxia inducible factor-1." Molecular Pharmacology 69(2): 411-418.

Brown, N. S. and R. Bicknell (2001). "Hypoxia and oxidative stress in breast cancer Oxidative stress-its effects on the growth, metastatic potential and response to therapy of breast cancer." Breast Cancer Research 3(5): 323.

Brunelle, J. K., E. L. Bell, N. M. Quesada, K. Vercauteren, V. Tiranti, M. Zeviani, R. C. Scarpulla and N. S. Chandel (2005). "Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation." Cell Metabolism 1(6): 409-414.

Brunelle, J. K. and A. Letai (2009). "Control of mitochondrial apoptosis by the Bcl-2 family." Journal of Cell Science 122(4): 437-441.

Burt, B. M., S. J. Rodig, T. R. Tilleman, A. W. Elbardissi, R. Bueno and D. J. Sugarbaker (2011). "Circulating and tumor-infiltrating myeloid cells predict survival in human pleural mesothelioma." Cancer 117(22): 5234-5244.

Calabrese, C., L. Iommarini, I. Kurelac, M. A. Calvaruso, M. Capristo, P. L. Lollini, P. Nanni, C. Bergamini, G. Nicoletti, C. D. Giovanni, A. Ghelli, V. Giorgio, M. F. Caratozzolo, F. Marzano, C. Manzari, C. M. Betts, V. Carelli, C. Ceccarelli, M. Attimonelli, G. Romeo, R. Fato, M. Rugolo, A. Tullo, G. Gasparre and A. M. Porcelli (2013). "Respiratory complex I is essential to induce a Warburg profile in mitochondria-defective tumor cells." Cancer and Metabolism 1(1): 11.

Campos, L., O. Sabido, J.-P. Rouault and D. Guyotat (1994). "Effects of BCL-2 antisense oligodeoxynucleotides on in vitro proliferation and survival of normal marrow progenitors and leukemic cells." Blood 84(2): 595-600.

Cannito, S., E. Novo, A. Compagnone, L. Valfrè di Bonzo, C. Busletta, E. Zamara, C. Paternostro, D. Povero, A. Bandino and F. Bozzo (2008). "Redox mechanisms switch on hypoxia-dependent epithelial-

mesenchymal transition in cancer cells." Carcinogenesis 29(12): 2267-2278.

Cao, J., Y. Liu, L. Jia, H.-M. Zhou, Y. Kong, G. Yang, L.-P. Jiang, Q.-J. Li and L.-F. Zhong (2007). "Curcumin induces apoptosis through mitochondrial hyperpolarization and mtDNA damage in human hepatoma G2 cells." Free Radical Biology and Medicine 43(6): 968-975.

Cao, W., S. Yacoub, K. T. Shiverick, K. Namiki, Y. Sakai, S. Porvasnik, C. Urbanek and C. J. Rosser (2008). "Dichloroacetate (DCA) sensitizes both wild-type and over expressing Bcl-2 prostate cancer cells in vitro to radiation." The Prostate 68(11): 1223-1231.

Cao, X., C. Rodarte, L. Zhang, C. D. Morgan, J. Littlejohn and W. R. Smythe (2007). "Bcl2/bcl-xl inhibitor engenders apoptosis and increases chemo-sensitivity in mesothelioma." Cancer Biology & Therapy 6(2): 246-252.

Carbone, M., R. A. Kratzke and J. R. Testa (2002). The pathogenesis of mesothelioma. Seminars in Oncology, Elsevier.

Carbone, M., P. Rizzo and H. Pass (2000). "Simian virus 40: the link with human malignant mesothelioma is well established." Anticancer Research 20(2A): 875-877.

Cardoso, A. R., B. Chausse, F. M. da Cunha, L. A. Luévano-Martínez, T. B. Marazzi, P. S. Pessoa, B. B. Queliconi and A. J. Kowaltowski (2012). "Mitochondrial compartmentalization of redox processes." Free Radical Biology and Medicine 52(11-12): 2201-2208.

Carmeliet, P., Y. Dor, J.-M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch and P. Maxwell (1998). "Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis." Nature 394(6692): 485-490.

Casarsa, C., N. Bassani, F. Ambrogi, G. Zabucchi, P. Boracchi, E. Biganzoli and D. Coradini (2011). "Epithelial-to-mesenchymal transition, cell polarity and stemness-associated features in malignant pleural mesothelioma." Cancer Letters 302(2): 136-143.

Cavalli, L. R., M. Varella-Garcia and B. C. Liang (1997). "Diminished tumorigenic phenotype after depletion of mitochondrial DNA." Cell Growth and Differentiation 8(11): 1189-1198.

Cavalli, L. R., M. Varella-Garcia and B. C. Liang (1997). "Diminished tumorigenic phenotype after depletion of mitochondrial DNA." Cell Growth and Differentiation 8: 1189-1198.

Cerutti, P. A. (1985). "Prooxidant states and tumor promotion." Science 227(4685): 375-381.

Chang, D., F. Wang, Y.-S. Zhao and H.-Z. Pan (2008). "Evaluation of oxidative stress in colorectal cancer patients." Biomedical and Environmental Sciences 21(4): 286-289.

Chen, C. D., L. N. Dai, M. Wang, X. H. Lai and J. Wang (2017). "Hypoxia induces autophagy in PA-1 ovarian teratoma cells and resistance to growth inhibition and apoptosis by chemotherapeutic agent cis-diamminedichloroplatinum." European journal of gynaecological oncology 38(2): 277-281.

Chen, J., Z. Ding, Y. Peng, F. Pan, J. Li, L. Zou, Y. Zhang and H. Liang (2014). "HIF-1α inhibition reverses multidrug resistance in colon cancer cells via downregulation of MDR1/P-glycoprotein." PLoS One 9(6): e98882.

Chen, L., P. Feng, S. Li, D. Long, J. Cheng, Y. Lu and D. Zhou (2009). "Effect of hypoxia-inducible factor-1α silencing on the sensitivity of human brain glioma cells to doxorubicin and etoposide." Neurochemical Research 34(5): 984-990.

Chen, Q., A. K. Camara, D. F. Stowe, C. L. Hoppel and E. J. Lesnefsky (2007). "Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion." American Journal of Physiology-Cell Physiology 292(1): C137-C147.

Chen, X., Z. Zhong, Z. Xu, L. Chen and Y. Wang (2010). "2′, 7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy." Free Radical Research 44(6): 587-604.

Chen, Z., T. Shi, L. Zhang, P. Zhu, M. Deng, C. Huang, T. Hu, L. Jiang and J. Li (2016). "Mammalian drug efflux transporters of the ATP binding cassette (ABC) family in multidrug resistance: A review of the past decade." Cancer Letters 370(1): 153-164.

Chew, S. H., Y. Okazaki, S. Akatsuka, S. Wang, L. Jiang, Y. Ohara, F. Ito, H. Saya, Y. Sekido and S. Toyokuni (2017). "Rheostatic CD44 isoform expression and its association with oxidative stress in human malignant mesothelioma." Free Radical and Biololgy Medicine 106: 91-99.

Chew, S. H. and S. Toyokuni (2015). "Malignant mesothelioma as an oxidative stress-induced cancer: An update." Free Radical Biology and Medicine 86: 166-178.

Cho, K., H.-W. Shin, Y.-I. Kim, C.-H. Cho, Y.-S. Chun, T.-Y. Kim and J.-W. Park (2013). "Mad1 mediates hypoxia-induced doxorubicin resistance in colon cancer cells by inhibiting mitochondrial function." Free Radical Biology and Medicine 60: 201-210.

Circu, M. L. and T. Y. Aw (2010). "Reactive oxygen species, cellular redox systems, and apoptosis." Free Radical Biology and Medicine 48(6): 749-762.

Clerkin, J., R. Naughton, C. Quiney and T. Cotter (2008). "Mechanisms of ROS modulated cell survival during carcinogenesis." Cancer Letters 266(1): 30-36.

Coffey, R. N., C. Morrissey, C. T. Taylor, J. M. Fitzpatrick and R. W. G. Watson (2005). "Resistance to caspase-dependent, hypoxia-induced apoptosis is not hypoxia-inducible factor-1 alpha mediated in prostate carcinoma cells." Cancer 103(7): 1363-1374.

Coleman, C. N. (1988). "Hypoxia in tumors: a paradigm for the approach to biochemical and physiologic heterogeneity." Journal of the National Cancer Institute 80(5): 310-317.

Comerford, K. M., T. J. Wallace, J. Karhausen, N. A. Louis, M. C. Montalto and S. P. Colgan (2002). "Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene." Cancer

Research 62(12): 3387-3394.

Cook, J. A., D. Gius, D. A. Wink, M. C. Krishna, A. Russo and J. B. Mitchell (2004). Oxidative stress, redox, and the tumor microenvironment. Seminars in Radiation Oncology, Elsevier.

Cortes-Dericks, L., L. Froment, R. Boesch, R. A. Schmid and G. Karoubi (2014). "Cisplatin-resistant cells in malignant pleural mesothelioma cell lines show ALDH high CD44+ phenotype and sphere-forming capacity." BMC Cancer 14(1): 304.

Cosse, J.-P. and C. Michiels (2008). "Tumour hypoxia affects the responsiveness of cancer cells to chemotherapy and promotes cancer progression." Anti-Cancer Agents in Medicinal Chemistry 8(7): 790-797.

Covello, K. L., J. Kehler, H. Yu, J. D. Gordan, A. M. Arsham, C.-J. Hu, P. A. Labosky, M. C. Simon and B. Keith (2006). "HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth." Genes & Development 20(5): 557-570.

Cowan, D. S. and I. F. Tannock (2001). "Factors that influence the penetration of methotrexate through solid tissue." International Journal of Cancer 91(1): 120-125.

Cullen, K. J., Z. Yang, L. Schumaker and Z. Guo (2007). "Mitochondria as a critical target of the chemotheraputic agent cisplatin in head and neck cancer." Journal of Bioenergetics and Biomembranes 39(1): 43-50.

Cunniff, B., K. Benson, J. Stumpff, K. Newick, P. Held, D. Taatjes, J. Joseph, B. Kalyanaraman and N. H. Heintz (2013). "Mitochondrial-targeted nitroxides disrupt mitochondrial architecture and inhibit expression of peroxiredoxin 3 and FOXM1 in malignant mesothelioma cells." Journal of Cellular Physiology 228(4): 835-845.

Cuvier, C., A. Jang and R. Hill (1997). "Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L+ B) secretion." Clinical & Experimental Metastasis 15(1): 19-25.

Daskalow, K., N. Rohwer, E. Raskopf, E. Dupuy, A. Kühl, C. Loddenkemper,

B. Wiedenmann, V. Schmitz and T. Cramer (2010). "Role of hypoxia-inducible transcription factor 1α for progression and chemosensitivity of murine hepatocellular carcinoma." Journal of Molecular Medicine 88(8): 817-827.

de Assis, L. V. M., J. Locatelli and M. C. Isoldi (2014). "The role of key genes and pathways involved in the tumorigenesis of Malignant Mesothelioma." Biochimica et Biophysica Acta-Reviews on Cancer 1845(2): 232-247.

Deben, C., V. Deschoolmeester, J. De Waele, J. Jacobs, J. Van den Bossche, A. Wouters, M. Peeters, C. Rolfo, E. Smits and F. Lardon (2018). "Hypoxia-Induced Cisplatin Resistance in Non-Small Cell Lung Cancer Cells Is Mediated by HIF-1α and Mutant p53 and Can Be Overcome by Induction of Oxidative Stress." Cancers 10(4): 126.

DeHaan, C., B. Habibi-Nazhad, E. Yan, N. Salloum, M. Parliament and J. Allalunis-Turner (2004). "Mutation in mitochondrial complex I ND6 subunit is associated with defective response to hypoxia in human glioma cells." Molecular Cancer 3(1): 19.

Denko, N. C. (2008). "Hypoxia, HIF1 and glucose metabolism in the solid tumour." Nature Reviews Cancer 8(9): 705-713.

Devadas, S., L. Zaritskaya, S. G. Rhee, L. Oberley and M. S. Williams (2002). "Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression." Journal of Experimental Medicine 195(1): 59-70.

Doktorova, H., J. Hrabeta, M. A. Khalil and T. Eckschlager (2015). "Hypoxia-induced chemoresistance in cancer cells: The role of not only HIF-1." Biomedical papers of the Medical Faculty of the University Palacky 159(2): 166-177.

Donaldson, K., F. A. Murphy, R. Duffin and C. A. Poland (2010). "Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma." Particle and fibre toxicology 7(1): 5.

Dong, H. Y., A. Buard, A. Renier, F. Lévy, L. Saint-Etienne and M.-C. Jaurand (1994). "Role of oxygen derivatives in the cytotoxicity and DNA damage produced by asbestos on rat pleural mesothelial cells in vitro." Carcinogenesis 15(6): 1251-1255.

Dong, Z. and J. Wang (2004). "Hypoxia selection of death-resistant cells. A role for Bcl-X(L)." Journal Of Biological Chemistry 279(10): 9215-9221.

Durand, R. E. (1986). "Chemosensitivity testing in V79 spheroids: drug delivery and cellular microenvironment." Journal of the National Cancer Institute 77(1): 247-252.

Edelman, G., W. Gallin, A. Delouvee, B. Cunningham and J. Thiery (1983). "Early epochal maps of two different cell adhesion molecules." Proceedings of the National Academy of Sciences 80(14): 4384-4388.

Erler, J. T., C. J. Cawthorne, K. J. Williams, M. Koritzinsky, B. G. Wouters, C. Wilson, C. Miller, C. Demonacos, I. J. Stratford and C. Dive (2004). "Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and-independent mechanisms and contributes to drug resistance." Molecular and Cellular Biology 24(7): 2875-2889.

Evans, M. D., M. Dizdaroglu and M. S. Cooke (2004). "Oxidative DNA damage and disease: induction, repair and significance." Mutation Research/Reviews in Mutation Research 567(1): 1-61.

Fassina, A., R. Cappellesso, V. Guzzardo, L. Dalla Via, S. Piccolo, L. Ventura and M. Fassan (2012). "Epithelial–mesenchymal transition in malignant mesothelioma." Modern Pathology 25(1): 86-99.

Fennell, D. A., G. Gaudino, K. J. O'byrne, L. Mutti and J. Van Meerbeeck (2008). "Advances in the systemic therapy of malignant pleural mesothelioma." Nature Reviews Clinical Oncology 5(3): 136-147.

Fennell, D. A. and R. M. Rudd (2004). "Defective core-apoptosis signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development." The Lancet Oncology 5(6): 354-362.

Ferecatu, I., F. Canal, L. Fabbri, N. M. Mazure, C. Bouton and M.-P.

Golinelli-Cohen (2018). "Dysfunction in the mitochondrial Fe-S assembly machinery leads to formation of the chemoresistant truncated VDAC1 isoform without HIF-1α activation." PloS One 13(3): e0194782.

Fiaschi, T. and P. Chiarugi (2012). "Oxidative stress, tumor microenvironment, and metabolic reprogramming: a diabolic liaison." International Journal of Cell Biology 2012: 1-8.

Fischer, B., C. Frei, U. Moura, R. Stahel and E. Felley-Bosco (2012). "Inhibition of phosphoinositide-3 kinase pathway down regulates ABCG2 function and sensitizes malignant pleural mesothelioma to chemotherapy." Lung Cancer 78(1): 23-29.

Forkink, M., G. R. Manjeri, D. C. Liemburg-Apers, E. Nibbeling, M. Blanchard, A. Wojtala, J. A. Smeitink, M. R. Wieckowski, P. H. Willems and W. J. Koopman (2014). "Mitochondrial hyperpolarization during chronic complex I inhibition is sustained by low activity of complex II, III, IV and V." Biochimica et Biophysica Acta (BBA)-Bioenergetics 1837(8): 1247-1256.

Francis, R. J., T. Segard, L. Morandeau, Y. G. Lee, M. J. Millward, A. Segal and A. K. Nowak (2015). "Characterization of hypoxia in malignant pleural mesothelioma with FMISO PET-CT." Lung Cancer 90(1): 55-60.

Franken, N. A., H. M. Rodermond, J. Stap, J. Haveman and C. Van Bree (2006). "Clonogenic assay of cells in vitro." Nature Protocols 1(5): 2315-2319.

Fruehauf, J. P. and F. L. Meyskens (2007). "Reactive oxygen species: a breath of life or death?" Clinical Cancer Research 13(3): 789-794.

Fuertes, M., J. Castilla, C. Alonso and J. Prez (2003). "Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways." Current Medicinal Chemistry 10(3): 257-266.

Fukuda, R., H. Zhang, J.-w. Kim, L. Shimoda, C. V. Dang and G. L. Semenza (2007). "HIF-1 regulates cytochrome oxidase subunits to optimize

efficiency of respiration in hypoxic cells." Cell 129(1): 111-122.

Fukumura, D. and R. K. Jain (2007). "Tumor microenvironment abnormalities: causes, consequences, and strategies to normalize." Journal of Cellular Biochemistry 101(4): 937-949.

Fulda, S., L. Galluzzi and G. Kroemer (2010). "Targeting mitochondria for cancer therapy." Nature Reviews Drug Discovery 9(6): 447-464.

Gatti, L. and F. Zunino (2005). Overview of tumor cell chemoresistance mechanisms. Chemosensitivity: Volume II, Springer: 127-148.

Geiger, T. R. and D. S. Peeper (2009). "Metastasis mechanisms." Biochimica et Biophysica Acta (BBA)-Reviews on Cancer 1796(2): 293-308.

Gergely Jr, P., C. Grossman, B. Niland, F. Puskas, H. Neupane, F. Allam, K. Banki, P. E. Phillips and A. Perl (2002). "Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus." Arthritis and Rheumatism 46(1): 175-190.

Ghani, F. I., H. Yamazaki, S. Iwata, T. Okamoto, K. Aoe, K. Okabe, Y. Mimura, N. Fujimoto, T. Kishimoto and T. Yamada (2011). "Identification of cancer stem cell markers in human malignant mesothelioma cells." Biochemical and Biophysical Research Communications 404(2): 735-742.

Giannoni, E., M. Parri and P. Chiarugi (2012). "EMT and oxidative stress: a bidirectional interplay affecting tumor malignancy." Antioxidants & Redox Signaling 16(11): 1248-1263.

Giovannetti, E., L. G. Leon, V. E. Gomez, P. A. Zucali, F. Minutolo and G. J. Peters (2016). "A specific inhibitor of lactate dehydrogenase overcame the resistance toward gemcitabine in hypoxic mesothelioma cells, and modulated the expression of the human equilibrative transporter-1." Nucleosides Nucleotides Nucleic Acids 35(10-12): 643-651.

Gius, D. and D. R. Spitz (2006). "Redox signaling in cancer biology." Antioxidants & Redox Signaling 8(7-8): 1249-1252.

Goodglick, L. A. and A. B. Kane (1990). "Cytotoxicity of long and short

crocidolite asbestos fibers in vitro and in vivo." Cancer Research 50(16): 5153-5163.

Gordan, J. D., J. A. Bertout, C.-J. Hu, J. A. Diehl and M. C. Simon (2007). "HIF-2α promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity." Cancer Cell 11(4): 335-347.

Gordan, J. D., C. B. Thompson and M. C. Simon (2007). "HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation." Cancer Cell 12(2): 108-113.

Gorowiec, M. R., L. A. Borthwick, S. M. Parker, J. A. Kirby, G. C. Saretzki and A. J. Fisher (2012). "Free radical generation induces epithelial-to-mesenchymal transition in lung epithelium via a TGF-β1-dependent mechanism." Free Radical Biology and Medicine 52(6): 1024-1032.

Gottesman, M. M. (2002). "Mechanisms of cancer drug resistance." Annual Review of Medicine 53(1): 615-627.

Goudarzi, H., Y. Hida, H. Takano, H. Teramae, H. Iizasa and J.-i. Hamada (2013). "Hypoxia affects in vitro growth of newly established cell lines from patients with malignant pleural mesothelioma." Biomedical Research 34(1): 13-21.

Goudarzi, H., H. Iizasa, M. Furuhashi, S. Nakazawa, R. Nakane, S. Liang, Y. Hida, K. Yanagihara, T. Kubo and K. Nakagawa (2013). "Enhancement of in vitro cell motility and invasiveness of human malignant pleural mesothelioma cells through the HIF-1α-MUC1 pathway." Cancer Letters 339(1): 82-92.

Graeber, T. G., C. Osmanian, T. Jacks, D. E. Housman, C. J. Koch, S. W. Lowe and A. J. Giaccia (1996). "Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours." Nature 379(6560): 88-91.

Grantab, R., S. Sivananthan and I. F. Tannock (2006). "The penetration of anticancer drugs through tumor tissue as a function of cellular adhesion and packing density of tumor cells." Cancer Research 66(2): 1033-1039.

Graves, J. A., Y. Wang, S. Sims-Lucas, E. Cherok, K. Rothermund, M. F. Branca, J. Elster, D. Beer-Stolz, B. Van Houten and J. Vockley (2012). "Mitochondrial structure, function and dynamics are temporally controlled by c-Myc." PloS One 7(5): e37699.

Graziani, I., S. Eliasz, M. A. De Marco, Y. Chen, H. I. Pass, M. Richard, P. R. Strack, L. Miele and M. Bocchetta (2008). "Opposite effects of Notch-1 and Notch-2 on mesothelioma cell survival under hypoxia are exerted through the Akt pathway." Cancer Research 68(23): 9678-9685.

Greijer, A. and E. Van der Wall (2004). "The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis." Journal of Clinical Pathology 57(10): 1009-1014.

Gross, A., J. M. McDonnell and S. J. Korsmeyer (1999). "BCL-2 family members and the mitochondria in apoptosis." Genes & Development 13(15): 1899-1911.

Guo, Q., F. Lan, X. Yan, Z. Xiao, Y. Wu and Q. Zhang (2018). "Hypoxia exposure induced cisplatin resistance partially via activating p53 and hypoxia inducible facor-1α in non-small cell lung cancer A549 cells." Oncology Letters 16(1): 801-808.

Gustafsson, M. V., X. Zheng, T. Pereira, K. Gradin, S. Jin, J. Lundkvist, J. L. Ruas, L. Poellinger, U. Lendahl and M. Bondesson (2005). "Hypoxia requires notch signaling to maintain the undifferentiated cell state." Developmental Cell 9(5): 617-628.

Guzy, R. D., B. Hoyos, E. Robin, H. Chen, L. Liu, K. D. Mansfield, M. C. Simon, U. Hammerling and P. T. Schumacker (2005). "Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing." Cell Metabolism 1(6): 401-408.

Guzy, R. D. and P. T. Schumacker (2006). "Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia." Experimental Physiology 91(5): 807-819.

Hao, J., X. Song, B. Song, Y. Liu, L. Wei, X. Wang and J. Yu (2008). "Effects of lentivirus-mediated HIF-1α knockdown on hypoxia-related

cisplatin resistance and their dependence on p53 status in fibrosarcoma cells." Cancer Gene Therapy 15(7): 449-455.

Harris, A. L. (2002). "Hypoxia—a key regulatory factor in tumour growth." Nature Reviews Cancer 2(1): 38-47.

Hashiguchi, K. and Q.-M. Zhang-Akiyama (2009). "Establishment of human cell lines lacking mitochondrial DNA." Mitochondrial DNA: methods and protocols: 383-391.

Heddleston, J. M., Z. Li, R. E. McLendon, A. B. Hjelmeland and J. N. Rich (2009). "The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype." Cell Cycle 8(20): 3274-3284.

Hensley, K., K. A. Robinson, S. P. Gabbita, S. Salsman and R. A. Floyd (2000). "Reactive oxygen species, cell signaling, and cell injury." Free Radical Biology and Medicine 28(10): 1456-1462.

Hirama, M., S. Isonishi, M. Yasuda and H. Ishikawa (2006). "Characterization of mitochondria in cisplatin-resistant human ovarian carcinoma cells." Oncology Reports 16(5): 997-1002.

Höckel, M., K. Schlenger, B. Aral, M. Mitze, U. Schäffer and P. Vaupel (1996). "Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix." Cancer Research 56(19): 4509-4515.

Hockel, M. and P. Vaupel (2001). "Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects." Journal of the National Cancer Institute 93(4): 266-276.

Holmquist-Mengelbier, L., E. Fredlund, T. Löfstedt, R. Noguera, S. Navarro, H. Nilsson, A. Pietras, J. Vallon-Christersson, Å. Borg and K. Gradin (2006). "Recruitment of HIF-1α and HIF-2α to common target genes is differentially regulated in neuroblastoma: HIF-2α promotes an aggressive phenotype." Cancer Cell 10(5): 413-423.

Hong, H. H. L., J. Dunnick, R. Herbert, T. R. Devereux, Y. Kim and R. C. Sills (2007). "Genetic alterations in K-ras and p53 cancer genes in lung neoplasms from Swiss (CD-1) male mice exposed transplacentally to

AZT." Environmental and Molecular Mutagenesis 48(3-4): 299-306.

Hopkins-Donaldson, S., R. Cathomas, A. P. Simões-Wüst, S. Kurtz, L. Belyanskya, R. A. Stahel and U. Zangemeister-Wittke (2003). "Induction of apoptosis and chemosensitization of mesothelioma cells by Bcl-2 and Bcl-xL antisense treatment." International journal of cancer 106(2): 160-166.

Hu, Y.-L., A. Jahangiri, M. De Lay and M. K. Aghi (2012). "Hypoxia-induced tumor cell autophagy mediates resistance to anti-angiogenic therapy." Autophagy 8(6): 979-981.

Hu, Y.-Y., L.-A. Fu, S.-Z. Li, Y. Chen, J.-C. Li, J. Han, L. Liang, L. Li, C.-C. Ji and M.-H. Zheng (2014). "Hif-1α and Hif-2α differentially regulate Notch signaling through competitive interaction with the intracellular domain of Notch receptors in glioma stem cells." Cancer Letters 349(1): 67-76.

Huang, C. Y., W. T. Kuo, Y. C. Huang, T. C. Lee and L. C. Yu (2013). "Resistance to hypoxia-induced necroptosis is conferred by glycolytic pyruvate scavenging of mitochondrial superoxide in colorectal cancer cells." Cell Death and Differentiation 4: e622.

Huang, J., U. M. Upadhyay and R. J. Tamargo (2006). "Inflammation in stroke and focal cerebral ischemia." Surgical Neurology 66(3): 232-245.

Huang, S. X., M. A. Partridge, S. A. Ghandhi, M. M. Davidson, S. A. Amundson and T. K. Hei (2012). "Mitochondria-derived reactive intermediate species mediate asbestos-induced genotoxicity and oxidative stress–responsive signaling pathways." Environmental Health Perspectives 120(6): 840-847.

Huber, H. J., H. Dussmann, S. M. Kilbride, M. Rehm and J. H. Prehn (2011). "Glucose metabolism determines resistance of cancer cells to bioenergetic crisis after cytochrome-c release." Molecular Systems Biology 7(1): 470.

Hunter Jr, F. E., J. Davis and L. Carlat (1956). "The stability of oxidative and phosphorylative systems in mitochondria under anaerobic conditions." Biochimica et Biophysica Acta 20(1): 237-242.

Iijima, T., T. Mishima, K. Akagawa and Y. Iwao (2003). "Mitochondrial hyperpolarization after transient oxygen-glucose deprivation and subsequent apoptosis in cultured rat hippocampal neurons." Brain Research 993(1-2): 140-145.

Indran, I. R., G. Tufo, S. Pervaiz and C. Brenner (2011). "Recent advances in apoptosis, mitochondria and drug resistance in cancer cells." Biochimica et Biophysica Acta-Bioenergetics 1807(6): 735-745.

Isonishi, S., M. Saitou, M. Yasuda and T. Tanaka (2001). "Mitochondria in platinum resistant cells." Human Cell 14(3): 203-210.

Ivan, M., K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane and W. G. Kaelin Jr (2001). "HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing." Science 292(5516): 464-468.

Jaakkola, P., D. R. Mole, Y.-M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. von Kriegsheim, H. F. Hebestreit, M. Mukherji and C. J. Schofield (2001). "Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation." Science 292(5516): 468-472.

Jain, R. K. (1988). "Determinants of tumor blood flow: a review." Cancer Research 48(10): 2641-2658.

Jain, R. K., V. P. Chauhan and D. G. Duda (2014). Vascular and interstitial biology of tumors. Abeloff's Clinical Oncology (Fifth Edition), Elsevier: 108-126.

Jampel, H. D. (1992). "Effect of brief exposure to mitomycin C on viability and proliferation of cultured human Tenon's capsule fibroblasts." Ophthalmology 99(9): 1471-1476.

Jangamreddy, J. R. and M. J. Los (2012). "Mitoptosis, a novel mitochondrial death mechanism leading predominantly to activation of autophagy." Hepatitis Monthly 12(8): e6159.

Janson, V. (2008). Cisplatin-resistance and cell death in malignant pleural mesothelioma cells. Doctoral thesis, Umeå University.

Järvinen, K., Y. Soini, K. Kahlos and V. L. Kinnula (2002). "Overexpression of ɣ-glutamylcysteine synthetase in human malignant mesothelioma." Human Pathology 33(7): 748-755.

Jean, D. and M.-C. Jaurand (2018). "Mesotheliomas in Genetically Engineered Mice Unravel Mechanism of Mesothelial Carcinogenesis." International Journal of Molecular Sciences 19(8): 2191.

JEWELL, U. R., I. KVIETIKOVA, A. SCHEID, C. BAUER, R. H. WENGER and M. GASSMANN (2001). "Induction of HIF-1α in response to hypoxia is instantaneous." The FASEB Journal 15(7): 1312-1314.

Jiang, J., Y.-l. Tang and X.-h. Liang (2011). "EMT: a new vision of hypoxia promoting cancer progression." Cancer Biology & Therapy 11(8): 714-723.

Jin, L., V. J. Amatya, Y. Takeshima, L. Shrestha, K. Kushitani and K. Inai (2010). "Evaluation of apoptosis and immunohistochemical expression of the apoptosis-related proteins in mesothelioma." Hiroshima Journals of Medical Sciences 59(2): 27-33.

Jo, A., S. Ham, G. H. Lee, Y.-I. Lee, S. Kim, Y.-S. Lee, J.-H. Shin and Y. Lee (2015). "Efficient mitochondrial genome editing by CRISPR/Cas9." BioMed Research International 2015: 1-10.

Jordan, C. T., M. L. Guzman and M. Noble (2006). "Cancer stem cells." New England Journal of Medicine 355(12): 1253-1261.

Kahlos, K., S. Anttila, T. Asikainen, K. Kinnula, K. O. Raivio, K. Mattson, K. Linnainmaa and V. L. Kinnula (1998). "Manganese superoxide dismutase in healthy human pleural mesothelium and in malignant pleural mesothelioma." American Journal of Respiratory Cell and Molecular Biology 18(4): 570-580.

Kahlos, K., Y. Soini, M. Säily, P. Koistinen, S. Kakko, P. Pääkkö, A. Holmgren and V. L. Kinnula (2001). "Up-regulation of thioredoxin and thioredoxin reductase in human malignant pleural mesothelioma." International Journal of Cancer 95(3): 198-204.

Kahlos, K., Y. Soini, R. Sormunen, R. Kaarteenaho-Wiik, P. Pääkkö, K.

Linnainmaa and V. L. Kinnula (2001). "Expression and prognostic significance of catalase in malignant mesothelioma." Cancer 91(7): 1349-1357.

Kai, K., S. D'Costa, R. C. Sills and Y. Kim (2009). "Inhibition of the insulin-like growth factor 1 receptor pathway enhances the antitumor effect of cisplatin in human malignant mesothelioma cell lines." Cancer Letters 278(1): 49-55.

Kai, K., S. D'Costa, B.-I. Yoon, A. R. Brody, R. C. Sills and Y. Kim (2010). "Characterization of side population cells in human malignant mesothelioma cell lines." Lung Cancer 70(2): 146-151.

Kaludercic, N., S. Deshwal and F. Di Lisa (2014). "Reactive oxygen species and redox compartmentalization." Frontiers in Physiology 5: 285.

Kamp, D. W., P. Graceffa, W. A. Pryor and S. A. Weitzman (1992). "The role of free radicals in asbestos-induced diseases." Free Radical Biology and Medicine 12(4): 293-315.

Kang, Y., M. Ding, G. Tian, H. Guo, Y. Wan, Z. Yao, B. Li and D. Lin (2013). "Overexpression of Numb suppresses tumor cell growth and enhances sensitivity to cisplatin in epithelioid malignant pleural mesothelioma." Oncology Reports 30(1): 313-319.

Kang, Y. and J. Massagué (2004). "Epithelial-mesenchymal transitions: twist in development and metastasis." Cell 118(3): 277-279.

Karicheva, O., J. M. Rodriguez-Vargas, N. Wadier, K. Martin-Hernandez, R. Vauchelles, N. Magroun, A. Tissier, V. Schreiber and F. Dantzer (2016). "PARP3 controls TGFbeta and ROS driven epithelial-to-mesenchymal transition and stemness by stimulating a TG2-Snail-E-cadherin axis." Oncotarget 7(39): 64109-64123.

Keith, B., R. S. Johnson and M. C. Simon (2012). "HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression." Nature Reviews Cancer 12(1): 9-22.

Kim, H. M., N. Haraguchi, H. Ishii, M. Ohkuma, M. Okano, K. Mimori, H. Eguchi, H. Yamamoto, H. Nagano and M. Sekimoto (2012). "Increased CD13 expression reduces reactive oxygen species, promoting

survival of liver cancer stem cells via an epithelial–mesenchymal transition–like phenomenon." Annals of Surgical Oncology 19(3): 539-548.

Kim, J.-w., I. Tchernyshyov, G. L. Semenza and C. V. Dang (2006). "HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia." Cell Metabolism 3(3): 177-185.

Kim, J.-Y. and J.-Y. Lee (2017). "Targeting Tumor Adaption to Chronic Hypoxia: Implications for Drug Resistance, and How It Can Be Overcome." International Journal of Molecular Sciences 18(9): 1854.

Kim, J.-Y. and J.-H. Park (2003). "ROS-dependent caspase-9 activation in hypoxic cell death." FEBS Letters 549(1-3): 94-98.

Kim, M.-C., F.-J. Cui and Y. Kim (2013). "Hydrogen peroxide promotes epithelial to mesenchymal transition and stemness in human malignant mesothelioma cells." Asian Pacific Journal of Cancer Prevention 14(6): 3625-3630.

Kim, M. C., S. H. Hwang, N. Y. Kim, H. S. Lee, S. Ji, Y. Yang and Y. Kim (2018). "Hypoxia promotes acquisition of aggressive phenotypes in human malignant mesothelioma." BMC Cancer 18(1): 819.

Kim, W. Y., S. Perera, B. Zhou, J. Carretero, J. J. Yeh, S. A. Heathcote, A. L. Jackson, P. Nikolinakos, B. Ospina and G. Naumov (2009). "HIF2α cooperates with RAS to promote lung tumorigenesis in mice." The Journal of Clinical Investigation 119(8): 2160-2170.

Kim, Y., Q. Lin, P. M. Glazer and Z. Yun (2009). "Hypoxic tumor microenvironment and cancer cell differentiation." Current Molecular Medicine 9(4): 425-434.

Kim, Y., T.-V. Ton, A. B. DeAngelo, K. Morgan, T. R. Devereux, C. Anna, J. B. Collins, R. S. Paules, L. M. Crosby and R. C. Sills (2006). "Major carcinogenic pathways identified by gene expression analysis of peritoneal mesotheliomas following chemical treatment in F344 rats." Toxicology and Applied Pharmacology 214(2): 144-151.

Kim, Y. J., S. H. Jeong, E. K. Kim, E. J. Kim and J. H. Cho (2017).

"Ursodeoxycholic acid suppresses epithelial-mesenchymal transition and cancer stem cell formation by reducing the levels of peroxiredoxin II and reactive oxygen species in pancreatic cancer cells." Oncology Reports 38(6): 3632-3638.

Kinnula, K., K. Linnainmaa, K. Raivio and V. Kinnula (1998). "Endogenous antioxidant enzymes and glutathione S-transferase in protection of mesothelioma cells against hydrogen peroxide and epirubicin toxicity." British Journal of Cancer 77(7): 1097-1102.

Kinnula, V., S. Lehtonen, R. Sormunen, R. Kaarteenaho-Wiik, S. Kang, S. Rhee and Y. Soini (2002). "Overexpression of peroxiredoxins I, II, III, V, and VI in malignant mesothelioma." The Journal of Pathology 196(3): 316-323.

Klabatsa, A., M. Sheaff, J. Steele, M. Evans, R. Rudd and D. Fennell (2006). "Expression and prognostic significance of hypoxia-inducible factor 1α (HIF-1α) in malignant pleural mesothelioma (MPM)." Lung Cancer 51(1): 53-59.

Kobayashi, C. I. and T. Suda (2012). "Regulation of reactive oxygen species in stem cells and cancer stem cells." Journal of Cellular Physiology 227(2): 421-430.

Koshiji, M., Y. Kageyama, E. A. Pete, I. Horikawa, J. C. Barrett and L. E. Huang (2004). "HIF-1α induces cell cycle arrest by functionally counteracting Myc." The EMBO Journal 23(9): 1949-1956.

Kovarova, J., M. Bajzikova, M. Vondrusova, J. Stursa, J. Goodwin, M. Nguyen, R. Zobalova, E. A. Pesdar, J. Truksa and M. Tomasetti (2014). "Mitochondrial targeting of α-tocopheryl succinate enhances its anti-mesothelioma efficacy." Redox Report 19(1): 16-25.

Kubo, T., S. Toyooka, K. Tsukuda, M. Sakaguchi, T. Fukazawa, J. Soh, H. Asano, T. Ueno, T. Muraoka and H. Yamamoto (2011). "Epigenetic silencing of microRNA-34b/c plays an important role in the pathogenesis of malignant pleural mesothelioma." Clinical Cancer Research 17(15): 4965-4974.

Kuh, H.-J., S. H. Jang, M. G. Wientjes, J. R. Weaver and J. L.-S. Au (1999).

"Determinants of paclitaxel penetration and accumulation in human solid tumor." Journal of Pharmacology and Experimental Therapeutics 290(2): 871-880.

Kulikov, A. V., A. S. Vdovin, B. Zhivotovsky and V. Gogvadze (2014). "Targeting mitochondria by α-tocopheryl succinate overcomes hypoxia-mediated tumor cell resistance to treatment." Cellular and Molecular Life Sciences 71(12): 2325-2333.

Kumar, A., M. C. Pant, H. S. Singh and S. Khandelwal (2012). "Assessment of the redox profile and oxidative DNA damage (8-OHdG) in squamous cell carcinoma of head and neck." Journal of Cancer Research and Therapeutics 8(2): 254-259.

Kumar, K., S. Wigfield, H. E. Gee, C. M. Devlin, D. Singleton, J.-L. Li, F. Buffa, M. Huffman, A. L. Sinn and J. Silver (2013). "Dichloroacetate reverses the hypoxic adaptation to bevacizumab and enhances its antitumor effects in mouse xenografts." Journal of Molecular Medicine 91(6): 749-758.

Kwong, J. Q., M. S. Henning, A. A. Starkov and G. Manfredi (2007). "The mitochondrial respiratory chain is a modulator of apoptosis." The Journal of Cell Biology 179(6): 1163-1177.

Lagadu, S., M. Lechevrel, F. Sichel, J. Breton, D. Pottier, R. Couderc, F. Moussa and V. Prevost (2010). "8-oxo-7, 8-dihydro-2'-deoxyguanosine as a biomarker of oxidative damage in oesophageal cancer patients: lack of association with antioxidant vitamins and polymorphism of hOGG1 and GST." Journal of Experimental and Clinical Cancer Research 29(1): 157.

Lamantea, E., F. Carrara, C. Mariotti, L. Morandi, V. Tiranti and M. Zeviani (2002). "A novel nonsense mutation (Q352X) in the mitochondrial cytochrome b gene associated with a combined deficiency of complexes I and III." Neuromuscular Disorders 12(1): 49-52.

Landriscina, M., F. Maddalena, G. Laudiero and F. Esposito (2009). "Adaptation to oxidative stress, chemoresistance, and cell survival." Antioxidants & Redox Signaling 11(11): 2701-2716.

Law, M., M. E. Hodson and B. Heard (1982). "Malignant mesothelioma of the pleura: relation between histological type and clinical behaviour." Thorax 37(11): 810-815.

Lazo, J. S., S.-M. Kuo, E. S. Woo and B. R. Pitt (1998). "The protein thiol metallothionein as an antioxidant and protectant against antineoplastic drugs." Chemico-Biological Interactions 111: 255-262.

Lee, H. B. and H. Ha (2007). "Mechanisms of epithelial-mesenchymal transition of peritoneal mesothelial cells during peritoneal dialysis." Journal of Korean Medical Science 22(6): 943-945.

Lee, J.-W., S.-H. Bae, J.-W. Jeong, S.-H. Kim and K.-W. Kim (2004). "Hypoxia-inducible factor (HIF-1) α: its protein stability and biological functions." Experimental & Molecular Medicine 36(1): 1-12.

Lee, J. G., J. H. Shin, H. S. Shim, C. Y. Lee, D. J. Kim, Y. S. Kim and K. Y. Chung (2015). "Autophagy contributes to the chemo-resistance of non-small cell lung cancer in hypoxic conditions." Respiratory Research 16: 138.

Lee, Y.-J., G. J. Lee, S. S. Yi, S.-H. Heo, C.-R. Park, H.-S. Nam, M.-K. Cho and S.-H. Lee (2016). "Cisplatin and resveratrol induce apoptosis and autophagy following oxidative stress in malignant mesothelioma cells." Food and Chemical Toxicology 97: 96-107.

Lee, Y.-J. and S.-H. Lee (2016). "Sulforaphane potentiates growth-inhibiting and apoptosis-promoting activities of cisplatin following oxidative stress and mitochondrial dysfunction in malignant mesothelioma cells." Molecular & Cellular Toxicology 12(3): 289-299.

Lee, Y. J. and S. H. Lee (2017). "Pro-oxidant activity of sulforaphane and cisplatin potentiates apoptosis and simultaneously promotes autophagy in malignant mesothelioma cells." Molecular Medicine Reports 16(2): 2133-2141.

Lenaz, G. (1998). "Role of mitochondria in oxidative stress and ageing." Biochimica et Biophysica Acta-Bioenergetics 1366(1-2): 53-67.

Leu, A. J., D. A. Berk, A. Lymboussaki, K. Alitalo and R. K. Jain (2000). "Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation." Cancer Research 60(16): 4324-4327.

Li, F., Y. Wang, K. I. Zeller, J. J. Potter, D. R. Wonsey, K. A. O'Donnell, J.-w. Kim, J. T. Yustein, L. A. Lee and C. V. Dang (2005). "Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis." Molecular and Cellular Biology 25(14): 6225-6234.

Li, G.-M. (2008). "Mechanisms and functions of DNA mismatch repair." Cell Research 18(1): 85-98.

Li, P., C. Zhou, L. Xu and H. Xiao (2013). "Hypoxia enhances stemness of cancer stem cells in glioblastoma: an in vitro study." International Journal of Medical Sciences 10(4): 399-407.

Liang, B., L. Miller and A. Weller (1999). "Ethyl-nitrosourea transformed astrocytes exhibit mitochondrial membrane hyperpolarization and constrained apoptosis." Apoptosis 4(2): 89-97.

Liao, J., F. Qian, N. Tchabo, P. Mhawech-Fauceglia, A. Beck, Z. Qian, X. Wang, W. J. Huss, S. B. Lele, C. D. Morrison and K. Odunsi (2014). "Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism." PLoS One 9(1): e84941.

Lim, C. B., C. M. Prêle, S. Baltic, P. G. Arthur, J. Creaney, D. N. Watkins, P. J. Thompson and S. E. Mutsaers (2015). "Mitochondria-derived reactive oxygen species drive GANT61-induced mesothelioma cell apoptosis." Oncotarget 6(3): 1519-1530.

Lin, Q., Y.-J. Lee and Z. Yun (2006). "Differentiation arrest by hypoxia." Journal of Biological Chemistry 281(41): 30678-30683.

Littlejohn, J. E., X. Cao, S. D. Miller, M. K. Ozvaran, D. Jupiter, L. Zhang, C. Rodarte and W. R. Smythe (2008). "Bcl-xL antisense oligonucleotide and cisplatin combination therapy extends survival in SCID mice with established mesothelioma xenografts." International Journal of

Cancer 123(1): 202-208.

Liu, B., Y. Chen and D. K. S. Clair (2008). "ROS and p53: a versatile partnership." Free Radical Biology and Medicine 44(8): 1529-1535.

Liu, L., X. Ning, L. Sun, H. Zhang, Y. Shi, C. Guo, S. Han, J. Liu, S. Sun and Z. Han (2008). "Hypoxia-inducible factor-1α contributes to hypoxia-induced chemoresistance in gastric cancer." Cancer Science 99(1): 121-128.

Liu, W., S.-M. Shen, X.-Y. Zhao and G.-Q. Chen (2012). "Targeted genes and interacting proteins of hypoxia inducible factor-1." International journal of biochemistry and molecular biology 3(2): 165-178.

Liu, X.-W., Y. Su, H. Zhu, J. Cao, W.-J. Ding, Y.-C. Zhao, Q.-J. He and B. Yang (2010). "HIF-1α-dependent autophagy protects HeLa cells from fenretinide (4-HPR)-induced apoptosis in hypoxia." Pharmacological Research 62(5): 416-425.

Liu, Z. and R. A. Butow (2006). "Mitochondrial retrograde signaling." Annual Reveiw of Genetics 40: 159-185.

Loboda, A., A. Jozkowicz and J. Dulak (2010). "HIF-1 and HIF-2 transcription factors—similar but not identical." Molecules and Cells 29(5): 435-442.

López-Lázaro, M. (2007). "Excessive superoxide anion generation plays a key role in carcinogenesis." International Journal of Cancer 120(6): 1378-1380.

Lu, C.-W., S.-C. Lin, K.-F. Chen, Y.-Y. Lai and S.-J. Tsai (2008). "Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance." Journal of Biological Chemistry 283(42): 28106-28114.

Lv, Y., S. Zhao, J. Han, L. Zheng, Z. Yang and L. Zhao (2015). "Hypoxia-inducible factor-1α induces multidrug resistance protein in colon cancer." OncoTargets and Therapy 8: 1941-1948.

Ma, Y., D. Liang, J. Liu, K. Axcrona, G. Kvalheim, T. Stokke, J. M. Nesland and Z. Suo (2011). "Prostate cancer cell lines under hypoxia exhibit

greater stem-like properties." PloS One 6(12): e29170.

Mahalingaiah, P. K., L. Ponnusamy and K. P. Singh (2015). "Chronic oxidative stress leads to malignant transformation along with acquisition of stem cell characteristics, and epithelial to mesenchymal transition in human renal epithelial cells." Journal of Cellular Physiology 230(8): 1916-1928.

Mahoney, B. P., N. Raghunand, B. Baggett and R. J. Gillies (2003). "Tumor acidity, ion trapping and chemotherapeutics: I. Acid pH affects the distribution of chemotherapeutic agents in vitro." Biochemical Pharmacology 66(7): 1207-1218.

Mancini, M., S. Camilleri-Bröet, B. O. Anderson and D. M. Hockenbery (2001). "Proliferative mitochondrial dysfunction and apoptosis." Advances in Cell Aging and Gerontology 5: 123-140.

Manente, A. G., G. Pinton, S. Zonca, M. Cilli, M. Rinaldi, A. Daga, S. Nilsson and L. Moro (2015). "Intracellular lactate-mediated induction of estrogen receptor beta (ERβ) in biphasic malignant pleural mesothelioma cells." Oncotarget 6(28): 25121-25134.

Manente, A. G., G. Pinton, S. Zonca, D. Tavian, T. Habib, P. V. Jithesh, D. Fennell, S. Nilsson and L. Moro (2016). "KDM6B histone demethylase is an epigenetic regulator of estrogen receptor β expression in human pleural mesothelioma." Epigenomics 8(9): 1227-1238.

Maples, K. and N. Johnson (1992). "Fiber-induced hydroxyl radical formation: correlation with mesothelioma induction in rats and humans." Carcinogenesis 13(11): 2035-2039.

Marczynski, B., P. Rozynek, T. Kraus, S. Schlösser, H. Raithel and X. Baur (2000). "Levels of 8-hydroxy-2′-deoxyguanosine in DNA of white blood cells from workers highly exposed to asbestos in Germany." Mutation Research-Genetic Toxicology and Environmental Mutagenesis 468(2): 195-202.

Marrache, S., R. K. Pathak and S. Dhar (2014). "Detouring of cisplatin to access mitochondrial genome for overcoming resistance." Proceedings of the National Academy of Sciences 111(29): 10444-

10449.

Martin, C. M., A. Ferdous, T. Gallardo, C. Humphries, H. Sadek, A. Caprioli, J. A. Garcia, L. I. Szweda, M. G. Garry and D. J. Garry (2008). "Hypoxia-inducible factor-2α transactivates Abcg2 and promotes cytoprotection in cardiac side population cells." Circulation Research 102(9): 1075-1081.

Maxwell, P. H., M. S. Wiesener, G.-W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher and P. J. Ratcliffe (1999). "The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis." Nature 399(6733): 271-275.

Maynard, S., S. H. Schurman, C. Harboe, N. C. de Souza-Pinto and V. A. Bohr (2008). "Base excision repair of oxidative DNA damage and association with cancer and aging." Carcinogenesis 30(1): 2-10.

Mazure, N. M., E. Y. Chen, K. R. Laderoute and A. J. Giaccia (1997). "Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element." Blood 90(9): 3322-3331.

McCord, A. M., M. Jamal, U. T. Shankavarum, F. F. Lang, K. Camphausen and P. J. Tofilon (2009). "Physiologic oxygen concentration enhances the stem-like properties of CD133+ human glioblastoma cells in vitro." Molecular Cancer Research 7(4): 489-497.

Mery, É., J. Hommell-Fontaine, M. Capovilla, A. Chevallier, F. Bibeau, S. Croce, P. Dartigues, R. Kaci, G. Lang-Averous and M.-H. Laverriere (2014). Peritoneal malignant mesothelioma: review and recent data. Annales de Pathologie.

Metcalf, R., J. Welsh, W. Bennett, M. Seddon, T. Lehman, K. Pelin, K. Linnainmaa, L. Tammilehto, K. Mattson and B. Gerwin (1992). "p53 and Kirsten-ras mutations in human mesothelioma cell lines." Cancer Research 52(9): 2610-2615.

Mimeault, M. and S. K. Batra (2013). "Hypoxia-inducing factors as master

regulators of stemness properties and altered metabolism of cancer-and metastasis-initiating cells." Journal of Cellular and Molecular Medicine 17(1): 30-54.

Miselis, N. R., Z. J. Wu, N. Van Rooijen and A. B. Kane (2008). "Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma." Molecular Cancer Therapeutics 7(4): 788-799.

Mitani, T., Y. Ito, N. Harada, Y. NAkANO, H. Inui, H. Ashida and R. Yamaji (2014). "Resveratrol reduces the hypoxia-induced resistance to doxorubicin in breast cancer cells." Journal of Nutritional Science and Vitaminology 60(2): 122-128.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell 80(2): 293-300.

Molinas, C. R. and P. R. Koninckx (2000). "Hypoxaemia induced by CO2 or helium pneumoperitoneum is a co-factor in adhesion formation in rabbits." Human Reproduction 15(8): 1758-1763.

Moolgavkar, S. H. and A. G. Knudson (1981). "Mutation and cancer: a model for human carcinogenesis." Journal of the National Cancer Institute 66(6): 1037-1052.

Morag, S., P. COHEN and D. R. ALESSI (1998). "The activation of protein kinase B by H2O2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2." Biochemical Journal 336(1): 241-246.

Morin, P. J. (2003). "Drug resistance and the microenvironment: nature and nurture." Drug Resistance Updates 6(4): 169-172.

Morishita, Y., S. Ookawara, I. Hirahara, S. Muto and D. Nagata (2016). "HIF-1alpha mediates Hypoxia-induced epithelial-mesenchymal transition in peritoneal mesothelial cells." Renal Failure 38(2): 282-289.

Morris, J. K. (1965). "A formaldehyde glutaraldehyde fixative of high osmolality for use in electron microscopy." Journal of Cell Biology 27(2): 137-139.

Mujoomdar, A. A., T. R. Tilleman, W. G. Richards, R. Bueno and D. J. Sugarbaker (2010). "Prevalence of in vitro chemotherapeutic drug resistance in primary malignant pleural mesothelioma: result in a cohort of 203 resection specimens." The Journal of Thoracic and Cardiovascular Surgery 140(2): 352-355.

Muller, F. L., Y. Liu and H. Van Remmen (2004). "Complex III releases superoxide to both sides of the inner mitochondrial membrane." Journal of Biological Chemistry.

Mutlu, A. G. (2012). Measuring of DNA damage by quantitative PCR. Polymerase Chain Reaction. A. G. Mutlu. Mehmet Akif Ersoy University, InTech: 283-284.

Nabavi, N., K. L. Bennewith, A. Churg, Y. Wang, C. C. Collins and L. Mutti (2016). "Switching off malignant mesothelioma: exploiting the hypoxic microenvironment." Genes & Cancer 7(11-12): 340-354.

Nagai, H., Y. Okazaki, S. H. Chew, N. Misawa, H. Yasui and S. Toyokuni (2013). "Deferasirox induces mesenchymal-epithelial transition in crocidolite-induced mesothelial carcinogenesis in rats." Cancer Prevention Research 6(11): 1222-1230.

Nardinocchi, L., R. Puca and G. D'Orazi (2011). "HIF-1α antagonizes p53-mediated apoptosis by triggering HIPK2 degradation." Aging 3(1): 33-43.

Nardinocchi, L., R. Puca, A. Sacchi and G. D'Orazi (2009). "Inhibition of HIF-1alpha activity by homeodomain-interacting protein kinase-2 correlates with sensitization of chemoresistant cells to undergo apoptosis." Molecular Cancer 8(1): 1.

Netti, P. A., D. A. Berk, M. A. Swartz, A. J. Grodzinsky and R. K. Jain (2000). "Role of extracellular matrix assembly in interstitial transport in solid tumors." Cancer Research 60(9): 2497-2503.

Nishi, H., T. Nakada, S. Kyo, M. Inoue, J. W. Shay and K. Isaka (2004). "Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT)." Molecular and Cellular Biology 24(13): 6076-6083.

O'Kane, S. L., R. J. Pound, A. Campbell, N. Chaudhuri, M. J. Lind and L.

Cawkwell (2006). "Expression of bcl-2 family members in malignant pleural mesothelioma." Acta Oncologica 45(4): 449-453.

Ohh, M., C. W. Park, M. Ivan, M. A. Hoffman, T.-Y. Kim, L. E. Huang, N. Pavletich, V. Chau and W. G. Kaelin (2000). "Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel–Lindau protein." Nature Cell Biology 2(7): 423-427.

Okamoto, A., C. Sumi, H. Tanaka, M. Kusunoki, T. Iwai, K. Nishi, Y. Matsuo, H. Harada, K. Takenaga and H. Bono (2017). "HIF-1-mediated suppression of mitochondria electron transport chain function confers resistance to lidocaine-induced cell death." Scientific Reports 7(1): 3816.

Oliva, C. R., D. R. Moellering, G. Y. Gillespie and C. E. Griguer (2011). "Acquisition of chemoresistance in gliomas is associated with increased mitochondrial coupling and decreased ROS production." PloS One 6(9): e24665.

Oliva, C. R., S. E. Nozell, A. Diers, S. G. McClugage, 3rd, J. N. Sarkaria, J. M. Markert, V. M. Darley-Usmar, S. M. Bailey, G. Y. Gillespie, A. Landar and C. E. Griguer (2010). "Acquisition of temozolomide chemoresistance in gliomas leads to remodeling of mitochondrial electron transport chain." Journal of Biologycal Chemistry 285(51): 39759-39767.

Padera, T. P., B. R. Stoll, J. B. Tooredman, D. Capen, E. di Tomaso and R. K. Jain (2004). "Cancer cells compress intratumour vessels." Nature 427(6976): 695.

Papp, T., H. Schipper, H. Pemsel, R. Bastrop, K.-M. Muller, T. Wiethege, D. G. Weiss, E. Dopp, D. Schiffmann and Q. Rahman (2001). "Mutational analysis of N-ras, p53, p16INK4a, p14ARF and CDK4 genes in primary human malignant mesotheliomas." International Journal of Oncology 18(2): 425-433.

Pasdar, E. A., M. Smits, M. Stapelberg, M. Bajzikova, M. Stantic, J. Goodwin, B. Yan, J. Stursa, J. Kovarova and K. Sachaphibulkij (2015). "Characterisation of mesothelioma-initiating cells and their susceptibility to anti-cancer agents." PLoS One 10(5): e0119549.

Peinado, H., D. Olmeda and A. Cano (2007). "Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?" Nature Reviews Cancer 7(6): 415-428.

Pellegrini, L., B. Pucci, L. Villanova, M. Marino, G. Marfe, L. Sansone, E. Vernucci, D. Bellizzi, V. Reali and M. Fini (2012). "SIRT3 protects from hypoxia and staurosporine-mediated cell death by maintaining mitochondrial membrane potential and intracellular pH." Cell Death and Differentiation 19(11): 1815-1825.

Peto, J., F. Matthews, J. Hodgson and J. R. Jones (1995). "Continuing increase in mesothelioma mortality in Britain." The Lancet 345(8949): 535-539.

Pfeiffer, T., S. Schuster and S. Bonhoeffer (2001). "Cooperation and competition in the evolution of ATP-producing pathways." Science 292(5516): 504-507.

Policastro, L. L., I. L. Ibañez, C. Notcovich, H. A. Duran and O. L. Podhajcer (2013). "The tumor microenvironment: characterization, redox considerations, and novel approaches for reactive oxygen species-targeted gene therapy." Antioxidants & Redox Signaling 19(8): 854-895.

Poppe, M., C. Reimertz, H. Dussmann, A. J. Krohn, C. M. Luetjens, D. Bockelmann, A. L. Nieminen, D. Kogel and J. H. Prehn (2001). "Dissipation of potassium and proton gradients inhibits mitochondrial hyperpolarization and cytochrome c release during neural apoptosis." Journal of Neuroscience 21(13): 4551-4563.

Pugh, C. W. and P. J. Ratcliffe (2003). "Regulation of angiogenesis by hypoxia: role of the HIF system." Nature Medicine 9(6): 677-684.

Qing, G. and M. C. Simon (2009). "Hypoxia inducible factor-2α: a critical mediator of aggressive tumor phenotypes." Current Opinion in Genetics & Development 19(1): 60-66.

Ramachandran, S., J. Ient, E.-L. Göttgens, A. J. Krieg and E. M. Hammond (2015). "Epigenetic therapy for solid tumors: highlighting the impact of tumor hypoxia." Genes 6(4): 935-956.

Ramos-Nino, M. E., L. Scapoli, M. Martinelli, S. Land and B. T. Mossman (2003). "Microarray analysis and RNA silencing link fra-1 to cd44 and c-met expression in mesothelioma." Cancer Research 63(13): 3539-3545.

Ramos-Nino, M. E., J. R. Testa, D. A. Altomare, H. I. Pass, M. Carbone, M. Bocchetta and B. T. Mossman (2006). "Cellular and molecular parameters of mesothelioma." Journal of Cellular Biochemistry 98(4): 723-734.

Ravenna, L., I. Cardillo, G. Curzio, A. Baldi, M. Mattioni, B. Vincenzi, M. Russo, S. Soddu and A. Verdina (2014). "Mesothelioma and hypoxia: modulation of the inflammation-related phenotype and identification of prognostic markers." Journal of Cancer Science and Therapy 6: 378-387.

Reddy, R. M., W.-S. Yeow, A. Chua, D. M. Nguyen, A. Baras, M. F. Ziauddin, S. M. Shamimi-Noori, J. B. Maxhimer, D. S. Schrump and D. M. Nguyen (2007). "Rapid and profound potentiation of Apo2L/TRAIL-mediated cytotoxicity and apoptosis in thoracic cancer cells by the histone deacetylase inhibitor Trichostatin A: the essential role of the mitochondria-mediated caspase activation cascade." Apoptosis 12(1): 55-71.

Rhee, S. G. (2006). "Cell signaling. H2O2, a necessary evil for cell signaling." Science 312(5782): 1882-1883.

Richard, D. E., E. Berra, E. Gothié, D. Roux and J. Pouysségur (1999). "p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1α (HIF-1α) and enhance the transcriptional activity of HIF-1." Journal of Biological Chemistry 274(46): 32631-32637.

Riganti, C., S. Doublier, E. Aldieri, S. Orecchia, P. G. Betta, E. Gazzano, D. Ghigo and A. Bosia (2008). "Asbestos induces doxorubicin resistance in MM98 mesothelioma cells via HIF-1α." European Respiratory Journal 32: 443-451.

Robinson, B. W., A. W. Musk and R. A. Lake (2005). "Malignant mesothelioma." The Lancet 366(9483): 397-408.

Robson, E. J., W. T. Khaled, K. Abell and C. J. Watson (2006). "Epithelial-to-mesenchymal transition confers resistance to apoptosis in three murine mammary epithelial cell lines." Differentiation 74(5): 254-264.

Rodríguez-Jiménez, F. J., V. Moreno-Manzano, R. Lucas-Dominguez and J. M. Sánchez-Puelles (2008). "Hypoxia causes downregulation of mismatch repair system and genomic instability in stem cells." Stem Cells 26(8): 2052-2062.

Rohwer, N. and T. Cramer (2011). "Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways." Drug Resistance Updates 14(3): 191-201.

Rohwer, N., C. Dame, A. Haugstetter, B. Wiedenmann, K. Detjen, C. A. Schmitt and T. Cramer (2010). "Hypoxia-inducible factor 1α determines gastric cancer chemosensitivity via modulation of p53 and NF-κB." PloS One 5(8): e12038.

Roth, M., J. Zhong, M. Tamm and J. Szilard (2009). "Mesothelioma cells escape heat stress by upregulating Hsp40/Hsp70 expression via mitogen-activated protein kinases." Journal of Biomedicine and Biotechnology 2009: 451084.

Rouault-Pierre, K., L. Lopez-Onieva, K. Foster, F. Anjos-Afonso, I. Lamrissi-Garcia, M. Serrano-Sanchez, R. Mitter, Z. Ivanovic, H. de Verneuil and J. Gribben (2013). "HIF-2α protects human hematopoietic stem/progenitors and acute myeloid leukemic cells from apoptosis induced by endoplasmic reticulum stress." Cell Stem Cell 13(5): 549-563.

Ruan, K., G. Song and G. Ouyang (2009). "Role of hypoxia in the hallmarks of human cancer." Journal of Cellular Biochemistry 107(6): 1053-1062.

Sakata, K., T. T. Kwok, B. Murphy, K. Laderoute, G. Gordon and R. Sutherland (1991). "Hypoxia-induced drug resistance: comparison to P-glycoprotein-associated drug resistance." British Journal of Cancer 64(5): 809-814.

Santamaría, G., M. Martínez-Diez, I. Fabregat and J. M. Cuezva (2005).

"Efficient execution of cell death in non-glycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H+-ATP synthase." Carcinogenesis 27(5): 925-935.

Sasabe, E., Y. Tatemoto, D. Li, T. Yamamoto and T. Osaki (2005). "Mechanism of HIF-1α-dependent suppression of hypoxia-induced apoptosis in squamous cell carcinoma cells." Cancer Science 96(7): 394-402.

Sasabe, E., Z. Yang, S. Ohno and T. Yamamoto (2010). "Reactive oxygen species produced by the knockdown of manganese-superoxide dismutase up-regulate hypoxia-inducible factor-1α expression in oral squamous cell carcinoma cells." Free Radical Biology and Medicine 48(10): 1321-1329.

Sawada, M., S. Nakashima, T. Kiyono, M. Nakagawa, J. Yamada, H. Yamakawa, Y. Banno, J. Shinoda, Y. Nishimura and Y. Nozawa (2001). "p53 regulates ceramide formation by neutral sphingomyelinase through reactive oxygen species in human glioma cells." Oncogene 20(11): 1368-1378.

Saxe, J. P., A. Tomilin, H. R. Schöler, K. Plath and J. Huang (2009). "Post-translational regulation of Oct4 transcriptional activity." PloS One 4(2): e4467.

Schieber, M. and N. S. Chandel (2014). "ROS function in redox signaling and oxidative stress." Current Biology 24(10): R453-R462.

Schoning, J. P., M. Monteiro and W. Gu (2017). "Drug resistance and cancer stem cells: the shared but distinct roles of hypoxia-inducible factors HIF1alpha and HIF2alpha." Clinical and Experimental Pharmacology and Physiology 44(2): 153-161.

Schramm, A., I. Opitz, S. Thies, B. Seifert, H. Moch, W. Weder and A. Soltermann (2010). "Prognostic significance of epithelial-mesenchymal transition in malignant pleural mesothelioma." European Journal of Cardio-Thoracic Surgery 37(3): 566-572.

Schwerdt, G., R. Freudinger, C. Schuster, F. Weber, O. Thews and M. Gekle (2005). "Cisplatin-induced apoptosis is enhanced by hypoxia and by

inhibition of mitochondria in renal collecting duct cells." Toxicological Sciences 85(1): 735-742.

Semenza, G. L. (2000). "HIF-1: mediator of physiological and pathophysiological responses to hypoxia." Journal of Applied Physiology 88(4): 1474-1480.

Semenza, G. L. (2007). "Hypoxia-inducible factor 1 (HIF-1) pathway." Science Signaling 2007(407): cm8.

Semenza, G. L. (2010). "Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics." Oncogene 29(5): 625-634.

Sendoel, A., I. Kohler, C. Fellmann, S. W. Lowe and M. O. Hengartner (2010). "HIF-1 antagonizes p53-mediated apoptosis through a secreted neuronal tyrosinase." Nature 465(7298): 577-583.

Sermeus, A., M. Genin, A. Maincent, M. Fransolet, A. Notte, L. Leclere, H. Riquier, T. Arnould and C. Michiels (2012). "Hypoxia-induced modulation of apoptosis and BCL-2 family proteins in different cancer cell types." PLoS One 7(11): e47519.

Shannon, A. M., D. J. Bouchier-Hayes, C. M. Condron and D. Toomey (2003). "Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies." Cancer Treatment Reviews 29(4): 297-307.

Shestov, A. A., X. Liu, Z. Ser, A. A. Cluntun, Y. P. Hung, L. Huang, D. Kim, A. Le, G. Yellen and J. G. Albeck (2014). "Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step." Elife 3: e03342.

Shimojo, Y., M. Akimoto, T. Hisanaga, T. Tanaka, Y. Tajima, Y. Honma and K. Takenaga (2013). "Attenuation of reactive oxygen species by antioxidants suppresses hypoxia-induced epithelial-mesenchymal transition and metastasis of pancreatic cancer cells." Clinical & Experimental Metastasis 30(2): 143-154.

Shin, D. H., Y.-J. Choi and J.-W. Park (2013). "SIRT1 and AMPK mediate hypoxia-induced resistance of non-small cell lung cancers to cisplatin and doxorubicin." Cancer Research 74(1): 298-308.

Singh, A. and J. Settleman (2010). "EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer." Oncogene 29(34): 4741-4751.

Sivertsen, S., R. Hadar, S. Elloul, L. Vintman, C. Bedrossian, R. Reich and B. Davidson (2006). "Expression of Snail, Slug and Sip1 in malignant mesothelioma effusions is associated with matrix metalloproteinase, but not with cadherin expression." Lung Cancer 54(3): 309-317.

Skulachev, V. P. (1988). Δ μ̄ H Consumers. Membrane Bioenergetics, Springer: 209-221.

Soini, Y., K. Järvinen, R. Kaarteenaho-Wiik and V. Kinnula (2001). "The expression of P-glycoprotein and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) in human malignant mesothelioma." Annals of Oncology 12(9): 1239-1245.

Sowter, H. M., R. Raval, J. Moore, P. J. Ratcliffe and A. L. Harris (2003). "Predominant role of hypoxia-inducible transcription factor (Hif)-1α versus Hif-2α in regulation of the transcriptional response to hypoxia." Cancer Research 63(19): 6130-6134.

Stewart IV, J. H., T.-L. Tran, N. Levi, W. S. Tsai, D. S. Schrump and D. M. Nguyen (2007). "The essential role of the mitochondria and reactive oxygen species in Cisplatin-mediated enhancement of fas ligand-induced apoptosis in malignant pleural mesothelioma." Journal of Surgical Research 141(1): 120-131.

Stiburek, L., J. Cesnekova, O. Kostkova, D. Fornuskova, K. Vinsova, L. Wenchich, J. Houstek and J. Zeman (2012). "YME1L controls the accumulation of respiratory chain subunits and is required for apoptotic resistance, cristae morphogenesis, and cell proliferation." Molecular Biology of the Cell 23(6): 1010-1023.

Sullivan, R. and C. H. Graham (2009). "Hypoxia prevents etoposide-induced DNA damage in cancer cells through a mechanism involving hypoxia-inducible factor 1." Molecular Cancer Therapeutics 8(6): 1702-1713.

Sullivan, R., G. C. Pare, L. J. Frederiksen, G. L. Semenza and C. H. Graham (2008). "Hypoxia-induced resistance to anticancer drugs is

associated with decreased senescence and requires hypoxia-inducible factor-1 activity." Molecular Cancer Therapeutics 7(7): 1961-1973.

Suzuki, E., S. Kim, H.-K. Cheung, M. J. Corbley, X. Zhang, L. Sun, F. Shan, J. Singh, W.-C. Lee and S. M. Albelda (2007). "A novel small-molecule inhibitor of transforming growth factor β type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection." Cancer Research 67(5): 2351-2359.

Suzuki, Y. and S. R. Yuen (2002). "Asbestos fibers contributing to the induction of human malignant mesothelioma." Annals of the New York Academy of Sciences 982(1): 160-176.

Szatrowski, T. P. and C. F. Nathan (1991). "Production of large amounts of hydrogen peroxide by human tumor cells." Cancer Research 51(3): 794-798.

Tabur, S., Ş. N. Aksoy, H. Korkmaz, M. Ozkaya, N. Aksoy and E. Akarsu (2015). "Investigation of the role of 8-OHdG and oxidative stress in papillary thyroid carcinoma." Tumor Biology 36(4): 2667-2674.

Tan, Q., A. M. Joshua, M. Wang, R. G. Bristow, B. G. Wouters, C. J. Allen and I. F. Tannock (2017). "Up-regulation of autophagy is a mechanism of resistance to chemotherapy and can be inhibited by pantoprazole to increase drug sensitivity." Cancer Chemotherapy and Pharmacology 79(5): 959-969.

Tanaka, M., Y. Miura, H. Numanami, S. Karnan, A. Ota, H. Konishi, Y. Hosokawa and M. Hanyuda (2015). "Inhibition of NADPH oxidase 4 induces apoptosis in malignant mesothelioma: Role of reactive oxygen species." Oncololy Reports 34(4): 1726-1732.

Tang, Y. A., Y. F. Chen, Y. Bao, S. Mahara, S. Yatim, G. Oguz, P. L. Lee, M. Feng, Y. Cai, E. Y. Tan, S. S. Fong, Z. H. Yang, P. Lan, X. J. Wu and Q. Yu (2018). "Hypoxic tumor microenvironment activates GLI2 via HIF-1alpha and TGF-beta2 to promote chemoresistance in colorectal cancer." Proceedings of the National Academy of Sciences of the United States of America 115(26): E5990-E5999.

Tannock, I. (1978). "Cell kinetics and chemotherapy: a critical review." Cancer Treatment Reports 62(8): 1117-1133.

Taylor, R. W. and D. M. Turnbull (2005). "Mitochondrial DNA mutations in human disease." Nature Reviews Genetics 6(5): 389-402.

Teicher, B. A. (1994). "Hypoxia and drug resistance." Cancer and Metastasis Reviews 13(2): 139-168.

Terauchi, M., H. Kajiyama, M. Yamashita, M. Kato, H. Tsukamoto, T. Umezu, S. Hosono, E. Yamamoto, K. Shibata and K. Ino (2007). "Possible involvement of TWIST in enhanced peritoneal metastasis of epithelial ovarian carcinoma." Clinical & Experimental Metastasis 24(5): 329-339.

Tomasetti, M., L. Nocchi, S. Staffolani, N. Manzella, M. Amati, J. Goodwin, K. Kluckova, M. Nguyen, E. Strafella and M. Bajzikova (2014). "MicroRNA-126 suppresses mesothelioma malignancy by targeting IRS1 and interfering with the mitochondrial function." Antioxidants & Redox Signaling 21(15): 2109-2125.

Tomasetti, M., M. Rippo, R. Alleva, S. Moretti, L. Andera, J. Neuzil and A. Procopio (2004). "α-Tocopheryl succinate and TRAIL selectively synergise in induction of apoptosis in human malignant mesothelioma cells." British Journal of Cancer 90(8): 1644-1653.

Trédan, O., C. M. Galmarini, K. Patel and I. F. Tannock (2007). "Drug resistance and the solid tumor microenvironment." Journal of the National Cancer Institute 99(19): 1441-1454.

Tse, J. C. and R. Kalluri (2007). "Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment." Journal of Cellular Biochemistry 101(4): 816-829.

Ujiie, H., K. Kadota, J.-i. Nitadori, J. G. Aerts, K. M. Woo, C. S. Sima, W. D. Travis, D. R. Jones, L. M. Krug and P. S. Adusumilli (2015). "The tumoral and stromal immune microenvironment in malignant pleural mesothelioma: a comprehensive analysis reveals prognostic immune markers." Oncoimmunology 4(6): e1009285.

Unruh, A., A. Ressel, H. G. Mohamed, R. S. Johnson, R. Nadrowitz, E. Richter, D. M. Katschinski and R. H. Wenger (2003). "The hypoxia-inducible factor-1α is a negative factor for tumor therapy." Oncogene 22(21): 3213-3220.

Uribe, D., A. Torres, J. D. Rocha, I. Niechi, C. Oyarzun, L. Sobrevia, R. San Martin and C. Quezada (2017). "Multidrug resistance in glioblastoma stem-like cells: Role of the hypoxic microenvironment and adenosine signaling." Molecular Aspects of Medicine 55: 140-151.

Valencia-Cervantes, J., S. Huerta-Yepez, G. Aquino-Jarquin, S. Rodriguez-Enriquez, D. Martinez-Fong, J. A. Arias-Montano and V. M. Davila-Borja (2018). "Hypoxia increases chemoresistance in human medulloblastoma DAOY cells via hypoxiainducible factor 1alphamediated downregulation of the CYP2B6, CYP3A4 and CYP3A5 enzymes and inhibition of cell proliferation." Oncology Reports 41(1): 178-190.

van Zandwijk, N., C. Clarke, D. Henderson, A. W. Musk, K. Fong, A. Nowak, R. Loneragan, B. McCaughan, M. Boyer and M. Feigen (2013). "Guidelines for the diagnosis and treatment of malignant pleural mesothelioma." Journal of Thoracic Disease 5(6): E254-E307.

Varin, E., C. Denoyelle, E. Brotin, M. Meryet-Figuiere, F. Giffard, E. Abeilard, D. Goux, P. Gauduchon, P. Icard and L. Poulain (2010). "Downregulation of Bcl-x L and Mcl-1 is sufficient to induce cell death in mesothelioma cells highly refractory to conventional chemotherapy." Carcinogenesis 31(6): 984-993.

Vaupel, P. (2004). Tumor microenvironmental physiology and its implications for radiation oncology. Seminars in Radiation Oncology, Elsevier.

Vaupel, P. (2008). "Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis." The Oncologist 13(Supplement 3): 21-26.

Vaupel, P. (2010). "Metabolic microenvironment of tumor cells: a key factor in malignant progression." Experimental oncology 32(3): 125-127.

Vaupel, P., H. Fortmeyer, S. Runkel and F. Kallinowski (1987). "Blood flow, oxygen consumption, and tissue oxygenation of human breast cancer xenografts in nude rats." Cancer Research 47(13): 3496-3503.

Vaupel, P., F. Kallinowski and P. Okunieff (1989). "Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review." Cancer Research 49(23): 6449-6465.

Vaupel, P. and A. Mayer (2007). "Hypoxia in cancer: significance and impact on clinical outcome." Cancer and Metastasis Reviews 26(2): 225-239.

Vaupel, P., O. Thews and M. Hoeckel (2001). "Treatment resistance of solid tumors." Medical Oncology 18(4): 243-259.

Vaupel, P. W. (1994). Blood flow, oxygenation, tissue pH distribution, and bioenergetic status of tumors, Ernst Schering Research Foundation.

Vega, S., A. V. Morales, O. H. Ocaña, F. Valdés, I. Fabregat and M. A. Nieto (2004). "Snail blocks the cell cycle and confers resistance to cell death." Genes & Development 18(10): 1131-1143.

Wagemaker, G. (1995). "Cell and cell system responses: Heterogeneity of radiation sensitivity of hemopoietic stem cell subsets." Stem Cells 13(S1): 257-260.

Wagner, J. C., C. Sleggs and P. Marchand (1960). "Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province." Occupational and Environmental Medicine 17(4): 260-271.

Walker, L. J., R. B. Craig, A. L. Harris and I. D. Hickson (1994). "A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress." Nucleic Acids Research 22(23): 4884-4889.

Wallace, D. C. (2012). "Mitochondria and cancer." Nature Reviews Cancer 12(10): 685-698.

Walsh, J. C., A. Lebedev, E. Aten, K. Madsen, L. Marciano and H. C. Kolb (2014). "The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities." Antioxidants & Redox Signaling 21(10): 1516-1554.

Wang, G. L., B.-H. Jiang, E. A. Rue and G. L. Semenza (1995). "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension." Proceedings of the National Academy of Sciences 92(12): 5510-5514.

Wang, G. L. and G. L. Semenza (1995). "Purification and characterization of hypoxia-inducible factor 1." Journal of Biological Chemistry 270(3): 1230-1237.

Wang, H. W., T. C. Zhu, M. E. Putt, M. Solonenko, J. Metz, A. Dimofte, J. Miles, D. L. Fraker, E. Glatstein, S. M. Hahn and A. G. Yodh (2005). "Broadband reflectance measurements of light penetration, blood oxygenation, hemoglobin concentration, and drug concentration in human intraperitoneal tissues before and after photodynamic therapy." Journal of Biomedical Optics 10(1): 14004.

Wang, J., M. P. Biju, M.-H. Wang, V. H. Haase and Z. Dong (2006). "Cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis: involvement of mitochondrial inhibition and p53 suppression." Journal of the American Society of Nephrology 17(7): 1875-1885.

Wang, L. C., A. Lo, J. Scholler, J. Sun, R. S. Majumdar, V. Kapoor, M. Antzis, C. E. Cotner, L. A. Johnson, A. C. Durham, C. C. Solomides, C. H. June, E. Pure and S. M. Albelda (2014). "Targeting fibroblast activation protein in tumor stroma with chimeric antigen receptor T cells can inhibit tumor growth and augment host immunity without severe toxicity." Cancer Immunol Research 2(2): 154-166.

Wang, T. and H. Wang (1996). "p53, apoptosis and human cancers." Journal of the Formosan Medical Association 95(7): 509-522.

Wardman, P. (2001). "Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia." Current Medicinal Chemstry 8(7): 739-761.

Waypa, G. B., J. D. Marks, R. Guzy, P. T. Mungai, J. Schriewer, D. Dokic and P. T. Schumacker (2010). "Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells." Circulation research 106(3): 526.

Weinberg, F. and N. S. Chandel (2009). "Reactive oxygen species-dependent signaling regulates cancer." Cellular and Molecular Life Sciences 66(23): 3663-3673.

Wenger, R. H., V. Kurtcuoglu, C. C. Scholz, H. H. Marti and D. Hoogewijs (2015). "Frequently asked questions in hypoxia research." Hypoxia 3: 35-43.

Wigerup, C., S. Påhlman and D. Bexell (2016). "Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer." Pharmacology & Therapeutics 164: 152-169.

Wirthner, R., S. Wrann, K. Balamurugan, R. H. Wenger and D. P. Stiehl (2008). "Impaired DNA double-strand break repair contributes to chemoresistance in HIF-1α-deficient mouse embryonic fibroblasts." Carcinogenesis 29(12): 2306-2316.

Wisnovsky, S. P., J. J. Wilson, R. J. Radford, M. P. Pereira, M. R. Chan, R. R. Laposa, S. J. Lippard and S. O. Kelley (2013). "Targeting mitochondrial DNA with a platinum-based anticancer agent." Chemistry & Biology 20(11): 1323-1328.

Wohlkoenig, C., K. Leithner, A. Deutsch, A. Hrzenjak, A. Olschewski and H. Olschewski (2011). "Hypoxia-induced cisplatin resistance is reversible and growth rate independent in lung cancer cells." Cancer Letters 308(2): 134-143.

Woraruthai, T., C. Charoenlap, C. Hongsaprabhas, A. Mutirangura and S. Honsawek (2018). "Alu hypermethylation and high oxidative stress in patients with musculoskeletal tumors." PeerJ 6: e5492.

Wu, H.-M., Z.-F. Jiang, P.-S. Ding, L.-J. Shao and R.-Y. Liu (2015). "Hypoxia-induced autophagy mediates cisplatin resistance in lung cancer cells." Scientific Reports 5: 12291.

Xu, J., S. Lamouille and R. Derynck (2009). "TGF-β-induced epithelial to mesenchymal transition." Cell Research 19(2): 156-172.

Xu, R.-h., H. Pelicano, Y. Zhou, J. S. Carew, L. Feng, K. N. Bhalla, M. J. Keating and P. Huang (2005). "Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with

mitochondrial respiratory defect and hypoxia." Cancer Research 65(2): 613-621.

Xuan, Y., H. Hur, I.-H. Ham, J. Yun, J.-Y. Lee, W. Shim, Y. B. Kim, G. Lee, S.-U. Han and Y. K. Cho (2014). "Dichloroacetate attenuates hypoxia-induced resistance to 5-fluorouracil in gastric cancer through the regulation of glucose metabolism." Experimental Cell Research 321(2): 219-230.

Yan, Y., F. Liu, L. Han, L. Zhao, J. Chen, O. I. Olopade, M. He and M. Wei (2018). "HIF-2α promotes conversion to a stem cell phenotype and induces chemoresistance in breast cancer cells by activating Wnt and Notch pathways." Journal of Experimental and Clinical Cancer Research 37(1): 256.

Yáñez-Mó, M., E. Lara-Pezzi, R. Selgas, M. Ramírez-Huesca, C. Domínguez-Jiménez, J. A. Jiménez-Heffernan, A. Aguilera, J. A. Sánchez-Tomero, M. A. Bajo and V. Álvarez (2003). "Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells." New England Journal of Medicine 348(5): 403-413.

Yang, J. and R. A. Weinberg (2008). "Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis." Developmental cell 14(6): 818-829.

Yang, M.-H. and K.-J. Wu (2008). "TWIST activation by hypoxia inducible factor-1 (HIF-1): implications in metastasis and development." Cell Cycle 7(14): 2090-2096.

Yang, M.-H., M.-Z. Wu, S.-H. Chiou, P.-M. Chen, S.-Y. Chang, C.-J. Liu, S.-C. Teng and K.-J. Wu (2008). "Direct regulation of TWIST by HIF-1α promotes metastasis." Nature Cell Biology 10(3): 295-305.

Yang, X., H. Yin, Y. Zhang, X. Li, H. Tong, Y. Zeng, Q. Wang and W. He (2018). "Hypoxia-induced autophagy promotes gemcitabine resistance in human bladder cancer cells through hypoxia-inducible factor 1α activation." International Journal of Oncology 53(1): 215-224.

Yang, Y., X. Pan, W. Lei, J. Wang and J. Song (2006). "Transforming growth

factor-β1 induces epithelial-to-mesenchymal transition and apoptosis via a cell cycle-dependent mechanism." Oncogene 25(55): 7235-7244.

Yang, Z., L. M. Schumaker, M. J. Egorin, E. G. Zuhowski, Z. Guo and K. J. Cullen (2006). "Cisplatin preferentially binds mitochondrial DNA and voltage-dependent anion channel protein in the mitochondrial membrane of head and neck squamous cell carcinoma: possible role in apoptosis." Clinical Cancer Research 12(19): 5817-5825.

Yap, T. A., J. G. Aerts, S. Popat and D. A. Fennell (2017). "Novel insights into mesothelioma biology and implications for therapy." Nature Reviews Cancer 17(8): 475-488.

Ye, X. and R. A. Weinberg (2015). "Epithelial–mesenchymal plasticity: a central regulator of cancer progression." Trends in Cell Biology 25(11): 675-686.

YEN, H. C., Y. C. TANG, F. Y. CHEN, S. W. CHEN and H. J. Majima (2005). "Enhancement of cisplatin-induced apoptosis and caspase 3 activation by depletion of mitochondrial DNA in a human osteosarcoma cell line." Annals of the New York Academy of Sciences 1042(1): 516-522.

Yeung, T. M., S. C. Gandhi and W. F. Bodmer (2011). "Hypoxia and lineage specification of cell line-derived colorectal cancer stem cells." Proceedings of the National Academy of Sciences 108(11): 4382-4387.

Yokomizo, A., M. Ono, H. Nanri, Y. Makino, T. Ohga, M. Wada, T. Okamoto, J. Yodoi, M. Kuwano and K. Kohno (1995). "Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide." Cancer Research 55(19): 4293-4296.

Young, J. H. (1973). "Membrane tubularization and osmotic swelling in mitochondria." Journal of Theoretical Biollogy 40(2): 329-335.

Yuan, F., H. A. Salehi, Y. Boucher, U. S. Vasthare, R. F. Tuma and R. K. Jain (1994). "Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial

windows." Cancer Research 54(17): 4564-4568.

Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J.-L. Vayssiere, P. X. Petit and G. Kroemer (1995). "Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo." Journal of Experimental Medicine 181(5): 1661-1672.

Zhang, H., M. Bosch-Marce, L. A. Shimoda, Y. S. Tan, J. H. Baek, J. B. Wesley, F. J. Gonzalez and G. L. Semenza (2008). "Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia." Journal of Biological Chemistry 283(16): 10892-10903.

Zhang, H., P. Gao, R. Fukuda, G. Kumar, B. Krishnamachary, K. I. Zeller, C. V. Dang and G. L. Semenza (2007). "HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity." Cancer Cell 11(5): 407-420.

Zhang, J., W. Zhang, T. Zhang, Q. Zhou, J. Liu, Y. Liu, D. Kong, W. Yu, R. Liu and C. Hai (2018). "TGF-beta1 induces epithelial-to-mesenchymal transition via inhibiting mitochondrial functions in A549 cells." Free Radical Research: 1-13.

Zhang, W., X. Wu, L. Wu, W. Zhang and X. Zhao (2015). "Advances in the diagnosis, treatment and prognosis of malignant pleural mesothelioma." Annals of Translational Medicine 3(13): 182.

Zhang, Y., Y. Du, W. Le, K. Wang, N. Kieffer and J. Zhang (2011). "Redox control of the survival of healthy and diseased cells." Antioxidants & Redox Signaling 15(11): 2867-2908.

Zhao, T., Y. Zhu, A. Morinibu, M. Kobayashi, K. Shinomiya, S. Itasaka, M. Yoshimura, G. Guo, M. Hiraoka and H. Harada (2014). "HIF-1-mediated metabolic reprogramming reduces ROS levels and facilitates the metastatic colonization of cancers in lungs." Scientific Reports 4: 3793.

Zhong, H., K. Chiles, D. Feldser, E. Laughner, C. Hanrahan, M.-M. Georgescu, J. W. Simons and G. L. Semenza (2000). "Modulation of hypoxia-inducible factor 1α expression by the epidermal growth

factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics." Cancer Research 60(6): 1541-1545.

Zhou, J., T. Schmid, S. Schnitzer and B. Brüne (2006). "Tumor hypoxia and cancer progression." Cancer Letters 237(1): 10-21.

Ziemann, C., A. Bürkle, G. F. Kahl and K. I. Hirsch-Ernst (1999). "Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures." Carcinogenesis 20(3): 407-414.

Zinkewich-Péotti, K. and P. A. Andrews (1992). "Loss of cis-diamminedichloroplatinum (II) resistance in human ovarian carcinoma cells selected for rhodamine 123 resistance." Cancer Research 52(7): 1902-1906.

Zonca, S., G. Pinton, Z. Wang, M. F. Soluri, D. Tavian, M. Griffin, D. Sblattero and L. Moro (2017). "Tissue transglutaminase (TG2) enables survival of human malignant pleural mesothelioma cells in hypoxia." Cell Death and Differentiation 8(2): e2592.

Zorov, D. B., M. Juhaszova and S. J. Sollott (2006). "Mitochondrial ROS-induced ROS release: an update and review." Biochimica et Biophysica Acta (BBA)-Bioenergetics 1757(5-6): 509-517.

국문초록

종양 미세환경 스트레스가 악성 중피종에 미치는 영향

김 명 철

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서울대학교 대학원 수의학과 임상수의학 전공

종양 세포는 정상 세포가 생존하기에 불리한 미세 환경에서 생존하며 고도의 악성도를 획득한다. 산화적 스트레스와 저산소증은 종양 미세환경에서 가장 만연해 있는 적대적인 미세환경요소이다. 악성 중피종은 석면 노출로 인해 발생하는 종양이다. 현재까지 적대적인 종양 미세환경과 악성 중피종의 진행에 대해 거의 연구가 되어 있지 않다. 본 연구는 산화적 스트레스와 저산소증이 악성 중피종에 미치는 영향에 대하여 조사하였다. 과산화수소 처리에 의한 산화적 스트레스는 악성 중피종의 상피간엽이행을 유도하였으며, E-cadherin의 발현 감소와 vimentin, *SLUG*, 그리고 TWIST의 발현 증가가 확인되었다. 과산화수소의 처치는 줄기세포능 유전자인 *OCT4*, *SOX2*, 그리고

*NANOG*의 발현을 유의적으로 증가시켰다. 이러한 유전자들의 발현 변화는 전사 인자인 HIF-1α와 TGF-β1의 활성에 의해 매개되었다. 특히, 면역조직화학적으로 TWIST 단백질이 간엽성 악성 중피종 세포의 핵에서만 발현되고 있음을 실제 악성 중피종 조직에서 확인하였다. 악성 중피종 세포를 저산소 (2.2% O_2) 환경에서 노출 시켰을 때, HIF-1α와 HIF-2α의 발현이 유도되었고 HIFα의 타겟 단백질인 GLUT-1의 발현 또한 함께 유도되었다. 저산소증은 악성 중피종 세포의 시험관 내 군집 형성능을 증강시켰으며, 세포사멸 저항성을 증가시켜 약물 저항성을 유도하였고, 종양 세포의 이동능과 침습능을 향상시켰다. 저산소증에 노출된 악성 중피종 세포는 줄기세포능과 관련된 단백질인 HIF-2α, OCT4, 그리고 CD44의 발현을 유의적으로 증가시켰다. 한편, 저산소증은 p-Akt 와 $NOTCH1^{IC}$의 발현에 유의적인 영향을 주지 않았다. 저산소 상태에서 항세포사멸 단백질 Bcl-2의 발현 증가가 확인되었다. Bcl-2는 악성 중피종의 세포사멸능을 감소시키는 것으로 보이며, Bax 대비 Bcl-2의 높은 비율은 저산소증에 의해 유도된 약물 저항성과 관련이 있었다. 한편, cisplatin은 HIF-1α와 HIF-2α 단백질을 빠르게 분해하였으며, HIFα의 발현은 저산소 상태에서 거의 확인되지 않았다. 저산소증에 노출된 악성 중피종 세포는 상피간엽이행을 유도하였으며, E-cadherin의 발현 감소와 vimentin의 발현 증가 그리고 간엽성 형태학적 변화를 통해 확인되었다. 저산소증에 의해 유도된 약물 저항성에 대해 미토콘드리아가 핵심적인 역할을 할 것이라는 가설을 설정하였다. 악성 중피종 세포를 저산소 (0.1% O_2) 실험 조건에서 배양하였을 때, 대조군에 비하여 cisplatin에 대한 약물 반응과 세포 사멸이 유의적으로 감소하였다. 저산소증은 미토콘드리아의 막 전위, 산화환원, 미토콘드리아 DNA, 그리고

미세구조에 미치는 cisplatin의 해로운 영향들을 유의적으로 억제하였다. Cisplatin은 미토콘드리아의 탈분극을 유도하였고, 저산소증은 미토콘드리아의 내막전위를 증가시켰다. 저산소에 의해 유도된 미토콘드리아의 과분극은 약물이 처리된 악성 중피종 세포에서 더욱 증강되었다. 미토콘드리아 과분극은 ATP 생산 또는 ATP 가수분해와 관련이 없었다. 또한, 미토콘드리아 탈분극은 미토콘드리아 투과성 변이공의 열림과 관련이 없었다. 저산소증은 cisplatin에 의한 미토콘드리아의 산화적 스트레스와 미토콘드리아 DNA 및 미세구조의 손상을 감소시켰다. 산화 환원 구획화가 저산소와 약물이 함께 처리된 악성 중피종 세포에서 확인되었다. 저농도의 ethidium bromide를 악성 중피종 세포에 장기간 노출시켰을 때, 미토콘드리아 DNA가 소실되었다. 미토콘드리아 DNA가 고갈된 악성 중피종 세포에서 세포 증식과 생존능이 유의적으로 감소되었다. 또한, 미토콘드리아 내막 전위가 발생되지 않았고 세포 내 ATP 양과 미토콘드리아 활성 산소 그리고 질량의 유의적인 감소가 확인되었다. 최종적으로, 미토콘드리아 DNA가 고갈된 악성 중피종 세포들은 저산소 상태에서 약물에 대한 저항성을 유도하지 못하였으며, 동시에 cisplatin에 의해 유도된 미토콘드리아 산화적 스트레스를 억제 시키지 못하였다. 본 연구의 결과는 산화적 스트레스와 저산소증이 악성 중피종의 공격적인 표현형 획득에 필수적이며 암 진행에 있어 중요한 역할을 한다는 것을 증명한다. 산화적 스트레스와 저산소 상태의 제어 방법은 악성 중피종 환자에서 종양 세포의 공격성을 감소시키는 효과적인 치료 전략이 될 수 있음을 제시한다. 더 나아가, 미토콘드리아의 전자전달계가 저산소 환경에서 악성 중피종의 약물 저항성을 유도하는데 핵심적인 요소라는 결론을 얻었다. 본 논문의 연구 결과는 악성 중피종의 질병 기전 및 치료 연구에 대한 이해를

넓힐 것이며, 미토콘드리아 표적이 저산소 환경에 노출된 악성 중피종의 치료 효율을 증가시킬 수 있다는 가능성을 타진한다. 또한, 본 논문의 연구 결과는 세포 또는 유기체가 정상 및 병태생리학적 상태에 기인한 저산소 스트레스에 적응하는 분자생물학적 기작을 이해하는데 학문적으로 공헌할 것이다.

핵심어: 악성 중피종, 적대적인 종양 미세 환경, 산화적 스트레스, 저산소증, 약물 저항성, 미토콘드리아, 미토콘드리아 DNA, 전자 전달계.

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감사의 글

서울대학교 수의과대학에 첫 발을 들여 놓은 지 12년이란 시간이 흘러가고 있습니다. 다양한 수의학 과목들을 접하고 작은 것 하나라도 놓치기 싫어 공부하던 학부 시절이 이제는 아련하기만 합니다.

종양을 진단하고 병태생리를 이해하고 연구를 하고 싶고 논문을 써 똑똑해지고 싶어서 대학원에 진학을 했고 이제 그 기나긴 시간의 끝이 다가왔습니다. 다양한 주제의 연구들을 수행하고 논문으로 출판하려고 부단히 애썼던 시간들이 너무나 행복했습니다. 그럼에도 불구하고 이전보다 더 명석하거나 독립적인 한 명의 학자로 충분히 성장하였다고 자신있게 대답할 수 있을지 아직 의문이 드는 것이 사실입니다. 다만, 항상 감사할줄 알며 그 누구보다 낮은 위치에서 겸손하고 우직하며 서두름없이 공부할 수 있는 사람이 되지 않았을까 조심스레 생각하며 자신감을 가져 봅니다.

정해진 답을 맞추는 것에 익숙해져 있던 저에게 새로운 사실을 찾아간다는 것은 결코 쉬운 일이 아니었습니다. 이 길이 내게 맞는지 끝없이 되물어야 했고, 때론 힘이 들어 그만두려는 생각도 해보았습니다. 그럼에도 부족한 제가 이 자리까지 올 수 있었던건 저를 믿고 격려해주신 많은 분들의 큰 도움이 있었기에 때문입니다.

자상함과 넓은 마음 그리고 날카로운 질문으로 성급하고 편협한 저를 사랑으로 지도해주신 김용백 교수님께 고개 숙여 진심으로 감사의 인사를 드립니다. 교수님께서 보여주신 모습들로부터 많은 것들을 배울 수 있었고, 그 가르침 항상 가슴 속에 새겨 어긋나지 않는 바른 삶을 살아가도록 하겠습니다. 가장 어려운 시기에 함께 실험실을 꾸려나가며 편안히 실험할 수 있도록 도와주고 든든한 모습으로 제 부족함을 감싸줬던 나연이에게 항상 고맙고, 또 미안한 마음이 큽니다. 실험에 큰 도움을 주신 최봉희 그리고 서유리 선생님 이 자리를 빌어 감사의 인사를 전합니다. 함께 동고동락하며 실험 결과를 해석해주고 더 좋은 방향으로 갈 수 있게 도와준 성현이와 홍석이에게 진심으로 감사의 말을 전합니다. 졸업 논문 발표라는 힘든 시기에 큰 힘이 되어준 수민이, 발표 자료의 사소한 부분까지 세심하게 조언해준 예슬이, 그리고 진심어린 따뜻한 말로 응원해준 예지와 햇살이에게 감사를 전합니다. 졸업을 앞두고 있는 지금까지, 한결같이 저를 도와주신 또 하나의 멘토 민석이 형에게도 진심으로 감사드립니다. 어려운 시간 내어 제가 쓴 부족한 논문이 제대로 된 모습의 논문이 되기까지 지도해주신 김대용 교수님, 류덕영 교수님, 백승준 교수님, 윤병일 교수님께 진심으로 감사드리며 부끄럽지 않은 제자가 되도록 늘 정진하겠습니다.

남들과 다른 길을 가고 있는 아들에게 항상 힘을 주시고 이겨낼 수 있도록 믿어주신 부모님께 너무 죄송하고 감사하고, 사랑합니다. 부모님께 배운, 무엇과도 바꿀 수 없는 삶의 지혜를 되새기여 더 좋은 아들이 되도록 하겠습니다. 항상 기도해주고 웃음과 예쁜 옷을 주며 힘을 준 동생 청아에게 감사의 말을 전합니다. 가족을 지켜주시고 기쁠 때나 슬플 때 제 마음 속에 항상 함께 계시고 기도와 간구에 응답해주시는 하나님께 이 모든 감사를 드립니다.

많은 도움에도 불구하고 일일이 감사함을 표현하지 못한 선후배와 동기들 그리고 여러 선생님들에게 이 자리를 빌어 죄송함을 표합니다.

다시 돌아가도 공부함에 있어 후회는 없을 것 같습니다. 박사라는 감투에 멋을 느끼지 않고 남을 도와주며 세상을 이롭게 하는 사람으로 남을 수 있도록 앞으로도 최선을 다하겠습니다. 감사합니다.