RESEARCH ARTICLE

Hydrogen Peroxide Promotes Epithelial to Mesenchymal Transition and Stemness in Human Malignant Mesothelioma Cells

Myung-Chul Kim, Feng-Ji Cui, Yongbaek Kim*

Abstract

Reactive oxygen species (ROS) are known to promote mesothelial carcinogenesis that is closely associated with asbestos fibers and inflammation. Epithelial to mesenchymal cell transition (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 human malignant mesothelioma (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, as indicated by increased expression levels of vimentin, SLUG and TWIST1, and decreased E-cadherin expression. Expression of stemness genes such as OCT4, SOX2 and NANOG was also significantly increased by treatment of HMM cells with H_2O_2. Alteration of these genes was mediated via activation of hypoxia inducible factor 1 alpha (HIF-1α) and transforming growth factor beta 1 (TGF-β1). Considering that treatment with H_2O_2 results in excess ROS, the present study suggests that oxidative stress may play a critical role in HMM carcinogenesis by promoting EMT processes and enhancing the expression of stemness genes.

Keywords: Mesothelioma - reactive oxygen species - epithelial to mesenchymal transition - stemness - HIF1α

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Introduction

Reactive oxygen species (ROS) play 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., 2009). Recently, it has been proposed that ROS signaling contributes to a phenotypic conversion- so called epithelial to mesenchymal transition (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 transforming growth factor β1 (TGF-β1) and hypoxia inducible factor 1 (HIF-1α) (Xu et al., 2009), mediated through EMT transcription factors such as SNAIL, SLUG, and TWIST1 (Cannito et al., 2008; Yang et al., 2008a). 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 et al., 2008b). 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 reactive oxygen species (ROS) induced by asbestos fiber promote EMT, producing more strong and resilient cells.

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, MMPs and transcriptional regulators of EMT in HMMs

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(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

Cell lines and 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) supplemented with 10% fetal bovine serum, 10mM HEPES, 1.5 g/L sodium bicarbonate, 2mM L-glutamine, 1 mM sodium pyruvate, and 100U/100ug/ml penicillin/ streptomycin 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 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 HM513 and MS1 cells, the cell proliferation assay using CellTiter 96 AQueous One Solution (Promega, Madison, WI) was performed according to the manufacturer's protocol. The 103 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 (20μl/well) was added and incubated for 1 h at 37°C, 5% CO_2. Absorbance was measured at 490 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT). The percentage of growth suppression for each dose was calculated by comparing to the same volume of media-treated control cells.

Quantitative real-time

RTPCR 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) 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) 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).

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) and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). The soluble protein concentrations were determined by the Bradford technique (Bio-Rad Laboratories, Hercules, CA). 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, Inc.) and tubulin (Santa Cruz Biotech, Inc.) 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

Table 1. Primer Sequences Used for Quantitative Real Time RT-PCR

Genes	Forward Primers	Backward Primers
TWIST1	5'-TCTCGGTCTGGAGGATGGAG-3'	5'-GTTATCCAGCTCCAGAGTCT-3'
SLUG	5'-GAGCATTTGCAGACAGGTCA-3'	5'-CCTCATGTTTGTGCAGGAGA-3'
β-Catenin	5'-GCCGGCTATTGTAGAAGCTG-3'	5'-GAGTCCCAAGGAGACCTTCC-3'
E-cadherin	5'-GATTCTGCTGCTCTTGCTGT-3'	5'-CCTGGT-CTTTGTCTGACTCTG-3'
OCT4	5'-ACATGTGTA AGCTGCGGCC-3'	5'-GTTGTGCATAGTCGCTGCTTG-3'
NANOG	5'-TTCAGTCTGGACACTGGCTG-3'	5'-CTCGCTGATTAGGCTCCAAC-3'
SOX2	5'-CGATGCCGACAAGAAAACTT-3'	5'-CAAACTTCCTGCAAAGCTCC-3'
NOTCH1	5'-GCAGTTGTGCTCCTGAAGAA-3'	5'-CGGGCGGCCAGAAAC-3'

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, Inc.) 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. 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 assay

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.

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.

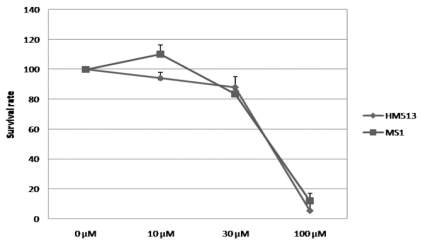

Figure 1. Cell Proliferation Assay on Mesothelial Cells Treated with H_2O_2. Cells were cultured in reduced serum media (0.5% FBS) containing 0, 10, 30 and 100 μM H_2O_2, followed by cell proliferation assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay. In MS1 and HM513 cells, treatment with 100 μM H_2O_2 significantly reduced cell proliferation

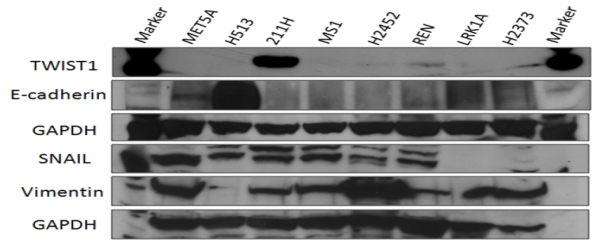

Figure 2. Expression of Emt-Related Molecules in Cultured Human Malignant Mesothelioma (Hmm) Cell Lines Determined by Western Blot Assay. TWIST1 is detected in MSTO-211H, REN and H2373, and E-cadherin is expressed in Met5A, HM513 and LRK1A. SNAIL. Vimentin is detected in most of HMM cell lines. Note the inverse relationship between TWIST1 and E-cadherin expression levels in HMM cell lines

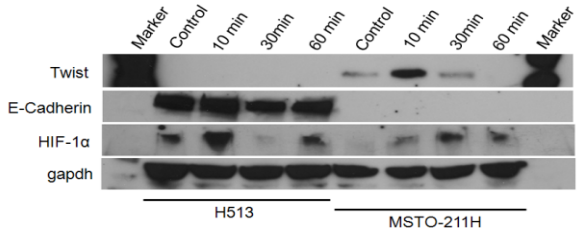

Figure 3. The Changes of Expression Levels of EMT-Related Genes in Human Mesothelioma Cells Following Treatment with 100 μM H_2O_2 for Indicated Periods. Lanes 2-4 are HM513 and lanes 5-8 are MSTO-211H. Note that TWIST1 expression was significantly increased in MSTO-211H cells at 10 min treatment, while E-cadherin expression was decreased in HM513 at 30 min treatment. HIF-1α expression was increased by H_2O_2 treatment in both HM513 and MSTO-211H cells

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.

Expression profile 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,

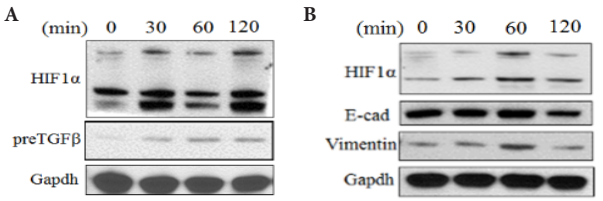

Figure 4. Western blot Analysis on the Expression Levels of Signaling Molecules Induced by H_2O_2. a) In HM513 cells, expression levels of HIF-1α and TGF-β1 were significantly increased by treatment with 100 μM H_2O_2 for 30 min. b) Following treatment of HM513 cells with 100 μM H_2O_2, expression level of E-cadherin was decreased, while vimentin expression was increased

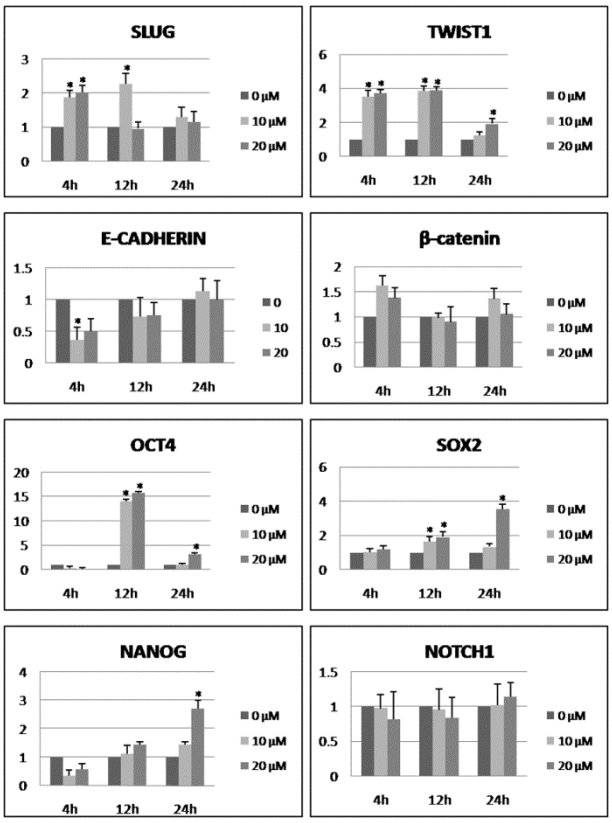

Figure 5. Expression Levels of EMT and Stemness-Related Genes Measured by Real Time Quantitative RTPCR in MS1 Cells Treated with 0, 10, or 20 μM H_2O_2 for Indicated Periods. H_2O_2 treatment significantly enhanced EMT promoting genes such as SLUG, TWIST1, and decreased E-cadherin. H_2O_2 treatment significantly 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 $p<0.05$)

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 4a). Following treatment with H_2O_2, expression level of E-cadherin was decreased, while vimentin was significantly increased (Figure 4b).

Following 100 μmol H_2O_2 treatment of MS1 and HM513, 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

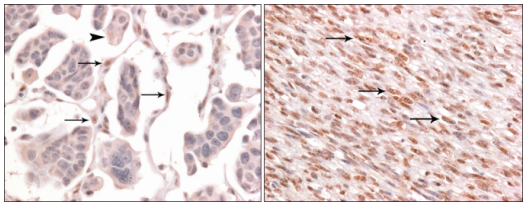

Figure 6. TWIST1 Expression Detected by Immunohistochemical Staining of Tissue Microarray Slides Containing Human Malignant Mesotheliomas (HMMs). Left panel: Epithelioid HMM cells did not show nuclear staining, instead weak diffuse cytoplasmic staining is present (arrowhead). A few spindle cells (arrows) separating the groups of neoplastic epithelioid cells exhibit positive nuclear staining for TWIST1. Right panel: Most of the sarcomatous mesothelial cells reveal positive staining in their nuclei (arrows)

was more dramatic in MS1 cells (Figure 5) than in HM513 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 HM513 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 HM513 cells, H_2O_2 treatment significantly increased the expression levels of OCT4 and NANOG, but SOX2 and NOTCH1 were not affected.

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.

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, epithelial to mesenchymal transition (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 et al., 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, 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 HM513 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 et al., 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 et al., 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.

Acknowledgements

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