Upregulation of FoxM1 by MnSOD Overexpression Contributes to Cancer Stem-Like Cell Characteristics in the Lung Cancer H460 Cell Line

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Abstract
Manganese superoxide dismutase promotes migration and invasion in lung cancer cells via upregulation of the transcription factor forkhead box M1. Here, we assessed whether upregulation of forkhead box M1 by manganese superoxide dismutase overexpression mediates the acquisition of cancer stem-like cell characteristics in non-small cell lung cancer H460 cells. The second-generation spheroids from H460 cells were used as lung cancer stem-like cells. The levels of manganese superoxide dismutase, forkhead box M1, stemness markers (CD133, CD44, and ALDH1), and transcription factors (Bmi1, Nanog, and Sox2) were analyzed by Western blot. Sphere formation in vitro and carcinogenicity of lung cancer stem-like cells were evaluated by spheroid formation assay and limited dilution xenograft assays. Knockdown or overexpression of manganese superoxide dismutase or/and forkhead box M1 by transduction with short hairpin RNA(shRNA) or complementary DNA were performed for mechanistic studies. We showed that manganese superoxide dismutase and forkhead box M1 amounts as well as the expression levels of stemness markers and transcription factors sphere formation in vitro, and carcinogenicity of lung cancer stem-like cells were higher than in monolayer cells. Lung cancer stem-like cells transduced with manganese superoxide dismutase shRNA or FoxM1 shRNA exhibited decreased sphere formation and lower amounts of stemness markers and transcription factors. Overexpression of manganese superoxide dismutase or FoxM1 in H460 cells resulted in elevated sphere formation rates and protein levels of stemness markers and transcription factors. Meanwhile, manganese superoxide dismutase knockdown or overexpression accordingly altered forkhead box M1 levels. However, forkhead box M1 knockdown or overexpression had no effect on manganese superoxide dismutase levels but inhibited or promoted lung cancer stem-like cell functions. Interestingly, forkhead box M1 overexpression alleviated the inhibitory effects of manganese superoxide dismutase knockdown in lung cancer stem-like cells. In a panel of non-small cell lung cancer cells, including H441, H1299, and H358 cells, compared to the respective monolayer counterparts, the expression levels of manganese superoxide dismutase and forkhead box M1 were elevated in the corresponding spheroids. These findings revealed the role of forkhead box M1 upregulation by manganese superoxide dismutase overexpression in maintaining lung cancer stem-like cell properties. Therefore, inhibition of forkhead box M1 upregulation by manganese superoxide dismutase overexpression may represent an effective therapeutic strategy for non-small cell lung cancer.

Keywords
cancer stem-like cell, FoxM1, lung cancer, MnSOD, self-renewal
Introduction

Advances in the understanding of tumor cell biology have focused on cancer stem cells (CSCs) or cancer stem-like cells (CSLCs), an etiogenic cell subset in tumorigenesis. Cancer stem-like cells are considered to be responsible for oncogenicity in a variety of carcinomas. Our research team previously demonstrated CSLCs contribute to self-renewal, migration, and invasion of the non-small cell lung carcinoma (NSCLC) A549 cell line. Therefore, elucidating the molecular mechanisms underlying the critical roles of CSLCs in NSCLC oncogenicity is extremely important. Because mitochondria either directly control cell metabolism or constitute a critical node in cell signaling, altered mitochondrial activity and uncontrolled cell division might lead to tumorigenesis and malignancy.

Manganese superoxide dismutase (MnSOD), a mitochondrial protein, catalyzes the transformation of the superoxide anion radical into H2O2 and oxygen to modulate cellular signaling pathways. The function of MnSOD in carcinogenesis and malignancy remains a subject of debate; however, a study by Chen et al demonstrated that MnSOD enhances migration and invasion by upregulating the forkhead box M1 (FoxM1) transcription factor, with elevated MnSOD amounts considered an independent predictive factor of survival and relapse in lung cancer. Nevertheless, whether and how MnSOD affects stem-like spheroid formation by lung cancer cells still requires adequate clarification.

Forkhead box M1 is highly expressed in proliferating cells and participates in cell cycle progression. Because FoxM1 is overexpressed in various human cancers, including lung cancer, it has been hypothesized to be implicated in tumorigenesis. It was shown that Mx-Cre FoxM1−/− mutant mice have significantly reduced lung adenomas, thanks to tumor cell inhibition. Conversely, FoxM1 overexpressing animals show more and larger tumors after treatment with 3-methylcholanthrene/butylated hydroxytoluene, as well as increased cell division in transplant tumors, in comparison to wild-type mice. In contrast, lung tumor number and size for FoxM1 knockdown mouse respiratory epithelial cells were shown to be significantly decreased. These findings clearly reveal an association of FoxM1 with lung oncogenicity. In addition, FoxM1 contributes to the acquisition of cancer stem properties and the epithelial–mesenchymal transition phenotype in several cancers. The abovementioned evidences prompted the hypothesis that MnSOD might upregulate FoxM1, thereby promoting the acquisition of cancer stem properties in NSCLC.

The present study demonstrated that elevated MnSOD levels resulted in increased FoxM1 amounts, conferring stemness to the NSCLC H460 cell line. We also demonstrated that MnSOD overexpression triggers FoxM1 upregulation to induce and maintain self-renewal features, thus promoting tumor development and progression in NSCLC.

Materials and Methods

Reagents

Invitrogen Life Technologies (Shanghai, China) manufactured Dulbecco modified Eagle medium (DMEM) and DMEM/F12 medium; Trypsin-EDTA, fetal bovine serum (FBS), and Penicillin-streptomycin. All cell culture dishes were provided by Corning Life Sciences (New York, USA).

Monoclonal antibodies raised against human anti-β-actin were manufactured by Sigma-Aldrich (Catalog No. A2066, St Louis, Missouri, USA). Rabbit polyclonal antibodies targeting CD44, ALDH1A1, CD133, Oct4, Nanog, and MnSOD were obtained from Abcam Company (Catalog No. ab24504, ab9883, ab19898, ab18976, ab109250, and ab13533; Cambridge, Massachusetts). Monoclonal antibodies raised against Bmi1 in mice were provided by Abcam Company (Catalog No. ab126783). Monoclonal antibodies raised against FoxM1 in rabbits (C-20) were manufactured by Santa Cruz Biotechnology, Inc. (Catalog No. sc-502; Beverly, Massachusetts).

The pHBad-MCMV-GFP, pHBad-U6-GFP, pHBad-MCMV-GFP-MnSOD, pHBad-MCMV-GFP-FoxM1, pHBad-U6-GFP-sh MnSOD, and pHBad-U6-GFP-sh FoxM1 plasmid packaging adenoviral particles were obtained from Hanbio Biotechnology Co Ltd (2.0 mL, 1 × 1011 PFU/mL; Shanghai, China).

Cell Culture and Sphere Formation Assay

Human NSCLC H460, H441, H1299, and H358 cells (Chinese Academy of Sciences, Shanghai, China) were maintained in DMEM containing 10% FBS with penicillin and streptomycin in a humid environment containing 5% CO2 at 37°C.

For sphere formation, serum-free culture medium containing 20 ng/mL of human recombinant basic fibroblast growth factor and human recombinant epidermal growth factor, 5 µg/mL insulin (Sigma-Aldrich), 0.4% bovine serum albumin

Abbreviations

CSCs, cancer stem cells; CSLCs, cancer stem-like cells; DMEM, Dulbecco modified Eagle medium; FBS, fetal bovine serum; FoxM1, forkhead box M1; H&E, hematoxylin and eosin; LCSLCs, lung cancer stem-like cells; MnSOD, manganese superoxide dismutase; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species.
Spheroids were obtained by centrifugation (200×g) and trypsin–EDTA digestion, followed by mechanical disruption. Single cells were washed and transferred into serum-free medium for sphere induction. Second-generation spheroids were used as a ratio of total number of spheroids generated by that of H460 cells seeded, multiplied by 100.

**In Vivo Tumorigenicity Experiments**

Balb/c-nu mice aged 4 weeks were purchased from the Animal Institute of the Chinese Academy of Medical Science. All animal experiments were performed in accordance with the institutional guidelines of the Hunan Normal University. The University Committee on Animal Care and Hunan Normal University approved the experimental protocols (No. 2015-146). Mice were randomly divided into 3 groups (4 mice/group) and maintained under standard conditions, according to standard protocols. Cells were suspended in serum-free DMEM/Matrigel (BD Biosciences) mixture (1:1 volume). Each recipient Balb/c-nu mouse was inoculated subcutaneously with H460-derived LCSLCs (1×10³, 1×10⁴, and 1×10⁵ cells) in 1 flank and monolayer H460 cells (1×10⁴, 1×10⁵, and 1×10⁶) in the other, respectively. Tumorigenicity experiments were terminated 1 month after cell inoculation. The harvested tumors were imaged and weighed immediately. After that, specimens from tumor tissue samples were fixed with 10% neutral-buffered formalin, processed in paraffin blocks, and sectioned. The sections were stained with hematoxylin and eosin (H&E) and examined for histopathology.

**Cell Transduction**

Human NSCLC H460 cells or LCSLCs were seeded in 24-well culture plates at 40% to 50% confluence and incubated overnight. Then, cells were transduced with several plasmids using the enhanced infection solution (ENi.s, Catalog No. REVG0002; GeneChem, Shanghai, China). After 4 hours of transduction, DMEM with 10% fetal calf serum (FCS) was added to replace the transduction medium; this was followed by 48 hours of incubation before gene and protein-level assessments.

**Western Blot**

Cells lysis was performed according to published protocols. Monoclonal anti-β-actin, anti-Bmi1, and anti-FoxM1 antibodies and polyclonal anti-CD44, anti-ALDH1A1, anti-CD133, anti-Oct4, anti-Nanog, and anti-MnSOD antibodies were used as primary antibodies for overnight incubation at 4°C. Appropriate horseradish peroxidase-conjugated secondary antibodies were used for detection: After incubation for 1 hour at room temperature, visualization of specific protein bands was carried out by enhanced chemiluminescence, with β-actin employed for normalization.

**Statistical Analysis**

SPSS version 20.0 for Windows (SPSS Inc, Chicago, Illinois, USA) was used for statistical analyses. Data are mean (standard deviation) and were assessed by 1-way analysis of variance. First, homogeneity of variance was determined. Least-Significant Difference (LSD) was used to perform pairwise comparisons among groups. In case of incomplete variance, control and experimental groups were analyzed with Tukey test. A value of $P < .05$ was considered statistically significant.
corresponding monolayer cells in vivo. Additionally, H&E staining was performed and revealed similar histological characteristics in tumor xenografts derived from H460 spheroids and the corresponding monolayer cells (Figure 1D). Collectively, these data demonstrated that H460 spheroids initiated tumors in vivo, suggesting that H460 spheroids may represent true LCSLCs in H460 cells.

Manganese Superoxide Dismutase and FoxM1 Protein Expression Levels Are Elevated in LCSLCs

The potential association of MnSOD with FoxM1 expression was assessed in both LCSLCs and H460 cells. As shown in Figure 2A, protein expression levels of MnSOD were markedly higher in LCSLCs compared to H460 cells. Interestingly,
FoxM1 protein expression was also elevated in cells expressing high levels of MnSOD than in those with reduced MnSOD amounts (Figure 2B). These findings suggested that elevated MnSOD expression might be associated with FoxM1 upregulation in LCSLCs.

We next assessed whether the expression levels of MnSOD and FoxM1 were also elevated in spheroids derived from other NSCLC cell lines, including H441, H1299, and H358. We found that the protein expression levels of MnSOD, FoxM1, and CSC-related biomarkers (CD133 and CD44) were markedly higher in second-generation spheroids compared to their respective monolayer cells in all 3 cell lines (Figure 2C-H). Together, these findings suggested that elevated expression levels of MnSOD and FoxM1 in spheroids were not specific to the H460 cell line.

**Manganese Superoxide Dismutase Transduction Promotes MnSOD and FoxM1 Expression as well as Spheroid Formation in H460 Cells**

Based on findings by Chen et al.,

Figure 2. Comparison of MnSOD (A) and FoxM1 (B) protein expression levels in H460 cells in monolayer growth and spheroids of different generations (mean [SD], n = 3; *P < .05 vs H460 cells in monolayer growth; **P < .05 vs first-, third-, or fourth-spheroid generation from the H460 cell line). The representative western blot image of MnSOD, FoxM1, CD133, and CD44 protein bands in H441 (C, F), H1299 (D, G), and H358 (E, H) cells in monolayer growth and second-generation spheroids. Data shown are the mean of 3 separate experiments. *P < .05 vs H441 or H1299 or H358 cells in monolayer growth. FoxM1, forkhead box M1; MnSOD, manganese superoxide dismutase; SD, standard deviation.
hypothesized that MnSOD could increase FoxM1 expression, thereby conferring self-renewal capability to LCSLCs.

To assess whether enhanced MnSOD expression affects FoxM1 expression levels and spheroid formation, MnSOD-carrying adenoviruses were used to generate MnSOD overexpressing H460 cells. Interestingly, MnSOD-expressing adenovirus-infected H460 cells showed markedly increased MnSOD and FoxM1 protein expression levels (Figure 3A). As shown in Figure 3B, elevated self-renewal potential was found in MnSOD overexpressing H460 cells. Furthermore, MnSOD expression modulated the protein expression levels of CD133, CD44, and ALDH1 (Figure 3C), and Bmi1, Nanog, and Oct4 (Figure 3D) in H460 cells. These findings suggested that MnSOD significantly regulated FoxM1 expression and LCSLC functions.

**Figure 3.** Effects of MnSOD transduction on protein expression of MnSOD and FoxM1 (A), spheroid formation (B), and expression levels of stem cell markers (C) and transcription factors (D) in H460 cells (mean [SD], n = 3; *P < .05 vs H460 cells; #P < .05 vs H460 cells expressing GFP). FoxM1 indicates forkhead box M1; MnSOD, manganese superoxide dismutase; SD, standard deviation.

**Effects of FOXM1 Transduction on MnSOD and FoxM1 Expression, and Spheroid Formation in H460 Cells**

To test the hypothesis that MnSOD may increase FoxM1 expression and confer self-renewal capability to LCSLCs, a FOXM1 expressing H460 cell clone was generated by infection with FOXM1-carrying adenoviruses. Overexpression of FoxM1 was not accompanied by increased MnSOD protein expression (Figure 4A). However, it increased the sphere formation rate (Figure 4B). In addition, FoxM1 overexpression upregulated CD133, CD44, and ALDH1 (Figure 4C) as well as Bmi1, Sox2, and Oct4 (Figure 4D) in H460 cells following infection with FOXM1-carrying adenoviruses.
Forkhead box M1 Expression and Spheroid Formation Are Dependent on MnSOD Expression in LCSLCs

To determine whether FoxM1 expression and spheroid formation are dependent on MnSOD expression, the MnSOD gene was silenced by infection with MnSOD short hairpin RNA (shRNA)-carrying adenoviruses.

MnSOD and FoxM1 protein levels in MnSOD knockdown LCSLCs were significantly reduced compared with the values of control LCSLCs (Figure 5A). In addition, sphere formation assay revealed that MnSOD silencing resulted in decreased self-renewal capability in LCSLCs (Figures 5B). Furthermore, compared to LCSLCs, MnSOD knockdown led to reduced protein expression levels of CD133, CD44, and ALDH1 (Figure 5C) as well as Bmi1, Nanog, and Oct4 (Figure 5D). These findings suggested that FoxM1 expression and stemness in LCSLCs from H460 cells were dependent upon MnSOD.

Knockdown of FOXM1 Has No Effect on MnSOD Expression But Inhibits CSLC Properties in LCSLCs

To further confirm that MnSOD may increase FoxM1 expression and subsequently confer self-renewal capability to LCSLCs, we next silenced the FOXM1 gene by infection with FOXM1 shRNA-carrying adenoviruses.

Forkhead box M1 gene silencing resulted in decreased FoxM1 protein amounts, but had no effect on MnSOD levels (Figure 6A). Meanwhile, FOXM1 knockdown resulted in decreased self-renewal capability of LCSLCs (Figure 6B). Furthermore, FOXM1 knockdown resulted in reduced protein

Figure 4. Effects of FOXM1 transduction on protein expression of MnSOD and FoxM1 (A), spheroid formation (B), and expression of stem cell markers (C), and transcription factors (D) in H460 cells (mean [SD], n = 3; *P < .05 vs H460 cells; †P < .05 vs H460 cells expressing GFP). FoxM1, forkhead box M1; MnSOD, manganese superoxide dismutase; SD, standard deviation.

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amounts of CD133, CD44, and ALDH1 (Figure 6C) as well as Bmi1, Sox2, and Oct4 (Figure 6D) in LCSLCs.

**Discussion**

This study first provided evidence, from assessing NSCLC H460 cells, that LCSLC stemness is promoted by MnSOD, via a mechanism involving up-regulation of the oncogenic transcription factor FoxM1. Our results showed that spheroids with LCSLC properties had upregulated MnSOD and FoxM1 protein expressions in parallel, compared to cells in monolayer growth from the H460, H441, H1299, and H358 cell lines. We also demonstrated that altering MnSOD and FoxM1 expression levels could change spheroid formation capabilities as well as the protein levels of CSC markers (CD133, CD44, and ALDH1) and multipotent transcription factors (Bmi1, Nanog, and OCT4). Importantly, FoxM1 protein expression, spheroid formation capability, and protein levels of CSC markers and multipotent transcription factors were dependent on MnSOD expression levels, as shown above, and FoxM1 overexpression alleviated the inhibitory effects of MnSOD silencing on LCSLC stemness in spheroids from H460 cells.

**Figure 5.** Effects of MnSOD short hairpin RNA (shRNA) transduction on protein expression of MnSOD and FoxM1 (A), spheroid formation (B), and expression of stem cell markers (C) and transcription factors (D) in LCSLCs (second-generation spheroids) from the H460 cell line (mean [SD], n = 3; *P < .05 vs LCSLCs from the H460 cell line; **P < .05 vs LCSLCs from the H460 cell line expressing GFP). FoxM1 indicates forkhead box M1; MnSOD, manganese superoxide dismutase; SD, standard deviation.
Although previous reports described MnSOD with tumor suppressive properties, multiple studies have demonstrated that it is highly expressed in multiple carcinomas such as gastric and colorectal carcinomas,16-18 glioblastoma,19 breast cancer,20,21 cervical cancer,22,23 prostate cancer,24 and lung cancer.8,25 Recently, it was shown that MnSOD expression and activity are associated with the functions and characteristics of CSCs or CSLCs.6,7,26-28 Here, we provided evidence that MnSOD participated in self-renewal capability as well as the expression of CSC markers and multipotent transcription factors in LCSLCs derived from NSCLC H460 cells. These results support the viewpoint that MnSOD induces CSLC properties in NSCLCs. A study by Meng et al29 showed that genetic and epigenetic downregulation of microRNA-212 promotes colorectal tumor metastasis via dysregulation of MnSOD. Therefore, miR-212 and MnSOD might be therapeutic targets for cancer.

Figure 6. Effects of FOXM1 short hairpin RNA (shRNA) transduction on protein expression of MnSOD and FoxM1 (A), spheroid formation (B), and expression of stem cell markers (C) and transcription factors (D) in lung cancer stem-like cells (LCSLCs) from the H460 cell line (mean [SD], n = 3; *P < .05 vs LCSLCs from the H460 cell line; #P < .05 vs LCSLCs from the H460 cell line expressing GFP). FoxM1 indicates forkhead box M1; MnSOD, manganese superoxide dismutase; SD, standard deviation.

Chen et al demonstrated that MnSOD expression is not changed by FoxM1-knockdown in A549 and H1355 cells, whereas MnSOD upregulates FoxM1 by releasing E2F1 and Sp1 transcription factors.8 As shown earlier, MnSOD protein levels did not change by FoxM1-silencing in spheroids derived from the H460 cell line. In human fibroblast cells, FoxM1 seems to regulate MnSOD expression at the messenger RNA and protein levels.30 Park et al described a negative feedback loop from FoxM1 to MnSOD that might control reactive oxygen species (ROS) in human fibroblast cell proliferation. Reactive oxygen species induce FoxM1, whereas elevated FoxM1, in turn, reduces ROS levels by promoting MnSOD expression.30 Based on the current findings and previous studies, we assume that FoxM1 upregulation by MnSOD is required to overcome ROS-induced oxidative stress occurring during lung cancer initiation and progression. How FoxM1 upregulation by
MnSOD promotes LCSLC characteristics of NSCLC remains to be further elucidated.

Studies have shown that FoxM1 has an important function in maintaining stem cell pluripotency via regulation of Oct4, Nanog, and Sox2, in association with Wnt/β-catenin, to upregulate CSC markers (CD133, CD44, and ALDH1), contributing to CSC or CSLC characteristics.10,31,32 The present report provided experimental evidence that FoxM1 protein expression, spheroid formation capability, and the expression of CSC markers and multipotent transcription factors are dependent on MnSOD expression; in addition, FoxM1 gene transduction was shown to alleviate the inhibitory effects of MnSOD silencing on LCSLC functions and characteristics in spheroids derived from H460 cells. Accordingly, FoxM1 seems to be a performance effector for MnSOD functions.

In summary, the current study provides a mechanistic evidence to support the possibility that lung cancer CSC or CSLC properties are enhanced by FoxM1 activation through MnSOD overexpression. Our findings in NSCLC H460 cells were further supported by the evidence that elevated MnSOD expression in spheroids is associated with lung cancer carcinogenicity and progression. Inhibiting FoxM1 activation by MnSOD overexpression may represent an important novel strategy for LCSLCs in human NSCLC treatment.

**Authors' Note**
Zhimin Fu and Xiaocheng Cao contributed equally to this work.

**Declaration of Conflicting Interests**
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