RESEARCH COMMUNICATION

Deletion of Forkhead Box M1 Transcription Factor Reduces Malignancy in Laryngeal Squamous Carcinoma Cells

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Abstract

   The transcription factor, Forkhead Box M1 (FoxM1), has a specific expression pattern during the cell cycle. It also plays an important role in cellular developmental pathways and in the maintenance of homeostasis between cell proliferation and apoptosis. However, the precise role and molecular mechanisms associated with FoxM1 in laryngeal squamous carcinoma remain unclear. Therefore, laryngeal squamous carcinoma cells were transfected with FoxM1-targeted small interfering RNA (siRNA) and compared with cells transfected with a control siRNA. Assays of these two treatment groups detected a decrease in cell viability associated with down-regulation of FoxM1 expression, and resulted in an inhibition of cell proliferation, migration, and invasion. These phenotypes were also associated changes in expression of VEGF and MMP-2, a decrease in expression of cyclin B, and an increase in expression of p27. These findings suggest that deregulation of FoxM1 protein signaling is sufficient to affect tumorigenesis and cancer progression. These results also indicate that inhibition of FoxM1 represents an attractive target for cancer therapy.

Keywords: SCC - transcription factor -malignancy - siRNA - cancer therapy

Introduction

   FoxM1 is a transcription factor of the Forkhead family and is characterized by a DNA-binding domain called the forkhead box, or winged helix domain (Laoukili et al., 2005). Previous studies have shown that FoxM1 signaling plays important roles in cellular developmental pathways, particularly in maintaining homeostasis between cell proliferation and apoptosis (Kalin et al., 2006). While mRNA and protein levels of FoxM1 are barely detectable in quiescent cells, these levels greatly increase when cells are stimulated to re-enter the cell cycle (Korver et al., 1997). For example, an increase in FoxM1 expression is initiated at the onset of the S-phase, and this increase is maintained throughout the G2-phase and mitosis (Laoukili et al., 2007).

   Previous studies have identified a novel set of genes that appear to be controlled by FoxM1, and these genes affect cell metabolism, cell signaling, extracellular matrix remodeling, and transcriptional regulation (Laoukili et al., 2005; Wonsey and Follettie 2005). Correspondingly, loss of FoxM1 expression has been associated with defects in mitotic spindles and an inappropriate accumulation of cells in mitosis, resulting in a mitotic catastrophe (Wonsey and Follettie 2005). In mice that have undergone a targeted deletion of FoxM1 in the liver, a decrease in bromodeoxyuridine (BrdU) incorporation was detected. In

addition, fewer mitotic cells were detected compared with wild-type controls following partial hepatectomy in this model (Wang et al., 2002). In combination, these results suggest that FoxM1 mediates a multiplicity of functions that contribute to regulation of the cell cycle machinery.

   FoxM1 has also been implicated in the process of tumor development, with abnormal activation of the FoxM1 gene representing one of the hallmarks of human malignancies (Laoukili et al., 2007). For example, up-regulation of FoxM1 has frequently been detected in lung cancer, glioblastoma, prostate cancer, basal cell carcinomas, hepatocellular carcinoma, and primary breast cancer (Teh et al., 2002; Kalinichenko et al., 2004; Katoh 2004; Wonsey and Follettie 2005; Kalin et al., 2006; Kim et al., 2006; Liu et al., 2006). In addition, overexpression of FoxM1 in laryngeal squamous carcinoma (Hep-2) cells has been associated with a cancerous phenotype and with increased expression of proliferating cell nuclear antigen (PCNA).

   However, the molecular mechanism(s) by which FoxM1 contributes to the aggressiveness of certain malignancies remains largely unknown. Therefore, in this study, down-regulation of FoxM1 was achieved using FoxM1-targeted small interfering RNA (siRNA) in Hep-2 cells. Assays of cell growth, migration, invasion, angiogenesis, and metastasis were then performed and compared with control cells.

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Materials and Methods

Cell lines and culture conditions.

The human laryngeal cancer cell line, Hep-2, was obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT) and 100 units/mL penicillin/streptomycin at 37°C in 5% CO2.

RNAi plasmid construction and transfection.

FoxM1-targeted and control siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A FoxM1 cDNA plasmid was purchased from OriGene Technologies (Rockville, MD). Hep-2 cells were transfected with plasmids using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells were harvested 48 h after transfection.

Assays of cell viability.

The viability of Hep-2 cells treated with FoxM1-targeted siRNA versus untreated (control) Hep-2 cells were measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) assays. Briefly, cells were plated at a density of 2×10^3 cells/well in 96-well plates and incubated for 24, 48, and 72 h in complete medium containing 0.5 mg/ml MTT (Sigma, St. Louis, MO). Four hours later, the medium was replaced with 100 μl dimethylsulfoxide (DMSO) (Sigma) and plates were vortexed for 10 min to dissolve crystals that formed. The absorbance of each well at 490 nm was recorded, and baseline readings were subtracted. Assays for each timepoint were performed in triplicate, and the mean and standard errors were calculated.

Reverse Transcription PCR (RT-PCR)

Total RNA was extracted from carcinoma cells using TRIzol Reagent (Invitrogen), and cDNA was synthesized using the Thermoscript RT-PCR System Kit (Invitrogen). The primer sets used included: FoxM1, 5'- ACC GCT ACT TGA CAT TGG-3' (forward) and 5'-GCA GTG GCT TCA TCT TCC-3' (reverse); VEGF, 5'-GAG CCT TGC CTT GCT GCT CTAC-3' (forward) and 5'- CAC CAG GGT CTC GAT TGG ATG -3' (reverse); MMP-2, 5'-TTC CCC CGC AAG CCC AAG TG-3' (forward) and 5'- GAG AAA AGC GCA GCG GAG TGA CG -3' (reverse). PCR products were separated on 5% non-denaturing polyacrylamide gels using a Protean-III electrophoresis chamber (Bio-Rad). Gels were stained with Vistra Green (Amersham Biosciences), visualized using a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA), and adapted for print using ImageQuant 5.0 (GE Healthcare, Piscataway, NJ).

Western blotting

SDS-PAGE was performed using standard techniques. An anti-FoxM1 polyclonal antibody (C-20, Santa Cruz Biotechnology) and a polyclonal anti-actin antibody (Santa Cruz Biotechnology) were purchased. Cell extracts were collected using a Nuclear Protein Extraction Kit (TaKaRa, Japan), analyzed by SDS-PAGE, and transferred to Hybond-enhanced chemiluminescence nitrocellulose membranes (GE Healthcare). Blocked membranes were then incubated with antibodies raised against cyclin B (H-433), VEGF (C-1), MMP-2 (I-14), p27 (C-19), and actin (I-19) (Santa Cruz Biotechnology).

Transwell migration assays

Transwells (8.0 μm membrane pores; Costar, Cambridge, MA) were coated with 2.5 μg/ml collagen overnight at 4°C. The invasive activity of FoxM1-targeted and control siRNA-transfected cells were assayed using the BD BioCoat Tumor Invasion Assay System (BD Biosciences). Briefly, cells (2×10^5) in 200 μl medium with 5% FCS were added to the top chamber. The bottom chamber was supplemented with platelet-derived growth factor (PDGF) (30 ng/ml) in medium containing 10% FCS. After 24 h at 37°C, non-migrating cells were removed from the upper side of transwells using cotton-tipped applicators. Transwell filter membranes were then fixed in 4% paraformaldehyde and stained with Giemsa (Amresco, OH, USA). The number of cells that migrated was quantified by counting the number of cells present in eight random microscopic fields per filter at a magnification of 200×. Statistical significance was determined using Student's two-tailed t-test, and the limit of statistical significance was $P < 0.05$.

In vivo tumor formation

Athymic nude mice (6 - 8 weeks old, 18 - 22 g, Institute of Zoology, Chinese Academy of Sciences) were maintained and used in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines (Fitzpatrick and Davies 1998). To induce tumors in vivo, 5×10^6 FoxM1-targeted siRNA or control siRNA-transfected cells were washed in PBS, resuspended in 200 μl PBS, and injected into each flank. Mice tumor xenografts were resected 42 days post-inoculation, and tumor volumes were calculated according to the following formula: tumor volume = (width^2 × length) / 2.

Results

Inhibition of cell proliferation by siRNA targeting FoxM1

Hep-2 cells were transfected with FoxM1-targeted siRNA and control siRNA. After 24 h, cells were assayed for viability using MTT assays. The resulting growth curves demonstrated that the proliferation rates of cells treated with FoxM1-targeted siRNA were significantly lower than those of control cells (Figure 1, $P < 0.05$). Furthermore, control cells exhibited normal proliferation and growth, while cells treated with FoxM1-targeted siRNA grew more slowly.

mRNA levels of FoxM1, MMP-2, and VEGF

In order to investigate gene-silencing effects in Hep-2 cells, RT-PCR was used to determine whether mRNA levels of FoxM1, MMP-2, and VEGF are affected by the introduction of FoxM1-targeted siRNA into Hep-2 cells. As shown in Figure 2, levels of all three mRNAs decreased 24 h after transfection, and at later timepoints, this decrease was more evident. The lowest levels of
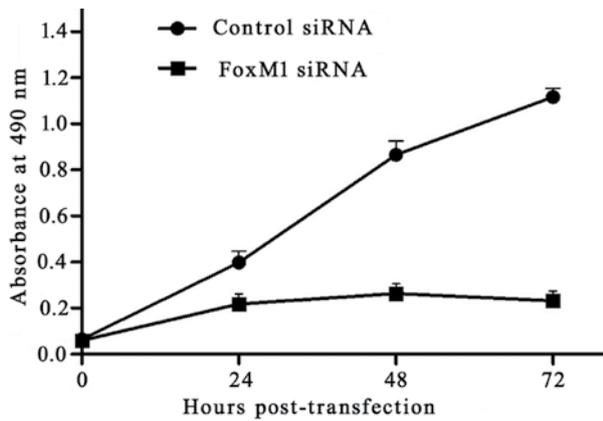
Figure 1. Growth Curves Calculated for Cells Treated with FoxM1-targeted RNAi and Control Cells. MTT data are presented and represent mean values ± SD. The growth rate of cells treated with FoxM1-targeted RNAi was significantly lower than that of control cells ($P < 0.01$)
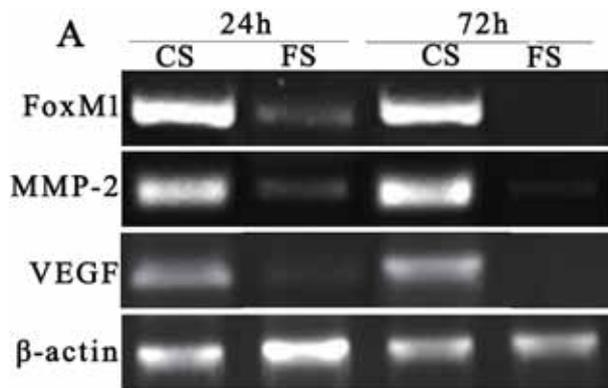
Figure 2. Detection of mRNA levels for FoxM1, MMP-2 and VEGF. Levels of FoxM1, MMP-2, and VEGF were found to significantly decrease 24 h after siRNA transfections were performed (chi-square test, $P < 0.01$). After 72 h, the lowest levels of FoxM1 mRNA were detected. CS: Control siRNA; FS: FoxM1 siRNA. ß-actin mRNA amplified as an internal standard
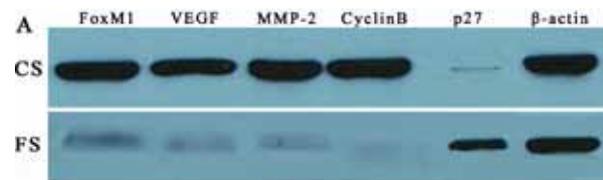
Figure 3. Protein Levels of FoxM1, VEGF, MMP-2, cyclin B, and p27. Forty-eight hours after transfection, protein levels of FoxM1, MMP-2, VEGF, and cyclin B significantly decreased, while levels of p27 increased ($P < 0.05$)

FoxM1 mRNA were detected 72 h after transfection.

Protein levels of FoxM1, VEGF, MMP-2, cyclin B, and p27

To further elucidate mechanisms involved in gene-silencing pathways of Hep-2 cells, western blotting was used to evaluate protein levels of FoxM1, VEGF, MMP-2, cyclin B, and p27 following the introduction of FoxM1-targeted siRNAs and control siRNAs. As shown in Figure 3, 48 h after transfection, protein levels of FoxM1, VEGF, MMP-2, and cyclin B were observed to decrease significantly, while levels of p27 increased, in cells treated with FoxM1-targeted siRNA ($P < 0.05$). These results are consistent with the RT-PCR results obtained as well.
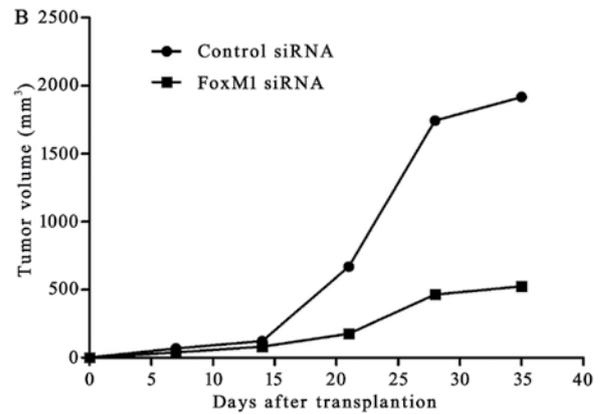
Figure 4. Tumor Growth Curves. Compared to control cells, mice injected with cells treated with FoxM1-targeted RNAi needed more days to form a tumor ($P < 0.05$)

Invasive ability of siRNA-treated cells

The invasive ability of siRNA-treated cells was also evaluated using transwell migration assays. After 24 h, cells treated with FoxM1-targeted siRNA exhibited a reduction in migration compared with control cells ($P < 0.05$).

Tumor formation assays

Tumorigenicity was evaluated in vivo using a xenograft mouse model. Hep-2 cells transfected with FoxM1-targeted siRNA that survived telomerase inactivation were injected into five mice to represent a test group. The same number of control mice received an injection of cells transfected with control siRNA. Tumor volume was measured after 35 days, and the average tumor volume was 1025.7 ± 97.5 mm^3 and 1796.4 ± 189.6 mm^3, respectively. Therefore, silencing of FoxM1 was associated with a decrease in tumor volume (Figure 4). Furthermore, the number of tumors that originated from FoxM1-siRNA treated cells was found to be significantly less than the number of tumors derived from control cells ($P < 0.05$).

Discussion

Studies of Fox protein function in mouse models have revealed both specific and redundant roles for Fox factors in development and tissue homeostasis. For example, FoxM1 transcription factors are critical for the G1–S and the G2–M cell cycle transitions, as well as for mitotic spindle integrity. Correspondingly, loss of FoxM1 in mouse models has been shown to be embryonic lethal in utero due to an inability of cells to enter mitosis., Ectopic expression of a FoxM1 isoform has also been shown to accelerate the development, proliferation, and growth of tumors in mouse models of prostate cancer (Kalin et al., 2006). In the present study, the role of FoxM1 in Hep-2 cells was investigated. Using RNA interference assays, a reduction in FoxM1 expression was found to be sufficient to inhibit Hep-2 cell proliferation, thereby affecting cell viability. Based on these results, it appears that a decrease in expression of FoxM1 represents an effective mechanism for eliminating transformed cells.

Tumor metastasis involves a series of steps that affect

cell invasion, degradation of basement membranes, and the stromal extracellular matrix. In combination, these affects ultimately facilitate tumor cell invasion and metastasis(Overall and Kleifeld 2006). Matrix metalloproteinases (MMPs) are a family of related enzymes that degrade the extracellular matrix, and are considered to be important factors in facilitating tumor invasion. In particular, MMP-2 facilitates the invasion and metastasis of human cancers by degrading collagen present in the basement membrane (Stetler-Stevenson 1994). In the present study, a significant decrease in expression of MMP-2 and VEGF was detected in Hep-2 cells following down-regulation of FoxM1. Many studies have shown that VEGF is a critical mediator of angiogenesis, with VEGF regulating many of the steps involved in cell proliferation, migration, and the tube formation of endothelial cells (Ellis and Hicklin 2008). Therefore, the results of this study strongly suggest that inhibition of FoxM1 represents a novel strategy to disrupt angiogenesis and tumor progression.

Functional characteristics of Hep-2 cells treated with FoxM1-targeted RNAi also included a decrease in the invasive potential of the surviving cells. As a result, the development of visible tumors was significantly delayed, and tumor volume was markedly reduced. Thus, cells treated with FoxM1-targeted siRNA were significantly less tumorigenic, consistent with the cell proliferation data obtained in vitro. In combination, these results suggest that directly, or indirectly, an absence of FoxM1 activity can lead to misregulation of cell homeostasis, thereby affecting cell proliferation, differentiation, and apoptosis.

Cyclin B has previously been shown to have an important role in cell cycle progression (Major et al., 2004). A decrease in cyclin B expression was detected following treatment of Hep-2 cells with FoxM1-targeted siRNA. An increase in expression of the cyclin-dependent kinase inhibitor, p27, was also detected in these cells. p27 has previously been shown to negatively regulate cell cycle progression (Lee and Yang 2001; Coqueret 2003), and FoxM1 expression has been shown to correlate with expression of many G2/M genes (Wang et al., 2002; Kalinichenko et al., 2004; Krupczak-Hollis et al., 2004; Kim et al., 2005).

Importantly, our results also identified a new set of genes that appear to be regulated by FoxM1. These include genes that affect cell metabolism, cell signaling, extracellular matrix remodeling, and transcriptional regulation. This implies that FoxM1 may exert a multiplicity of functions in regulating the cell cycle machinery. Correspondingly, our experimental evidence strongly supports a role for FoxM1 down-regulation as an anti-tumor and anti-metastatic mechanism in Hep-2 cells. Therefore, the FoxM1 signaling pathway may represent a promising therapeutic target, and the development of agents that target FoxM1 could provide a significant therapeutic impact on the treatment of human cancers.

Acknowledgements

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