FoxM1c Counteracts Oxidative Stress-induced Senescence and Stimulates Bmi-1 Expression*

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The Forkhead box transcription factor FoxM1 is expressed in proliferating cells. When it was depleted in mice and cell lines, cell cycle defects and chromosomal instability resulted. Premature senescence was observed in embryonic fibroblasts derived from FoxM1 knock-out mice, but the underlying cause has remained unclear. To investigate whether FoxM1 can protect cells against stress-induced premature senescence, we established NIH3T3 lines with doxycycline-inducible overexpression of FoxM1c. Treatment of these lines with sublethal doses (20 and 100 μM) of H2O2 induced senescence with senescence-associated β-galactosidase expression and elevated levels of p53 and p21. Induction of FoxM1c expression markedly suppressed senescence and expression of p53 and p21. Consistent with down-regulation of the p19Arf-p53 pathway, p19Arf levels decreased while expression of the Polycomb group protein Bmi-1 was induced. That Bmi-1 is a downstream target of FoxM1c was further supported by the dose-dependent induction of Bmi-1 by FoxM1c at both the protein and mRNA levels, and FoxM1 and Bmi-1 reached maximal levels in cells at the G2/M phase. Depletion of FoxM1c by RNA interference decreased Bmi-1 expression. Using Bmi-1 promoter reporters with wild-type and mutated c-Myc binding sites and short hairpin RNAs targeting c-Myc, we further demonstrated that FoxM1c activated Bmi-1 expression via c-Myc, which was recently reported to be regulated by FoxM1c. Our results reveal a functional link between FoxM1c, c-Myc, and Bmi-1, which are major regulators of tumorigenesis. This link has important implications for the regulation of cell proliferation and senescence by FoxM1 and Bmi-1.

Foxhead box (Fox) M1, known previously as WIN, HFH-11, and Trident, is a transcription factor ubiquitously expressed in proliferating cells (1–4). This proliferation, rather than tissue-specific expression, indicates a role in the regulation of cell division (5). Three isoforms of FoxM1, FoxM1a, FoxM1b, and FoxM1c, have been reported previously (4), but only FoxM1b and FoxM1c exhibit transactivation activity in reporter assays (2, 6, 7). The physiological significance of FoxM1a is not clear, as this isoform is not conserved in mouse species (7).

Reverse transcription PCR (RT-PCR) and RNase protection analyses indicated that FoxM1c is the predominant form expressed in various primary and secondary cell lines (e.g. human BJ1 and mouse NIH3T3 cells) and neonatal tissues rich in mitotically active cells. FoxM1b is the major isoform expressed in skin and testis (1, 7, 8). When overexpressed in liver, FoxM1b stimulated regeneration after partial hepatectomy and in old-aged mice (9–12). A similar cell cycle stimulatory effect of FoxM1b overexpression could also be demonstrated in multiple cell types of Rosa26-FoxM1b transgenic mice (13), and FoxM1b overexpression has been shown to enhance tumor growth in transgenic mouse models of prostate cancer and glioma (14–16). In HeLa cells, elevated levels of FoxM1c were found to accelerate transition through the G2/M phase of the cell cycle (6). The effect of overexpression of FoxM1c in mice has not been reported.

FoxM1 levels display cell cycle phase-dependent changes; its expression is initiated just before entry into S phase and peaks at the G2/M phase of the cell cycle (3, 6). Consistent with the predominant G2/M expression of FoxM1, loss-of-function analyses in FoxM1−/− mouse embryonic fibroblasts (MEFs) and in cancer cell lines by RNA interference have established a requirement for FoxM1 in mitosis (17–19). FoxM1-depleted cells have difficulty executing mitosis and exhibit chromosomal instability and polyploidy. Further microarray and chromatin immunoprecipitation analyses revealed important G2/M-specific genes like cyclin B1, Cdc25B, CENPA, and Aurora B as direct targets of FoxM1 (17–19). Consistent with the M phase role of FoxM1, depletion of FoxM1 expression in various mouse models perturbed the development of multiple tissues (20–23) and suppressed tumor formation (15, 24–26).

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Besides exhibiting cell cycle arrest, FoxM1<sup>−/−</sup> MEFs show increased expression of p19<sup>Arf</sup> and senescence-associated (SA) β-galactosidase, which are typical markers of senescent cells (18, 27). The occurrence of senescence in FoxM1-deficient cells could be an indirect consequence of increased DNA damage (28). Alternatively, FoxM1 may have a direct protective role against senescence that is lost in FoxM1<sup>−/−</sup> MEFs. To address whether FoxM1 can protect normal MEFs from stress-induced premature senescence (SIPS), we established doxycycline-inducible NIH3T3 lines to test the ability of elevated FoxM1c levels to protect against H<sub>2</sub>O<sub>2</sub>-induced premature senescence. Induction of FoxM1c expression dramatically suppressed SIPS and expression of p53 and p21<sup>Cip1</sup>. Consistent with a down-regulation of p53, expression of p19<sup>Arf</sup>, a well known positive cline (tet) repressor under the regulation of the human cytomegalovirus (CMV) promoter, was decreased while levels of the Polycomb group protein Bmi-1 were induced. Bmi-1 is a major negative regulator of the Ink4a/Arf/Ink4b locus that encodes p19<sup>Arf</sup> as well as the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup> and p15<sup>INK4b</sup> (31, 32). Our analysis indicates an active role for FoxM1c in protecting against senescence as increased FoxM1c expression in MEFs exerted an antagonistic effect against oxidative SIPS by suppressing the p19<sup>Arf</sup>/p53 pathway via induction of Bmi-1.

**EXPERIMENTAL PROCEDURES**

**Generation of Doxycycline-inducible FoxM1c-expressing NIH3T3 Cells**—The NIH3T3 mouse fibroblastic cell line was purchased from The American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% bovine serum (Invitrogen). The pcDNA6/TR vector (Invitrogen), which expresses the tetracycline (tet) repressor under the regulation of the human cytomegalovirus promoter, was stably transfected into NIH3T3 cells using Lipofectamine<sup>TM</sup> 2000 (Invitrogen) to establish lines constitutively expressing the tet repressor. These lines were tested for tet repressor function by introducing the reporter construct pcDNA<sup>TM</sup>4/TO-Luc by transient transfection. pcDNA<sup>TM</sup>4/TO-Luc contains a luciferase gene under the control of a modified cytomegalovirus promoter carrying tet operator sequences, and its expression is therefore dox-inducible. One line (3T3/6TR) was selected based on strong inducibility of luciferase activity. The expression plasmid pcDNA<sup>TM</sup>4/TO-FoxM1c was generated by replacing the Luc coding sequence with full-length FoxM1c coding sequence as reported previously (33). pcDNA<sup>TM</sup>4/TO-FoxM1c was then transfected into the 3T3/6TR line that expresses the tet repressor. Stable transfecants were selected and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% Tet-On<sup>TM</sup> approved fetal bovine serum (Clontech) and in the presence of 4 µg/ml blasticidin (Invitrogen) and 200 µg/ml zeocin (Invitrogen) as described by the manufacturer.

**Immunoblotting**—Immunoblotting was performed as described previously (7). Protein concentration was measured using the Bio-Rad protein assay kit according to the manufacturer’s instruction. Protein samples were separated by SDS-PAGE in 7–12% Laemmli gels and electroblotted onto Hybond<sup>TM</sup>-C Super nitrocellulose membranes (Amersham Biosciences). Blots were incubated with anti-FoxM1 antiserum (1:200), MPP2–C20 from Santa Cruz Biotechnology), anti-Cdc25B antiserum (1:200, H-85 from Santa Cruz), anti-cyclin B1 antiserum (1:200, GNS1 from Santa Cruz), anti-α-tubulin antiserum (1:500, DM1A from NeoMarker), anti-p53 (1:500, Pab1801 from Santa Cruz), anti-p21 (1:500, F5 from Santa Cruz), anti-p16 (1:500, F12 from Santa Cruz), anti-p19<sup>Arf</sup> antiserum (1:500, 54–57 from Calbiochem), anti-Bmi-1 antiserum (1:500, F6 from Upstate Biotechnology), or anti-c-Myc antiserum (1:500, C-33 from Santa Cruz).

Senescence-associated β-Galactosidase Staining and Flow Cytometry—Phosphate-buffered saline-washed cells were fixed for 5 min (room temperature) in 2% formaldehyde with 0.2% glutaraldehyde. After washing with phosphate-buffered saline, cells were incubated at 37 °C for 12 h with fresh SA β-galactosidase stain solution (1 mg of X-gal/ml, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 40 mM citric acid in phosphate-buffered saline, pH 6.0). DNA analysis by flow cytometry was performed as described previously (7).

**RT-PCR**—Total RNA was extracted from samples of ~1 × 10<sup>6</sup> cells using TRIzol<sup>®</sup> reagent (Invitrogen). The quantity and quality of RNA samples were determined by absorbance at 260 and 280 nm. RNA samples with an A<sub>260</sub>/A<sub>280</sub> ratio between 1.90 and 2.10 were stored at ~80 °C until use. For synthesis of first-strand cDNAs, 2 µg of total RNA was incubated at 65 °C for 5 min and then chilled on ice immediately. The heat-denatured total RNA was used to perform the reverse transcription reaction using SuperScript<sup>™</sup> III reverse transcriptase (Invitrogen) and oligo(dT)<sub>20</sub> primer according to the manufacturer’s instructions. For a typical semiquantitative RT-PCR reaction, 30 µl of master mix containing 1 × reaction buffer IV, 0.2 mM of each dNTPs, 1.25 units of DyNAzyme<sup>TM</sup> II DNA polymerase (Finnzymes), and 1 × PCR buffer for DyNAzyme<sup>TM</sup> II DNA polymerase was added into 20 µl of boiled RT sample containing 0.1 µg of total RNA. PCR was performed by initial denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. Mouse Bmi-1 and glyceraldehyde-3-phosphate dehydrogenase cDNAs were amplified using the following primers and cycle numbers: Bmi-1, 5′-agcagcatgtgcgatga-3′ and 5′-gtctccagctgtcagtc-3′, 26 cycles; glyceraldehyde-3-phosphate dehydrogenase, 5′-gacctggagacgcccggg-3′ and 5′-acggacacacctggctg-3′, 23 cycles. cDNA amounts and cycle numbers were optimized to ensure that amplification was within the linear range for quantitative analysis. Ten microliters of PCR-amplified products were electrophoresed in 1.5% (w/v) agarose gels with 0.25 µg/ml ethidium bromide. Experiments were repeated twice.

**RNA Interference**—A plasmid-based approach by transient transfection using Lipofectamine<sup>™</sup> 2000 was employed to deplete FoxM1 and c-Myc expression from different NIH3T3-derived cell lines. Each gene was silenced with two short hairpin RNA (shRNA)-expressing plasmids, and a control plasmid expressing an unrelated shRNA was used as negative control. For silencing FoxM1 expression, pTER A and pTER B were generated by cloning the two complementary hairpin sequences of 5′-acggtccctctccaag-3′ and 5′-aataggagagcctgct-3′, respectively, into the BglII and HindIII sites of the
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**RESULTS**

Establishment of Doxycycline-inducible FoxM1c-expressing NIH3T3 Cell Lines—FoxM1 function is essential for the proper execution of mitosis in MEFs (17, 18). Early passage FoxM1Δ/- MEFs exhibited premature senescence characterized by increased expression of SA-β-galactosidase and p19Arf. This senescent phenotype suggests that FoxM1 expression is required to protect MEFs from senescence. To investigate the ability of FoxM1 in counteracting SIPS in MEFs, we established two NIH3T3 lines (A and B) that exhibited doxycycline (a tetracycline derivative)-inducible FoxM1c expression (Fig. 1). The c instead of the b isoform was investigated because FoxM1c is the predominant isoform expressed in mitotically active cells (7). For both lines, treatment with dox from 2 to 8 μg/ml for 24 h gradually induced FoxM1 expression. FoxM1 induction was dose-dependent; stimulation at 8 μg/ml was 8.6- and 7.0-fold over the uninduced levels in lines A and B, respectively (Fig. 1). The uninduced levels in both lines were higher than the basal levels in the parental lines (NIH3T3 with no introduced plasmid and NIH3T3/6TR stably transfected with the tet repressor construct), which required longer exposure time to visualize (data not shown). The functional effect of FoxM1c overexpression was reflected by parallel increases in levels of Cdc25B and cyclin B1 (Fig. 1), both of which are direct transcriptional targets of FoxM1c (7, 17, 18).

**FoxM1 Overexpression Protects Cells against Oxidative Stress-induced Senescence**—We subjected both FoxM1c-inducible NIH3T3 cell lines to low doses of H2O2 to cause SIPS (35, 36). Treatment with H2O2 at 20 and 100 μM for 48 h followed by 24 h of incubation in H2O2-free medium induced SA-β-galactosidase expression in ~40–60% of cells (Fig. 2, histograms). The blue senescent cells became larger, flatter, and more vacuolated in appearance (compare Fig. 2, A and B). To address whether FoxM1c induction could slow down the senescence process, both lines were incubated for 24 h in the presence of dox (4 μg/ml) (after 48 h of H2O2 treatment) before their harvest for X-gal staining. Dox-induced FoxM1c expression in both lines clearly decreased the percentage of senescent cells (Fig. 2), and the majority of cells retained the spindle shape characteristic of fibroblasts (Fig. 2, and L). DNA analysis of non-treated and dox-treated cells of line A using flow cytometry did not reveal a decrease in percentage of cells at the G2/M phase upon FoxM1c induction, suggesting that the protective function was due to a direct effect on cellular senescence rather than an indirect effect of a perturbation in cell cycle progression (Fig. 2, M and N).
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**FIGURE 2.** Induced FoxM1c expression counteracts premature senescence induced by H2O2 treatment. Control (NIH3T3/6TR; A, B, G, and H), line A (C–F), and line B (I–L) cells were seeded onto coverslips and untreated (A, B, G, and H) or treated with H2O2 at 20 μM (C, D, I, and J) or 100 μM (E, F, K, and L) for 48 h. Afterward, cells were incubated in H2O2-free medium for 24 h in the absence (A, C, E, G, I, and K) or presence (B, D, F, H, J, and L) of dox (4 μg/ml) before their fixation for assay of senescent cells by SA-β-galactosidase staining. The blue senescent cells became larger, flatter, and more vacuolated in appearance. The histograms show the percentage of senescent cells and indicate a clear suppression of stress-induced premature senescence with dox-induced FoxM1c overexpression. The mean ± S.E. of three independent experiments are shown. M and N, DNA analyses of line A cells before and after dox induction. Untreated and line A cells treated with dox (4 mg/ml for 24 h) were harvested and stained with propidium iodide for flow cytometric analysis.

FoxM1c Suppresses the p19arf-p53 Pathway and Induces Bmi-1 Expression—To study the molecular mechanism underlying the counteractive effect of FoxM1c on SIPS, immunoblotting was performed to measure the levels of p53, p21Cip1, p19Arf, and Bmi-1, which are all components within the p19Arf-p53 pathway believed to be dominant in MEFs (37–39). H2O2 treatment at 20 and 100 μM was found to stimulate expression of p53, p21Cip1, and p19Arf in line A, and this up-regulation indicated activation of the p19Arf-p53 pathway of senescence (Fig. 3). In line with previous reports that the p16Ink4a pathway does not play a significant role in senescence in MEFs, we failed to detect p16Ink4a expression in our cellular system using multiple anti-p16Ink4a antibodies (Fig. 3 and results not shown). Activation of the p19Arf-p53 pathway was attenuated by the dox induction of FoxM1c expression, and there was a concomitant up-regulation of Bmi-1 (Fig. 3), which is known to inhibit expression of the Ink4a/Arf/Ink4b locus (31, 32). These findings suggested that FoxM1c counteracts SIPS by suppression of the p19Arf-p53 pathway via activation of Bmi-1.

**Bmi-1 Is a Downstream Target of FoxM1c and Is Required to Protect against Senescence**—To verify the regulation of Bmi-1 by FoxM1c, we tested whether Bmi-1 expression in line A is FoxM1c-dependent. As shown in Fig. 4A, FoxM1c overexpression, induced by different doses of dox, up-regulated Bmi-1 expression. Because FoxM1c is a transcription factor, we expect that the stimulatory effect was mediated at the transcriptional level. Indeed, a dox-dependent up-regulation of Bmi-1 mRNA was revealed using semi-quantitative RT-PCR analysis (Fig. 4B). If Bmi-1 is a physiological target of FoxM1, we would expect suppression of Bmi-1 expression upon knock down of FoxM1 function. To this end, we transiently transfected NIH3T3 cells with pTER plasmids expressing shRNAs (pTER A and pTER B) (33) to deplete FoxM1 function. When compared with cells transfected with the control pTERGL3 plasmid that targets firefly luciferase, knock down of FoxM1 expression resulted in a decrease in Bmi-1 level with a concomitant up-regulation of p19Arf and also p53 and p21Cip1 expression (Fig. 4C).

FoxM1c expression levels exhibit cell cycle phase-dependent changes in synchronized fibroblasts (3, 6, 7), being very low in the G1 phase, gradually increasing during the S phase, and peaking at the G2/M phase. In this study, NIH3T3 cells, similarly synchronized by serum deprivation, expressed a low level of FoxM1 at 8 h after arrest when most cells were at G1, or early S phase (Fig. 5A), similar to the level in an asynchronous culture. FoxM1 was found to stimulate expression at 16 and 24 h after arrest when most cells were in the G2/M phase. Analysis of protein extract from line A (after dox induction at 4 μg/ml for 24 h) indicated that the FoxM1c levels induced in our lines were comparable with the peak endogenous levels of FoxM1 found in G2/M cells. The close correlation of FoxM1c and Bmi-1 expression levels supported the view that FoxM1c is a physiological downstream target of FoxM1c.

To test the ability of Bmi-1 in protecting against oxidative stress-induced senescence, we overexpressed Bmi-1 in NIH3T3 cells by transient transfection. As shown in Fig. 5B, Bmi-1 overexpression decreased the percentage of senescent...
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In this study, we demonstrated using inducible FoxM1-expressing NIH3T3 cell lines that increased FoxM1 expression had an antagonistic effect against SIPS by suppressing the p19<sup>Arf</sup>-p53 pathway via activation of Bmi-1 expression. Our data suggest that the onset of senescence and the associated up-regulation of p53 and p19<sup>Arf</sup> in FoxM1-deficient MEFs (18, 28) results from the down-regulation of Bmi-1. This is in line with our finding that senescence increased with knock down of Bmi-1 expression in NIH3T3 cells. Bmi-1 levels display cell cycle phase-dependent changes. Expression is initiated just before entry into S phase, is maintained during G1 phase progression, and increases dramatically in G2 and mitotic cells (3, 6, 7). Expression of FoxM1 and Bmi-1 at S and G2/M phases during normal cell cycle progression in MEFs explains why senescence is usually trig-

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**DISCUSSION**

Sequence inspection of the Bmi-1 promoter did not reveal any putative Forkhead binding sites within the FoxM1c-responsive region in pGL3-BmiPrWT. As indicated earlier, the promoter fragment in the pGL3-BmiPrWT construct contains a conserved E-box sequence (Fig. 6A). The E-box has been shown to bind and confer responsiveness to c-Myc (40). To determine the role of c-Myc in FoxM1c-induced Bmi-1 expression, we performed reporter assays using pGL3-BmiPrMut and pGL3-BmiPrΔMyc (40), two reporter constructs that contain a point mutation (CACGTG changed to CGCGTG) and deletion of the c-Myc binding site, respectively. Dox-inducible activation of Bmi-1 promoter activity was abolished in these mutant reporter constructs (Fig. 6B). Moreover, the mutant constructs were not activated by co-transfection with the FoxM1c expression plasmid (Fig. 6C). These findings indicated that FoxM1c activates the Bmi-1 promoter via c-Myc.

FoxM1c has recently been shown to transactivate the human c-Myc promoter in transient reporter assays, and direct binding of the c-Myc promoter has been demonstrated using electrophoretic mobility shift and chromatin immunoprecipitation assays (41). Consistent with these findings, dox-induced FoxM1c overexpression stimulated c-Myc expression (Fig. 7A), and knock down of FoxM1 expression using shRNAs (expressed by pTER A and pTER B) led to decreased levels of c-Myc (Fig. 7B). To test the notion that FoxM1c directly activates c-Myc expression, which in turn leads to enhanced Bmi-1 expression, we depleted c-Myc in line A using two shRNA-expressing plasmids, c-Myc sh1 and c-Myc sh2, and tested the resulting effect on dox-induced Bmi-1 expression (Fig. 7C). As expected, suppression of c-Myc expression attenuated the stimulation of Bmi-1 expression by FoxM1c upon dox induction. Taken together, our data showed that FoxM1c stimulates Bmi-1 expression via up-regulation of c-Myc levels.
Gerated in cells at G0/G1 phase when the senescence-counteracting effect of FoxM1 is absent in MEFs. In our inducible cell lines, FoxM1c levels in G1 and S cells were raised to be comparable with those in G2/M cells. We are of the opinion that the senescence-counteracting effect of induced FoxM1c

**FIGURE 4.** FoxM1c regulates Bmi-1 expression. A, line A cells induced with dox at the indicated concentrations (0 – 8 mg/ml) for 24 h were harvested for immunoblot analysis of FoxM1, Bmi-1, and tubulin expression. B, levels of Bmi-1 and glyceraldehyde-3-phosphate dehydrogenase transcripts (Gadph) were determined by semiquantitative RT-PCR analysis. Untreated and dox-treated NIH3T3/6TR cells were analyzed in parallel as controls. C, knock down of FoxM1 expression using shRNAs decreased Bmi-1 expression. pTER plasmids encoding hairpins A and B that target against FoxM1 were separately transfected into NIH3T3 cells and the cell lysates harvested for Western blot analysis of FoxM1, p53, p21, p19ARF, Bmi-1, and tubulin expression. pTERGL3, which targets against firefly luciferase, was included as negative control.

**FIGURE 5.** Bmi-1 is a downstream target of FoxM1 and is required to protect against senescence. A, a tight correlation between FoxM1 and Bmi-1 levels was observed. NIH3T3 cells, asynchronized or synchronized by serum deprivation, were analyzed for FoxM1 and Bmi-1 expression by immunoblot analysis. Asynchronized and synchronized cells at 8 h after arrest were mostly in G1 and S phases, whereas synchronized cells at 16 and 24 h after arrest were predominantly at G2/M phase. Levels were compared with line A cells induced with dox at 4 μg/ml for 24 h. B, Bmi-1 overexpression decreased H2O2-induced senescence. NIH3T3 cells were untransfected or transiently transfected with the Bmi-1-expressing plasmid pBabe Bmi-1 or the parental plasmid pBabe. C, FoxM1 depletion led to increased senescence. NIH3T3 cells were untransfected or transiently transfected with shRNA-expressing plasmids targeted against Bmi-1 or an unrelated gene. The effect of Bmi-1 overexpression or knock down was confirmed by immunoblot analysis. Transfected cells were treated with H2O2 at 20 or 100 μM for 48 h followed by SA-β-galactosidase staining to detect senescent cells. The histograms show the percentage of senescent cells and indicate a clear inverse relationship between Bmi-1 levels and H2O2-induced senescence. The mean ± S.E. of three independent experiments is shown.

transfected into NIH3T3 cells and the cell lysates harvested for Western blot analysis of FoxM1, p53, p21, p19ARF, Bmi-1, and tubulin expression.
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is exerted mostly in G₁ cells where endogenous FoxM1 expression is not yet initiated.

Recent studies have established cellular senescence as an important tumor suppressor mechanism (42–46). Functional restoration of p53 as the master regulator of senescence in various tumor types is enough to promote tumor regression by triggering senescence (47, 48). It would be worth testing whether the tumor-promoting effect of FoxM1 detected in mouse glioma, prostate carcinoma, and colorectal cancer (14, 15, 26) also involves activation of Bmi-1 and suppression of senescence mediated by the p19ARF/p53 pathway. The p16-pRb pathway is an alternative pathway commonly activated in human senescent cells. It remains to be tested whether elevation of FoxM1c would also antagonize the p16-pRb pathway and protect cells from SIPS triggered by other stimuli like oncogenic activation, ionizing radiation, and replicative senescence induced by telomere attrition in human cells (38). The likelihood of FoxM1c having a general senescence-counteracting effect is high, based on our finding that FoxM1c up-regulates the expression of Bmi-1, which is a major negative regulator of the Ink4a/Arf/Ink4b locus (31, 32).

The following lines of evidence have led us to conclude that regulation of Bmi-1 expression by FoxM1c is mediated by c-Myc. First, dox-induced up-regulation of FoxM1c stimulated expression of both c-Myc and Bmi-1. Second, shRNA-mediated depletion of FoxM1c down-regulated the expression of both c-Myc and Bmi-1. Third, endogenous expression of FoxM1c, c-Myc, and Bmi-1 were closely correlated with one another upon cell cycle re-entry after serum deprivation. In fact, all three genes behave like classical late early genes inducible by serum activation in MEFs. Fourth, dox-induced activation of Bmi-1 expression by FoxM1c was abrogated when c-Myc levels were suppressed using shRNAs. FoxM1c has recently been shown to bind the c-Myc promoter in vitro (41), and the conserved c-Myc binding site within the Bmi-1 promoter has been demonstrated to be critically required for its activity (Ref. 40 and this study). Thus, it is very likely that the positive regulation of Bmi-1 by FoxM1c is mediated via c-Myc.

It is worth noting that FoxM1, c-Myc, and Bmi-1 have all been shown to exhibit tumor-promoting function in various cell and animal models. Moreover, both c-Myc and Bmi-1 have been found to be important for the maintenance of stem cell function (49–54). In our opinion, FoxM1, as upstream regulator, would also be important for the maintenance of stem cell function (49–54). In our opinion, FoxM1, as upstream regulator, would also be important for the maintenance of stem cell function (49–54).
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A

| 3T3+/Dox 4μg/ml | 3T3/16TR + Dox 4μg/ml | Dox 0μg/ml | Dox 2μg/ml | Dox 4μg/ml | Dox 8μg/ml |
|-----------------|------------------------|-----------|-----------|-----------|-----------|
| c-Myc           |                        |           |           |           |           |
| Tubulin         |                        |           |           |           |           |

B

| pTER A | pTER B | pTERG1.3 |
|--------|--------|-----------|
| FoxM1  | c-Myc  | Tubulin   |

C

| Control sh | c-Myc sh1 | c-Myc sh2 |
|------------|-----------|-----------|
| Tubulin    |           |           |

FIGURE 7. FoxM1c activates Bmi-1 expression via c-Myc. A, dox-induced FoxM1c overexpression in line A cells led to stimulation of c-Myc expression. Line lysates as described in Fig. 4 were analyzed for c-Myc expression by immunoblot analysis. B, NIH3T3 cells were depleted of FoxM1c expression using shRNA-expressing pTER plasmids as described in Fig. 5. C, NIH3T3 cells were depleted of FoxM1c expression using shRNA-expressing pTER plasmids before their harvest for Western blot analysis of FoxM1c, c-Myc, Bmi-1, and tubulin expression. FoxM1c-mediated stimulation of Bmi-1 expression was strongly suppressed by the knock down of c-Myc expression.

Bmi-1. Additionally, the recent discovery of stabilization of the FoxM1 protein upon DNA damage implicates its functional involvement in checkpoint signaling (28).

In summary, we have provided the first evidence that FoxM1c, via up-regulation of c-Myc and Bmi-1, suppresses SIPS in MEFs. The regulatory interactions among FoxM1c, c-Myc, and Bmi-1 bring new insight into the molecular mechanism of tumorigenesis. Further understanding of the molecular basis of regulation of senescence by FoxM1c will be helpful in developing therapies to treat cancer and age-related diseases.

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