FoxM1, a critical regulator of oxidative stress during oncogenesis

Hyun Jung Park, Janai R Carr, Zebin Wang,
Veronique Nogueira, Nissim Hay,
Angela L Tyner, Lester F Lau,
Robert H Costa and Pradip Raychaudhuri*

Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, College of Medicine and University of Illinois Cancer Center, Chicago, IL, USA

The transcription factor FoxM1 is over-expressed in most human malignancies. Although it is evident that FoxM1 has critical functions in tumour development and progression, the mechanisms by which FoxM1 participates in those processes are not understood. Here, we describe an essential role of FoxM1 in the regulation of oxidative stress that contributes to malignant transformation and tumour cell survival. We identify a negative feedback loop involving FoxM1 that regulates reactive oxygen species (ROS) in proliferating cells. We show that induction of FoxM1 by oncogenic Ras requires ROS. Elevated FoxM1, in turn, downregulates ROS levels by stimulating expression of ROS scavenger genes, such as MnSOD, catalase and PRDX3. FoxM1 depletion sensitizes cells to oxidative stress and increases oncogene-induced premature senescence. Moreover, tumour cells expressing activated AKT1 are 'addicted' to FoxM1, as they require continuous presence of FoxM1 for survival. Together, our results identify FoxM1 as a key regulator of ROS in dividing cells, and provide insights into the mechanism how tumour cells use FoxM1 to control oxidative stress to escape premature senescence and apoptosis.

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Introduction

Reactive oxygen species (ROS) are continuously generated in cells largely by the mitochondria and membrane-bound NADPH oxidases. Owing to their highly reactive nature towards cellular components including proteins, lipids and DNA, ROS are generally considered as harmful byproducts of cellular metabolism. However, ROS have been recognized as an important mediator

of cellular events, such as intracellular signal transduction, metabolism, proliferation and apoptosis (Sundaresan *et al*, 1995; Irani *et al*, 1997; Finkel, 2000; Pham *et al*, 2004). Effects of ROS depend on the redox status of the intracellular milieu, which is tightly regulated by the balance between production and detoxification of ROS (Gutteridge and Halliwell, 2000; Droge, 2002). Cells have evolved mechanisms to protect themselves from ROS-induced damage by expressing various detoxifying enzymes such as superoxide dismutase, catalase, glutathione peroxidases and peroxiredoxins.

In *Caenorhabditis elegans* (*C. elegans*), the activated forkhead box transcription factor DAF-16 upregulates the transcription of genes encoding the anti-oxidant enzymes and extends the lifespan of the worm (Ogg *et al*, 1997). The mammalian DAF-16 homologue, forkhead box 3a (FoxO3a) has been reported also to protect quiescent cells from oxidative stress by activating the transcription of manganese superoxide dismutase (MnSOD) (Kops *et al*, 2002). In contrast to *C. elegans*, which consists of mostly post-mitotic cells, many mammalian progenitor cells in regenerating tissues proliferate throughout life. Proliferating cells are metabolically more active and subsequently produce more ROS. Increased ROS generation is also common in cancer cells resulting from activation of various oncogenes (Irani *et al*, 1997; Sattler *et al*, 2000). However, the FoxO transcription factors that activate detoxifying enzymes are inactivated in proliferating cells by PI3K-AKT/ PKB pathway. Moreover, this PI3K-AKT/ PKB pathway is often mutated and highly activated in various tumours (Biggs *et al*, 1999; Brunet *et al*, 1999; Carnero *et al*, 2008). The elevation of ROS by AKT through the inactivation of FoxO transcription factors were recently shown to promote cellular senescence and to sensitize cells to ROS-mediated cell death (Nogueira *et al*, 2008). Therefore, proliferating cells or tumour cells are expected to accumulate ROS and may have acquired alternative mechanisms that protect cells from oxidative stress. However, the exact mechanisms by which proliferating normal cells or tumour cells defend themselves from increased ROS are not well understood.

FoxM1, similar to the FoxO transcription factors, is a member of the forkhead box transcription factor family. However, unlike FoxO transcription factors, which are activated in quiescent cells and inhibit cell proliferation, FoxM1 is only expressed in proliferating cells and has critical functions in cell-cycle progression (Laoukili *et al*, 2005; Wang *et al*, 2005). Several studies implicated FoxM1 in tumourigenesis, as it is over-expressed in various human malignancies. For example, gene expression profiles in a number of carcinomas, including prostate, breast, lung, ovary, colon, pancreas, stomach, bladder, liver and kidney showed that FoxM1 is over-expressed in all the carcinomas investigated (Pilarsky *et al*, 2004). High expression of FoxM1 in glioblastoma correlates with the tumourigenicity of the glioma cells (van den Boom *et al*, 2003; Liu *et al*, 2006). Moreover, in prostate

*Corresponding author. Department of Biochemistry and Molecular Genetics (M/C 669), University of Illinois at Chicago, College of Medicine, University of Illinois Cancer Center, MBRB 2302, 900 South Ashland Avenue, Chicago, IL 60607, USA. Tel.: +1 312 413 0255; Fax: +1 312 355 3847; E-mail: pradip@uic.edu

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cancer, over-expression of FoxM1 strongly correlates with metastasis (Chandran *et al*, 2007). FoxM1 is also required for invasion and angiogenesis of pancreatic cancer cells (Wang *et al*, 2007). Together, these observations indicate a strong reliance of tumour cells on FoxM1.

Interestingly, FoxM1 was shown to counteract oxidative stress-induced premature senescence (Li *et al*, 2008). Authors of that study suggested that FoxM1 inhibits premature senescence by stimulating expression of Bmi1. In this study, we show that expression of FoxM1 is induced by increased oncogenic stress requiring ROS, and that the upregulated FoxM1 in a negative feedback loop counteracts elevated intracellular ROS levels by stimulating the expression of anti-oxidant enzyme genes to protect dividing cells and tumour cells from oxidative stress.

Results

Oncogenic H-RasV12 stimulates FoxM1 expression by increasing ROS

A moderate increase in ROS on growth factor stimulation is known to promote proliferation in various types of cells (Burdon, 1995; Lo and Cruz, 1995; Sundaresan *et al*, 1995). Moreover, a direct involvement of ROS, especially superoxide (O_2^-), in oncogenic Ras-induced proliferation and tumorigenesis was reported (Irani *et al*, 1997). As FoxM1 is critical for proliferation and often over-expressed in tumours, we investigated whether oncogene-induced ROS has a role in the over-expression of FoxM1 to promote cellular transformation. To examine the effect of oncogenic Ras on FoxM1 expression, we infected immortalized mouse embryonic fibroblasts (MEFs) with H-RasV12 retrovirus. FoxM1 expression was significantly increased in H-RasV12 expressing cells compared with that in the control cells, which coincided with increased intracellular superoxide (O_2^-) levels (Figure 1A). To examine whether the induction of FoxM1 by H-RasV12 involved increased ROS, we treated cells with superoxide dismutase (SOD) mimetic anti-oxidants, Tempol (1 mM) or MnTM-2-PyP (50 μ M). Under these conditions, Tempol caused a significant reduction in endogenous superoxide without any significant increase in peroxides (Supplementary Figure 1A). When H-RasV12 expressing cells were treated with Tempol or MnTM-2-PyP, the induction of FoxM1 was markedly reduced (Figure 1B). Similar results were obtained using immortalized MEFs (Supplementary Figure 1B) and three different primary human fibroblasts (Supplementary Figure 1C) expressing a 4-hydroxytamoxifen (4-OHT)-inducible H-RasV12-ER. A significant increase in FoxM1 levels was observed on H-RasV12 induction by 4-OHT (Supplementary Figure 1B and C). Moreover, the increase in FoxM1 expression was inhibited by Tempol. Other anti-oxidants, including catalase and 10 mM N-acetyl cysteine (NAC), also blocked the induction of FoxM1 (Supplementary Figure 1B). Together, these observations indicated that ROS is required for an increase in FoxM1 levels on H-Ras activation.

Next, we examined whether ROS is critical also for serum-induced expression of FoxM1. Cells were maintained in serum-free media for 48 h and then stimulated with serum containing media with or without anti-oxidants. Anti-oxidant treatment abolished serum-induced expression of FoxM1 (Supplementary Figure 1D). Earlier, it was reported that increased ROS is critical for G1/S transition to inactivate

Figure 1 Oncogenic Ras-induced FoxM1 expression requires ROS. (A) Immortalized MEFs infected with control or H-RasV12 retrovirus. Four days after selection total cell extracts were analysed for expression of FoxM1 by immunoblotting (upper) and for ROS levels by DHE fluorescence, as described in 'Materials and methods' (lower). (B) Cells expressing H-RasV12 were treated with DMSO, 1 mM Tempol or 50 μ M MnTM-2-PyP. Total cell extracts and total RNA from the treated cells were analysed for the FoxM1 protein (upper) and mRNA by RT-PCR (lower). Bar graph presents mean $\pm$ standard deviation (s.d.) of three experiments (* P < 0.05). (C) Immortalized MEFs were incubated with Tempol containing media for indicated times. Cell extracts were then subjected to immunoblotting (upper) or BrdU-incorporation assay (lower). Bar graph presents mean $\pm$ s.d. (n = 3; * P < 0.05). (D) Cells were treated with superoxide dismutase (SOD) inhibitor, diethyldithiocarbamate (DDC) for 16 h and collected for immunoblotting to analyse FoxM1 levels. (E) Cells were maintained in low serum media (2% FBS) for 48 h and treated with hydrogen peroxide (H_2O_2) for the next 16 h. FoxM1 levels were analysed by western blotting. β -actin serves as loading control.

APC/Cdh1 ubiquitin ligases and allow accumulation of the cell-cycle regulators (Havens *et al*, 2006). In addition, we recently showed that FoxM1 is a target of the APC/Cdh1 complex (Park *et al*, 2008a). However, we observed no significant effect of the anti-oxidants on the half-life of the FoxM1 protein under our condition (data not shown). To confirm that the decrease in FoxM1 levels on anti-oxidant treatment is not caused by cell-cycle arrest or proliferation defects, we performed a time-course experiment. A decrease in FoxM1 protein was observed as early as 6 h after Tempol treatment, whereas the inhibition of proliferation was observed 24 h after the treatment as determined by decreased BrdU incorporation (Figure 1C). To further validate the ROS-dependent expression of FoxM1, we treated cells with 10 μ M diethyldithiocarbamate (DDC), a well-known inhibitor of SOD that is expected to increase endogenous superoxide levels. Clearly, treatment with DDC increased expression of FoxM1 (Figure 1D). Moreover, consistent with an earlier study in endothelial cells (Ye *et al*, 1997; Zhao *et al*, 2006), treatments with hydrogen peroxide (H_2O_2) also increased expression of FoxM1 (Figure 1E).

FoxM1 is critical for transformation by oncogenic Ras

As FoxM1 is induced by the oncogenic Ras (H-RasV12), we investigated whether FoxM1 is required for the transforming function of Ras. We introduced the H-RasV12 transgene into immortalized MEFs harbouring floxed alleles of FoxM1. The H-RasV12-transformed cells were infected with Ad-LacZ (control) or Ad-Cre to delete the floxed alleles of FoxM1. Deletion of FoxM1 caused a transient activation of the stress kinase, p38 mitogen-activated protein kinase (MAPK) (Supplementary Figure 1E). The cells were subjected to soft agar colony formation assay. Deletion of FoxM1 caused a huge decrease in the number and size of the colonies (Figure 2A), suggesting that FoxM1 is critical for H-RasV12-induced transformation. As FoxM1 stimulates proliferation genes, it is likely that reduced proliferation has a function in reducing the colonies in FoxM1-deleted cells.

To further investigate the mechanisms by which FoxM1 participates in H-RasV12-induced transformation, we inhibited FoxM1 by siRNA transfection. It was shown that expression of oncogenic Ras in primary fibroblasts causes premature senescence, which is known to be a barrier for Ras-induced transformation. We found that siRNA-mediated depletion of FoxM1 exacerbated the H-RasV12-induced premature senescence in primary fibroblasts. Cells expressing a tamoxifen-inducible H-RasV12 were transfected with control or two different FoxM1-siRNAs. The cells were then treated with 4-OHT for 3 days and subjected to senescence-associated β -galactosidase (SA- β -Gal) assay to score senescent cells. The FoxM1-depleted cells exhibited four- to five-fold increases in senescent cells (Figure 2B). Together, these results suggest that FoxM1, in addition to stimulation of the cell-cycle genes, contributes to the transforming pathway of Ras by attenuating the senescence programme induced by oncogenic Ras.

FoxM1 protects cells from oxidative stress

IMR90 cells depleted of FoxM1 exhibited a large and flat-cell morphology, and there was an increase in SA- β -Gal-positive

cells (Figure 3A), which are consistent with our earlier reports with FoxM1^{-/-} MEFs (Wang *et al*, 2005). Interestingly, we observed that the level of phospho-p38 MAPK was increased in FoxM1 siRNA-transfected cells, coinciding with the senescence-like phenotype (Figure 3A, upper panel). The FoxM1-siRNAs had very little effects on the levels of the other forkhead family factors FoxO1 and FoxA (Figure 3A). The p38 MAP kinase is known to be activated by various stresses, including ROS (Ono and Han, 2000), and activation of p38 MAPK was reported to induce premature senescence (Haq *et al*, 2002; Wang *et al*, 2002). It is believed that p38 MAPK mediates ROS-induced premature senescence. Recently, p38 MAPK- α was identified as a sensor of oxidative stress that suppressed oncogene-mediated transformation (Dolado *et al*, 2007). Therefore, we investigated the possibility that FoxM1 depletion might activate ROS-p38 MAPK pathway to cause the increase in senescence-like phenotype. To explore this possibility, we treated cells with anti-oxidants. The anti-oxidants inhibited activation of p38 MAPK in FoxM1-depleted cells (Figure 3B, upper panel). Moreover, treatments with a p38 MAPK inhibitor (SB203580) or catalase decreased a senescence-like phenotype induced by the depletion of FoxM1 (Figure 3B). Interestingly, however, p38 inhibitor or catalase did not rescue the cell-cycle arrest induced by the depletion of FoxM1 (data not shown).

We further investigated whether FoxM1 protects cells from oxidative stress. A recent study (Li *et al*, 2008) indicated that FoxM1 could overcome premature senescence induced by H₂O₂ by inducing expression of Bmi1. We measured the effect H₂O₂ on p38 MAPK activation. We observed stronger activation of p38 MAPK in FoxM1-depleted IMR90 cells than that in control cells on H₂O₂ treatment (Figure 3C, upper panel). Furthermore, FoxM1-depleted cells were more sensitive to H₂O₂-induced cell death (Figure 3C, lower panel) and to senescence induced by sub-lethal concentration of H₂O₂ (Figure 3D), indicating that FoxM1 is important for protecting cells from oxidative stress, which is consistent with the earlier study (Li *et al*, 2008). In addition, we observed that

Figure 2 FoxM1 induction is critical for cellular transformation by H-RasV12. (A) FoxM1^{fl/fl} MEFs expressing H-RasV12 were infected with AdLacZ or AdCre. The MEF cells were then grown on soft agar for 14 days. Bar graph is expressed as a percentage of LacZ-infected cells (mean $\pm$ s.d.; $n = 3$). (B) The IMR90 cells stably expressing H-RasV12-ER were transfected with control or two different FoxM1 siRNA duplexes (siFoxM1#1 and siFoxM1#2) and incubated with or without 4-OHT for indicated times. Efficient knockdown of FoxM1 was assayed by immunoblotting (upper panel). SA- β -Gal assay was performed and positively stained cells were counted (mean $\pm$ s.d. of three experiments; ** $P < 0.01$) (lower panel).

Figure 3 FoxM1 protects cells from oxidative stress. (A–D) IMR90 cells were transfected with control siRNA or FoxM1 siRNA (50 nM) for 72 h. (A) Cell extracts (150 μg) were prepared for western blotting to analyse FoxM1 knockdown efficiency and phospho-p38 (pp38) levels. In addition, FoxO1 and FoxA levels were determined by western analysis demonstrating specificity of the FoxM1-siRNAs (upper panel). Cells were fixed and subjected to senescence-associated-β-Gal (SA-β-Gal) staining (lower panel). (B) Cells were treated with 10 μM p38 inhibitor (SB203580), NAC (2 mM) or 50 units/ml catalase after 4 h of siRNA transfection. Activation of p38 MAPK was analysed by immunoblotting (upper panel). SA-β-Gal staining was performed and positive cells were counted. Bar graph presents mean ± s.d. ($n = 3$; $*P < 0.05$) (lower panel). (C) Cells were treated with indicated concentrations of H₂O₂ for 3 h. The total cell extracts (75 μg) were subjected to western blotting to measure the levels of phospho-p38 and total p38 (upper panel). For the viability assay, cells were washed with PBS and incubated with fresh media for 24 h, and viable cell counts are plotted (lower panel) (mean ± s.d. of three experiments; $**P < 0.01$). (D) The siRNA-transfected cells were treated with hydrogen peroxide (100 μM) for 1 h and then washed with PBS twice. Cells were maintained in fresh media for 7 days and then subjected to SA-β-Gal assay, as described in ‘Materials and methods’.

expression of the wild type or a constitutively activate FoxM1 markedly attenuated p38 MAPK activation induced by H₂O₂ (Supplementary Figure 2, upper panel). Accordingly, FoxM1 expressing cells were more resistant to the oxidative stress-induced cell death (Supplementary Figure 2, lower panel).

FoxM1 regulates intracellular levels of ROS

To investigate the molecular basis of FoxM1-mediated cellular protection from oxidative stress, we investigated the possibility that FoxM1 might be a regulator of ROS. We observed that the FoxM1-deficient cells exhibited higher ROS levels compared with the control cells, as measured by flow cytometry

using dichlorodihydrofluorescein diacetate (DCF-DA) fluorescent probe (Figure 4A; Supplementary Figure 3A). In addition, we measured intracellular superoxide (O₂^{•−}) levels using dihydroethidium (DHE) and found that FoxM1-depleted cells also accumulated more O₂^{•−} (Figure 4A; Supplementary Figure 3A), which is consistent with the notion that FoxM1 is a regulator of ROS.

As FoxM1 is a transcription factor, we sought to identify FoxM1 target genes involved in ROS regulation. Earlier, the FoxO transcription factors were implicated in regulating antioxidant genes such as MnSOD, catalase and PRDX3 (Nemoto and Finkel, 2002; Burgering and Medema, 2003; Chiribau

Figure 4 FoxM1 induces anti-oxidant genes by directly binding to their promoter: (A, B) IMR90 cells were transfected with control or FoxM1 siRNA for 72 h. (A) Intracellular ROS level was measured using DCF-DA or DHE fluorescence probes, as described in 'Materials and methods' (left). ROS amounts were presented as mean $\pm$ s.d. of fluorescence intensity of cells. *, *** P < 0.05, 0.001 as compared with control siRNA-transfected cells. Quantitative RT-PCR was performed using FoxM1, catalase, MnSOD and PRDX3 primers. RNA was prepared from control or FoxM1 siRNA-transfected cells. The data are mean $\pm$ s.d. of triplicate (* P < 0.05 as compared with AdLacZ-infected cells) (right). (B) The protein expression of anti-oxidant genes was analysed by western blotting with the indicated antibodies. (C) IMR90 fibroblasts were infected with LacZ or FoxM1 adenovirus for 48 h. Catalase and MnSOD mRNA levels were measured by quantitative RT-PCR. The data are mean $\pm$ s.d. of triplicate (* P < 0.05). (D) IMR90 cells were transfected with plasmid expressing T7-epitope-tagged FoxM1. The transfected cells were subjected to chromatin immunoprecipitation (ChIP) assays using a previously described procedure. A monoclonal antibody against T7-epitope or isotype-matched IgG was used for immunoprecipitation. The primers used to detect FoxM1 interaction with catalase promoter (D), MnSOD promoter (E) and the negative controls are indicated by arrows. The primer sequences are included in 'Materials and methods'.

et al, 2008). Therefore, we investigated whether FoxM1 regulates transcription of the same detoxifying genes in proliferating cells, where FoxO is inactive. The mRNA levels of those genes were compared by quantitative RT-PCR in control and FoxM1 siRNA-transfected cells (Figure 4A, right panel). We found that the anti-oxidant genes were expressed at much lower levels in FoxM1-deficient cells than in control cells. The reduction in the anti-oxidant enzymes was also detected at the protein level (Figure 4B).

To confirm that FoxM1 actively regulates intracellular ROS levels, we infected cells with FoxM1 expressing adenovirus and measured the levels of ROS. Cells over-expressing FoxM1 had significantly lower levels of ROS than control cells

(Supplementary Figure 3B). Moreover, we observed that increased expression of FoxM1 significantly upregulated expression level of MnSOD and catalase (Figure 4C). To investigate whether FoxM1 directly stimulates the expression of the ROS-regulatory genes *in vivo*, we performed chromatin immunoprecipitation assay (ChIP). Database analyses allowed us to identify consensus FoxM1-binding sites in the first intron of the human catalase promoter and at -4972 upstream of the transcription start site in the human MnSOD promoter. ChIP assay confirmed that FoxM1 binds to MnSOD and catalase promoter regions containing the putative FoxM1-binding site (Figure 4D and E). The results are consistent with the notion that FoxM1 regulates the level of ROS

by stimulating expression of the anti-oxidant genes through direct interactions with the regulatory regions of those genes.

As FoxO transcription factors are known to regulate the same set of genes to protect quiescent cells from oxidative stress, we tested whether the expression of the FoxO transcription factors could alleviate the oxidative stress-sensitive phenotype observed in FoxM1-deficient cells. We expressed a constitutively active form of FoxO1 (FoxO1-3A) in the FoxM1-depleted cells. FoxO1-3A expression significantly reduced phospho-p38 levels and cell death induced by H₂O₂ in FoxM1-deficient cells (Supplementary Figure 4). Similar results were observed with catalase expression (data not shown). By contrast, cell-cycle deficiency observed from FoxM1-depleted cells was not rescued by either FoxO expression (Laoukili *et al*, 2005) or catalase expression (data not shown).

Tumour cells expressing activated AKT1 are addicted to FoxM1

A growing body of evidence suggests that increased levels of ROS contribute to oncogene-induced senescence (Lee *et al*, 1999; Nogueira *et al*, 2008). Recently, it was reported that AKT has a critical function in oncogene-induced senescence through increasing ROS levels partly by inhibiting FoxO (Nogueira *et al*, 2008). As ROS increases FoxM1, we investigated whether activated AKT1 (4-OHT-inducible myristylated AKT1 (mAKT1)-ER) could increase expression of FoxM1. Surprisingly, unlike RasV12, activated mAKT1 failed to stimulate expression of FoxM1 (Supplementary Figure 5A). We think that the lack of stimulation of FoxM1 expression is related to lack of JNK activation in the mAKT1-expressing cells (Supplementary Figure 5C; also see Kim *et al*, 2001; Song and Lee, 2005), as JNK is critical for the RasV12-induced expression of FoxM1 (Supplementary Figure 5D). Next, we examined whether FoxM1 facilitates oncogenic AKT-induced transformation by reducing intracellular ROS levels. We used

the 4-OHT-inducible mAKT1-ER system in IMR90 cells. Enforced expression of FoxM1 by retrovirus infection attenuated the senescence-like phenotype in the mAKT1-induced cells, concomitantly with a decrease in the cellular ROS levels (Figure 5A). Moreover, FoxM1 expression also increased the tumorigenic activity of mAKT1, as evidenced by increased colony formation on soft agar (Figure 5B).

Recent studies showed that tumour cells tend to be addicted to oncogenes, as the tumour cells underwent apoptosis or senescence when the oncogenes were inactivated (Felsher and Bishop, 1999; Sharma *et al*, 2006). Therefore, we examined whether cells harbouring hyper-activated AKT, which increases ROS production and decreases detoxifying enzyme synthesis by inactivating FoxOs, are addicted to FoxM1. We depleted FoxM1 by siRNA transfection and performed cellular senescence assay. On FoxM1 depletion the U2OS osteosarcoma cells displayed increased senescence-like phenotype compared with the control siRNA-transfected cells. Interestingly, the mAKT1-over-expressing U2OS cells exhibited more severe senescence phenotype than the parental U2OS cells (Figure 6A), suggesting that cells harbouring hyper-activated AKT1 are more dependent on the FoxM1 for protection from oxidative stress. The reduction in growth rate in the mAKT1-expressing cells by FoxM1-siRNA was observed also in cell-proliferation assays (compare the two panels in Figure 6B after 5 days of FoxM1-siRNA transfection, the siRNA inhibition of FoxM1 persists for 4–5 days), as well as in BrdU-incorporation assays 5 days after FoxM1-siRNA transfection (Figure 6C). Furthermore, we observed that transient FoxM1 depletion caused permanent growth arrest and significantly reduced colony formation in mAKT1-expressing cells, whereas parental U2OS cells were able to recover to regrow 4 days after transfection and form colonies, albeit less than control siRNA-transfected cells (Figure 6B and D). As a control, we transfected cells with Aurora B kinase siRNA, which arrests cells at G2/M phase of cell cycle to

Figure 5 FoxM1 enhances mAKT-induced transformation. (A) IMR90 cells stably expressing mAKT-ER were infected with FoxM1 expressing retrovirus. mAKT was induced by adding 4-OHT for 24 h. ROS levels were determined by FACS after DCF-DA treatment and presented as mean $\pm$ s.d. ** $P < 0.01$; NS, not significant (upper). The cells were maintained in culture for 3 or 7 days after 4-OHT treatment, and then subjected to SA- β -Gal assay. The data show a percentage of β -Gal-positive cells (mean $\pm$ s.d.; $n = 3$; * $P < 0.05$) (bottom). (B) Immortalized MEFs stably expressing mAKT-ER was infected with Mock or FoxM1 retrovirus. The cells were subjected to soft agar assay with or without 4-OHT. Data shown are representatives of three independent experiments (* $P < 0.05$).

Figure 6 Tumour cells expressing hyper-activated AKT are dependent on FoxM1. (A–D) U2OS or mAKT-expressing U2OS cells were transfected with control or FoxM1 siRNA for 72 h. Insert (A) shows the efficient knockdown of FoxM1 by siRNA analysed by western blotting. Cells were subjected to SA-β-Gal staining (A). (B) 5×10^5 cells were plated and proliferation rates were measured by counting number of cells for 8 consecutive days. Culture medium was replaced every 3 days. (C) Cells were re-plated on gelatin-coated coverslips at 4 days after siRNA transfection. The next day, cells were subjected to BrdU-incorporation assay. Data represent mean $\pm$ s.d. of three independent experiments ($*P < 0.05$). (D) Clonogenic survival was determined using the strategy depicted in the upper panel. Data shown are representative pictures of colonies from at least three independent experiments.

confirm that permanent growth arrest after transient inactivation of FoxM1 was not a consequence of growth arrest, but caused by the damage induced by FoxM1 inactivation. These results suggest that the tumour cells expressing hyper-activated AKT are addicted to FoxM1 for proliferation and clonogenic survival.

Discussion

Low levels of ROS, induced by a number of growth factors, function as signalling messengers to increase cell growth and proliferation. However, higher concentration of ROS induces growth arrest, apoptosis or necrosis. The biphasic effects of ROS on cells have emphasized the necessity for precision in regulating the intracellular ROS levels. In this study, we showed that FoxM1 transcription factor is a sensor of ROS induced by oncogenic Ras and serum. Cellular proliferation

requires high level of energy supply. Therefore, it is very important for cells to monitor environmental cues, such as nutrients and oxygen to decide whether to proliferate or to rest. The requirement of ROS for expression of FoxM1, a critical factor for cellular proliferation, ensures that cells proliferate only when the conditions are favourable with regards to energy metabolism and oxygen supply. This suggests interesting interplays between cellular metabolism and proliferation.

Given the critical role of FoxM1 in proliferation, it is conceivable that ROS elevated by mitogenic stimuli utilize FoxM1 to promote proliferation. Our results show that increased FoxM1 not only accelerates cellular proliferation but also negatively regulates intracellular ROS levels (see model in Figure 7) by stimulating expression of the detoxifying enzymes, such as catalase, MnSOD and PRDX3. Interestingly, those genes are also known as targets of FoxO

Figure 7 Model depicting FoxM1 as a sensor and regulator of oncogene-induced ROS to protect cells from oxidative stress and promote cellular proliferation and transformation.

transcription factors. On the basis of similarity of FoxM1 and FoxO DNA-binding domains and consensus binding sequences, it was speculated that they could regulate the same target genes. Indeed, here we showed that FoxM1 regulates the expression of the same anti-oxidant genes, to protect cells from oxidative stress, when FoxO is off duty. However, it is less likely that the same DNA-binding sites are involved because the established FoxO-binding site on MnSOD promoter region is located at -1249 and the element TTGTTTAC (Kops *et al*, 2002) is not a FoxM1 cognate site (Ye *et al*, 1997). Interestingly, FoxO1 is a more potent activator of those anti-oxidant genes (data not shown). Given that moderate levels of ROS are required for proliferation, it is conceivable that FoxM1 has lower anti-oxidative capacity than FoxO1 so as to maintain sufficient ROS levels to support proliferation.

It is still unclear whether FoxM1 and FoxO have redundant functions in oxidative stress signalling. As activation of FoxM1 and FoxO transcription factors has opposite effects on cell fate, proliferation versus cell-cycle arrest or apoptosis, the selective activation of FoxM1 and FoxO could serve as important determinant of cell fate after redox signalling or oxidative stress. In that regard, it is interesting to note that FoxO3a has been shown to inhibit expression of FoxM1, which would contribute to the mechanism by which FoxO3a inhibits cell proliferation (Delpuech *et al*, 2007). FoxO3a is a potent regulator of ROS, as it stimulates expression of MnSOD, catalase, PRDX3 and Sestrin3 (Nemoto and Finkel, 2002; Burgering and Medema, 2003; Chiribau *et al*, 2008; Nogueira *et al*, 2008). Therefore, it is tempting to speculate that FoxO3a inhibits FoxM1 expression by inhibiting ROS. However, it was shown that FoxO3a inhibits FoxM1 gene expression through induced expression of the transcriptional repressor Mxi-SR α that displaces c-Myc from the FoxM1 promoter (Delpuech *et al*, 2007). Nevertheless, the inhibition of FoxM1 by FoxO3a is consistent with the notion that the two genes function at different physiological conditions. For example, FoxM1 is expected to be active in regulating ROS when mitogenic stimuli activate the PI3K/AKT pathway, which inactivates FoxO factors (Greer and Brunet, 2005). Consistent with that notion, we observed that cells expressing active AKT depend on FoxM1 for survival (Figure 6).

MnSOD and PRDX3 are predominantly localized to the mitochondria. Given that mitochondria are one of the major sources of ROS during apoptosis (Cai and Jones, 1999), it is compelling to speculate that FoxM1 might be also able to attenuate apoptosis, involving induction of mitochondrial

ROS, by upregulating detoxifying enzymes, such as MnSOD and PRDX3.

Cancer cells generally produce high level of ROS resulting from increased cellular metabolism and activation of oncogenes. In this study, we showed that enforced FoxM1 expression protects cells from ROS-induced senescence and cell death, implying that tumour cells might adapt to the increased ROS by over-expressing FoxM1. The elevated ROS, however, also make tumour cells more vulnerable to oxidative stress and more dependent on cellular anti-oxidant systems. Earlier studies showed that we could use such vulnerabilities to selectively kill cancer cells by increasing oxidative stress (Trachootham *et al*, 2006; Nogueira *et al*, 2008). We observed that FoxM1-depleted cells were more sensitive to oxidative stress and underwent senescence, partly because of increased intracellular ROS. In agreement with these observations, tumour cells with activated AKT, and therefore high ROS levels, were more dependent on FoxM1. Our observations suggest a clinical strategy in which FoxM1 inhibitor would cause senescence preferentially in AKT-activated cells or in cells with high ROS levels. Moreover, a combination of FoxM1 inhibitor with anti-cancer drugs that increase cellular ROS levels would be more effective and improve the therapeutic outcomes.

Materials and methods

Cell culture

Human primary fibroblasts IMR90, BJ and 1064SK, mouse fibroblasts NIH3T3 and human osteosarcoma U2OS cells were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium supplemented 10% foetal bovine serum (FBS) and penicillin-streptomycin. BJ cells were maintained in MEM medium (with Earl's salt, 2 mM L-glutamine, and 0.1 mM non-essential amino acid) supplemented with 10% FBS and penicillin-streptomycin. NIH3T3 cells expressing wild-type FoxM1b (WT-FoxM1b) and constitutively active FoxM1b (CA-FoxM1b) were generated by infecting cells with retrovirus carrying WT-FoxM1b or CA-FoxM1b (Park *et al*, 2008b) and selection with hygromycin. After selection, cells were pooled for further analysis.

Anti-oxidants

Cells were treated with various anti-oxidants to decrease intracellular levels of ROS. Included are 1 mM Tempol (Calbiochem), 50 μ M MnTM-2-PyP (Calbiochem), 10 mM NAC (Sigma), 50 units/ml catalase (Sigma). The anti-oxidants were added 1 h before serum induction of cells or activation of Ras by 4-OHT.

Immortalized FoxM1^{fl/fl} MEFs, expression of H-RasV12 and deletion of FoxM1

Generation of C57BL/6 mice containing FoxM1^{fl/fl} was described earlier (Krupczak-Hollis *et al*, 2004). To isolate MEFs from embryos, the liver, heart and head were removed from 13 d embryo, and the remaining embryo was digested with 0.25% Trypsin/EDTA to isolate single-cell suspension of MEFs. The MEFs were maintained in DMEM supplemented with 10% FBS, penicillin-streptomycin, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acid and 55 μ M 2-mercaptoethanol. The MEFs were immortalized by infecting with a retrovirus expressing dominant negative p53 followed by selection. To generate immortalized FoxM1^{fl/fl} MEFs expressing H-RasV12, cells (5×10^5) were plated in 10 cm dishes. The next day, cells were infected with retrovirus carrying H-RasV12 for 24 h. Stably transduced cells were generated after selection with 50 μ g/ml Hygromycin B (Invitrogen) until complete loss of uninfected cells. For deletion of FoxM1, pools of stably transfected FoxM1^{fl/fl} MEFs were infected with adenovirus expressing Cre or LacZ in serum-free medium for 1 h, followed by incubation in complete medium for 48 h.

Cell-proliferation assay

5-bromo-2-deoxyuridine (BrdU) (Sigma) was added to culture media for 1 h at the concentration of 10 μ M. Cells were fixed and stained with anti-BrdU antibody (1:200) followed by anti-mouse FITC (Dako) and DAPI (Molecular Probes). The means of BrdU-positive cells were obtained from two independent triplicate experiments.

Western blot analysis

Western blotting was performed as described earlier (Major *et al*, 2004). Whole-cell extracts were prepared in lysis buffer consisting of 50 mM Tris-HCl pH7.5, 100 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol and freshly added 1 $\times$ complete mini protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail set II (Calbiochem). Rabbit polyclonal antibody against FoxM1 was described (Major *et al*, 2004). The following antibodies were also used: phospho-p38 (1:500 dil.; Cell Signaling Technology), phospho-JNK (1:500 dil.; Cell Signaling Technology), p38 (1:2000 dil.; Cell Signaling Technology), JNK (1:500 dil.; Cell Signaling Technology), phospho-AKT (1:500 dil.; Cell Signaling Technology), H-Ras (1:500 dil.; Santa Cruz Technology), catalase (1:1000 dil.; Sigma), PRDX3 (1:5000; Sigma), MnSOD (1:2000 dil.; Stressgen).

siRNA transfection

Two siRNA duplexes specific to human FoxM1 mRNA named siFoxM1#1 (CAACAGGAGUCUAAUCAAAG) and siFoxM1#2 (GGAC CACUUUCCUACUUU) containing 2-uracil (U) 3' overhangs were synthesized (Dharmacon Research). Human Aurora B siRNA duplex was purchased from Cell Signaling Technology. These siRNA duplexes (50 nM) were transfected using Lipofectamine 2000 reagent (Invitrogen) in serum-free media following manufacturer's protocol. Four hours after transfection, medium containing 10% FBS was added. Cells were collected 72 h after transfection to prepare total RNA, protein extracts, ROS measurement or senescence assay. Adenovirus expressing FoxM1 was a gift from Dr Guy Adami (UIC—College of Dentistry).

Measurement of intracellular ROS by FACS analysis

Cells were incubated with either 5 μ M DCF-DA (Molecular Probes) or 10 μ M DHE (Molecular Probes) for 30 min. Cells were then washed with PBS, trypsinized and immediately analysed by a flow cytometer (Bass *et al*, 1983).

SA- β -Gal staining

Cells were transfected with 50 nM FoxM1 siRNA. After 4 h transfection, cells were treated with 10 μ M p38 inhibitor (SB203580; Calbiochem) or 50 units/ml catalase (Sigma). SB203580 and catalase were freshly added every day. Cells were collected at 72 h after siRNA transfection. Cells were washed with PBS and fixed in 2% formaldehyde and 0.2% glutaraldehyde solution in PBS. Cells incubated overnight in staining solution containing 1 mg/ml X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM MgCl₂.

Colony formation assay

U2OS cells were transfected with 50 nM control or FoxM1 siRNA duplex using Lipofectamine 2000 (Invitrogen) as described above. The cells (1500 cells/plate) were seeded on to 6 cm plates. After 7–10 days, cells were fixed with methanol and stained with crystal violet.

Chromatin immunoprecipitation

ChIP assay were performed as described earlier (Wang *et al*, 2005). IMR90 cells were transfected with T7-FoxM1b. Forty-eight hours

after transfection, formaldehyde was added to the medium to a final concentration of 1%, and gently swirled the plate for 10 min to crosslink proteins and DNA. The crosslinking reaction was terminated by Glycine to a final concentration of 125 nM for 5 min. The cells were washed twice with ice-cold PBS and collected with cold PBS containing protease and phosphatase inhibitor. After centrifugation at 2000 r.p.m. for 10 min, the cells were re-suspended in 1 ml of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH8.1) and kept on ice for 1 h. The lysate was then sonicated using a Misonix 600 W Sonicator (Misonix Inc, six pulses of 20 s) at a power setting of 30%. For immunoprecipitation, anti-T7 antibody (Novagen) or same concentration of mouse IgG was incubated with the sonicated lysates at 4°C with rocking overnight. The immune complexes were collected by incubation with 30 μ l of salmon sperm DNA-saturated Protein A- and Protein G-sepharose (Upstate) for 1 h. The samples were washed and the crosslinks were reversed by addition of 100 μ l TE (1 mM EDTA, 10 mM Tris-HCl, pH7.4) containing 200 mM NaCl and 100 μ g/ml Proteinase K (Sigma) at 65°C overnight. The DNA was extracted using PCR purification kit (Qiagen) and 2.5 μ l of the purified DNA was used for real-time PCR. The following primers were used: catalase forward, 5'-GATGGGCGGATTCTTGAGG-3'; catalase reverse, 5'-AGTAGTTGGG ATTACAGGCGA-3'; MnSOD forward, 5'-TTGACACTGAGTCCCACT TGTGCG-3'; MnSOD reverse, 5'-GCTGAGGCAGAAGAATCGCTT-3'.

Real-Time PCR

Quantitative RT-PCR was performed as described earlier (Wang *et al*, 2005). Briefly, cells were collected at 72 h after transfection using Trizol reagent (Invitrogen). After DNaseI digestion, 1 μ g of total RNA was used to synthesize cDNA using Bio-Rad cDNA synthesis kit. Real-time PCR was performed using following mixture: 1 $\times$ IQ SybrGreen supermix (Bio-Rad), 100 nM of each primers and 1 μ l of cDNA in a 25 μ l total volume. Reactions were amplified and analysed in triplicate using a MyiQ single-colour real-time PCR detection system (Bio-Rad). The following primers were used: human FoxM1 forward, 5'-GGAGGAAATGCCACACTTAGCG-3'; FoxM1 reverse, 5'-TAGGACTTCTTGGGTCTTGGGGTG-3'; human catalase forward, 5'-TGATTACACTCCACGCTGGTGAG-3'; catalase reverse, 5'-CATAGATGCCCTCTGAGACTCTGC-3'; human MnSOD forward, 5'-GGCTTGTTTCAATAAGGAACGG-3'; MnSOD reverse, ATCCCCAGCAGTGAATAAGG-3'; human PRDX3 forward, 5'-GTT GTCGAGTCTCAGTGGAATC-3'; PRDX3 reverse, 5'-TTCTAACAGCA CACCGTAGTCTCG-3'.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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