FOXO Transcription Factors Enforce Cell Cycle Checkpoints and Promote Survival of Hematopoietic Cells after DNA Damage

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Abstract

The PI3K/AKT signaling pathway contributes to cell cycle progression of cytokine-dependent hematopoietic cells under normal conditions, and it is absolutely required to override DNA damage–induced cell cycle arrest checkpoints in these cells. Phosphatidylinositol-3-kinase (PI3K)/AKT activity also correlates with Cdk2 activity in hematopoietic cells, suggesting that Cdk2 activation may be a relevant end point for this signaling pathway. However, mediators downstream of AKT in this pathway have not been defined. The forhead transcription factor O (FOXO) family are negatively regulated by AKT-dependent phosphorylation and are known regulators of genes affecting cell cycle progression. We show that enhanced FOXO activity replicates the effect of PI3K inhibitors in enforcing G1 and G2 phase arrest after DNA damage. Conversely, knockdown of endogenous FOXO proteins increased Cdk2 activity and overrode DNA damage checkpoints in cells lacking PI3K activity. Moreover, loss of FOXO activity caused an increase in sensitivity to cisplatin–induced cell death, which was associated with failure to arrest cell cycle progression in the face of DNA damage caused by this chemotherapeutic agent. These cell cycle arrests were dependent on p27 expression when mediated by FOXO3a alone, but also involve p27-independent mechanisms when promoted by endogenous FOXO proteins. Together, these observations show that FOXO proteins enforce DNA damage–induced cell cycle arrest in hematopoietic cells. Inhibition of FOXO activity by cytokine–induced PI3K/AKT signaling is sufficient to override these DNA damage–induced cell cycle checkpoints, but may negatively impact hematopoietic cell viability. (Mol Cancer Res 2009;7(8):1294–303)

Introduction

Proliferation and survival of most normal hematopoietic cells is controlled by cytokine growth factors, which inhibit apoptosis and are required to promote transit through G1 phase of the cell cycle (1). Cells that have sustained DNA damage have additional impediments to proliferation that result in cell cycle arrest at G1 and G2 phase checkpoints (2). However, these growth arrest checkpoints can be overridden in hematopoietic cells by cytokines, which promote continued proliferation even in the face of DNA damage (3). This potent proliferative activity is clinically useful and cytokines, such as erythropoietin, are used to stimulate repopulation of specific hematopoietic lineages. However, forced proliferation of cells that have sustained irreparable DNA damage can cause cell death and enhance sensitivity to DNA damaging agents, such as cisplatin (4-6). Thus, depending on context, proliferative signals can have either positive or negative effects on hematopoietic lineage expansion.

Many cytokines, such as interleukin (IL)-3 and erythropoietin, act through type I cytokine receptors and regulate multiple signaling pathways with established links to cell cycle regulation, including the Jak/Stat pathway, the Ras pathway, and phosphoinositide 3-kinase (PI3K) signaling pathways (7). We have shown that PI3K signaling is required for erythropoietin and IL-3 to override G1 and G2 phase growth arrest checkpoints that are induced by DNA damage (4, 8-10). Similarly, PI3K or its downstream targets have been shown to regulate stress response checkpoints in a variety of cell types (11-16), and cytokine-induced PI3K activity contributes to efficient proliferation of hematopoietic cells in the absence of DNA damage (17-19). Thus, this signaling pathway has significant effects on hematopoietic cell proliferation under normal conditions and in cells with damaged DNA. How PI3K signaling overrides DNA damage–induced cell cycle arrest in hematopoietic cells has not been determined, but we have consistently associated the lack of this activity with a failure to activate Cdk2 complexes (4, 9, 10, 19), suggesting that Cdk2 activation is an ultimate target of this signaling pathway.

PI3K activity produces phosphatidylinositol-3,4,5-trisphosphates at the plasma membrane, which act as activation sites for a variety of signaling proteins, including the serine/threonine kinases AKT (20). Importantly, AKT activity is also required for IL-3 to override DNA damage checkpoints in hematopoietic cells (8). Thus, PI3K-dependent control of hematopoietic cell cycle progression is likely regulated through phosphorylation targets of AKT (21). One such target is the Forkhead O (FOXO) family of transcription factors, which
are inactivated and exported from the nucleus following phosphorylation by AKT (22). The mammalian FOXO family includes three members (FOXO1, FOXO3a, and FOXO4) that are expressed in hematopoietic cell lineages and which regulate expression of proteins affecting apoptosis and survival, such as Bim and MnSOD (23–25), as well as proteins controlling cell cycle progression, such as p27 (23, 26, 27), p130 (28), cyclin G2 (29), and Gadd45 (12). Moreover, lacking all three FOXO proteins display an increased cellularity in hematopoietic compartments (30), suggesting a potential role in restricting proliferation in these cell types. Thus, inhibition of FOXO proteins may be a likely target of the cytokine-induced PI3K/AKT signaling pathway that promotes proliferation and overrides DNA damage checkpoints in hematopoietic cells.

In the present study, we have investigated the potential role of FOXO proteins as a target of PI3K/AKT in the pathway leading to Cdk2 activation and the ability to override DNA damage–induced arrest checkpoints in hematopoietic cells. We show that FOXO activity is sufficient to enforce these checkpoints and to inhibit Cdk2 activity. Moreover, loss of endogenous FOXO activity reduces the requirement for PI3K/AKT to override DNA damage checkpoints and maintain normal proliferation. Finally, we show that the failure of cell cycle checkpoints resulting from loss of FOXO activity leads to increased death of hematopoietic cells exposed to cisplatin.

Results
Enhanced FOXO Activity and PI3K Inhibition Have Similar Effects on Hematopoietic Cell Cycle Progression

To examine the role of FOXO activity in the PI3K/AKT signaling pathway that overrides DNA damage–induced checkpoints in hematopoietic cells, we prepared a tamoxifen-inducible FOXO3a-ER fusion constructs similar to those previously described (12). IL-3–dependent 32D cells were used to establish stable cell lines expressing similar levels of the myc-tagged FOXO3a-ER construct or a myc-ER control construct (Fig. 1A). Under normal culture conditions, there were no significant differences in the cell cycle distribution between FOXO3a-ER and control cells (Fig. 1B and C, −IR), suggesting that the FOXO3a-ER fusion proteins did not affect proliferation when localized to the cytoplasm in the absence of tamoxifen. By contrast, treatment with 4-hydroxy-tamoxifen (4OHT) to induce nuclear localization of estrogen receptor (ER) fusion proteins reduced the S phase content of the FOXO3a cells but did not affect control cells, indicating that the effects of 4OHT on proliferation were dependent on the FOXO3a activities of the FOXO3a-ER fusion construct. Interestingly, the reduction in S phase content produced by FOXO3a-ER activation was quantitatively and qualitatively similar to the effect produced by the PI3K inhibitor, LY294002. Consistent with our previous results (8), PI3K inhibition produced a more dramatic cell cycle arrest in cells that sustained DNA damage through γ-irradiation, as indicated by the accumulation of cells in G1 and G2-M phases (Fig. 1B, +IR). A similar arrest was also produced by 4OHT treatment of FOXO3a, but not control cells (Fig. 1B and C, +IR). Similar effects of FOXO3a activation on cell cycle progression were obtained when BrdUrd incorporation was used to directly assess S phase content in cultured cell populations (Fig. 1D). We also assessed the activity of the FOXO3a-ER construct when transiently expressed by retroviral infection of IL-3–dependent BaF3 cells (Supplementary Fig. S1). In these experiments, 4OHT-induced activation of FOXO3a-ER reduced S phase content and promoted G1 and G2-M phase arrest of infected cells after γ-irradiation, recapitulating the FOXO3a-dependent effects observed in the stable 32D cells lines. Together, these observations show that increased FOXO3a activity can mimic the effect of PI3K inhibition in hematopoietic cells in normal culture or following DNA damage, and suggest that inhibition of FOXO3a is a target of PI3K/AKT signaling in hematopoietic cells.

To determine if FOXO3a activity and PI3K inhibition have similar effects on molecular regulators of cell cycle progression, we assessed their effects on Cdk2 activity (Fig. 1E). Consistent with previous results (9, 10), LY294002 inhibited Cdk2 activity in irradiated cells. A similar inhibition of Cdk2 activity resulted from 4OHT treatment of FOXO3a-ER cells, whereas 4OHT treatment had no detectable effect on Cdk2 activity in control cells.

Together, these observations show that increased FOXO3a activity inhibits proliferation and enhances DNA damage–induced cell cycle arrest in IL-3–dependent hematopoietic cells, suggesting that PI3K inhibitors may enforce these checkpoints by protecting endogenous FOXO activity. Such a relationship predicts that enhanced FOXO activity would cooperate with PI3K inhibition to reduce proliferation. Indeed, 4OHT treatment of irradiated cells potentiated the cell cycle arrest induced by submaximal doses of LY294002 (Fig. 2A). Similarly, 4OHT enhanced the dose-dependent arrest of γ-irradiated FOXO3a-ER cells (Fig. 2B). Although 4OHT decreased the S phase content of FOXO3a-ER cells under all treatment conditions, it did not alter the dose response to γ-irradiation, suggesting that molecular mechanisms initiating the DNA damage response were not affected by FOXO activity. These data indicate that activation of FOXO3a and inhibition of PI3K cooperate to restrain proliferation, and are consistent with their involvement in the same pathway responsible for overriding DNA damage–induced arrest checkpoints.

Endogenous FOXO Proteins Are Required to Enforce DNA Damage Checkpoints

If the effects associated with FOXO3a-ER activation reflect activities of endogenous FOXO proteins, the effects of PI3K inhibition should be dependent on the level of endogenous FOXO activity in hematopoietic cells. There are no completely conserved 19 bp sequences shared by all murine FOXO family members, but one was identified that is shared by FOXO1 and FOXO3a. One mismatch within this sequence should prevent effective knockdown of FOXO4 by a corresponding short hairpin RNA (shRNA), but knockdown of the majority of FOXO proteins may allow a reduction in total FOXO activity sufficient to assay its requirement in reducing proliferation in the absence of PI3K activity. Stable expression of this shRNA in 32D cells produced several clones exhibiting reduced levels of FOXO1 and FOXO3a without significantly altering endogenous levels of FOXO4 expression (Fig. 3A). Knockdown of FOXO protein did not alter IL-3–dependent proliferation of 32D cells under normal culture conditions, or the ability...
of IL-3 to override γ-irradiation–induced growth arrest (Fig. 3B and D). However, FOXO-knockdown (FOXOkd) cell lines treated with LY294002 exhibited a significantly smaller reduction in S phase content (Fig. 3B and C) and proliferated at a faster rate (Fig. 3D) than did cells expressing a control shRNA. Moreover, FOXOkd cells were significantly less arrested by γ-irradiation in the presence of LY294002 (Fig. 3B and C). In addition to the reduced arrest at early time points (24-48 hours) after γ-irradiation and PI3K inhibition, FOXOkd cells also exhibited an increasing level of S phase content at later time points (Fig. 4A and B). By contrast, control cells remained in G1 and G2-M phase arrest through 6 days after irradiation in the absence of PI3K activity. Consistent with this long-term arrest, LY294002-treated control cells retained little Cdk2 activity after irradiation (Fig. 4C and D). Although PI3K-inhibition continued to reduce Cdk2 activity in irradiated FOXOkd cells, these cells retained 4-fold more activity than was observed in control cells. These data indicate that continued high levels of endogenous FOXO activity are required to enforce the inhibition of Cdk2 and long-term arrest of hematopoietic cells after DNA damage, suggesting that PI3K/AKT signaling overrides these checkpoints by inhibiting FOXO activity.

**FIGURE 1.** Enhanced FOXO activity and PI3K inhibition have similar effects on cell cycle regulation. A. Equal numbers of 32D cell clones stably expressing a myc-ER vector control (control) or a myc-FOXO3a-ER (FOXO3a) construct were lysed and Western blotted with myc antibodies. Panels showing the migratory positions of myc-ER and myc-FOXO3a-ER were taken from the same exposure of the blot. B. 32D cell clones shown in A were suspended in media with IL-3 (1.4 ng/mL) and were divided into parallel cultures supplemented with 4OHT (1 μmol/L), LY294002 (LY; 10 μmol/L), or without additional supplements (−). Parallel cultures were then treated with (+γIR) or without (−γIR) 4Gy γ-irradiation. Twenty-four hours after irradiation, cells from each culture were assayed for DNA content by PI staining and flow cytometry. Histograms represent at least 3 × 10^6 events with the percentage of cells in each cell cycle phase indicated. C. S phase percentages obtained as described for B were averaged from at least three experiments involving two independent control and two independent FOXO3a-ER cell lines. Columns, mean; bars, SE. 4OHT treatment did not significantly effect control cultures (P > 0.05), but 4OHT significantly reduced S-phase content in FOXO3a-ER clones compared with controls (P < 0.005). D. Cells treated as described for B were pulsed with BrdUrd (1 μmol/L; 30 min), and were stained with BrdUrd antibodies and PI before analysis by flow cytometry. The percentage of S-phase cells was determined from the gate shown. E. Vector control (control) or myc-FOXO3a-ER cell lines (FOXO3a) were cultured in IL-3 and exposed to 4Gy γ-irradiation in the presence or absence of LY294002 or 4OHT, as described in B. Cells were lysed and subjected to a Cdk2 kinase assay 24 h after γ-irradiation.
was determined by PI staining and flow cytometry. Twenty-four hours after irradiation, the S phase content of each culture was determined by PI staining and flow cytometry.

FIGURE 2. FOXO3α potentiates the growth arrest induced by PI3K inhibition and γ-irradiation. A, 32D cell lines expressing the myc-ER control construct (control) or myc-FOXO3α-ER (FOXO3α) were suspended in media containing IL-3 (1.4 ng/mL) alone or supplemented with 4OHT (+4OHT; 1 μmol/L). Parallel cultures were also supplemented with varying concentrations of LY294002, and all cultures were treated with 4 Gy γ-irradiation. Twenty-four hours after irradiation, the S phase content of each culture was determined by PI staining and flow cytometry. B, Control and FOXO3α cell lines used in A were suspended in media containing IL-3 (1.4 ng/mL) with or without 4OHT (1 μmol/L). Parallel cultures were treated with various dosages of γ-irradiation and cultured for 24 h, after which the S phase content of each culture was determined by PI staining and flow cytometry.

Although proliferation of FOXOkd cells did not differ from control cells in the absence of PI3K-inhibition and DNA damage (Fig. 3B and D), the FOXOkd cell lines did retain modestly higher levels of S phase at later time points after irradiation even without PI3K-inhibition (Fig. 4B, γIR). This suggests that even fully active cytokine-induced PI3K/AKT signaling is not sufficient to completely prevent FOXO-dependent inhibition of proliferation in DNA-damaged hematopoietic cells.

FOXO Activity Promotes Resistance to Cisplatin-Induced Death in Hematopoietic Cells

Previously, we have shown that cytokines also override growth arrest checkpoints induced by cisplatin (4). In contrast to the continued proliferation of γ-irradiated cells, overriding checkpoints after cisplatin-induced DNA damage caused hematopoietic cells to accumulate in G2 phase and subsequently die by a nonapoptotic mechanism. However, inhibition of PI3K enforced a G1 phase arrest and was shown to preserve hematopoietic cell viability after cisplatin treatment. To assess the role of FOXO proteins in this response, control and FOXOkd clones were exposed to cisplatin for 24 hours while being maintained in the presence or absence of LY294002 (Fig. 5A and B). Consistent with our previous results, inhibition of PI3K increased the G1 phase arrest and overall viability of control cells. By contrast, FOXOkd clones displayed an increased sensitivity to cisplatin treatment and a reduced ability to be rescued by PI3K-inhibition. Indeed, although removal of LY294002 after cisplatin-treatment allowed the resumption of proliferation and recovery of reasonably viable populations of control cells, few viable cells were identifiable after identical treatment of FOXOkd clones (Fig. 5C). Thus, reduced FOXO expression increased the overall sensitivity of 32D cells to cisplatin-induced death and prevented the rescue of these cells by inhibition of PI3K, suggesting that endogenous FOXO proteins are important mediators of these effects in hematopoietic cells.

FOXO-Dependent Effects on Mediators of Proliferation and Survival

The ability of active FOXO proteins to enforce cell cycle arrest and preserve viability in DNA-damaged cells suggests that targets of FOXO may alter the activity of cell cycle regulators, or effectors of cell death. As shown in Fig. 6A, 4OHT-induced activation of FOXO3α-ER significantly increased expression of p27 and Bim and this activity was modestly enhanced in cells cotreated with cisplatin or γ-irradiation, suggesting that the presence of DNA damage may affect the activity of the FOXO3α-ER fusion protein. Because expression of p27 protein can be regulated through diverse mechanisms, we also assessed the effect of FOXO3α activation on expression of p27 mRNA. Consistent with its potential activity as a transcription factor promoting p27 expression, 4OHT-induced activation of FOXO3α-ER significantly increased expression of this mRNA (3.7-fold increase, \(P = 0.0003\); Fig. 6B). Expression of Bim and p27 proteins were also enhanced by PI3K inhibition after γ-irradiation of 32D control cells and this effect was reduced in FOXOkd clones (Fig. 6C and D). Although p27 and Bim seemed to be generally induced by activation of FOXO3α-ER or endogenous FOXO proteins (activated through PI3K inhibition) in DNA-damaged 32D cells, we were not able to detect expression of some potential FOXO targets, such as Gadd45 (data not shown). Another potential target, MnSOD, was not detectably altered by activation of the FOXO3α-ER fusion protein (Fig. 6A). However, MnSOD expression was increased by PI3K-inhibition after γ-irradiation, and this effect was reduced in FOXOkd cell lines (Fig. 6C and D). Therefore, some but not all reported targets of FOXO-mediated expression were consistently regulated by PI3K signaling and inducible FOXO activity in settings where PI3K signaling and inhibited FOXO activity override arrest checkpoints and reduce survival after exposure to DNA damaging agents.

Because cell cycle arrest is a prominent feature associated with increased FOXO activity in DNA-damaged hematopoietic cells, we tested if this effect was mediated through FOXO-dependent expression of p27. Stable expression of a p27
shRNA in 32D-FOXO3a-ER cells produced several clones that maintained expression of the myc-FOXO3a-ER transgene, but lacked 4OHT-induced expression of p27 protein (Fig. 7A and B). Importantly, 4OHT-induced Bim expression was not noticeably altered in these p27 knockdown (p27kd) clones (Fig. 7A), indicating that the FOXO3a-ER transgene remained functional. Knockdown of p27 did not significantly alter the proliferation of cells under normal culture conditions where the FOXO3a-ER transgene was not activated (Fig. 7C and D). However, loss of p27 expression resulted in a near complete abrogation of 4OHT effects promoting cell cycle arrest in irradiated cells and reducing proliferation in nonirradiated cells. Therefore, these effects of FOXO3a activation seem to depend on its ability to promote p27 expression. Knockdown of p27 also prevented p27 protein expression induced by LY294002 treatment (Fig. 7B). Loss of p27 expression was associated with a modest but reproducible increase in S phase content (Fig. 7D) and a corresponding reduction in G1 phase (Fig. 7E) after irradiation and LY294002 treatment, suggesting a reduced efficiency of the DNA damage–induced G1 phase arrest. Nonetheless, LY294002 treatment of irradiated p27kd cell lines produce a G2–M phase accumulation that did not differ significantly from those observed in 32D cell clones retaining normal p27 expression. Therefore, a knockdown of p27 that is sufficient to abrogate FOXO3a-specific effects in 32D cells had only minor effects on the cell cycle arrests that depend on endogenous FOXO proteins, suggesting that other targets of these transcription factors can be sufficient to enforce DNA damage arrest checkpoints in hematopoietic cells.

Discussion

In this study, we have identified the FOXO family of transcription factors as a target of PI3K/AKT signaling effecting Cdk2 activation and overriding DNA damage–induced cell cycle checkpoints in cytokine-dependent hematopoietic cells. This was shown by the ability of overexpressed FOXO3a to prevent PI3K/AKT signaling from overrides DNA damage–induced cell cycle arrest (Fig. 1), whereas knockdown of endogenous FOXO proteins overrode this arrest in the absence of PI3K/AKT activity (Figs. 3 and 4). Therefore, continued FOXO activity is required to maintain DNA damage–induced arrest, which can be overridden by mechanisms that inhibit FOXO activity, such as AKT phosphorylation of FOXO proteins.

We have previously reported that levels of phosphorylated p53 and expressed p21 protein remain high in hematopoietic cells that override arrest checkpoints and continue to proliferate after DNA damage (9, 10), suggesting that overriding these checkpoints through PI3K/AKT-dependent inactivation of FOXO proteins does not suppress the activities of DNA damage response mediators. If FOXO activity does not enforce arrest checkpoints by promoting the response to DNA damage, it may do so by directly regulating the expression of genes controlling cell cycle progression. We have previously found that the status of PI3K signaling correlates with the activity of Cdk2 in DNA-damaged hematopoietic cells (4, 9, 10). Here, we found a reciprocal relationship between FOXO and Cdk2 activities, where activation of FOXO3a-ER was associated with inactive Cdk2 (Fig. 1) and knockdown of endogenous FOXO

![FIGURE3.](image-url)  
Knockdown of endogenous FOXO proteins in hematopoietic cell lines. A. Parental 32D cells, or subclones transfected with plasmid expressing a FOXO family shRNA (FOXOkd) or a control shRNA (control), were screened for FOXO protein expression by Western blotting with specific antibodies. Two representative control and FOXOkd clones are shown. Antibodies to γ-tubulin were used to assess loading. B. Control and FOXOkd cell lines were suspended in media with IL-3 (1.4 ng/mL) and divided into parallel cultures supplemented with LY294002 (LY; 10 μmol/L) or without additional supplements (−). Parallel cultures were then treated with (+IR) or without (−IR) 4 Gy γ-irradiation. Twenty-four hours after irradiation, cells from each culture were assayed for DNA content by PI staining and flow cytometry. Histograms represent at least 3 × 10^6 events with the percentage of cells in each cell cycle phase indicated. C. S phase percentages obtained as described for B were used to compute the relative reduction in S phase induced by LY294002 treatment (S+/IR/S−IR). These values were averaged from at least three independent FOXOkd and control cell lines. Columns, mean; bars, SE. LY294002-induced reductions in S phase for control and FOXOkd cell lines were significantly different (P < 0.001). D. Doubling times for exponentially growing cultures of control and FOXOkd cell lines in media with 1.4 ng/mL IL-3 (−LY) or in IL-3 media supplemented with 10 μmol/L LY294002 (LY) were calculated from the fold increase in cell number over a 24-h period [Doubling time = 24 *Log(2)/Log(fold-increase in cell number)]. Values shown are averaged from at least three independent cell lines. Doubling times for control and FOXOkd cell lines were significantly different in the presence of LY294002 (P < 0.0001), but were not significantly different in the absence of LY (P = 0.32).
proteins prevented complete inactivation of Cdk2 by PI3K inhibitors (Fig. 4). This suggests that inhibition of Cdk2 is a target of FOXO activities that enforce G1 and G2-M phase arrest after DNA damage. A potential mediator of this effect is p27, a Cdk inhibitor that has been reported to reduce survival and proliferation in hematopoietic cells in the absence of DNA damage (27, 28). We show that p27 expression is enhanced in DNA-damaged cells where FOXO activity is not fully controlled by PI3K signaling (Fig. 6), and that knockdown of p27 expression largely eliminates the ability of activated FOXO3a to reduce proliferation and enforce DNA damage arrest checkpoints in hematopoietic cells (Fig. 7). Therefore, FOXO-induced p27 expression can produce all of the effects associated with PI3K inhibition in irradiated hematopoietic cells. Nonetheless, loss of p27 expression was not sufficient to significantly attenuate the arrest checkpoints that are enforced by endogenous FOXO proteins in 32D cells, suggesting that other targets of FOXO-mediated transcription also have the potential to inhibit cell cycle progression. Therefore, the individual or combined roles of other FOXO targets will need to be assessed before the mediator(s) of FOXO-dependent arrest in DNA-damaged hematopoietic cells can be clearly defined. The distinction between the p27-dependent arrest induced by activated FOXO3a-ER and the lack of p27 dependence for the arrests enforced by PI3K inhibition also suggests that the individual FOXO proteins may make distinct contributions toward promoting these arrests. Indeed, our experiments linking reduced FOXO activity with reduced arrest after DNA damage (Figs. 3 and 4) were made in cell with targeted knockdown of both FOXO1 and FOXO3a, suggesting the possibility that p27-independent arrests may be mediated by FOXO1 compared with the p27-dependent arrest mediated by activated FOXO3a. In this regard, we also observed differences in protein expression associated with targeting different FOXO proteins. Specifically, knockdown of both FOXO1 and FOXO3a nearly eliminated MnSOD expression induced by PI3K inhibition (Fig. 6C), whereas FOXO3a-specific activation did not noticeably effect MnSOD expression (Fig. 6A), suggesting that MnSOD expression may be a more specific target of FOXO1 in 32D cells. Whether this or other distinctions between FOXO1 and FOXO3a activities produce the p27-independent arrest of DNA-damaged hematopoietic cells remains to be determined. Because knockdown of both FOXO1 and FOXO3a did not completely eliminate the arrest produced by PI3K inhibition in DNA-damaged cells, it is also unclear at present if FOXO4 might make unique or redundant contributions to these arrests.

FOXO activity has previously been shown to promote death and inhibit cell cycle progression in hematopoietic cells withdrawn from cytokine (24, 27, 28). Similarly, we found that FOXO activity reduced cytokine-dependent proliferation (Fig. 1) whereas reduced FOXO expression protected cells from the effects of PI3K inhibitors (Fig. 3). However, enhanced proliferation was only observed in FOXO-kd cells when PI3K activity was inhibited. Bone marrow cells from FOXO-deficiency mice also do not display alterations in their primary growth characteristics, despite the expansion of myeloid cell lineages in these mice (30). These observations suggest that cytokine-induced PI3K signaling may normally keep FOXO activity under control, but that cells...
deficient in FOXO activity might have a proliferative advantage in settings where PI3K signaling is absent or insufficiently antagonistic. The presence of DNA damage may represent such a circumstance, because knockdown of endogenous FOXO proteins seemed to enhance long-term proliferation of irradiated cells even in the face of unimpeded PI3K/AKT signaling (Fig. 4B).

Although FOXO activity seems to restrict proliferation of normal and DNA-damaged hematopoietic cell, the nature of this activity is clearly altered by DNA damage. Specifically, FOXO activity produced a modest reduction in S phase and accumulation in G1 phase in normal cells compared with the stable G1 and G2-M phase arrests induced after DNA damage (Fig. 1). Also, FOXO3a-induced expression of p27 and Bim proteins was enhanced in cells that also sustained DNA damaging (Fig. 6A). The fact that these differing effects resulted from equivalent 4OH-induced activation of the FOXO3a-ER fusion protein suggests that FOXO activity is differently regulated or has different consequences in the presence or absence of DNA damage. Indeed, FOXO-regulated genes differ significantly between cell types (31), and FOXO subcellular localization and acetylation can alter its transcriptional activity within a cell during responses to cellular stress (32-38). Such context-specific regulation could explain differing effects of FOXO activity in DNA-damaged and normal hematopoietic cells. However, mechanisms effecting expression or nuclear localization would seem unlikely to affect the activity of 4OH-induced FOXO3a-ER and differences in FOXO acetylation have not been reproducibly observed in conjunction with irradiation-induced DNA damage (36, 37). Alternatively, FOXO gene targets, such as p27 and gadd45, are also regulated through posttranscriptional mechanisms, some of which can be affected by DNA damage (39). So, even invariant FOXO activity could potentially produce different cellular effects if its transcriptional targets are differently regulated in response to DNA damage. Nonetheless, our findings indicate that inhibition of these FOXO activities is a primary target of cytokine-induced PI3K/AKT signaling in hematopoietic cells under both normal conditions and in the context of DNA damage.

Although context affects the response to FOXO activation, the consequences resulting from reduced FOXO activity also differ with circumstance. This is evident in the enhanced proliferation of FOXOkd cell lines after γ-irradiation (Fig. 4) compared with the increased sensitivity of these cells to cisplatin-induced death (Fig. 5). The increased death in FOXOkd cells occurred despite a reduced ability to promote Bim expression when PI3K signaling was inhibited (Fig. 6), suggesting that the effect may not be directly related to the activity of apoptotic pathways. Indeed, these effects of FOXO activity are consistent with our previous assessment of PI3K activity in DNA-damaged hematopoietic cells, where PI3K signaling increased non-apoptotic death of hematopoietic cells exposed to cisplatin (4). By contrast, PI3K signaling overrode cell cycle arrest without dramatically reducing survival in γ-irradiated cell populations (8, 9). Therefore, the ability of FOXO activity to promote survival in DNA-damaged hematopoietic cells seems to be more specific to cisplatin treatment compared with γ-irradiation. We have shown that inhibition of PI3K signaling can enhance the survival of a fraction of hematopoietic cells exposed to γ-irradiation, but this occurs only in those cells that initially arrest in G2 phase after irradiation, whereas survival was not affected in cell that initially arrested in G1 phase (10). Interestingly, hematopoietic cells treated with cisplatin also accumulate in a G2 phase arrest and subsequently die when PI3K signaling overrides arrest checkpoints (4). Thus, it is possible that forms of DNA damage that promote more dominant G2 phase arrests are more toxic to hematopoietic cells when the checkpoints

FIGURE 5. FOXOkd enhances sensitivity to cisplatin and prevents protection by PI3K inhibition. A, 32D shRNA control (control) and FOXOkd cell lines were cultured in media supplemented with 1.4 ng/mL IL-3 or IL-3 media supplemented with 10 μmol/L LY294002 (IL-3+LY). All cultures were then treated with cisplatin (5 μmol/L). After 24 h, cisplatin was removed and cultures were maintained in their original media formulation (IL-3 or IL-3+LY) for an additional 24 h. DNA content of each sample was assessed by PI staining and flow cytometry. Viability of each culture was assessed by trypan blue exclusion and is indicated in the histogram (%v). B, Viabilities were averaged from five experiments similar to A. Columns, mean; bars, SE. Viabilities were significantly reduced (P < 0.01) in FOXOkd cells compared with controls. C, Cultures from the IL-3+LY arm shown in A were washed to remove LY294002 and were returned to culture in media supplemented with IL-3 (1.4 ng/mL). After 96 h, DNA content and viability were assessed as in B.
are overridden. Alternatively, overriding a G2 phase arrest may be less sustainable than overriding G1 phase arrests in DNA-damaged hematopoietic cells. Regardless of the specific mechanism leading to cell death, activity of the PI3K/AKT/FOXO signaling pathway significantly influences the fate of hematopoietic cells after DNA damage. Therefore, the state of this pathway in patients treated with radiation therapies or chemotherapies may have bearing on their efficacy and/or toxicity toward hematopoietic cell lineages.

Materials and Methods

Expression Constructs

A tamoxifen inducible vector, MSCV-myc-ER-IRES-GFP, was derived from the MSCV-IRES-GFP retroviral vector (40) by inserting a myc-encoding oligo (AATTATGGCGGAA-CAAAAACTCATCTCAGAAGAGGATCTGAATTCAGGCTAGATCTAATT) at the EcoRI site, and the ligand binding domain of a mutant ER at the BglII site. This mutant ER domain (41) lacks transcriptional activity and does not bind estrogen, but is induced to translocate from the cytoplasm to the nucleus when bound by 4OHT. A cDNA inserted in-frame between the myc-epitope and ER coding sequence will be expressed as a myc-tagged fusion protein with the 4OHT-inducible ER domain. Wild-type human FOXO3a (12) was amplified by PCR from plasmid constructs (kindly provided by Michael Greenberg, Harvard Medical School, Boston, MA) and cloned into the StuI site of MSCV-myc-ER-IRES-GFP. shRNA expression constructs were prepared in pSUPER.retro.neo (Oligoengine) as recommended by the manufacturer. Constructs were prepared containing a sequence conserved in murine FOXO1 and FOXO3a (GTGCCCTACTTCAAGGA-ATA), or a sequence specific to murine p27 (GGGCCAACA-GAAGAAGA). A control construct contained a sequence not present in any known murine RNA (GTTGACGGCA-GAAGTTGTG).

Cell Lines and Culture Conditions

32D murine myeloid cells and BaF3 murine pre-B-cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L glutamine, and recombinant murine IL-3 (1.4 ng/mL). In certain experiments, a PI3K inhibitor (LY294002; Calbiochem), an ER agonist (4OHT; Sigma-Aldrich), or cisplatin (Calbiochem) were added. Cells exposed to γ-irradiation received a single calibrated dose from a 137Cs source. All cultures were incubated at 37°C with 5% CO2.

Stable expression of plasmid constructs in 32D cells was accomplished by electroporation (Bio-Rad Gene Pulser II) of pSuper.retro.neo plasmids, or coelectroporation of MSCV constructs with pSV2-neo. Electroporated cells were selected by culture in 1 mg/mL G418 (Calbiochem), and multiple clonal lines were obtained by limiting dilution.
**Cell Cycle Analysis**

Cells were suspended in propidium iodide staining solution (0.05 mg/mL propidium iodide, 0.1% sodium citrate, 0.1% triton X-100, 1 μg/mL RNAase) and incubated in the dark for 30 min at room temperature before analysis on a Becton Dickinson FACS-can. At least $1 \times 10^4$ events were recorded for each sample. The percentage of cells in each phase of the cell cycle was determined by ModFit Cell Cycle Analysis Software (Verity).

**Western Blot Analysis**

Cells were lysed directly in sample buffer [125 mmol/L Tris (pH 6.8), 10% glycerol, 5% 2-mecaptoethanol, 2% SDS, 0.2 mg/mL bromophenol blue] and were resolved (1 x $10^6$ cell equivalents per lane) by SDS-PAGE. Proteins were transferred to nitrocellulose membranes, probed with appropriate antibodies, and visualized by enhanced chemiluminescence (Amersham) or the Odyssey IR imaging system (LI-COR Biosciences). Antibodies used included those specific for the myc epitope (Novus Biologicals), p27 (Santa Cruz Biotechnology), Bim (Affinity BioReagents), MnSOD (University of Iowa Radiation and Free Radical Research Core), and γ-tubulin (Sigma). Antibodies to FOXO1, FOXO3a, and FOXO4 were from Cell Signaling Technology.

**FIGURE 7.** FOXO-dependent arrest of DNA-damaged 32D cells is mediated through p27-dependent and p27-independent mechanisms. 

A. myc-FOXO3a-ER expressing 32D cells (FOXO3a) or subclones stably expressing a p27 shRNA (FOXO3a/p27kd) were treated with or without 1 μm/40 HT for 24 h, after which total cell lysates were Western blotted with the specified antibodies. Two representative FOXO3a/p27kd clones are shown. B. Parallel cultures of FOXO3a or FOXO3a/p27kd cells were treated as indicated with 4Gy γ-irradiation (γIR), 10 μmol/L LY294002 (LY), or 40 HT. After 24 h, total cell lysates were Western blotted with antibodies to p27 or γ-tubulin. C. Cells treated as described for B were assayed for DNA content by PI staining and flow cytometry. The percentage of cells in each cell cycle phase is indicated for each histogram. D. S phase percentages obtained as described for C were averaged from multiple experiments involving two parental FOXO3a clones and nine FOXO3a/p27kd subclones. Columns, mean; bars, SE. 40 HT treatment did not significantly change S phase content in FOXO3a/p27kd cultures (P > 0.2), whereas S phase was significantly reduced in 40 HT-treated FOXO3a cells (P < 0.001). E. The average G1 and G2-M phase content are graphed for cells treated with LY294002 and 40 Gy γ-irradiation, as described for C.
Cdk2 Kinase Assay

Cells were lysed [0.5% IGPAL, 10% glycerol, 50 mmol/L Tris (pH 8.0), 0.1 mmol/L EDTA, 150 mmol/L NaCl, 0.1 mmol/L NaN3 VO4, 50 mmol/L NaF, 1 mmol/L DTT and 50 μg/mL phenylmethylsulfonyl fluoride] at 4°C, cleared of insoluble material (10,000 × g for 15 min), and were normalized for protein content using the BCA protein assay (Pierce). Cdk2 complexes were precipitated with agarose-conjugated anti-Cdk2 (Santa Cruz Biotechnology) and were suspended in kinase buffer [50 mmol/L HEPES (pH 8.0), 10 mmol/L MgCl2] plus 0.1 pmol ATP, 10 μCi [γ-32P] ATP (MP Biomedical), and 10 μg histone H1 (Roche). After 30 min at 30°C, reactions were stopped by addition of 5× sample buffer and were resolved by SDS-PAGE. Reaction products were visualized on a PhosphorImager (Molecular Dynamics).

Statistical Analysis

Comparisons between cell lines and responses to different treatments were evaluated using a Student’s t test. Significant differences were indicated by P values of <0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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