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A New Fork for Clinical Application: Targeting Forkhead Transcription Factors in Cancer

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Abstract

Forkhead O transcription factors (FOXO) play a pivotal role in the regulation of a myriad of cellular functions including cell cycle arrest, cell death, and protection from stress stimuli. Activation of cell survival pathways such as phosphoinositide-3-kinase/AKT/IKK or RAS/mitogen-activated protein kinase are known to phosphorylate FOXOs at different sites which cause FOXOs nuclear exclusion and degradation, resulting in the suppression of FOXO's transcriptional activity. Perturbation of FOXO's function leads to deregulated cell proliferation and accumulation of DNA damage, resulting in diseases such as cancer. Emerging evidence shows that active FOXO proteins are crucial for keeping cells in check; and inactivation of FOXO proteins is associated with tumorigenesis, including breast cancer, prostate cancer, glioblastoma, rhabdomyosarcoma, and leukemia. Moreover, clinically used drugs like paclitaxel, imatinib, and doxorubicin have been shown to achieve their therapeutic effects through activation of FOXO3a and FOXO3a targets. In this review, we will focus the novel functions of FOXOs revealed in recent studies and further highlight FOXOs as new therapeutic targets in a broad spectrum of cancers.

Background

Forkhead proteins, the transcription factors of winged-helix domain, are characterized by a conserved DNA-binding domain—the forkhead box among invertebrate and mammalian cells (1-3). Based on the forkhead box domain, the fork-head genes are grouped into 19 subclasses of *FOX* genes (4). Forkhead O transcription factors (FOXO) is one of the largest subgroups of forkhead family members. The FOXO sub-family contains four members (FOXO1, FOXO3, FOXO4, and FOXO6), which activate or repress multiple genes such as Bim and FasL involved in apoptosis (5,6), p27kip (7) and cyclin D (8) in cell cycle regulation, GADD45a in DNA damage repair (2,5,6,9), manganese superoxide dismutase in stress response (10), and glucose-6-phosphatase in metabolism (11).

FOXO3a overexpression has been shown to inhibit tumor growth *in vitro* and tumor size *in vivo* in breast cancer cells (12,13). Furthermore, cytoplasmic location of FOXO3a seems to correlate with poor survival in patients with breast cancer (12). In addition, genetic deletion of five FOXOs alleles (FOXO1, FOXO3 and FOXO4) gives modest neoplastic phenotypes, whereas deletion of all of the FOXOs alleles generates progressive cancerous phenotypes, such as thymic lymphomas and hemangiomas. These data elucidated FOXOs as bona fide tumor

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suppressor genes (14). Recent studies also reveal the importance of FOXOs in preserving the self-renewal capacity of hematopoietic stem cells (15,16), however, the details of these mechanisms are currently under work in progress.

Signals Regulating FOXO Activation and Destruction

Growth factors have been shown to down-regulate the expression and activity of FOXOs. During tumor development, the inhibition of FOXO3a's transcriptional activity promotes cell transformation, tumor progression, and angiogenesis (1,2,12,17). FOXOs are regulated by a broad variety of external stimuli, such as epidermal growth factor receptors (EGFR), insulin, insulin-like growth factor, neurotrophins, nutrients, cytokines, and oxidative stress. These stimuli control FOXO protein expression, subcellular localization, or DNA binding and transcriptional activity. FOXO proteins are primarily regulated through posttranslational modifications, including phosphorylation, acetylation, ubiquitination, and possibly other modifications yet to be identified.

Many kinase pathways have been identified to activate FOXO activity, including the stress-activated c-*Jun*-NH₂-kinase (JNK; refs. 18,19), the mammalian orthologue of the Ste20-like protein kinase (MST1; ref. 20), and AMP-activated protein kinase (AMPK; refs. 21,22). The phosphoinositide 3-kinase (PI3K)/AKT pathway was the first inhibitory pathway unraveled for FOXO factors, in addition to AKT, serum and glucocorticoid-induced kinase (SGK), casein kinase 1 (CK1), dual-specificity tyrosine-phosphorylated-regulated kinase 1A (DYRK1A), IκB kinase β (IKKβ; ref. 12), and extracellular signal-regulated kinases 1 and 2 (ERK1/2; ref. 13; please see the following discussion) also suppressed FOXOs activity (Table 1).

Three Oncokinases Targeting FOXO3a

It is worth mentioning that AKT, IKK, and ERK are three commonly activated oncogenic kinases in human cancers. Interestingly, all three kinases target the same tumor suppressor gene, *FOXO3a* (Fig. 1). It is known that AKT, IKK, and ERK phosphorylate FOXO3a at different phosphorylation sites (Table 1) in response to growth factor and insulin stimulation from *Caenorhabditis elegans* to mammals. Similarly, phosphorylation of FOXO3a by these three oncogenic kinases results in FOXO3a translocation from the nucleus to the cytoplasm, and subsequent degradation. AKT-dependent phosphorylation of FOXO3a (Thr³², Ser²⁵³, and Ser³¹⁵ for human FOXO3) enhances FOXO3a/14-3-3 interaction and promotes FOXO3a nuclear export to the cytoplasm, resulting in the repression of FOXO3a transcriptional function (2,23). IKK and ERK phosphorylate FOXO3a at Ser⁶⁴⁴ and Ser²⁹⁴, Ser³⁴⁴, and Ser⁴²⁵, respectively. All three kinase-mediated phosphorylations stimulate FOXO3a ubiquitination, resulting in its proteasomal degradation. Phosphorylation of FOXO3a by ERK may induce its conformational change and promote the physical interaction of FOXO3a with the E3 ubiquitin ligase MDM2, leading to subsequent FOXO3a ubiquitination and proteasomal degradation (13). MDM2 does not seem to serve as the E3 ligase responding to the degradation of AKT- and IKK-phosphorylated FOXO3a. However, another E3 ligase, Skp2, was reported to mediate AKT-dependant phosphorylation of FOXO1 ubiquitination and degradation. FOXO1 loss in which Skp2 was overexpressed has been found in a mouse lymphoma model (24). Noting this, it would be interesting to see whether Skp2 also serves as an E3 ligase for FOXO3a degradation. At this moment, it is still not yet clear which E3 ligase responds to IKKβ-mediated degradation of FOXO3a.

Additionally, CK1, DYRK1A, and CDK2 also phosphorylate FOXOs at various sites to inhibit FOXOs activity. It is interesting to note that AKT and ERK were shown to up-regulate CK1 (25) and CDK2 (26) activity, respectively, and DYRK1A was reported to prolong ERK activity by forming and stabilizing the RAS/B-RAF/MEK1 complex (27). Therefore, it would be attractive to address whether DYRK1A inactivated FOXO3a may be mediated by ERK.

Phosphorylation of FOXOs by AKT, IKK, ERK, CK1, CDK2, and DYRK1A universally leads to FOXO's inhibition. However, whether CK1, DYRK1A, and CDK2 are also involved in tumorigenesis is yet to be determined. Because AKT, IKK, and ERK govern most of the signaling pathways in regulating FOXOs on top of the other kinases, inhibition of AKT, IKK, and ERK is likely to be sufficient to restore FOXO's function.

A similarly interesting story is that, in addition to FOXO3a, AKT, ERK, and IKK β also inactivate another tumor suppressor, the tuberous sclerosis 1/2 (TSC1/2) complex. Phosphorylation of TSC2 (by AKT and ERK; refs. 28,29) and TSC1 (by IKK β ; ref. 30) results in the disruption of the TSC1/2 complex, and thereby activates the oncogenic mTOR signaling contributing to tumor progression. Similar to FOXO3a, the non-phosphorylation-mimick mutations of TSC1/2 resist inhibition of the activated kinases and block cell growth and tumorigenesis. It would be interesting to understand how these three oncogenic kinases coordinately regulate FOXO3a and TSC1/2 or if other modulations are also involved. The fact that AKT, IKK β , and ERK can phosphorylate, down-regulate, and inactivate the tumor suppressors, FOXO3a and TSC, raises an attractive question of whether the three oncogenic kinases also target other common tumor suppressors to exert their oncogenicity. It is also appealing to ask whether other FOXO-targeting kinases, such as CK1, CDK2, and DYRK1A, can phosphorylate and regulate TSC. In all, targeting AKT, ERK, and IKK β with inhibitors to allow the reactivation of FOXOs, TSC, and maybe more, may provide an attractive strategy for anticancer therapeutics.

Clinical-Translational Advances

FOXO factors have been shown to be deregulated in several tumor types including breast cancer, prostate cancer, glioblastoma, rhabdomyosarcoma, and leukemia (1,31). Loss of FOXO1a through chromosomal deletion (13q14) was shown to promote androgen-independent prostate cancers (32). In addition, cytoplasmic localization or down-regulation of FOXOs via AKT, IKK, and ERK-mediated phosphorylation was observed in breast cancers (12,13). Inactivation of FOXOs seems to be an important step in carcinogenic transformation, increasing the activity of FOXOs represents a reasonable therapeutic strategy. Although targeting oncoproteins with small molecule inhibitors is a prevailing strategy for current cancer therapy, developing small molecules that restore the function of tumor suppressors is equally important and even more challenging.

Therapeutics and Compounds Enhancing FOXO's Nuclear Localization

It was shown that the FOXO families can serve as therapeutic targets in various cancers by mediating the cytostatic and cytotoxic effects of various chemotherapeutic drugs (Table 2).

For example, the chemotherapeutic drugs paclitaxel (33,34) and KP372-1 (a multiple kinase inhibitor; ref. 35), which are currently used in the treatment of breast carcinoma and acute myeloid leukemia, can activate FOXO3a by reducing AKT activity. Paclitaxel also activates *c-Jun*-NH₂-kinase, which phosphorylates several sites within the DNA-binding domain of FOXO3a (34). These phosphorylation residues reduce the interaction between FOXO3a with 14-3-3 protein, leading to impaired nuclear export of FOXO3a mediated by 14-3-3, and thereby increasing FOXO3a activity (23,34).

Some attempts were made to promote FOXO's function by regulating its subcellular localization. For example, screening of marine natural product extracts identified a bromotyrosine derivative, psammaphysene A, that can cause the relocalization of FOXO1 to the nucleus in PTEN-deficient cells (36). In addition, CRM1 inhibitors have also been discovered to enhance FOXO1 activity by blocking FOXO1 nuclear export (37).

Therapeutics Activating FOXOs and Downstream Genes

Several findings have concluded the clinical importance of activating FOXO3a and its downstream genes in various anticancer therapeutics (31). TP53, a well-known tumor suppressor gene, is mutated in 50% of human cancers. These p53 mutations play an essential role in tumorigenesis and drug resistance. Different from p53, FOXO3a mutation has not yet been found in human cancer, which makes therapeutics activating FOXO3a more appealing than others. And indeed, activation of FOXO3a in p53-mutated cancer cells is sufficient for tumor suppression (12,13). For example, doxorubicin has shown to activate FOXO3a to induce the expression of the multidrug resistance gene *ABCB1* (*MDR1*) in K562 doxorubicin-sensitive leukemic cells (38). In chronic myeloid leukemia, inhibition of BCR-ABL by imatinib activates FOXO3a and induces Bim-dependent apoptosis (39). Imatinib also induces erythroid differentiation through repression of the *ID1* gene transcription by FOXO3a (40).

Due to the potent antitumor activity of FOXO3a, drugs that activate FOXO3a can be used in combination with other therapeutic agents to sensitize tumor cells. A novel phosphoinositide-dependent protein kinase-1/AKT inhibitor, OSU-03012, was recently reported to sensitize ER-negative breast cancer cells to tamoxifen through desuppressing AKT downstream effectors, including FOXO3a and p27 (41). It was also shown that EGFR family blockade by the blocking antibodies (such as trastuzumab or cetuximab) could inhibit the PI3K pathway and induce FOXO3a activity, which leads to transcriptional activation of the proapoptotic *BNIP3L* gene (42). Blocking EGFR/HER2 was used as single agent and in combination with other agents in clinical trials against breast, prostate, kidney, ovarian, and lung cancers (31). It would also be intriguing to see if the re-introduction of active FOXO3a could sensitize resistant cancer cells to small molecules targeting the EGFR family (such as lapatinib and gefitinib; ref. 31).

Activation of FOXO3s can also be effective in overcoming tumor resistance against radiotherapy (31). It was reported that FOXO3a activation efficiently induced Bim and apoptosis in p53-null osteosarcoma cell lines exposed to ionizing radiation (9). Recently, FOXO3a was shown to enhance ataxia telangiectasia mutated (ATM) activity through the binding of the carboxyl terminal domain of FOXO3a to the FAT (FRAP, ATM, and TRRAP; ref. 43) domain of ATM, thereby contributing to the activation of ATM (44). These data suggest that FOXO3a may be an important factor for radiation-inducing apoptosis. A combination therapy of radiation with chemo-therapy targeting FOXO3a may be able to sensitize resistant tumor cells to radiotherapy. Especially with our recent findings of FOXO3a regulation by the RAS-MEK-ERK pathway, AZD6244, a MEK1/2 inhibitor that is currently used in phase II clinical trials in various cancers (45), might serve as a potential candidate to synergize with other anticancer therapeutics through activating FOXO3a.

It was reported that adenovirus-mediated transfer of constitutively active FOXO3a induces apoptosis in melanoma cells (46). Further investigation needs to be done *in vivo* with various cancer types (31). Gene therapy using cancer-specific expression vector has shown both effectiveness and specificity in killing human tumors (47-50). Therefore, using a cancer-specific gene therapy to deliver functional FOXO3a may also provide a potential anticancer therapeutic strategy.

However, it is worth noting that targeting FOXO proteins may be complicated by potential feedback mechanisms. Most current evidence supports the notion that activation of FOXO3a is an important player in mediating cell apoptosis in a broad spectrum of tumors (31). Alternatively, FOXO1 has recently been shown to increase protein kinase B phosphorylation by repressing expression of TRB3, a pseudokinase inhibiting protein kinase B phosphorylation (31,51). The FOXO homologue, DAF16, has been shown to induce the expression of the insulin receptor and increase PI3K activity in *C. elegans* (52). Thus, FOXO transcription factors may

engage in a feedback regulation towards the upstream PI3K/AKT pathway. Therefore, while exploring FOXOs as a therapeutic agent, one may need to consider prevention of FOXO's feedback loop. Our knowledge of FOXO is continually advancing and recent studies reveal great rationales and opportunities to adopt FOXO family members as therapeutic targets in cancers. AKT, IKK α , and ERK phosphorylate and down-regulate the tumor suppressors FOXO3a and TSC. Restoring FOXO's and TSC's activity from the regulation of these kinases can be effective for cancer treatment. In recent years, the development and success of multitargeted agents has shed light on treating cancers by inhibiting more than one receptor tyrosine kinase at once. For example, lapatinib, a small molecule inhibitor, has shown effectiveness in targeting two EGFR family members, EGFR and HER2, which used to be therapeutically aimed by single targeting reagents, such as gefitinib (a receptor tyrosine kinase inhibitor) and trastuzumab (a humanized monoclonal antibody), respectively (53,54). Herein, based on the previous findings in our model, developing therapeutic agents to multitarget AKT, IKK β , and ERK will be expected to boost antitumor activity through the maximal activation of FOXOs and TSC, and also achieve maximal therapeutic efficacy with the least resistance to cancer treatment.

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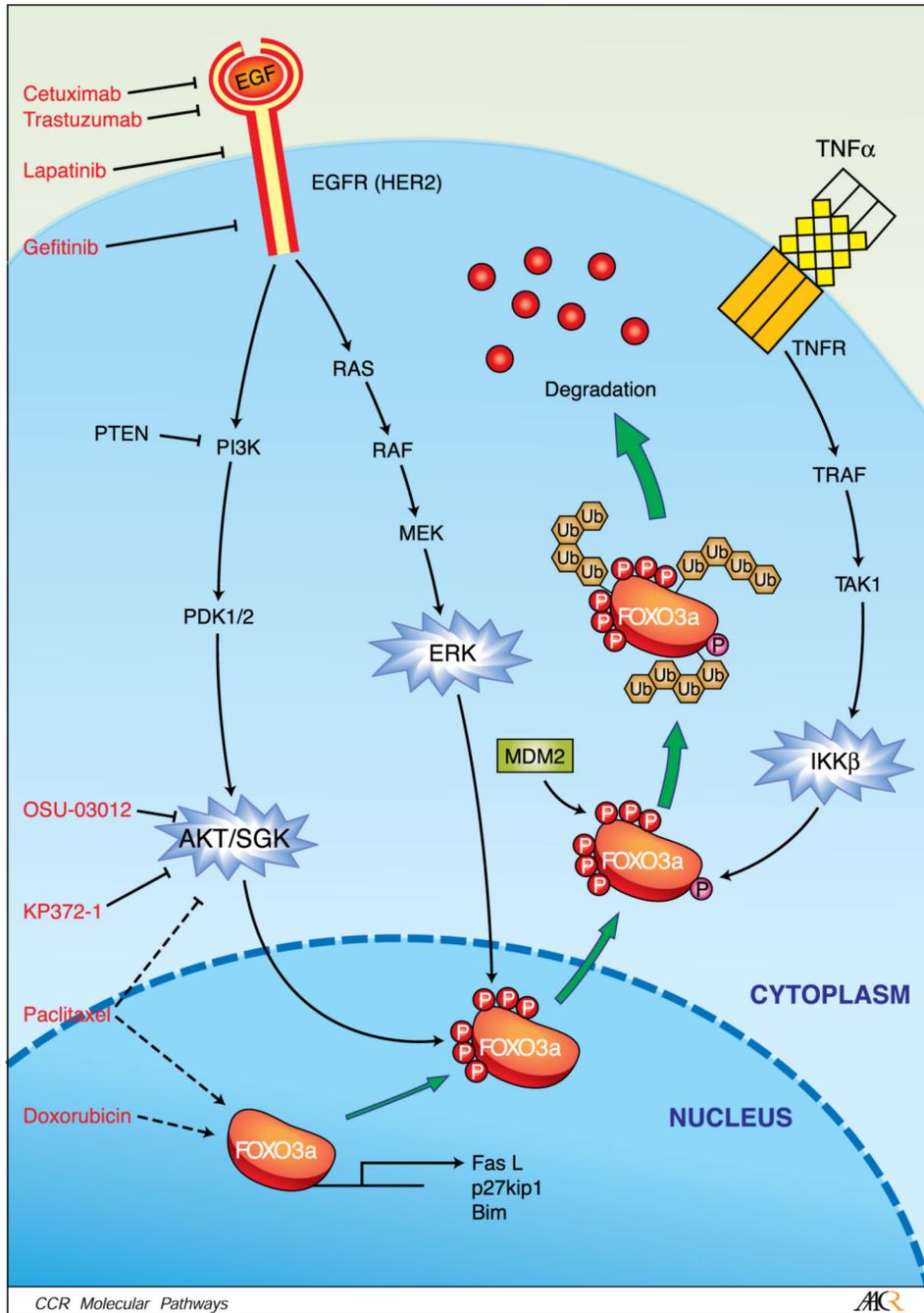


Fig. 1. PI3K/AKT, RAS/ERK, and TNFR/IKK pathways are known to phosphorylate FOXO3a at different sites (indicated by different colors; see Table 1). Phosphorylation of FOXO3a causes FOXO3a nuclear exportation, thereby suppressing FOXO's transcriptional activity. Recently, FOXO3a phosphorylation by ERK was found to lead to FOXO3a down-regulation via MDM2-mediated proteasome degradation. Inhibition of the EGFR family that governs PI3K and RAS pathways by clinically used drugs, such as cetuximab, trastuzumab, lapatinib, and gefitinib effectively up-regulates FOXO3a. Moreover, targeting AKT/SKG by OSU-03012, paclitaxel, KP372-1, and doxorubicin (see Table 2), has also been shown to achieve their therapeutic effects through activation of FOXO3a and FOXO3a targets such as FasL, Bim, and p27.

Table 1
Phosphorylation and activity alteration of FOXO members are regulated by an array of protein kinases

Kinase	FOXO1	FOXO3	FOXO4	FOXO6	E3 ligase	Effect
AKT/SGK	T24	T32	T28	T26	Skp2 (FOXO1)	Inhibition
	S256	S253	S193	S184		
	S319	S315	S258			
IKK β		S644			?	Inhibition
		S294, S344, S425			MDM2 (FOXO3a)	Inhibition
ERK1/2						Inhibition
CK1	S322	S318	S261		?	Inhibition
	S325	S321	S264			
DYRK1A	S329	S325	S268		?	Inhibition
CDK2	S249				?	Inhibition
AMPK		T179, S399, S413, S555, S588, S626				Activation
JNK			T447, T451			Activation
MST1	S207					Activation

Table 2

Clinical anticancer treatments target FOXO3a through three oncogenic kinases (AKT, IKK, and ERK) and various signaling pathways

Cancer type	Drug or therapy	Protein targeted	AKT, IKK, and ERK affected	Signal pathway affected
Breast cancer	Paclitaxel	FOXO3a	AKT	Effected tubulin, decrease in AKT and increase in JNK activity, leading to FOXO3a activation and induction of Bim (33,34)
Acute myeloid leukemia	KP372-1	FOXO3a	AKT	Inhibition of AKT activity, activating FOXO3a and inducing apoptosis (35)
Chronic myeloid leukemia	Imatinib (STI571 or Glivec)	FOXO3a	AKT and ERK	BCR-ABL inhibition, leading to FOXO3a dephosphorylation and Bim-dependent apoptosis; down-regulation of ID1 and erythroid differentiation (39,40)
Osteosarcoma, breast cancer	Ionizing radiation (IR or UV)	FOXO3a	?	Induction of FOXO3a in p53-null cells and enhanced ATM activity (9,44)
Breast, prostate, kidney, ovarian, non-small cell lung cancer	Trastuzumab, cetuximab, lapatinib, gefitinib	FOXO3a	AKT and ERK	EGFR blockade; induction of FOXO3a and expression of proapoptotic FOXO3a target BNIP3L1 (42)
Leukemia	Doxorubicin	FOXO3a	?	Activation of FOXO3a and MDR1 (38)
Melanoma	Adenovirus-mediated transfer of constitutively active FOXO3A	FOXO3a	?	Activation of FOXO3a and induction of apoptosis (46)
Breast cancer	OSU-03012/tamoxifen	FOXO3a	AKT	Inhibition of PDK-1/AKT activity, activating FOXO3a and sensitizing ER-negative breast cancer cells to tamoxifen (41)