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Clever cancer strategies with FoxO transcription factors

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Abstract

Given that cancer and related disorders affect a wide spectrum of the world's population, and in most cases are progressive in nature, it is essential that future care must overcome the present limitations of existing therapies in the absence of toxic side effects. Mammalian forkhead transcription factors of the O class (FoxOs) may fill this niche since these proteins are increasingly considered to represent unique cellular targets directed against human cancer in light of their pro-apoptotic effects and ability to lead to cell cycle arrest. Yet, FoxOs also can significantly affect normal cell survival and longevity, requiring new treatments for neoplastic growth to modulate novel pathways that integrate cell proliferation, metabolism, inflammation and survival. In this respect, members of the FoxO family are extremely compelling to consider since these transcription factors have emerged as versatile proteins that can control angiogenesis, stem cell proliferation, cell adhesion and autoimmune disease. Further elucidation of FoxO protein function during neoplastic growth should continue to lay the foundation for the successful translation of these transcription factors into novel and robust clinical therapies for cancer.

Keywords

angiogenesis; cancer; immune system; oxidative stress; stem cells

Introduction

Mammalian forkhead transcription factors of the O class (FoxOs) that include FoxO1, FoxO3, FoxO4 and FoxO6 have emerged as critical regulators of cellular growth and proliferation and are therefore considered to be potential targets for therapeutic strategies directed against cancer. FoxO proteins are found throughout the body and are expressed in tissues of the reproductive system of males and females, skeletal muscle, the cardiovascular system, lung, liver, pancreas, spleen, thymus and the nervous system.[1-6] The prior nomenclature for these proteins, such as

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forkhead in rhabdomyosarcoma (*FKHR*), the Drosophila gene fork head (*fkh*), and Forkhead RElated ACtivator (FREAC)-1 and -2, has been replaced. The current nomenclature for human Fox proteins places all letters in uppercase, otherwise only the initial letter is listed as uppercase for the mouse, and for all other chordates the initial and subclass letters are in uppercase.[7] Initially, the FoxOs were first reported in fusion genes in human soft-tissue tumors and leukemias. FOXO1, termed forkhead in rhabdomyosarcoma (FKHR), and FOXO3a, also known as FKHRL1 (forkhead in rhabdomyosarcoma like protein 1), and their genes were identified through chromosomal translocations in alveolar rhabdomyosarcoma tumors.[8] The acute leukemia fusion gene located in chromosome X (*AFX*), also known as the *FOXO4* gene, was described as a gene that fused to MLL transcription factor as a result of the *t(X; 11)* chromosomal translocation in acute lymphoblastic leukemia.[9] A fusion between FOXO2 and MLL also occurs in some cases of acute myeloid leukemia that also is believed to be identical to FOXO3a.[10]

FoxO Proteins as Transcription Factors

At least 100 forkhead genes and 19 human subgroups that range from *FOXA* to *FOXS* are now known to exist since the initial discovery of the fly *Drosophila melanogaster gene forkhead*.[11] Forkhead proteins function as transcription factors to either inhibit or activate target gene expression and therefore, these proteins must bind to DNA through the forkhead domain that relies upon fourteen protein-DNA contacts. The forkhead domain in Fox proteins consists of three α-helices, three β-sheets, and two loops that are referred to as the wings,[12] but not all winged helix domains are considered to be Fox proteins.[13] On X-ray crystallography[12] or nuclear magnetic resonance,[14] the forkhead domain is described as a "winged helix" as a result of a butterfly-like appearance. High sequence homology is present in the α-helices and β-sheets with variations described in either absent β-sheets and loops or additional α-helices. Although both the first and second loops make contact with DNA, it is the second loop that can influence the stability of DNA binding. In addition, post-translational modification of FoxO proteins, such as phosphorylation or acetylation that block FoxO activity, alter the binding of the C-terminal basic region to DNA to prevent transcriptional activity.[15] However, other mechanisms may influence DNA binding of forkhead proteins, such as variations in the N-terminal region of the DNA recognition helix, changes in electrostatic distribution, and the ability of forkhead proteins to be shuttled to the cell nucleus.[5,16]

FoxO Pro-apoptotic Pathways and Cell Cycle Regulation Block Neoplastic Progression

Genes linked to apoptosis may not necessarily lead to cell death since current studies have suggested additional roles for genes normally associated with apoptosis that can involve cellular replication and transcription. Yet, cellular apoptosis can lead to several degrees of pathology in diseases such as neurodegenerative disease, diabetes mellitus (DM) and cardiovascular injury.[17] More importantly, regulation of apoptotic pathways appears to serve a critical juncture for the control of tumor growth and unregulated cell proliferation.[5,18] Apoptotic cell death is considered to be a dynamic process that involves both early and late events. Membrane phosphatidylserine (PS) externalization is an early event during cell apoptosis that assists microglia to target cells for phagocytosis.[19,20] This process occurs with the expression of the phosphatidylserine receptor (PSR) on microglia during oxidative stress,[21-23] since blockade of PSR function in microglia prevents the activation of microglia.[24,25] As an example, externalization of membrane PS residues occur in cells during periods of oxidative stress that involve anoxia,[26] reactive oxygen species (ROS) exposure,[27] and with agents that produce ROS, such as 6-hydroxydopamine.[28] In contrast to cells with PS exposure, the cleavage of genomic DNA into fragments is considered to be a later event during apoptotic injury.[17,29] Endonucleases responsible for DNA degradation have been identified and include

the acidic, cation independent endonuclease (DNase II), cyclophilins, and the 97 kDa magnesium—dependent endonuclease. In the nervous system, endonucleases include a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium dependent endonuclease.[17,29]

Interestingly, the induction of apoptosis in cells through FoxO proteins may require pathways aligned with oxidative stress (Figs. 1 and 3). Oxidative stress occurs during the release of ROS that consist of oxygen free radicals and other chemical entities. Oxygen free radicals and mitochondrial DNA mutations have become associated with tissue injury, aging and accumulated toxicity for an organism.[17] ROS include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide and peroxynitrite.[29] Most reactive species are produced at low levels during normal physiological conditions and are scavenged by endogenous antioxidant systems that include superoxide dismutase, glutathione peroxidase, catalase and small molecules, such as vitamins C, E, D_3 and nicotinamide, the amide form of niacin or vitamin B_3.[20,30,31] During periods of oxidative stress, FoxO transcription factors can lead to cell injury and apoptosis,[32] since forkhead transcription factors such as FoxO1 and FoxO3a must be present for oxidative stress to result in apoptotic cell injury.[33] Under other conditions of oxidative stress, FoxO3a in conjunction with c-Jun N-terminal kinase (JNK) have been shown to modulate an apoptotic ligand activating a Fas-mediated death pathway in cultured motoneurons,[34] to lead to apoptosis through tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) and BH3-only proteins Noxa and Bim in neuroblastoma cells,[35] and to promote pro-apoptotic activity of p53.[36] Additional work shows that loss of FoxO expression during oxidative stress is protective to cells. For example, protein inhibition or gene knockdown of FoxO1 or FoxO3a can lead to reduction in ischemic infarct size in the brain,[37] mediate protection of metabotropic glutamate receptors during vascular injury,[38] enhance pancreatic β-cell or neuronal survival through NAD^+ precursors during oxidative stress,[39] and provide trophic factor protection with erythropoietin (EPO)[40] and neurotrophins.[41]

FoxO proteins also appear to be ideal to regulate tumor growth not only through pro-apoptotic pathways, but also through the blockade of cell cycle progression (Fig. 3). For example, FoxO3a and FoxO4 can promote cell cycle arrest in mouse myoblastic cell lines through modulation of growth-arrest and DNA-damage-response protein 45.[5,42] Treatment of chronic myelogenous leukemia cell lines with the Bcr-Abl tyrosine kinase inhibitor imatinib requires FoxO3a activation to antagonize cell proliferation and promote apoptotic cell death through increased TRAIL production[43] (Fig. 2). In addition, the transcription factor E2F-1 that controls the induction of the cell cycle has been reported in cell lines to increase the endogenous expression of FoxO1 and FoxO3a to lead to cell cycle arrest.[44] In contrast, the loss of FoxO3a activity in association with c-myc, p27 and nuclear factor-κB (NFκB) can result in cell cycle induction and malignant transformation of mouse cells in the presence of oncogene activation[5,8] (Fig. 2). Other work suggests that FoxO proteins utilize the p53 upstream regulator p19(Arf) through myc to block cell cycle induction and lymphoma progression.[45]

FoxO Proteins, Post-translational Control and Integration with Novel Cellular Signaling

Post-translational modification of FoxO proteins involves pathways associated with phosphorylation, acetylation and ubiquitylation[8,46-48] (Fig. 2). In regards to the inhibition of FoxO protein activity, the serine-threonine kinase protein kinase B (Akt) is a primary mediator of phosphorylation of FoxO1, FoxO3a and FoxO4.[8,49] Activation of Akt is usually cytoprotective, such as during hyperglycemia,[50] hypoxia,[51] β-amyloid (Aβ) toxicity,[52] cardiomyopathy[53] and oxidative stress.[19,25,54] Akt can prevent cellular apoptosis through the phosphorylation of FoxO proteins.[32] Post-translational phosphorylation of FoxO proteins will maintain FoxO transcription factors in the cytoplasm by association with 14-3-3 proteins

and prevent the transcription of pro-apoptotic target genes.[40,55] An exception in regards to the subcellular trafficking of FoxO proteins involves FoxO6. This FoxO protein usually resides in the nucleus of cells and is phosphorylated by Akt in the nucleus. FoxO6 does not contain a conserved C-terminal Akt motif which limits nuclear shuttling of this protein, but FoxO6 transcriptional activity can be blocked by growth factors independent of shuttling to the cytosol through a FoxO6 N-terminal Akt site.[56]

Modulation of Akt activity also oversees apoptotic pathways of caspases that may offer an alternative mechanism to regulate FoxO proteins. Caspases are a family of cysteine proteases that are synthesized as inactive zymogens that are proteolytically cleaved into subunits at the onset of apoptosis.[57-59] The caspases 1 and 3 have been linked to the apoptotic pathways of genomic DNA cleavage, cellular membrane PS exposure, and activation of inflammatory cells[24,60,61] (Fig. 2). Caspase pathways may be tied to the forkhead transcription factor FoxO3a since increased activity of FoxO3a can result in cytochrome *c* release and caspase-induced apoptotic death.[35,38-40] Pathways that can inhibit caspase 3 activity appear to offer a unique regulatory mechanism. For example, caspase 3 cleavage of Fox3a can lead to pro-apoptotic amino-terminal (Nt) fragments that can lead to cell death. However, during caspase 3 inhibition, inactive phosphorylated FoxO3a remains intact and does not lead to apoptotic cell injury during oxidative stress.[38-40]

Post-translational modification of FoxO proteins also relies upon pathways associated with ubiquitylation and acetylation.[62,63] Akt phosphorylation of FoxO proteins not only retains these transcription factors in the cytoplasm, but also leads to ubiquitination and degradation through the 26S proteasome.[46,63] In the absence of Akt, IκB kinase (IKK) also can directly phosphorylate and block the activity of FoxO proteins, such as FoxO3a.[5,8] This leads to the proteolysis of FoxO3a via the Ub-dependent proteasome pathway.[8,46-48] The serum- and glucocorticoid-inducible protein kinase (Sgk), a member of a family of kinases termed AGC (protein kinase A/ protein kinase G/protein kinase C) kinases which includes Akt, also can phosphorylate and retain FoxO3a in the cytoplasm.[64] Knowledge that Sgk and Akt can phosphorylate FoxO3a at different sites may offer new opportunities to more effectively prevent apoptotic cell injury that may be mediated by FoxO3a activity. Yet, phosphorylation of FoxO proteins does not always lead to negative regulation. The protein kinase mammalian sterile 20-like kinase-1 also can phosphorylate FoxO proteins directly and lead to their activation.[65] The ability of sterile 20-like kinase-1 to activate FoxO proteins may be linked to JNK, since sterile 20-like kinase-1 can increase JNK activation.[66] FoxO proteins also are acetylated by histone acetyltransferases that include p300, the CREB-binding protein (CBP), and the CBP-associated factor and are deacetylated by histone deacetylases, such as SIRT1, a NAD^+-dependent deacetylase and the mammalian ortholog of the silent information regulator 2 (Sir2) protein[5] (Fig. 2). Acetylation of FoxO proteins provides another avenue for the control of these proteins. Once acetylated such as by CBP, FoxO proteins may translocate to the cell nucleus but have diminished activity since acetylation of lysine residues on FoxO proteins has been shown to limit the ability of FoxO proteins to bind to DNA.[67] In addition, acetylation can increase phosphorylation of FoxO proteins by Akt.[67]

Interestingly, FoxO proteins are associated with other novel signal transduction pathways tied to cell death. One pathway in particular involves proteins derived from the Drosophila *Wingless* (*Wg*) and the mouse *Int-1* genes. The Wnt proteins are secreted cysteine-rich glycosylated proteins that can control cell proliferation, differentiation, survival and tumorigenesis.[68,69] More than eighty target genes of Wnt signaling pathways have been demonstrated in human, mouse, Drosophila, Xenopus and zebrafish. These genes are present in several cellular populations, such as neurons, cardiomyocytes, endothelial cells, cancer cells and pre-adipocytes.[70] At least nineteen of twenty-four Wnt genes that express Wnt proteins have been identified in the human.[68,69,71]

One Wnt pathway controls target gene transcription through β-catenin, generally referred to as the canonical pathway.[68,69] It is the β-catenin pathway that appears to tie FoxO proteins and Wnt signaling together.[18] For example, in relation to Alzheimer's disease, A βis toxic to cells[26,52] and is associated with the phosphorylation of FoxO1 and FoxO3a that can be blocked with ROS scavengers.[72] A common denominator in the pathways linked to Aβ toxicity involves Wnt signaling through β-catenin. β-catenin may increase *FoxO* transcriptional activity and competitively limit β-catenin interaction with members of the lymphoid enhancer factor/T cell factor family[73] and β-catenin also has been demonstrated to be necessary for protection against Aβ toxicity in neuronal cells.[26]

Additional shared signal transduction pathways between Wnt and FoxO proteins involve Akt. Processes that involve cellular proliferation, injury and immune system modulation with Akt and FoxO proteins also have parallel cellular pathways with Wnt. For example, Wnt relies upon Akt for the proliferation and differentiation of cardiomyocytes.[74] In addition, reduction in tissue injury during pressure overload cardiac hypertrophy and the cytoprotective benefits of cardiac ischemic preconditioning also appear to depend upon Akt.[68,69] Furthermore, Wnt overexpression can independently increase the phosphorylation and the activation of Akt to promote cellular protection and control microglial activation.[26]

Yet, other members of the forkhead family in addition to FoxOs also rely upon Wnt signaling in several scenarios that involve regulated as well as unchecked cell proliferation.[68,69,75] For example, FoxD3 is activated by the Wnt pathway to control neural plate development[76] and Foxl1 activates the Wnt/β-catenin pathway to increase extracellular proteoglycans, promote gastrointestinal cell proliferation, and possibly foster carcinogenesis.[77] The Wnt pathway also utilizes forkhead members to modulate endocrine activity and can activate Foxn1 for regulatory control of thymic function.[78] In other examples of cell development, Wnt signaling has been shown to rely upon Foxf1 and Foxf2 during intestinal maturation in murine models.[79] In addition, Foxa2 in mice may be a significant component in early anterior-posterior axis polarization.[80]

In regards to tumorigenesis, deregulation of Wnt alone promotes activation of β-catenin that has been associated with the proliferation of medulloblastoma tumors.[81] In addition, reduced expression of inhibitors of the Wnt pathway, such as axin, may foster lung cancer cell invasion.[82] Multiple other studies also point to the activation of the Wnt pathway during gastric cancer. For example, Wnt5a expression has been correlated with advanced gastric cancer stages and a poor prognosis[83] while experimental activation of the β-catenin pathway leads to the development of gastric tumors.[84] In conjunction with forkhead proteins, loss of Foxl1 that can regulate the Wnt pathway and prevent β-catenin nuclear accumulation is believed to be a significant etiology for gastrointestinal tumorigenesis.[77]

FoxO Proteins, Stem Cells and Angiogenesis

The ability of FoxO proteins to block tumor progression most likely is tightly coupled to the modulation of stem cell proliferation and new vessel growth. The initial identification of FoxO proteins in soft-tissue tumors and leukemias, neoplasms now believed to harbor cancer stem cells for tumor self-renewal,[81] suggests that FoxO proteins may be closely associated with the oversight of stem cell proliferation and differentiation (Fig. 3). For example, either simultaneous deletion of *Foxo1*, *Foxo3a* and *Foxo4* or single deletion of *Foxo3a* in mice prevents the repopulation of hematopoietic stem cells and leads to apoptosis in these stem cell populations.[85,86] Furthermore, vascular cytoprotective agents, such as the growth factor EPO,[55,87,88] also may be required to modulate FoxO protein activity (Fig. 1) such as during erythroid progenitor cell development,[42,89] suggesting that current clinical use of agents such as EPO during anemia or cancer may have less defined treatment implications for patients than

originally anticipated.[55,89] In cell culture and animal studies, EPO is cytoprotective in vascular cells and can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow.[42,89,90] Interestingly, the ability of EPO to foster eythroid progenitor cell development is dependent upon the inhibition of FoxO3a activity,[55,89] but also may require regulation of specific gene expression through an EPO-FoxO3a association to promote erythropoiesis in cultured cells.[91] In relation to the reproductive potential of an organism, deletion of the *FoxO3a* gene results in the depletion of oocytes and subsequent infertility.[92] Other work using a mouse model of FoxO3a overexpression in oocytes further suggests that FoxO3a retards oocyte growth and follicular development and leads to anovulation and luteinization of unruptured follicles.[93] These studies may suggest a role for FoxO proteins, and specifically FoxO3a, in relation to not only the development of cancer stem cell niches, but also in regards to oocyte and follicular cell maturation. For example, in a small percentage of women who suffer from premature ovarian failure mutations in *FOXO3a* and *FOXO1a* have been observed.[94]

In addition to the modulation of stem cell development, FoxO proteins play a significant role to govern new vessel growth that can impact upon tumor cell growth and dispersion (Fig. 3). New capillary formation from pre-existing vessels into an avascular area is a process known as angiogenesis that is present during embryogenesis, during menstruation and during pathological processes that involve wound healing, chronic inflammation and tumor growth.[69,89] FoxO proteins are intimately involved in endothelial cell development and angiogenesis. For example, *Foxo3a*$^{-/-}$ and *Foxo4*$^{-/-}$ mice develop without incidence and are indistinguishable from control littermates. Yet, mice that are singly deficient in *Foxo1* die by embryonic day eleven and lack development of the vascular system.[95] Other work illustrates that endothelial cell colonies in *Foxo1*-deficient mice fail to respond to vascular endothelial growth factor in a manner similar to wild-type endothelial cells,[96] suggesting that FoxOs are necessary not only for the development of vascular cells, but also for the biological response to cellular mediators.

FoxO Proteins and Immune System Surveillance

In general, forkhead transcription factors have a vital role in maintaining immune system function and may influence the progression of tumor growth. For example, the forkhead family member FoxP3 can control the development and function of thymic-derived CD4(+) CD25(+) regulatory T cells (Treg) that impart autoimmunity. Loss of FoxP3 can result in autoimmune disorders.[97] In addition, recent work identifies the expression of FoxP3 in tumor cells, such as melanoma,[98] as well as in Tregs which may significantly affect patient mortality since the increased presence of Tregs in cancer patients combined with FoxP3 expression in tumors may impair antitumor autoimmune responses and lead to high mortality.[99]

In regards to FoxO proteins, these forkhead transcription factors also may impact upon neoplastic progression since they lead to the induction of apoptotic pathways and may influence early apoptotic membrane PS externalization (Fig. 3). The ability to regulate early apoptotic membrane PS exposure[24] and inflammatory cell activity[19] can ultimately impact upon cell survival since activated immune cells can lead to the phagocytic removal of tumor cells.[21,29] Inflammatory cells, such as macrophages or microglia, require the activation of intracellular cytoprotective pathways to proliferate and remove injured cells.[22,100] At times, this can be a beneficial process and form a barrier for the removal of foreign microorganisms and promote tissue repair during cell injury.[30,42] However, inflammatory cells also may lead to cellular damage through the generation of ROS and through the production of cytokines.[42] Interestingly, in mice deficient for *Foxo3a*, lymphoproliferation, organ inflammation of the salivary glands, lung and kidney, and increased activity of helper T cells results, supporting an important role for FoxO3a in preventing T cell hyperactivity.[101] FoxO3a also appears to be

necessary for neutrophil activity, since *Foxo3a* null mice are resistant to models of neutrophilic inflammation that involve immune complex-mediated inflammatory arthritis.[102]

In clinical studies, patients with rheumatoid arthritis and osteoarthritis show phosphorylation of FOXO3a in T lymphocytes as well as FOXO1 and FOXO4 in synovial macrophages, suggesting that loss of functional FOXO family members may lead to inflammatory cell activation in these disorders.[103] *FOXO1* gene transcript levels also are downregulated in peripheral blood mononuclear cells of patients with systemic lupus erythematosus and rheumatoid arthritis,[104] illustrating a potential etiology through the loss of functional FOXO proteins for these disorders and possibly providing a biomarker of disease activity. Other work has demonstrated that FOXO1 protein regulates L-selectin expression that can regulate human T lymphocyte trafficking.[105] More importantly, studies suggest a relationship between the regulation of immune system activity and the induction of apoptotic pathways that are dependent upon FoxO proteins. Prevention of inflammatory activation and apoptosis in the nervous system such as in systemic lupus erythematosus in animal models may require the upregulation of different Fox proteins, such as FoxJ1 and FoxO3a, that can block NFκB activation and interferon-gamma secretion.[106] FoxO proteins also may work in concert with Fas signaling to clear activated T cells following a decrease in cytokine stimulation in patients with autoimmune lymphoproliferative syndromes,[107] suggesting that activation of specific FoxO proteins may be beneficial for autoimmune disorders but may impair treatments designed to target tumor cells through immune mediated pathways.

FoxO Proteins and Cellular Metabolism

Clinical and experimental studies highlight the role of FoxO proteins during cellular metabolism that may affect the course of patients with cancer. Early work with FoxO proteins has shown that metabolic signaling with these transcription factors is conserved among multiple species including *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals. FoxO proteins are homologous to the transcription factor DAuer Formation-16 (DAF-16) in the worm *Caenorhabditis elegans* that can determine metabolic insulin signaling and lead to lifespan extension,[108,109] suggesting a significant role for FoxO proteins in relation to mammalian cell function.[5,8] In fact, FoxO proteins can stimulate the insulin-like growth factor binding protein-1 (IGFBP1) promoter by binding to the insulin-responsive sequence (IRS) [110] (Fig. 3). Both insulin and insulin-like growth factor-1 (IGF-1) can suppress this activity through activation of Akt.[110,111]

In clinical studies, analysis of the genetic variance in *FOXO1a* and *FOXO3a* on metabolic profiles, age-related diseases, fertility, fecundity and mortality have observed higher HbA_{1c} levels and increased mortality risk associated with specific haplotypes of *FOXO1a*.[112] These clinical observations may coincide with the demonstration in human endothelial progenitor cells that elevated glucose levels can reduce post-translational phosphorylation of FOXO1, FOXO3a and FOXO4 and allow for the nuclear translocation of these proteins to initiate an apoptotic program in endothelial progenitor cells.[113] In experimental models, FoxO proteins may prevent the toxic effects of high serum glucose levels. Interferon-gamma driven expression of tryptophan catabolism by cytotoxic T lymphocyte antigen 4 may activate Foxo3a to protect dendritic cells from injury in nonobese diabetic mice.[114] Additional studies have demonstrated that adipose tissue-specific expression of Foxo1 in mice improved glucose tolerance and sensitivity to insulin during an elevated fat diet.[115] FoxO proteins also may protect against diminished mitochondrial energy levels known to occur during insulin resistance such as in the elderly populations.[30,116,117] In caloric restricted mice that have decreased energy reserves, Foxo1, Foxo3a and Foxo4 mRNA levels were noted to progressively increase over a two year course.[3] These observations complement studies in

Drosophila and mammalian cells that demonstrate an increase in insulin signaling to regulate cellular metabolism during the upregulation of FoxO1 expression.[118]

However, the ability for FoxO proteins to maintain proper physiologic controls over cellular metabolism may be limited and occur only during specific circumstances. For example, mice with a constitutively active Foxo1 transgene have increased microsomal triglyceride transfer protein and elevated plasma triglyceride levels.[119] Studies in cardiomyocytes also suggest detrimental results with enhanced FoxO activity. Increased transcriptional activity of FoxO1, such as by the Sirt1 activator resveratrol, can diminish insulin mediated glucose uptake and result in insulin resistance.[120] In addition, overexpression of Foxo1 in skeletal muscles of mice can lead to reduced skeletal muscle mass and poor glycemic control,[121] illustrating that activation of FoxO proteins also may impair cellular energy reserves. Additional investigations that block the expression of Foxo1 in normal and cachectic mice[122] or reduce FoxO3 expression[123] show the reverse with an increase in skeletal muscle mass or resistance to muscle atrophy. These results become especially relevant in patients with cancer and cachexia, since FoxO protein expression may further muscle wasting for these individuals. Given these concerns, one potential agent to consider for the maintenance of cellular metabolism in cancer patients is nicotinamide,[20,57] an agent that also can inhibit FoxO protein activity.[39] In patients with DM, oral nicotinamide protects β-cell function, prevents clinical disease in islet-cell antibody-positive first-degree relatives of type-1 DM, and can reduce HbA_{1c} levels.[20,57,116] Nicotinamide, which is closely linked to cell longevity pathways,[124,125] may derive its protective capacity through two separate mechanisms of post-translational modification of FoxO3a. Nicotinamide not only can maintain phos-phorylation of FoxO3a and inhibit its activity, but also can preserve the integrity of the FoxO3a protein to block FoxO3a proteolysis that can yield pro-apoptotic amino-terminal fragments.[39]

FoxO Proteins and Therapeutic Strategies for Cancer

Obviously, one of the most important clinical strategies for FoxO proteins involves treatments designed to control human cancer progression in light of the pro-apoptotic effects of FoxO proteins and their ability to block cell cycle progression. For example, studies with prostate cancer have shown that the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) is mutated in approximately eighty percent of tumors with the loss of FOXO1 and FOXO3a activity. In cell cultures, overexpression of FoxO1 and FoxO3a in prostrate tumor cell lines also leads to apoptosis, suggesting that FoxO1 and FoxO3a are necessary for limiting prostate cell tumor growth.[6] In addition, it has been shown that inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth[126] while agents that increase FoxO3a activity in both androgen sensitive and androgen insensitive prostate cell lines prevent prostate cancer cell progression.[127] Furthermore, therapeutic strategies that rely upon the overexpression of a non-phosphorylatable form of FoxO3a that cannot be inactivated can sensitize prostate cancer cells to androgen-withdrawal-induced apoptosis.[128] Yet, it should be noted that in prostate cell lines FoxO3a can be a positive regulator of androgen receptor expression and therefore may play a complex role in prostate cancer cell proliferation and growth inhibition.[129] Other factors that control FoxO protein function also may play a role during prostate tumor progression. In prostate cancer cells, cyclin-dependent kinase 1 (CDK1) can become overexpressed and subsequently phosphylate FOXO1 to block its transcriptional activity and contribute to prostate tumorigenesis.[130] In a similar manner, it has been shown that astrocyte-elevated gene-1 (AEG-1) can be upregulated in clinical prostate cancer,[131] possibly lead to activation of Akt that suppresses FOXO3a[132] and apoptosis in prostate tumor cells.

Initial investigations of FOXO3a in clinical breast cancer suggested that activation of FOXO3a was associated with lymph nodal metastasis and a poor prognosis.[133] In contrast to these

observations, other studies reported that FOXO3a was inactivated by IKK and that inactivation of FOXO3a was associated with a poor prognosis in breast cancer,[134] suggesting that FOXO3a sub-cellular localization and pathways that enhance its activity could be used not only as prognostic assays but also as therapeutic targets. Other work in breast cancer cells demonstrate the tumor repressive ability of FoxOs by illustrating that increased activity of FoxO3a in association with JNK in breast cancer cell lines[135] or in association with cyclin-dependent kinase inhibitor p27 in isolated human breast cancer cells can suppress breast cancer progression.[136] In addition, FoxO proteins may be able to modulate estrogen function and indirectly block breast cancer growth. Overexpression of FoxO3a in breast cancer cell lines can decrease the expression of estrogen receptor regulated genes and inhibits 17beta-estradiol (E2)-dependent breast cancer growth.[137]

In addition to the ability to inhibit prostate and breast tumor growth, FoxO proteins may represent a viable option to control tumor progression in other tissues. FoxO proteins can function as redundant repressors of tumor growth. For example, somatic deletion in mice of *Foxo1*, *Foxo3a* and *Foxo4* results in the growth of thymic lymphomas and hemangiomas.[138] Other work illustrates that FoxO3a activation in colon carcinoma cell lines prevents tumor proliferation through Myc target genes that involve the Mad/Mxd family of transcriptional repressors.[139] In addition, the loss of FoxO3a activity may participate in oncogenic transformation in B-chronic lymphocytic leukemia[140] and in the progression of chronic myelogenous leukemia cell lines.[43] Furthermore, studies suggest that some proteins, such as the Kaposi's sarcoma-associated herpesvirus latent protein LANA2, may specifically block the transcriptional activity of FoxO3a to lead to tumor growth.[141] In cell models of endometrial cancer, pre-sensitization of cells to block Akt activation and foster transcription activity of FoxO1 enhances the effect of chemotherapy to limit tumor growth.[142]

Conclusions

The potential translation of FoxO proteins and their signal transduction pathways into viable therapeutic strategies against cancer offer exciting prospects for the future. FoxO proteins control several vital cellular pathways in relation to cell proliferation, metabolism, inflammation and survival. As a result, the ability of FoxO proteins to control cell cycle progression and promote apoptosis highlights the potential of FoxOs to become at the very least an important component for the development of new strategies against tumorigenesis. For example, use of triple mutant FoxO1 or FoxO3a expression in which three phosphorylation sites have been altered to prevent inactivation of this protein has been proposed as a potential therapeutic agent against melanoma tumors[143] and endometrial cancer.[144] Interestingly, new work suggests that the utilization and combination of multiple biomarkers may improve risk assessment for patients.[145] Studies such as this offer additional support for the use of FoxO proteins as biomarkers of cancer progression. As an example, down-regulation of the phosphatidylinositol 3 kinase and Akt pathways have been associated with increased transcript levels for FOXO1a and FOXO3a in clinical prostate cancer samples and may indicate the onset of pre-cancerous changes or the progression of on-going tumor growth.[146] Although loss of Akt activity in prostate cancer cells can result in enhanced FoxO3a activity and subsequent apoptosis of tumor cells,[131] it is conceivable that early stages of cancer may lead to reduced Akt activity with insufficient levels of active forkhead transcription factors to limit tumor progression. In addition, the early and persistent expression of phosphorylated FOXO1a in gastric tumors may not only indicate the onset of cancer, but also suggest an improved prognosis for patients.[147]

Despite the presently known attributes of FoxO proteins to potentially treat a number of cancers, FoxO transcription factors also may have a “dark side” that can limit clinical utility. Further investigations are required since FoxO protein inhibition of cell cycle progression may

not consistently lead to apoptotic cell death. Some investigations suggest that during oxidative stress, FoxO3a activation in association with SIRT1 can lead to cell cycle arrest, but not result in apoptotic cell injury.[148] Furthermore, during hypoxic stress, forkhead transcription factors, such as FOXO3a, may potentiate anti-apoptotic pathways in breast cancer cells to further tumor growth.[149] FoxO proteins also have been linked to potential chemotherapy drug resistance.[150] Increased expression of MDR1 (P-glycoprotein) has been associated with chemotherapy drug resistance in breast cancer cells and recent work shows that FoxO1 can stimulate the transcriptional activity of MDR1 that may promote increased tolerance of tumor cells.[151] In addition, the common pathways shared between Wnt and forkhead proteins may have another side that impacts upon the ability to control tumor growth.[68,75] FoxO proteins may assist with β-catenin activation in the Wnt pathway and lead to tumor cell proliferation.[69] In the presence of Wnt deregulation and increased β-catenin activity, tumorigenesis may ensue, such as with the proliferation of medulloblastoma tumors.[81] It is clear that future basic and clinical investigations are necessary to continue to elucidate the immense potential of FoxO proteins as well as to understand the potential limits of these transcription factors.

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Abbreviations

Aβ, β-amyloid
Akt, protein kinase B
AFX, acute leukemia fusion gene located in chromosome X
AGC, protein kinase A/protein kinase G/protein kinase C
CBP, CREB-binding protein
DAF-16, dauer formation-16
DM, diabetes mellitus
EPO, erythropoietin
FKHR, forkhead in rhabdomyosarcoma
FKHRL1, forkhead in rhabdomyosarcoma like protein 1
IKK, IκB kinase
IGF-1, insulin-like growth factor-1
IGFBP1, insulin-like growth factor binding protein-1
IRS, insulin-responsive sequence
JNK, Jun N-terminal kinase
NFκB, nuclear factor-κB
PS, phosphatidylserine
PTEN, tumor suppressor phosphatase and tensin homolog deleted on chromosome ten
PSR, phosphatidylserine receptor
ROS, reactive oxygen species
Wg, drosophila *wingless*

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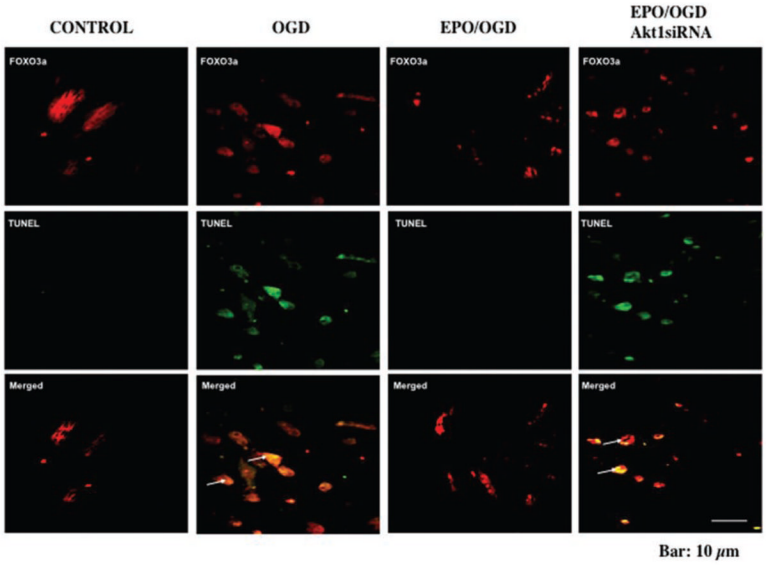

Figure 1.

FoxO3a leads to apoptotic cell injury that can be prevented by erythropoietin (EPO) though Akt dependent pathways. Immunofluorescent double staining for FoxO3a and TUNEL was performed at 6 hours after oxidative stress with oxygen-glucose deprivation (OGD). EPO (10 ng/ml) during OGD prevents nuclear DNA degradation and FoxO3a nuclear translocation in the same rat brain endothelial cells (ECs) with no overlap of staining in merged images. In contrast, white arrows in merged images show both nuclear FoxO3a and TUNEL staining (yellow) in ECs with OGD alone or with combined EPO/OGD and Akt1 siRNA gene silencing, illustrating that EPO requires Akt1 to prevent FoxO3a nuclear translocation that leads to apoptotic DNA degradation. Control equals untreated ECs.

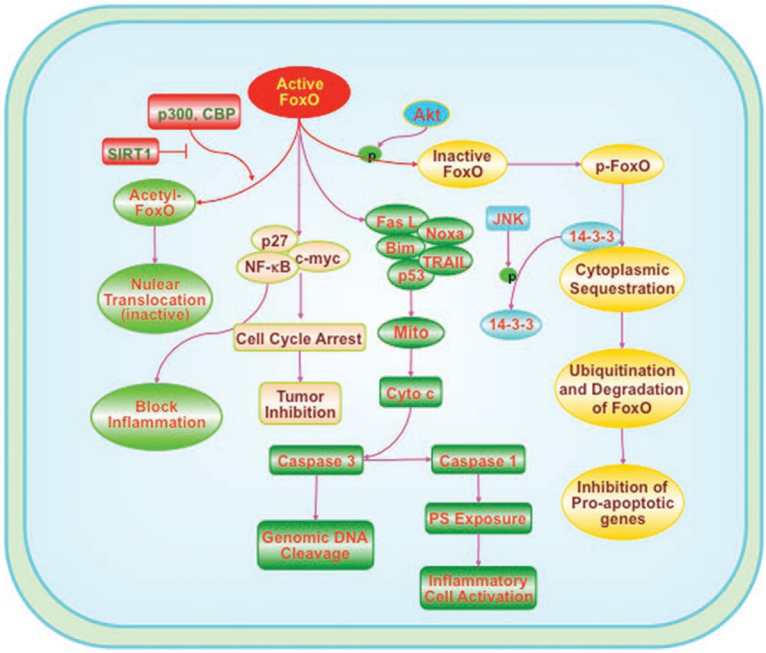

Figure 2.

FoxO proteins employ multiple signal transduction pathways that affect cell proliferation, survival and immune system function. Post-translational modification of FoxO proteins involves pathways associated with phosphorylation, acetylation and ubiquitylation. The serine-threonine kinase protein kinase B (Akt) can prevent cellular apoptosis through the phosphorylation of FoxO proteins. Post-translational phosphorylation (p) of FoxO proteins will maintain FoxO transcription factors in the cytoplasm by association with 14-3-3 proteins and prevent the transcription of pro-apoptotic target genes. During FoxO protein activation, FoxOs can prevent inflammatory cell activation through the inhibition of nuclear factor-κB (NFκB). FoxO proteins can lead to apoptotic death pathways that involve mitochondrial (Mito) release of cytochrome *c* (Cyto *c*) and caspase activation through a Fas-mediated ligand (Fas L) death pathway, tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), BH3-only proteins Noxa and Bim, or p53. Cell cycle inhibition that blocks tumor growth through FoxO protein activation can rely upon c-myc, p27 and NFκB. FoxO proteins may influence inflammatory cell activation through phosphatidylserine (PS) externalization on cells.

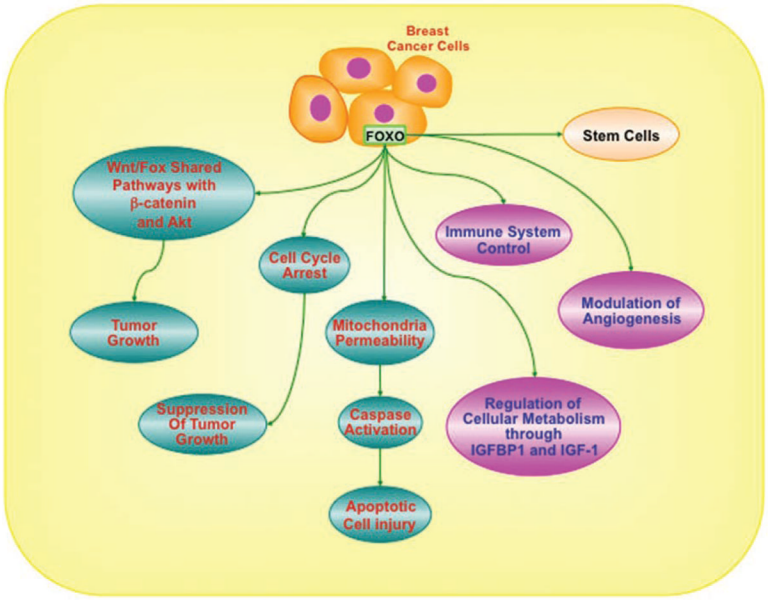

Figure 3.

As an example in breast cancer cells, FoxO proteins influence of number of cell pathways that impact upon the immune system, cellular metabolism, angiogenesis, stem cell development, cell cycle regulation and apoptosis. FoxO proteins form an important component for processes that involve apoptosis following increased mitochondrial membrane permeability with subsequent caspase activation. FoxO proteins suppress tumor growth through cellular mechanisms that require cell cycle arrest, angiogenesis and immune system regulation. FoxO proteins also are intimately involved with pathways that control cellular metabolism that include insulin-like growth factor binding protein-1 (IGFBP1) and insulin-like growth factor-1 (IGF-1). In relation to the integration with novel signal transduction pathways, FoxO proteins interface with Wnt and Akt to modulate cellular proliferation and survival with common mechanisms that can impact upon pathways such as those associated with β-catenin.