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A "FoxO" in Sight: Targeting FoxO Proteins from Conception to Cancer

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Synopsis

The successful treatment for multiple disease entities can rest heavily upon the ability to elucidate the intricate relationships that govern cellular proliferation, metabolism, survival, and inflammation. Here we discuss the therapeutic potential of the mammalian forkhead transcription factors predominantly in the O class, FoxO1, FoxO3, FoxO4, and FoxO6, which play a significant role during normal cellular function as well as during progressive disease. These transcription factors are integrated with several signal transduction pathways, such as Wnt proteins, that can regulate a broad array of cellular process that include stem cell proliferation, aging, and malignancy. FoxO transcription factors are attractive considerations for strategies directed against human cancer in light of their pro-apoptotic effects and ability to lead to cell cycle arrest. Yet, FoxO proteins can be associated with infertility, cellular degeneration, and unchecked cellular proliferation. As our knowledge continues to develop for this novel family of proteins, potential clinical applications for the FoxO family should heighten our ability to limit disease progression without clinical compromise.

Keywords

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

1. Origin and structure of FoxO transcription factors

More than 100 forkhead genes and 19 human subgroups that extend from FOXA to FOXS are now known to exist since the initial discovery of the fly *Drosophila melanogaster gene fork head* 1›2. A current nomenclature has replaced prior terms, such as forkhead in rhabdomyosarcoma (*FKHR*), the Drosophila gene fork head (*fkh*), and Forkhead RElated

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ACtivator (FREAC)-1 and -2. Within the subclasses of the Fox proteins that are each designated by a letter, an Arabic number is provided such that the actual name of a Fox protein would follow the designation of “Fox”, then a subclass or subgroup “Letter” is provided, and finally the member “Number” is listed. In relation to the nomenclature for human Fox proteins, all letters are capitalized, 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.

Of the mammalian forkhead transcription factors in the O class, FoxO1, FoxO3, FoxO4, and FoxO6 proteins can play a significant role during normal cellular function as well as during progressive disease. The most recently cloned member is FoxO6, but progressive interest in FoxO1, FoxO3, and FoxO4 has shown that these transcription factors can promote cell proliferation as well as cell death 3. For example, 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 2‚4. It is believed that FoxO proteins can influence cellular function in multiple species, since metabolic signaling with FoxO proteins is conserved among *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammals.

The forkhead box (FOX) family of genes have a conserved forkhead domain (the “forkhead box”) described as a “winged helix” as a result of the butterfly-like appearance on X-ray crystallography 5 and nuclear magnetic resonance 6. The forkhead domain in FoxO proteins consists of three α-helices, three β-sheets, and two loops that are referred to as the wings 2‚3, but it should be noted that not all winged helix domains are considered to be Fox proteins 7. 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. FoxO proteins bind DNA through the FoxO-recognized element with the consensus sequence T/C-G/A-A-A-A-C-A-A in the C-terminal basic region of the forkhead DNA binding domain8‚9. The forkhead proteins either activate or repress target gene expression through fourteen protein-DNA contacts with the primary recognition site located at α-helix H3 5. Although both the first and second loops make contact with DNA, it is the second loop that can enhance the specificity and stability of the binding. It is believed that 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 10. Yet, the mechanisms that lead to DNA binding with FoxO proteins are not completely defined and may depend upon several factors, such as variations in the N-terminal region of the recognition helix, changes in electrostatic distribution, and the ability of FoxO proteins to be shuttled to the cell nucleus that can be controlled by the C-terminal region of the forkhead domain 11‚12.

2. Tissue expression of FoxO proteins

FoxO proteins are expressed throughout the body and are found in the ovary, prostate, skeletal muscle, brain, heart, lung, liver, pancreas, spleen, thymus, and testis 3‚13¯17. Initially, 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 2. 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 18. A fusion between FOXO2 and MLL also occurs in some cases of acute myeloid leukemia that also is believed to be identical to FOXO3a 19.

Interestingly, FoxO proteins are not equally expressed in all tissues, suggesting that individual FoxO proteins may have specificity in regards to cellular function. For example, Foxo6 expression is found throughout several regions of the brain that play a significant role in cognitive function and emotion, such as the hippocampus, the amygdala, and the nucleus accumbens 16. However, Foxo1 may have a greater role in motor pathways with some memory formation, since its expression is primarily in the striatum and sub-regions of the hippocampus 16. On the other hand, Foxo3 is more diffusely represented in the hippocampus, cortex, and cerebellum, suggesting a complementary role for this FoxO protein to control cognitive and motor function. Furthermore, in mouse embryos and adults, mRNA expression of Foxo1, Foxo3a, and Foxo4 have a significant presence in muscle, adipose tissue, and liver with Foxo3a displaying a greater distribution in the heart, brain, and kidney 14.

3. FoxO proteins and cellular signaling

3.2 Post-translational control of FoxO proteins

Post-translational modification of FoxO proteins is critical for the regulation of these transcription factors and employs the biochemical pathways associated with phosphorylation, acetylation, and ubiquitylation 2,4,20,21. 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 2,22. Activation of Akt is usually cytoprotective, such as during free radical exposure 23,24, hyperglycemia 25, hypoxia 26,27, β-amyloid toxicity 28–30, and oxidative stress 31–33. Akt can prevent cellular apoptosis through the phosphorylation of FoxO proteins 34. 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 35,36. 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. Yet, FoxO6 transcriptional activity can be blocked by growth factors independent of shuttling to the cytosol through a FoxO6 N-terminal Akt site 37.

Modulation of Akt activity during oxidative stress can control the apoptotic pathways of the caspase family that may offer an alternative mechanism to regulate FoxO proteins. Caspases are a family of cysteine proteases that are synthesized as inactive zymogens which are proteolytically cleaved into subunits at the onset of apoptosis 38,39. The caspases 1 and 3 have each been linked to the apoptotic pathways of genomic DNA cleavage and cellular membrane PS exposure 23,40–42. These caspases, in addition to caspase 8 and 9, are also tied to the direct activation and proliferation of microglia 23,32,33. Furthermore, caspase 9 is activated through a process that involves the cytochrome c -apoptotic protease-activating factor-1 (Apaf-1) complex 43,44. 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 35,45–47. Pathways that can inhibit caspase 3 activity appear to offer a unique regulatory mechanism for FoxO3a that blocks the proteolytic degradation of inactive phosphorylated FoxO3a to prevent apoptotic cell injury during oxidative stress 35,45,46.

In addition to phosphorylation of forkhead transcription factors, post-translational modification of FoxO proteins also relies upon biochemical pathways associated with ubiquitylation and acetylation 48,49. 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 4,49. In the absence of Akt, IκB kinase (IKK) also can directly

phosphorylate and block the activity of FoxO proteins, such as FoxO3a. This leads to the proteolysis of FoxO3a via the Ub-dependent proteasome pathway 50. 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 51. 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. Interestingly, c-Jun N-terminal kinase (JNK) phosphorylates 14-3-3 protein leading to the nuclear localization of FoxO proteins, such as FoxO3a 52, suggesting that JNK promotes apoptosis through increased FoxO protein activity. The protein kinase mammalian sterile 20-like kinase-1 also can phosphorylate FoxO proteins directly and lead to their activation 53. 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 54. 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 2‚4‚20‚21. 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 55. In addition, acetylation can increase phosphorylation of FoxO proteins by Akt 55.

3.2 FoxO proteins, oxidative stress, and apoptosis

In many diseases, cellular survival and cellular longevity are intimately dependent upon exposure to oxidative stress and the induction of apoptotic pathways. Oxidative stress is a result of the release of reactive oxygen species (ROS) that include superoxide free radicals, hydrogen peroxide, singlet oxygen, nitric oxide, and peroxynitrite 56. Oxygen free radicals and mitochondrial DNA mutations have become associated with tissue injury, aging, and accumulated toxicity for an organism 1‚57‚58. Most ROS 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 molecule substances, such as vitamins C, E, D_3 59 and nicotinamide, the amide form of niacin or vitamin B_3 60-63.

Genes involved in apoptosis have recently been found to be involved in processes of cell replication and transcription, suggesting that apoptotic pathways may be involved in multiple cellular events that do not necessarily lead to cell death 64. However, during conditions that inadequately control the production of ROS and lead to oxidative stress, cell apoptotic injury can ensue and contribute to disease pathology in disorders such as diabetes, Alzheimer's disease, and cardiovascular injury 1‚57‚65‚66. Apoptotic cell death is a dynamic process that entails both early and late events. Membrane phosphatidylserine (PS) externalization is an early event during cell apoptosis 67‚68 that assists microglia to target cells for phagocytosis 32‚33‚62‚69‚70. This process occurs with the expression of the phosphatidylserine receptor (PSR) on microglia during oxidative stress 71-73, since blockade of PSR function in microglia prevents the activation of microglia 23‚33. As an example, externalization of membrane PS residues occur in neurons during anoxia 74-76, nitric oxide exposure 77‚78, and during the administration of agents that induce the production of ROS, such as 6-hydroxydopamine 79. The cleavage of genomic DNA into fragments 80-82 is considered to be a later event during apoptotic injury 41. Several enzymes responsible for DNA degradation have been identified and include the acidic, cation independent endonuclease (DNase II), cyclophilins, and the 97 kDa magnesium - dependent endonuclease 1‚56. Three separate endonuclease activities are present in neurons

that include a constitutive acidic cation-independent endonuclease, a constitutive calcium/magnesium-dependent endonuclease, and an inducible magnesium dependent endonuclease 83,84.

Cell culture and animal studies that examine the effects of oxidative stress illustrate that FoxO proteins are closely tied to apoptotic injury (Table 1). It appears that FoxO1 and FoxO3a must be present for oxidative stress to result in apoptotic cell injury 85 and that the conditional deletion of FoxO1, FoxO3a, and FoxO4 can lead to an increase in ROS 86. Furthermore, FoxO3a in conjunction with JNK has been shown to modulate an apoptotic ligand activating a Fas-mediated death pathway in cultured motoneurons 87, to lead to apoptosis through tumor-necrosis-factor-related apoptosis-induced ligand (TRAIL) and BH3-only proteins Noxa and Bim in neuroblastoma cells 47, and to promote pro-apoptotic activity of p53 88. In addition, loss of FoxO protein activity can result in cytoprotection. Protein inhibition or gene knockdown of FoxO proteins, such as FoxO1 or FoxO3a, increases neuronal survival through NAD^+ precursors 46, leads to stroke reduction by estradiol 89, mediates the protective effects of metabotropic glutamate receptors 45, and provides trophic factor protection with erythropoietin (EPO) 35 and neurotrophins 90. This cytoprotection, such as with EPO, involves both inhibition of nuclear shuttling (Figure 1) as well as phosphorylation by Akt 35.

3.3 FoxO proteins and integration with novel cellular pathways

FoxO proteins are integrated with multiple signal transduction pathways which regulate cellular apoptosis and longevity during oxidative stress. 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 91,92. 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 61. At least nineteen of twenty-four Wnt genes that express Wnt proteins have been identified in the human 91-93.

Wnt proteins are generally divided into functional classes based on their ability to induce a secondary body axis in *Xenopus* embryos and to activate certain signaling cascades that consist of the Wnt1 class and the Wnt5a class 61,92. These involve intracellular signaling pathways that are critical for Wnt signal transduction. One Wnt pathway involves intracellular calcium release and is termed the non-canonical or Wnt/calcium pathway consisting primarily of Wnt4, Wnt5a, and Wnt11. The non-canonical system functions through non-β-catenin-dependent pathways and also includes the planar cell polarity (PCP) pathway or the Wnt-calcium-dependent pathways 91-93. A second pathway controls target gene transcription through β-catenin, generally referred to as the canonical pathway that involves Wnt1, Wnt3a, and Wnt8. It is the β-catenin pathway that appears to tie FoxO proteins and Wnt signaling together. For example, in relation to Alzheimer's disease, amyloid is toxic in cell culture 28,94 and is associated with the phosphorylation of FoxO1 and FoxO3a that can be blocked with ROS scavengers 95 (Table 1). Interestingly, a common denominator in the pathways linked to amyloid 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 96 and β-catenin also has been demonstrated to be necessary for protection against amyloid toxicity in neuronal cells 94.

In addition to shared signal transduction pathways between Wnt and FoxO proteins that involve β-catenin, Akt is intimately tied to both Wnt and FoxO signaling. As previously described, Akt phosphorylates and blocks the activity of the FoxO proteins FoxO1, FoxO3a,

and FoxO4 2,22. In relation to Wnt signaling, Wnt relies upon Akt for cell differentiation and cytoprotection. For example, neuronal cell differentiation that is dependent upon Wnt signaling and trophic factor induction is blocked during the repression of Akt activity 97. Furthermore, Wnt differentiation of cardiomyocytes does not proceed without Akt activation 98 while reduction in tissue injury during pressure overload cardiac hypertrophy 99 and the benefits of cardiac ischemic preconditioning also appear to rely upon Akt 100. In addition, Wnt over-expression can independently increase the phosphorylation and the activation of Akt to promote neuronal protection and control microglial activation 94.

4. FoxO, stem cells, and cellular development

As our knowledge of FoxO proteins continues to grow, new work provides evidence for the role of FoxO proteins in vascular system development, fertility, and progenitor cell differentiation. Studies have shown that *Foxo3a* -/- and *Foxo4* -/- mice develop without incidence and are indistinguishable from control littermates, but mice singly deficient in *Foxo1* die by embryonic day eleven and lack development of the vasculature 101. Furthermore, *Foxo3a* -/- mice become infertile and experience follicular activation to the extent that ovarian follicles are depleted of oocytes 13. Other work using a mouse model of Foxo3a over-expression in oocytes further suggests that Foxo3a retards oocyte growth and follicular development and leads to anovulation and luteinization of unruptured follicles 102. The studies with Foxo3a null mice may suggest a role for FoxO3a in addition to FoxO1 in relation to oocyte and follicular development. For example, in a small percentage of women who suffer from premature ovarian failure, mutations in *FOXO3a* and *FOXO1a* have been observed 103 (Table 1). If one examines hematopoietic stem cell development, studies suggest that FoxO3a alone may play a role in maintaining hematopoietic stem cells, since these cells are significantly decreased in aged *Foxo3*-/- mice compared to the littermate controls 104. Yet, other work indicates that the combined loss of *Foxo1*, *Foxo3a*, and *Foxo4* in mice is required to lead to defective repopulation of hematopoietic stem cells with resultant apoptosis 86.

A number of cellular agents, such as the growth factor and cytokine EPO 36,105, also may determine whether FoxO proteins function in concert or independently to progenitor cell growth. In cell culture and animal studies, EPO is cytoprotective in neuronal and vascular cells and can stimulate postnatal neovascularization by increasing endothelial progenitor cell mobilization from the bone marrow 44,106,107. Interestingly, the ability of EPO to foster eythroid progenitor cell development is dependent upon the inhibition of FoxO3a activity 36,108, but also may require regulation of specific gene expression through an EPO-FoxO3a association to promote erythropoiesis in cultured cells 109. In addition, rat enteric nervous system precursor development that occurs in the presence of the growth factor glial cell line-derived neurotrophic factor appears to require the inactivation of FoxO1 and FoxO3a 110.

5. FoxO proteins, diabetes, and cellular metabolism

Both clinical and experimental studies exemplify the role of FoxO proteins in cellular metabolism and disorders such as diabetes mellitus (DM). DM is a significant health concern for both young and older populations 111,112. Approximately 16 million individuals in the United States and more than 165 million individuals worldwide suffer from DM. By the year 2030, it is predicted that more than 360 million individuals will be afflicted with DM and its debilitating conditions 113. Type 2 DM represents at least 80 percent of all diabetics and is dramatically increasing in incidence as a result of changes in human behavior and increased body mass index 114. Type 1 insulin-dependent DM is present in 5-10 percent of all diabetics 112, but is increasing in adolescent minority groups

115. Furthermore, the incidence of undiagnosed diabetes, impaired glucose tolerance, and fluctuations in serum glucose in the young raises additional concerns 116.

Patients with DM can develop severe neurological and vascular disease 117,118 that can lead to an increased risk for cognitive decline 1,38,119. Interestingly, the development of insulin resistance and the complications of DM in the nervous and vascular systems can be the result of cellular oxidative stress 111,112. Hyperglycemia can lead to increased production of ROS in endothelial cells, liver and pancreatic β-cells 120-123. Recent clinical correlates support these experimental studies to show that elevated levels of ceruloplasmin are suggestive of increased ROS 124. Furthermore, acute glucose swings in addition to chronic hyperglycemia can trigger oxidative stress mechanisms, illustrating the importance for therapeutic interventions during acute and sustained hyperglycemic episodes 125.

Both clinical and experimental studies exemplify the role of FoxO proteins during cellular metabolism and DM. FoxO proteins can stimulate the insulin-like growth factor binding protein-1 (IGFBP1) promoter by binding to the insulin-responsive sequence (IRS) 126. Insulin and insulin-like growth factor-1 (IGF-1) can suppress FoxO protein activity through activation of Akt 126,127. In a clinical study of 734 individuals, the *c. −343–1582C>T* polymorphism of *FOXO3a* displayed a significant association with body mass index such that the highest body mass index was present in individuals homozygous for this allele 128. Analysis of the genetic variance in *FOXO1a* and *FOXO3a* on metabolic profiles, age-related diseases, fertility, fecundity, and mortality illustrated higher HbA_{1c} levels and increased mortality risk associated with specific haplotypes of *FOXO1a*. There also was an increased risk of stroke in two haplotypes of *FOXO3a* block-A, suggesting an association with cerebral oxidative stress disorders such as diabetes and stroke with *FOXO1a* and *FOXO3a* 129 (Table 1).

In some animal and cell culture studies, modulation of forkhead transcription factors, such as FoxO3a, may counteract the detrimental effects of high serum glucose levels. For example, 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 130. Additional investigations have associated diabetic nephropathy to post-translational changes in FoxO3a by demonstrating that inhibitory phosphorylation of FoxO3a increases in rat and mouse renal cortical tissues two weeks after the induction of diabetes by streptozotocin 131, suggesting that the loss of FoxO3a activity can lead to renal disease. The human immunodeficiency virus (HIV) -1 accessory protein Vpr also has been reported in human hepatoma cells to contribute to insulin resistance by interfering with FoxO3a signaling with protein 14-3-3 132. Yet, other work suggests that inactivation of FoxO proteins may foster cytoprotection. For example, enteric neurons can be protected from hyperglycemia by glial cell line-derived neurotrophic factor that can affect Akt signaling and prevent FoxO3a activation 25.

The preservation of cellular energy reserves and mitochondrial function also may be a critical factor for FoxO proteins to regulate cellular metabolism during DM. Chronic exposure to elevated levels of free fatty acids can increase ROS production in cells and has been shown to lead to mitochondrial DNA damage and impaired pancreatic β-cell function 133. In patients with type 2 DM, skeletal muscle mitochondria have been described to be smaller than those in control subjects 134. In addition, a decrease in the levels of mitochondrial proteins and mitochondrial DNA in adipocytes has been correlated with the development of type 2 DM 135. Insulin resistance in the elderly also has been associated with elevation in fat accumulation and altered mitochondrial oxidative and phosphorylation activity 136,137. In caloric restricted mice that have decreased energy reserves, mRNA expression is progressively increased for Foxo1, Foxo3a, and Foxo4 over a two year course

15. This work is complementary to investigations in *Drosophila* and mammalian cells that show up-regulation of FoxO1 expression leads to increased insulin signaling to regulate cellular metabolism 138. Yet, other studies such as with Foxo1 have shown that over-expression of this transcription factor in skeletal muscles of mice can lead to reduced skeletal muscle mass and poor glycemic control 139, illustrating that activation of FoxO proteins may also impair cellular energy reserves. As a result, one potential agent to consider for the maintenance of cellular metabolism in DM is nicotinamide 61،140،141, an agent that also can inhibit FoxO protein activity 46. 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 38،62،111. It is of interest to note that nicotinamide may derive its protective capacity through two separate mechanisms of post-translational modification of FoxO3a. Nicotinamide not only can maintain phosphorylation 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 46 (Table 1).

6. FoxO proteins, cellular longevity, and immune system function

As an extension of the studies examining apoptotic cell injury, FoxO proteins also have been tied to cell longevity and aging as shown by early studies linking DAF-16 in *Caenorhabditis elegans* to increased longevity 38،142. However, the relationship between FoxO transcription factors and proteins that increased cellular lifespan has been met with controversy. SIRT1 is an NAD^+-dependent deacetylase and the mammalian ortholog of the silent information regulator 2 (Sir2) protein associated with increased lifespan in yeast. Some studies suggest that stimulation of SIRT1 during starvation is dependent upon FoxO3a activity as well as p53 143. In contrast, other work has shown in cell culture that SIRT1 may repress the activity of FoxO1, FoxO3a, and FoxO4, suggesting that cellular longevity may benefit from reduction in FoxO protein generated apoptosis 144. Additional studies offer alternative views to illustrate that SIRT1 binds to FoxO proteins, such as FoxO4, to catalyze its deacetylation and enhance FoxO4 activity while acetylation of FoxO4 by cyclic-AMP responsive element binding (CREB)-binding protein serves to inhibit FoxO4 transcriptional activity 145،146.

FoxO proteins also have been linked to cell aging and senescence. In cultured human dermal fibroblasts, gene silencing of FoxO3a protein results in cell morphology consistent with cell senescence, cell population doubling times, and the generation of ROS, suggesting that FoxO protein activity may be required to extend cell longevity and limit oxidative stress 147. Additional work in animal models of aging demonstrates a reduction in SIRT1 in the heart, but no significant change in FoxO3a expression with advanced age. However, during exercise training, an up-regulation of FoxO3a and SIRT1 activity is observed in the heart 148, suggesting that the benefits of physical activity for the cardiovascular system may be associated with FoxO proteins. In addition, FoxO proteins may be protective during aging, since loss of FoxO3a activity in explanted vascular smooth muscle of aged animals may limit tissue antioxidant properties through decreased manganese-superoxide dismutase and lead to enhanced cell injury with aging 149. Extension of cellular lifespan that depends upon the prevention of cell senescence at least in primary human cultured vascular cells also may require the negative regulation of Akt to allow for the activation of FoxO3a 150.

Given that inflammatory cell modulation has a significant impact upon cellular apoptosis, FoxO proteins also function as critical components for modulation of immune cell function. The ability to regulate early apoptotic membrane PS externalization and subsequent inflammatory cell activity can ultimately impact upon cell survival and longevity since activated immune cells can lead to the phagocytic removal of both neurons and vascular

cells 56,71. Inflammatory cells, such as macrophages or microglia, require the activation of intracellular cytoprotective pathways to proliferate and remove injured cells 72,151. Inflammatory cells can be beneficial and form a barrier for the removal of foreign microorganisms and promote tissue repair during cell injury 39,152. Yet, inflammatory cells also may lead to cellular damage through the generation of ROS and through the production of cytokines 41,71,153.

Clinical studies have demonstrated in synovial biopsy tissue of patients with rheumatoid arthritis and osteoarthritis the phosphorylation of FOXO1 and FOXO4 in macrophages and the phosphorylation of FOXO3a in T lymphocytes, suggesting that inhibitory post-translational phosphorylation of these FOXO family members may lead to inflammatory cell activation 154 (Table 1). Additional work has shown that *FOXO1* gene transcript levels are down-regulated in peripheral blood mononuclear cell of patients with systemic lupus erythematosus and rheumatoid arthritis 155, illustrating a potential etiology through the loss of functional FOXO proteins for these disorders and possibly providing a biomarker of disease activity. Clinical work also suggests a relationship between the regulation of immune system activity and the induction of apoptotic pathways that are dependent upon FoxO proteins. FoxO proteins may work in concert with Fas signaling to clear activated T cells following a decrease in cytokine stimulation in patients with autoimmune lymphoproliferative syndromes, suggesting that specific FoxO proteins may be targeted for treatment of autoimmune disorders 156. 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 157. FoxO3a also appears to be necessary for neutrophil activity, since *Foxo3a* -/- mice are resistant to models of neutrophilic inflammation that involve immune complex-mediated inflammatory arthritis 158. Prevention of inflammatory activation and apoptosis in the nervous system such as in systemic lupus erythematosus in animal models may require the up-regulation of different Fox proteins, such as FoxJ1 and FoxO3a, that can block nuclear factor-κB (NF-κB) activation and interferon-gamma secretion 159 (Table 1). Animal studies using experimental autoimmune encephalomyelitis to mimic multiple sclerosis and myelin injury also have shown that osteopontin, a protein expressed in multiple sclerosis lesions, leads to the prolonged survival of myelin-reactive T cells and disease progression through a combination of events that involve FoxO3a inhibition, NF-κB activation, and modulating the expression of the pro-apoptotic proteins Bim, Bak, and Bax 160.

7. FoxO proteins and tumorigenesis

One of the most interesting therapeutic applications for FoxO proteins involves strategies directed against human cancer, since the pro-apoptotic effects of FoxO proteins and their ability to block cell cycle progression make these transcription factors almost ideal therapeutic targets to control tumorigenesis. 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 45 161. 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 162. 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 163 (Table 1). However, the loss of FoxO3a activity in association with c-myc, p27, and NF-κB can result in cell cycle induction and malignant transformation of mouse cells in the presence of oncogene activation 164. It should be noted that 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 the Sir2 homolog SIRT1 can lead to cell cycle arrest, but not result in apoptosis 165.

Early clinical studies of breast cancer in relation to FOXO3a suggested that activation of FOXO3a was associated with lymph nodal metastasis and a poor prognosis 166. However, other studies reported that FOXO3a was confined to the cytoplasm of human tumor cells, inactivated by IKK, and that this inactivation of FOXO3a was associated with a poor prognosis in breast cancer 50, 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. In animal studies, somatic deletion in mice of *Foxo1, Foxo3a*, and *Foxo4* results in the growth of thymic lymphomas and hemangiomas, further illustrating the potential of FoxO proteins to function as redundant repressors of tumor growth 167. Studies in breast cancer cells parallel this work and show that increased activity of FoxO3a in association with JNK in breast cancer cell lines 168 or in association with cyclin-dependent kinase inhibitor p27 in isolated human breast cancer cells can suppress breast cancer progression 169.

Studies with prostate cancer have shown that the tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) was mutated in almost eighty percent of tumors with the loss of FOXO1 and FOXO3a activity. In cell culture work, over-expression of FoxO1 and FoxO3a in prostrate tumor cell lines could result in apoptosis, suggesting that FoxO1 and FoxO3a were necessary for limiting prostate cell tumor growth 17. In further support of this work, inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth 170 while agents that increase FoxO3a activity in both androgen sensitive and androgen insensitive prostate cell lines prevent prostate cancer cell progression 171 (Table 1).

In addition to neoplasms in breast and prostate, FoxO proteins also may represent a viable option to control tumor growth in tissues throughout the body. FoxO3a activation in colon carcinoma cell lines prevents tumor proliferation through Myc target genes that involve the Mad/Mxd family of transcriptional repressors 172. Other investigations illustrate that the loss of FoxO3a activity may participate in oncogenic transformation in B-chronic lymphocytic leukemia 173 and in the progression of chronic myelogenous leukemia cell lines 162. 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 174. Yet, FoxO proteins may have a complex role during tumor growth. FoxO3a is a positive regulator of androgen receptor expression and therefore may also assist with prostate cancer cell proliferation 175. In addition, loss of functional FoxO3a in human ovarian cancer cell lines can limit the sensitivity of ovarian cancer cells to chemotherapy 176, suggesting that FoxO proteins may be responsible for altered treatment outcomes in the presence of combined therapeutic approaches (Table 1).

8. Perspectives and future considerations: Targeting FoxO proteins

In light of the robust ability of FoxO proteins to oversee cell proliferation, cell metabolism, cell survival, and immune system function, these transcription factors may be enthusiastically considered for the treatment of a wide variety of disorders. For example, the known mutations in FoxO proteins that exist in several disease entities may provide novel insights for therapeutic strategies that can address a broad range of disorders. Although theses mutations are considered to represent one of multiple factors responsible for disorders such as premature ovarian failure, further analysis in larger populations of patients with premature ovarian failure could enhance our understanding of the role of FoxO proteins in disorders of human fertility. In relation to the immune system, recent work has suggested

that FoxO proteins may function as biomarkers of disease activity and also offer a potential target for the treatment of autoimmune disorders. Furthermore, the ability of FoxO proteins to control cell cycle progression and promote apoptotic cell death suggests that FoxO transcription factors may be developed for new advances against tumorigenesis. As an example, triple mutant FoxO3a expression that cannot be inhibited through phosphorylation has been proposed as a potential therapeutic target against melanoma tumors 177.

Yet, it must be realized that the causal relationships between FoxO proteins and cellular metabolism, apoptotic injury, immune system function, and cancer are not well defined and that protocols to modulate FoxO proteins may yield a double-edge sword for both beneficial and detrimental clinical results. For example, the common pathways shared between Wnt and forkhead proteins may have another side that relates to tumorigenesis 91،178. Fox transcription factors can activate the Wnt/β-catenin pathway to increase extracellular proteoglycans and promote gastrointestinal cell proliferation 179. In the presence of Wnt deregulation and increased β-catenin activity, tumorigenesis may ensue 92. Deregulation in the Wnt pathway that promotes activation of β-catenin and cell survival also has been associated with the proliferation of medulloblastoma tumors 180. In addition, Wnt expression has been correlated with advanced gastric cancer stages and a poor prognosis 181 while experimental activation of the β-catenin pathway leads to the development of gastric tumors 182. Irrespective of the role of Wnt signaling, conditions also can exist that allow FoxO proteins to prevent cell cycle progression in cells without leading to apoptotic injury. Although this result may be considered beneficial to block degenerative disorders, in the setting of cancer, these results would severely limit clinical utility. Furthermore, FoxO transcription factors can foster apoptosis in prostate cancer cells, but also may contribute to androgen receptor expression and potentially diminish any clinical benefits.

As a result, it becomes essential to promote both basic as well as clinical research with FoxO proteins to comprehend the complex role played by these transcription factors. For example, FoxO proteins are expressed throughout the brain and particularly in sensitive cognitive and motor regions, but it is unclear how FoxO transcription factors may influence neuronal plasticity, angiogenesis, and immune system function that alter the progression of dementias or behavioral abnormalities. Investigations also are required to clarify both independent and shared signal transduction pathways of FoxO proteins, such as with Wnt signaling, to further understand the cellular processes that can influence gene transcription and intracellular trafficking of these pathways. In some scenarios, Fox proteins may prevent tumorigenesis during Wnt deregulation, but in other examples, Fox proteins may assist with β-catenin activation and potentially tumor cell proliferation. A number of currently unknown parameters may account for these discrepancies, such as the role of FoxO post-translational state, factors that control FoxO protein DNA binding, intracellular signaling and tissue specifics during oxidative stress, age of the organism, and cellular metabolic state. As new studies continue to unfold, the clinical applications for FoxO proteins should develop at a surprisingly fast pace to not only foster health maintenance, but also limit disease progression.

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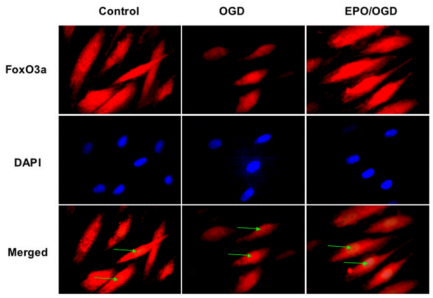

Figure 1. Erythropoietin (EPO) excludes FOXO3a from nuclear translocation during oxidative stress

Application of EPO (10 ng/ml) during an 8 hour period of oxygen-glucose deprivation (OGD), OGD alone, or in untreated primary rat cerebral endothelial cells (Control) was followed at 6 hours with immunofluorescent staining with primary rabbit anti-FoxO3a antibody then by Texas red conjugated anti-rabbit secondary antibody. Nuclei of endothelial cells were counterstained with DAPI and are highlighted in the bottom panels for Control, OGD, and EPO/OGD with green arrows. In merged images, endothelial cells with combined EPO and OGD demonstrate minimal FoxO3a staining in the nuclei of cells (white) and show the cytoplasm of endothelial cells with significant FoxO3a staining (red). These observations are in contrast to cells with OGD alone with significant FoxO3a staining in both the cytoplasm and the nuclei of endothelial cells, demonstrating the ability of EPO to prevent the translocation of FoxO3a to the nucleus to initiate a pro-apoptotic program.

Table 1
FoxO signaling and related pathways in disease

Pathological modalities	FoxO proteins expression and post-translational effects	Selected references
Alzheimer's disease and aging	Amyloid leads to phopshorylarion of FoxO1 and FoxO3a	95
	β-catenin can modulate FoxO transcriptional activity	96
	Wnt signaling relies upon β-catenin to prevent neuronal amyloid toxicity	94
	Benefits of physical cardiovascular activity may be tied to FoxO protein expression	148
	Prevention of FoxO protein translational activity can reduce cell loss during oxidative stress and neurodegeneration	35, 45, 89-90
Cancer	FoxO proteins are attractive strategies against cancer cell proliferation through cell cycle regulation	161-164
	Absence of functional FoxO proteins leads to breast, thymic, and prostate tumors	17, 167-171
	FoxO3a transcriptional activity may be required to prevent B-chronic lymphocytic leukemia and chronic myelogenous leukemia	162. 173
	FoxO proteins may have a complex role in cancers during modulation of androgen receptor expression and chemotherapy sensitivity	175-176
Diabetes mellitus	FoxO1 and FoxO3a associated with increased HbA_{1c} and increased mortality	128
	FoxO1 and FoxO3a linked to increased risk of diabetes and stroke	129
	In some animal models of diabetes, loss of FoxO3a activity may contribute to disease complications	131
	NAD^+ precursors protect β-cell function and reduce clinical disease during diabetes that may require removal of FoxO3a activity	38, 46, 62, 111
Immune system dysfunction	Post-translational phosphorylation of FoxO proteins may promote inflammatory cell activation during rheumatoid arthritis and osteoarthritis	154-156
	Animal studies suggest that Fox proteins are required to modulate T-cell activity, neutrophil activity, NF-κB activity, and interferon-gamma secretion	157-159
Premature ovarian failure	FoxO3a can prevent oocyte growth and follicular development	13, 102
	In some women who experience premature ovarian failure, mutations in FOXO1a and FOXO3a have been reported	103

Nomenclature: “FoxO” refers to cell culture, tissue, or animal studies; “FOXO” refers to human studies