OutFOXing Myeloid Cells in Atherosclerosis With FoxOs

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Compelling experimental and human studies implicate myeloid-driven inflammation as a significant component in the initiation and progression of atherosclerosis and a range of other chronic inflammatory diseases, including obesity, type 2 diabetes mellitus, and metabolic syndrome. Indeed, elevated circulating white blood cell (WBC) count is a strong predictor of coronary heart disease and all-cause mortality independent of traditional risk factors, an association that has been observed across sexes, age, and diverse ethnic populations over the past 4 decades. Elevated monocyte counts may also correlate with cardiovascular risk better than other WBC subsets. Accumulating studies have recognized specific circulating myeloid cells, such as Ly-6C+ monocytes in mice and CD14+CD16+ subsets in humans, as potential instigators of atherosclerotic lesion formation and insulin resistance. The importance of the number of peripheral mononuclear cells as a contributor to lesion formation was highlighted, in part, using op/op mice that bear a mutation in the coding region for macrophage colony-stimulating factor; op/op mice bred onto atherosclerotic-prone mice exhibited marked reductions in lesion formation. Subsequent experimental studies demonstrated that reduction in the number of peripheral myeloid cells in the circulation pharmacologically also attenuated the development of vascular inflammatory disease. However, identification of the downstream transcriptional regulators that govern peripheral myeloid cell numbers and function in the context of atherosclerosis remain poorly understood.

In this issue of Circulation Research, Tsuchiya reveals an important role for the forkhead box (FoxO) family of transcription factors, namely FoxO1, FoxO3a, and FoxO4, in controlling the number of circulating myeloid cells and effects on atherosclerosis and insulin resistance. Specifically, myeloid ablation of all 3 FoxO isoforms increased the proliferation rate of granulocyte-macrophage progenitors (GMPs) in the bone marrow, resulting in elevated peripheral number of monocytes and granulocytes, and also enhanced atherosclerotic lesion formation in low density lipoprotein receptor (LDLR)−/− mice. In addition, these FoxO-deficient macrophages exhibited reduced sensitivity to cholesterol-induced apoptosis and generated increased markers of oxidative stress. Furthermore, macrophages from the compound triple FoxO knockout (MYFKO)/LDLR−/− mice demonstrated impaired insulin signaling, a hallmark of the insulin resistant state. Similarly, reduced hepatic insulin signaling was observed in Western diet-fed MYFKO/LDLR−/− mice associated with inducible nitric oxide synthase–dependent NO production and a generalized increase of protein cysteine nitrosylation and tyrosine nitration. Impressively, neutralization of reactive oxygen species (ROS) in these mice with the antioxidant N-acetyl-L-cysteine reversed the increase in peripheral WBC and monocytes, atherosclerotic lesion formation, and features of the insulin-resistant phenotype. Collectively, these findings provide a significant step forward in our understanding of myeloid transcriptional regulation in atherosclerotic lesion formation.

Although on the surface these findings increase enthusiasm for identifying mechanisms to restore FoxO expression as a protective mechanism in the development of atherosclerosis, a few important reservations merit consideration. In particular, these provocative findings from myeloid triple FoxO–deficient mice contrast starkly with ablation of the same triple FoxO isoforms in the vascular endothelium, an effect that led to reduced atherosclerosis and insulin resistance in LDLR−/− mice (Figure). Furthermore, these findings are even more surprising because 2 recent reports demonstrated that in response to FoxO1 activation, macrophages increase inflammation by enhancing interleukin-1β expression or toll-like receptor-4 signaling. To understand the paradoxical phenotypes, one should consider cell-type–specific effects, subcellular localization, targets, interactors, and post-translational modifications of the FoxO family of transcription factors in related biological paradigms.

The FOX family of proteins constitute a group of transcription factors defined by an evolutionarily conserved 80- to 110-aa DNA–binding motif known as the winged helix because of the similarity to a butterfly among the helix-turn-helix structure. FOX transcription factors bind to DNA as monomers and may act as transcriptional activators or repressors. The founding member was identified from a mutant of the Drosophila gene, Forkhead, bearing ectopic head structures. FOX members are broken down into 19 subfamilies FOX A to S phylogenetically across 10 species; there are at least 44 FOX members in humans and mice. The forkhead transcription factors of the O subgroup (FoxO) have garnered significant attention because of their functional roles in regulating cell growth, differentiation, metabolism, and oxidative stress. Although there are 4 FoxO family members in mice (FoxO1, FoxO3a, FoxO4, and FoxO6), FoxO6 expression is restricted to the brain, whereas the others have a broader and more varied expression pattern in tissues and cell types.
Several lines of evidence support critical roles for FoxO1, FoxO3a, or FoxO4 in diverse aspects of cardiovascular and immune biology, in part, because of their ability to serve as targets of insulin and growth factor signaling pathways. For example, in response to activation of the insulin/insulin-like growth factor signaling pathway, activation of phosphoinositide 3-kinase/Akt leads to phosphorylation of these FoxOs that contain 3 conserved Akt phosphorylation motifs. On Akt-mediated FoxO phosphorylation, they are excluded or exported from the nucleus into the cytoplasm leading to their inactivation, whereas unphosphorylated FoxOs remain in the nucleus to mediate transcriptional responses. Careful attention is required for how FoxOs are phosphorylated because free cholesterol loading in macrophages can lead to activation of Jun NH2-terminal kinase, and in other cell types, Jun NH2-terminal kinase may phosphorylate FoxOs on different amino acids than Akt, an effect resulting in increased nuclear localization and activity. This point is highlighted in a recent study by Kawano et al demonstrating increased expression of nuclear FoxO1 in adipose tissue macrophages in response to a high-fat diet in mice. Myeloid-specific deletion of 3-phosphoinositide–dependent protein kinase 1 (thereby reducing FoxO1 Akt–mediated phosphorylation) or overexpression of a constitutively active nuclear FoxO1 in mice increased FoxO1 nuclear localization, transcriptional activity, and insulin resistance, an effect mediated in part by increased expression of the chemokine receptor cer2 and accumulation of adipose M1 proinflammatory macrophages. Thus, not only is FoxO expression an important determinant for functional effects in cells, but also FoxO subcellular localization.

FoxOs bind DNA by either positively or negatively regulate target gene expression. One of the major phenotypic differences between the cell-specific FoxO knockout mice is the induction of inducible nitric oxide synthase (iNOS)–dependent protein nitrosylation in myeloid FoxO–deficient LDLR−/− mice, an effect associated with increased reactive oxygen species (ROS) production, whereas endothelial FoxO–deficient LDLR−/− mice have increased endothelial nitric oxide synthase (eNOS) expression and eNOS–dependent vasorelaxation with reduced ROS generation. FoxO cofactors may include p300/CBP, PGC-1, SIRT1, or HDACs. Akt is also known as Protein Kinase B. Cdkns indicates cyclin-dependent kinase inhibitors; ERK, extracellular signal–regulated kinases; FC, free cholesterol; GPx, one of the glutathione peroxidases; ICAM, intercellular adhesion molecule; InhA, inhibitor of HDACs; IRS, insulin receptor substrate; ICAM, intercellular adhesion molecule; LPS, lipopolysaccharide; NADPHox, NADPH oxidase; PI3K, phosphoinositide 3-kinase; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.
or high-fat diet–fed MYFKO mice. These findings align well with a recent study demonstrating that bone marrow–deficiency of FoxO4 accelerated atherosclerotic lesion formation in apolipoprotein E (ApoE)^−/− mice, an effect associated with increased macrophage ROS and interleukin-6 expression.¹⁵ Taken together, these findings are consistent with the premise that under stringent conditions of LDLR–deficiency, myeloid FoxOs may target antioxidant gene programs to establish cellular defense against oxidative stress, a significant contributor to atherogenesis.

If FoxOs typically function to reduce ROS, then why is the observed phenotype using myeloid triple FoxO ablation (increased atherosclerosis) antithetical to the endothelial triple FoxO ablation (reduced atherosclerosis)? To paraphrase John F. Kennedy, “Ask not what your transcription factor can do for your cell, ask what your cell can do with your transcription factor.” Accordingly, cellular context is important and transcription factor gene targets may be differentially expressed in specific cell types at baseline or by pathophysiological stimuli. Indeed, in vascular endothelial–specific triple-deficient FoxO/LDLR^−/− mice, the relative expression of endothelial nitric oxide synthase, a known FoxO target gene that is repressed by FoxO1 and FoxO3a,¹⁶ was significantly increased on the mRNA and protein level, an effect associated with increased endothelial nitric oxide synthase–dependent vasorelaxation and NO production.⁸ Although FoxO1 was found to bind directly to the gene promoters of the adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, alternative NO–dependent inhibition of nuclear factor-kappa B (NF-kB) signaling and leukocyte adhesion may have also been operative as described.¹⁷ Conversely, expression of NADPH oxidase subunits p22phox, p67phox, and gp91phox, which are responsible for ROS generation, were decreased in the vascular endothelial FoxO–deficient/LDLR−/− mice. Moreover, endothelial nitric oxide synthase–derived NO can inhibit endothelial cell senescence and apoptosis. Taken together, these findings underscore the importance of cell-type–specific effects dependent on the relative expression of FoxO target genes.

Similarly, differential expression of FoxO gene targets in the myeloid compartment may underlie the effects observed in MYFKO/LDLR−/− mice on the higher proliferation rate for GMPs in the bone marrow and increased peripheral WBC and monocytes. Macrophages from the MYFKO/LDLR−/− mice exhibited significantly decreased expression of cell cycle regulatory genes, including Cdkn1b, Cdkn2a, Cdkn2b, Ccng2, and p53. Indeed, absence of macrophage p53 or bone marrow–derived Cdkn2a increases lesion formation in atherosclerotic-prone mice.¹⁸,¹⁹ In addition, macrophages from MYFKO/LDLR−/− mice exhibited increased expression of the chemo kinase receptors Ccr5 and Cx3cr1, adhesion molecule CD44, integrins Itgα1, Itgβ1 and -2, and Psgl-1; consequently, these macrophages adhered readily to endothelial monolayers.

Alterations in the abundance of GMPs in the bone marrow and subsequent numbers of peripheral monocytes have been linked to effects on atherosclerotic lesion formation by other molecules. Angiotensin II type I receptor–deficiency in the bone marrow reduced the number of GMPs, peripheral monocytes, and accelerated atherosclerotic lesion formation.²⁰ Conversely, loss of the transporters ABCA1 and ABCG1 in the bone marrow markedly increased GMPs, peripheral monocytes, and accelerated atherosclerosis.²¹ Similar to FoxO-deficient hematopoietic stem cells (HSCs), myeloid-specific FoxO ablation in LDLR−/− mice harbored GMPs with increased number of cells in S/G2/M.²² GMPs are hierarchically more mature than common myeloid progenitors or HSCs. Because lyszyme M (Lys) is expressed higher in GMPs than other hematopoietic stem/progenitors, this may account for the more pronounced proliferative phenotype in GMPs in the MYFKO/LDLR−/− mice that were generated using the Lys-Cre driver mice.

Reduced macrophage apoptosis in the MYFKO/LDLR−/− mice may also indirectly contribute to the increased peripheral WBC, macrophage-derived ROS, and lesion progression. Although FoxO-deficient HSCs demonstrated a 2-fold increase in apoptosis, FoxO-deficient macrophages from MYFKO/LDLR−/− mice had decreased apoptosis in response to cholesterol loading with reduced expression for the proapoptotic ligand FasL.⁷ These findings are consistent with studies demonstrating that FoxOs promote apoptosis of insulin-resistant macrophages during cholesterol loading, an effect mediated by induction of the NF-κB inhibitor IκBα.²³ Although expression for IκBα was not assessed in macrophages from MYFKO/LDLR−/− mice, there were no significant differences in p65 phosphorylation or p105 expression in macrophages from Western diet-fed MYFKO mice. Macrophage apoptosis has been implicated as having dual roles in atherosclerosis depending on the phase examined. A protective role for macrophage apoptosis on lesion formation has been observed after long-term high-fat diet, thereby suggesting that the reduced apoptosis in MYFKO/LDLR−/− mice may contribute to lesion progression. However, endothelial cells isolated from endothelial–specific triple FOXO–deficient LDLR−/− mice also exhibited reduced apoptosis and increased cellular proliferation, suggesting that apoptosis is not likely the dominant mechanism underlying the different phenotypes. Furthermore, despite differences of effects on apoptosis in HSC-deficient and myeloid-deficient FoxO cells (in the LDLR−/− background), both elaborated increased ROS. Moreover, administration of N-acetyl-L-cysteine to HSC-deficient or myeloid-specific FoxO–deficient mice rescued their respective phenotypes, indicating a critical antioxidant role for these FoxOs in stage-specific hematopoietic cells.

Although the findings by Tsuchiya⁷ also extend a participatory role of FoxO-mediated regulation of myeloid ROS, oxidative stress may also, in turn, regulate FoxOs in a feed-forward regulation by affecting post-translational modifications, including FoxO acetylation, phosphorylation, ubiquitination, and, perhaps, methylation.²² These effects may modulate FoxO subcellular localization, protein–protein interactions, or direct FoxO transcriptional activity. For example, exposure to hyperglycemia and free cholesterol leads to FoxO1 acetylation. Macrophages expressing a constitutively active deacetylated FoxO1 retained anti-inflammatory properties in response to free cholesterol loading, without inducing apoptosis, an effect associated with reduced Akt phosphorylation and NF-κB and mitogen activated protein kinase/extracellular signal–regulated kinases activation.²³ In contrast, macrophages harboring a FoxO1 phosphorylation mutant not only inhibited inflammation but also promoted apoptosis.²⁴ Indeed, ubiquitination and methylation may also regulate FoxO protein activity. Although poly-ubiquitination of FoxO results in its degradation, mono-ubiquitination promotes FoxO nuclear translocation and
activity. In contrast, methylation of FoxO interferes with Akt-mediated FoxO phosphorylation. Thus, FoxO function may be altered in response to multiple levels of post-translational modifications, particularly under oxidative stress.

Differential expression of FoxO interactors in myeloid and endothelial cells is yet another potential important component to consider for the cell-type–specific effects in response to insulin/phosphoinositide 3-kinase/Akt signaling. For example, an emerging paradigm from studies of quiescent naïve T cells is that Kruppel-like factor 2 is a direct target of FoxO1, an effect that induces Kruppel-like factor 2–dependent expression of L-selectin and the lymphocyte homing receptors sphingosine-1-phosphate receptor 1 and CCR7, which control T cell entry and egress in lymphatic tissues. Might similar paradigms exist for FoxO-mediated Kruppel-like factor 2 effects on myeloid egress from the bone marrow or tissues that harbor myeloid cells/progenitors, including the spleen? Several other possibly relevant FoxO interactors have been reported, including NRF-κB. Some studies of FoxO and NF-κB have demonstrated parallel effects on atherosclerotic lesion formation on their inhibition in the vascular endothelium (reduces) and myeloid cells (increases), raising the possibility of functional interactions with these factors or their signaling pathways. In addition, FoxO3a and the coactivator PGC-1α directly interact in endothelial cells to promote a cooperative induction of antioxidant genes, raising the possibility of metabolic dysregulation in myeloid cells via FoxO–PGC-1α interactions. Collectively, additional insights into the regulation of FoxO interactors, which may be cell-type specific, require further study.

Finally, the study by Tsuchiya elicits important insights for therapeutic targeting of FoxOs. Currently, preclinical studies are investigating FoxO1 inhibitors as potential antiatherogenic therapies. Although myeloid-specific single FoxO1–deficiency did not reveal an increase in peripheral WBC or monocytes when placed on a high-fat diet, careful attention to cardiovascular risk may be warranted if these molecules are to be examined in human subjects that have dyslipidemia or insulin resistance. Several mechanistic questions remain: (1) What is the full repertoire of genes regulated by FoxOs in myeloid cells versus endothelial cells? (2) Which FoxO isoforms are responsible for regulation of these targets? (3) Which of the 3 FoxO isoforms may dominantly contribute to the observed cell-type–specific phenotypes? (Rescue of individual constitutively active FoxOs may be helpful in this regard); and (4) What FoxO interactors and post-translational modifications may be operative in myeloid versus endothelial cells?

Understanding how and which FoxOs regulate seemingly disparate pathways, such as oxidative stress protection, proapoptotic programs, and cell cycle proliferation, will be crucial to our understanding of FoxO function in response to metabolic stress. Because proinflammatory macrophages are linked to obesity, diabetes mellitus, and atherosclerosis, the findings by Tsuchiya, as well as the new MYFKO mice, will no doubt serve as a strong platform for interrogating these issues in future studies.

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None.

**References**


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