FOXO3a Turns the Tumor Necrosis Factor Receptor Signaling Towards Apoptosis Through Reciprocal Regulation of c-Jun N-Terminal Kinase and NF-κB

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Objective—We evaluated the full range effects of FOXO3a in endothelial cells (ECs) by microarray analysis and investigated the role of FOXO3a regulating TNF receptor signaling pathway.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were transfected with adenoviral vectors expressing constitutively active FOXO3a (Ad-TM-FOXO3a). Ad-TM-FOXO3a transfection caused remarkable apoptosis, which were accompanied with upregulation of genes related with TNF receptor signaling, such as TNF-α, TANK (TRAF-associated NF-κB activator), and TTRAP (TRAF and TNF receptor-associated protein). Furthermore, κB-Ras1 (IκB-interacting Ras-like protein-1) which is known to block IκB degradation was found increased, and intranuclear translocation of NF-κB was inhibited. GADD45β and XIAP, negative regulators of c-Jun N-terminal kinase (JNK), were suppressed and JNK activity was increased. Attenuation of TNF signaling pathway either by blocking antibody for TNF receptor or by blocking JNK with DMAP (6-dimethylaminopurine) or Ad-TAM67 (dominant negative c-Jun) cotransfection, significantly reduced FOXO3a-induced apoptosis. Finally, treatment of vasculature with heat shock, an activator of endogenous FOXO3a, resulted in EC apoptosis, which was completely rescued by Ad-TAM67.

Conclusion—FOXO3a promotes apoptosis of ECs, through activation of JNK and suppression of NF-κB. These data identify a novel role of FOXO3a to turn TNF receptor signaling to a proapoptotic JNK-dependent pathway. (***Arterioscler Thromb Vasc Biol.*** **2008;28:112–120.**)

Key Words: FOXO3a ■ JNK ■ NF-κB ■ apoptosis

The status of the external environment influences cell survival via various signaling mechanisms. Tumor necrosis factor (TNF) is a potent cytokine that serves pleiotropic function in inflammation, cell proliferation, and apoptosis.[1,2] NF-κB and c-Jun are known as 2 major transcription factors mediating the TNF receptor signaling.[3] In this cascade, NF-κB plays a dominant role mediating inflammatory response while blocking apoptosis via inhibition of the c-Jun-N-terminal kinase (JNK).[4]

Forkhead transcription factors are emerging as key factors, under regulation of Akt, to play various roles ranging from cellular proliferation, metabolism,[5] apoptosis,[6,7] and even adaptation to cellular stress.[8,9] Regarding endothelial cells (ECs), previous studies by our group[10,11] and others[12] have suggested a proapoptotic role of FOXO3a, one of main forkhead transcription factors expressed in ECs. But the molecular mechanism of FOXO3a in ECs has not been fully defined yet.

In this study, we evaluated the full range effects of FOXO3a on human umbilical vein endothelial cells (HUVECs) by microarray analysis, in which we found that several genes involved in the TNF receptor pathway were upregulated. Therefore we investigated the role of FOXO3a in the TNF receptor signaling and its implication of the proapoptotic action of FOXO3a in ECs in vitro and in vivo.

Materials and Methods

For expanded methods, please see the supplemental materials, available online at http://atvb.ahajournals.org.

EC Culture and Gene Transfer Using Adenoviral Vectors

Four to 6 passage of HUVECs (Clonetics) were cultured as previously described.[13]

Oligonucleotide Microarray

Magic-II 10K oligonucleotide microarray (Macrogen) was used as previously described.[10,14]

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Real-Time Quantitative RT-PCR Analysis

Changes in RNA-expression of TANK (TRAF-associated NF-κB activator), and TTRAP (TRAF and TNF receptor-associated protein) were determined by real-time QRT-PCR as previously described.[10]

RT-PCR and Immunoblot Analysis

Changes in RNA expression of TNF-α, κB-Ras1, GADD-45β, and XIAP were determined by RT-PCR as previously described.[15]

Chromatin Immunoprecipitation (ChIP) Assay

A chromatin immunoprecipitation (ChIP) assay was performed.

Immunofluorescent Staining

For NF-κB localization, HUVECs were immunostained with mouse monoclonal antibody against p65.

Immunoprecipitation/In Vitro JNK Kinase Assay

Immunoprecipitated proteins with anti-JNK1 antibody were incubated with anti-phospho-c-jun antibody to compare phosphorylating activity of JNK1 as previously described.[16]

Electrophoretic Mobility Shift Assay

The DNA binding activity of the nuclear protein was evaluated according to the established method with modifications.[17]

Evaluation of In Vivo Endothelial Denudation and Apoptosis After Heat Shock Treatment on Vessel

Male Sprague-Dawley rats, 13 weeks old, weighing about 400 g (Daehan Biolink Co, Chung-Buk, Korea) were used for animal experiment as previously described.[18]

FACS Analysis of EC Apoptosis

Apoptosis was quantified by measuring the hypodiploid DNA content using flow cytometry (FACS) analysis.[18]

Statistical Analysis

All data are expressed as mean±SD.

Results

FOXO3a-Regulated Genes Identified by Oligonucleotide Microarray

To identify the FOXO3a-regulated genes in ECs, we compared the gene expression profile of Ad-TM-FOXO3a–transfected HUVECs with a control vector (Ad-GFP) or Ad-DN-FOXO3a-transfected HUVECs. From our previous experiments, genes transfected by adenoviral vectors are appreciably expressed approximately 12 hours after transfection.[10] Therefore, we decided to evaluate at 16, 24, and 40 hours to include both early and late response to FOXO3a activation. The activation or suppression of genes was evaluated by measuring the fold ratio between Ad-TM-FOXO3a or Ad-DN-FOXO3a–transfected HUVECs with Ad-GFP–transfected HUVECs. A cDNA was considered a putative FOXO3a-regulated target if the hybridization signal was more than 2 folds of the control.

Constitutive activation of FOXO3a increased the expression of 73 genes and decreased 59 genes out of ≈10 000 genes analyzed. The Table lists some of the genes that were strongly regulated by FOXO3a. As expected from the previously reported proapoptotic action of FOXO3a, several genes associated with apoptosis or cell cycle arrest were upregulated, whereas most of the genes that were significantly downregulated were those known to be associated with extracellular matrices, heat shock proteins, and growth factors (supplemental Figure I, available online at http://atvb.ahajournals.org). From this data, we found that Fas ligand, which is a well-known effecter molecule in forkhead transcription factor–induced apoptosis of neural cells and fibroblasts,[19] was not upregulated in ECs, suggesting that, at least in ECs, there exists another mechanism which mediates apoptotic signaling. Interestingly, we found that, in the highly upregulated genes, there were several genes related to TNF receptor signaling pathway, including TNF-α, TANK, TTRAP, and κB-Ras1 (IκB-interacting Ras-like protein-1), suggestive of a link between FOXO3a and the TNF receptor signaling.

FOXO3a Induces and Amplifies TNF Receptor Signaling

To validate the microarray data, we evaluated TANK and TTRAP mRNAs, which were found to be significantly upregulated (Figure 1A). The relative fold elevation compared with GAPDH was 1±0.3 at 16 hours, 122±40 at 24 hours, and 293±110 at 40 hours in case of TANK, and 1±0.3 at 16 hours, 186±60 at 24 hours, and 2999±1100 at 40 hours in case of TTRAP, respectively. Changes of TNF-α were analyzed in 2 ways; the secreted TNF-α by ELISA of the cell culture supernatant and the intracellular TNF-α by immunoblot of the cell lysates. FOXO3a activation significantly increased TNF-α in both instances (Figure 1B). Immunoblot analysis confirmed the increased protein synthesis of TANK and TTRAP, which was completely reversed by Ad-DN-FOXO3a (Figure 1C). Next, we performed ChIP analysis to investigate the transcriptional regulation of those molecules by FOXO3a and found that FOXO3a was bound to the promoter sites of TANK, whereas was not bound to either TNF-α or TTRAP (Figure 1D), of which pattern was confirmed by electrophoretic mobility shift assay using the putative FOXO3a binding site oligonucleotides (supplemental Figure II).

FOXO3a Induced κB-Ras1, Suppressing NF-κB Activation

κB-Ras1 (IκB-interacting Ras-like protein-1) is known as an inhibitor of NF-κB by regulating IκB degradation.[20,21] FOXO3a activation increased κB-Ras1 both in mRNA level (supplemental Figure IIIA) and in protein level (Figure 2A, upper panel), which was reversed by Ad-DN-FOXO3a. The increment of κB-Ras1 corresponded to the significant increase of IκBβ protein but not to the change of IκBα amount (data not shown), which suggested that degradation of IκBβ was prevented by κB-Ras1 (Figure 2A, middle panel). The ChIP analysis also suggested that κB-Ras1 expression was transcriptionally regulated by FOXO3a (Figure 2C).

Next, NF-κB activity in response to FOXO3a overexpression was evaluated by immunofluorescent staining for p65 subunit of NF-κB after 24 hours of adenoviral transfection. NF-κB primarily remained in the cytoplasm either in Ad-GFP or Ad-TM-FOXO3a transfected HUVECs (Figure 2B, upper panels). When stimulated with TNF-α, a well-known positive regulator of NF-κB, p65 still remained in the cytoplasm in FOXO3a-transduced ECs, whereas it was swiftly translocated

Table. FOXO3a-Regulated Genes After Adenoviral Gene Transfer in HUVECs (partial list)

Accession No.	Gene Symbol	Gene Description	TM-16 hr	TM-24 hr	TM-40 hr	DN-16 hr	DN-24 hr	DN-40 hr	Function
NM_016508	CDKL3	Cyclin-dependent kinase-like 3	93.33		295.1	7.41			Cell cycle
AK128396	TRAF6	TNF receptor-associated factor 6	35.48		6.46				Signaling
NM_005310	GRB7	Growth factor receptor-bound protein 7	33.88	10.72	40.74	24.55		3.24	Signaling
NM_003640	IKBKG	Inhibitor of kappa light polypeptide enhancer in B cells	30.73	0.85		0.33	0.06		Signaling/immune
NM_002423	MMP7	Matrix metalloproteinase matrilysin uterine	16.70	32.02	32.63		43.65	3.89	Matrix
NM_005527	HSPA1L	Heat shock 70kD protein-like 1	15.14		28.18				Regulation of apoptosis
NM_001334	CTSO	Cathepsin O	13.80		47.86		0.95	0.09	Proteolysis/peptidolysis
NM_002422	MMP3	Matrix metalloproteinase 3 (stromelysin 1)	7.06	15.45	4.10	4.30	1.32	8.15	Matrix
NM_000594	TNF	Tumor necrosis factor	6.03	4.14	3.01	0.50	0.34	1.32	Signaling
NM_006256	PKN2	Protein kinase N2	6.03	7.84	9.80	1.34	0.43	0.81	Signaling
AK074480	ANXA1	Annexin A1	5.90	8.66	7.90	0.98	0.79	0.75	Intracellular signaling
NM_002425	MMP10	Matrix metalloproteinase 10 (stromelysin 2)	5.87	6.60	4.79	0.19			Matrix/proteolysis
NM_006218	PIK3CA	Phosphoinositide −3-kinase, catalytic, alpha,	4.75	5.87	1.05	1.17			Signaling/akt block
NM_015645	CIQTNF5	C1q and tumor necrosis factor related protein 5	4.66	8.35	0.48	0.40	0.57	1.44	Apoptosis
NM_002838	PTPRC	Protein tyrosine phosphatase, receptor type, C	4.67	2.14			0.18	0.26	Proliferation
NM_002731	PRKACB	Protein kinase, cAMP-dependent, catalytic, beta	4.57	4.57	21.38				Signaling
NM_153425	TRADD	TNFRSF1A-associated via death domain	4.32	0.59	0.06	0.39	0.32	0.50	Apoptosis/signaling
NM_020345	KBRAS1	NFKB inhibitor interacting Ras-like 1	4.27	3.72	35.48	0.34		1.51	Signaling
NM_016614	TTRAP	TRAF and TNF receptor-associated protein	3.23	2.88	3.83	1.08	0.84	0.89	Transcription
NM_004180	TANK	TRAF family member-associated NFKB activator	3.11	2.82	1.70	1.04	0.63	1.10	Apoptosis/signaling
NM_001903	CTNNA1	Catenin (cadherin-associated protein), α1	0.47	0.37	0.04	0.67	0.59	1.08	Growth/adhesion
NM_183422	TSC-22	Transforming growth factor beta-stimulated CLONE 22	0.45	0.43	0.17	0.59	0.91	1.44	Transcription
NM_001226	CASP6	Caspase 6, apoptosis-related cysteine protease	0.44	0.39	0.10	0.59	2.24	1.62	Apoptosis
NM_001554	CYR61	Cysteine-rich, angiogenic inducer, 61	0.37	0.16	0.59	0.81	0.71	0.84	Adhesion/proliferation
NM_016292	TRAP1	Heat shock protein 75	0.29	0.05		1.04	6.35	1.41	Regulation of apoptosis
NM_002587	PCDH-1	Protocadherin 1	0.27	0.45	0.28	1.03	1.67	1.41	Adhesion/signaling
NM_006597	HSPA8	Heat shock 70kD protein 8	0.21	0.27	0.04	0.77	0.72	1.12	Regulation of apoptosis

Blank sections are not determined in microarray.

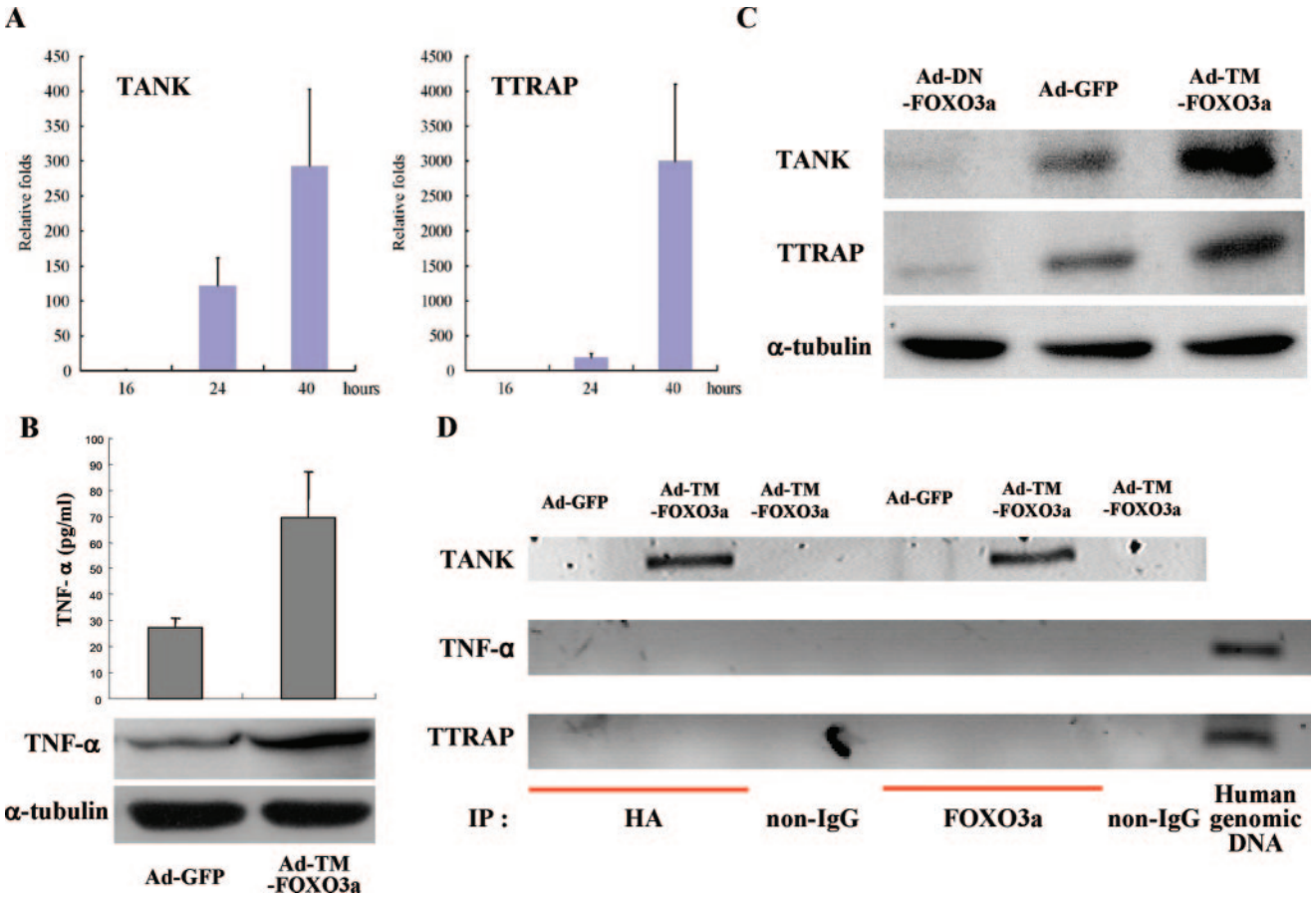

Figure 1. FOXO3a induces TNF-α, TANK, and TTRAP expression. A, Quantification of real-time quantitative RT-PCR using gene-specific human TANK and TTRAP primers. Data are expressed as mean±SE for 3 independent experiments. B, ELISA in supernatant (upper) and immunoblot of whole cell lysates for TNF-α (lower). C, Immunoblot of TANK and TTRAP. D, Chromatin immunoprecipitation assays of TNF-α, TANK, and TTRAP. Those showed FOXO3a bound to the promoter region of TANK, not to the promoter regions of TNF-α or TTRAP.

into the nucleus in the control ECs (Figure 2B, lower panels). This suppression of NF-κB activation by FOXO3a was confirmed by electrophoretic mobility shift assay, which showed reduced nuclear translocation of NF-κB in TM-FOXO3a transduced HUVECs (supplemental Figure IIIB). Because NF-κB is reported to regulate JNK via GADD45β and XIAP,[22] we evaluated the expression of GADD45β and XIAP after FOXO3a activation. The expression of GADD45β and XIAP were significantly downregulated following FOXO3a activation (Figure 2D, supplemental Figure IIIC).

FOXO3a Activates JNK, Turning TNF Receptor Pathway Toward Apoptosis

JNK activity after FOXO3a activation was evaluated using an in vitro kinase assay. Strong activation of JNK was detected after 24 hours of TM-FOXO3a gene transduction compared with the control, which was near completely reversed by Ad-DN-FOXO3a (Figure 3A). Then, the role of the increased JNK activity on TNF-α expression was evaluated. As shown in Figure 3B, the induction of TNF-α expression by FOXO3a was completely reversed by Ad-TAM67 in ECs under stimulation of lipopolysaccharide (LPS) or heat shock. These findings indicate that the increased c-Jun after FOXO3a activation might be the regulatory mechanism of FOXO3a-induced TNF-α expression especially in the activated ECs.

Next, we performed blocking experiments of TNF receptor signaling pathway to investigate how much TNF receptor signaling to JNK contributes to proapoptotic action of FOXO3a. When TNF-α signal was inhibited by a blocking antibody against TNF receptor, FOXO3a-induced apoptosis was significantly attenuated. Moreover, when JNK was blocked either by 6-Dimethylaminopurine (DMAP), a JNK inhibitor, or by cotransfection with Ad-TAM67, apoptosis was reduced in a similar extent to TNF-α receptor blockage (Figure 3C, supplemental Figure IVA).

To elucidate the implication of native FOXO3a–JNK signaling during the stress response, HUVECs were subjected to heat shock at 42°C for 4 hours (supplemental Figure IVB). In resting state, native FOXO3a located in cytoplasm, thus remained inactive. Heat shock rapidly induced intranuclear translocation thus activation of native FOXO3a from 30 minutes, which was accompanied with increased cytotoxicity (Figure 3D). Transduction with Ad-TAM67 or DN-FOXO3a significantly reduced cytotoxicity under these conditions, suggesting the hypothesized FOXO3a - JNK signaling pathway contributes to the cytotoxicity of ECs during stress condition.

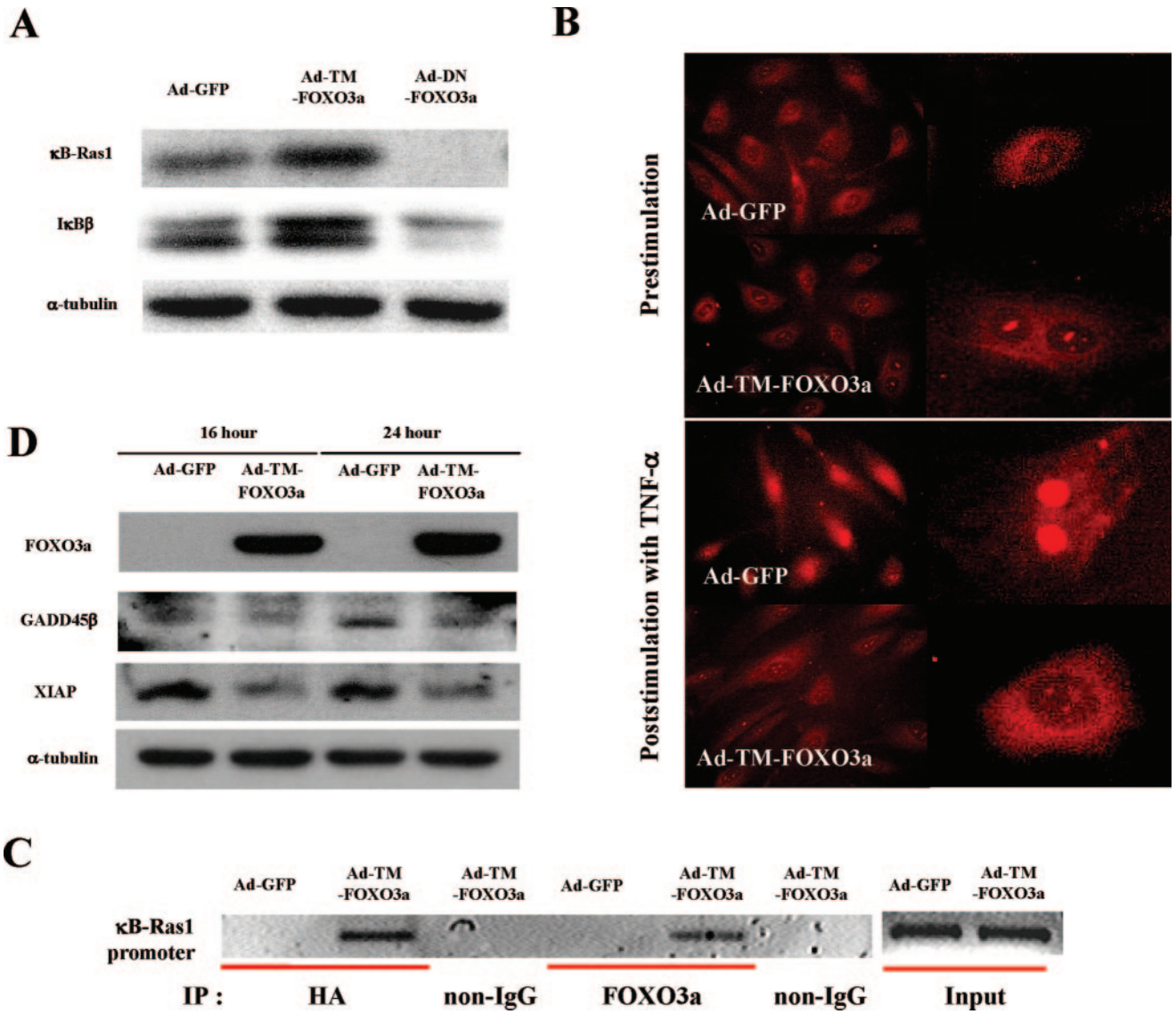

Figure 2. FOXO3a induces κB-Ras1, leading to suppression of NF-κB. A, Immunoblot of the whole cell lysates for κB-Ras1 and IκBβ. B, Immunocytochemistry and fluorescent microscopy for p65 subunit of NF-κB. After adenoviral transduction, cells were either unstimulated or stimulated with TNF-α for 45 minutes. In unstimulated state, NF-κB was primarily detected in the cytoplasm of HUVECs transfected with either Ad-GFP or Ad-TM-FOXO3a (upper panels). When stimulated with TNF-α, p65 NF-κB in the control group translocate to the nucleus, whereas in TM-FOXO3a transduced HUVECs p65 still remained in the cytoplasm (lower panels). C, Chromatin immunoprecipitation assays of κB-Ras1. D, Immunoblot of whole cell lysates of ECs for GADD45β and XIAP.

In Vivo Evidence of FOXO3a-Induced JNK-Mediated Endothelial Damage in Heat-Shock Treated Vessel

Finally, to show that the activation of FOXO3a is a physiologically relevant phenomenon, we performed experiments where rat carotid arteries were exposed to heat shock with or without prior blockade of FOXO3a signaling by in vivo gene delivery of the indicated adenoviral vectors. Then, the heat-shocked vessels in organ culture were immunostained and the luminal surface of the endothelial lining of the vessel was observed en face by the confocal microscopy. Double immunostaining of VE-cadherin and activated caspase-3 visualized apoptosis of ECs on the luminal surface of blood vessel after heat shock, which was reduced by pretreatment with Ad-TAM67 and was aggravated by Ad-TM-FOXO3a (Figure 4A). In immunostaining of PECAM-1 with DAPI staining, we quantitated the endothelial damage by counting the round nuclei with PECAM-1 positivity as viable ECs on the luminal surface of vessel (Figure 4B). Heat shock treatment significantly reduced the number of viable ECs on luminal surface of vessel compared with normal temperature control group. Pretreatment with Ad-TAM67 significantly protected endothelial lining from heat shock-induced damage. Conversely, pretreatment with Ad-TM-FOXO3a significantly aggravated damage on the endothelial lining of heat-shocked vessel (Figure 4C, supplemental Figure VII).

Discussion

The important finding of our study is that the forkhead transcription factor, FOXO3a, activates JNK, while suppressing NF-κB in ECs, which turns the TNF receptor signaling from survival to apoptosis. In addition, this study has several

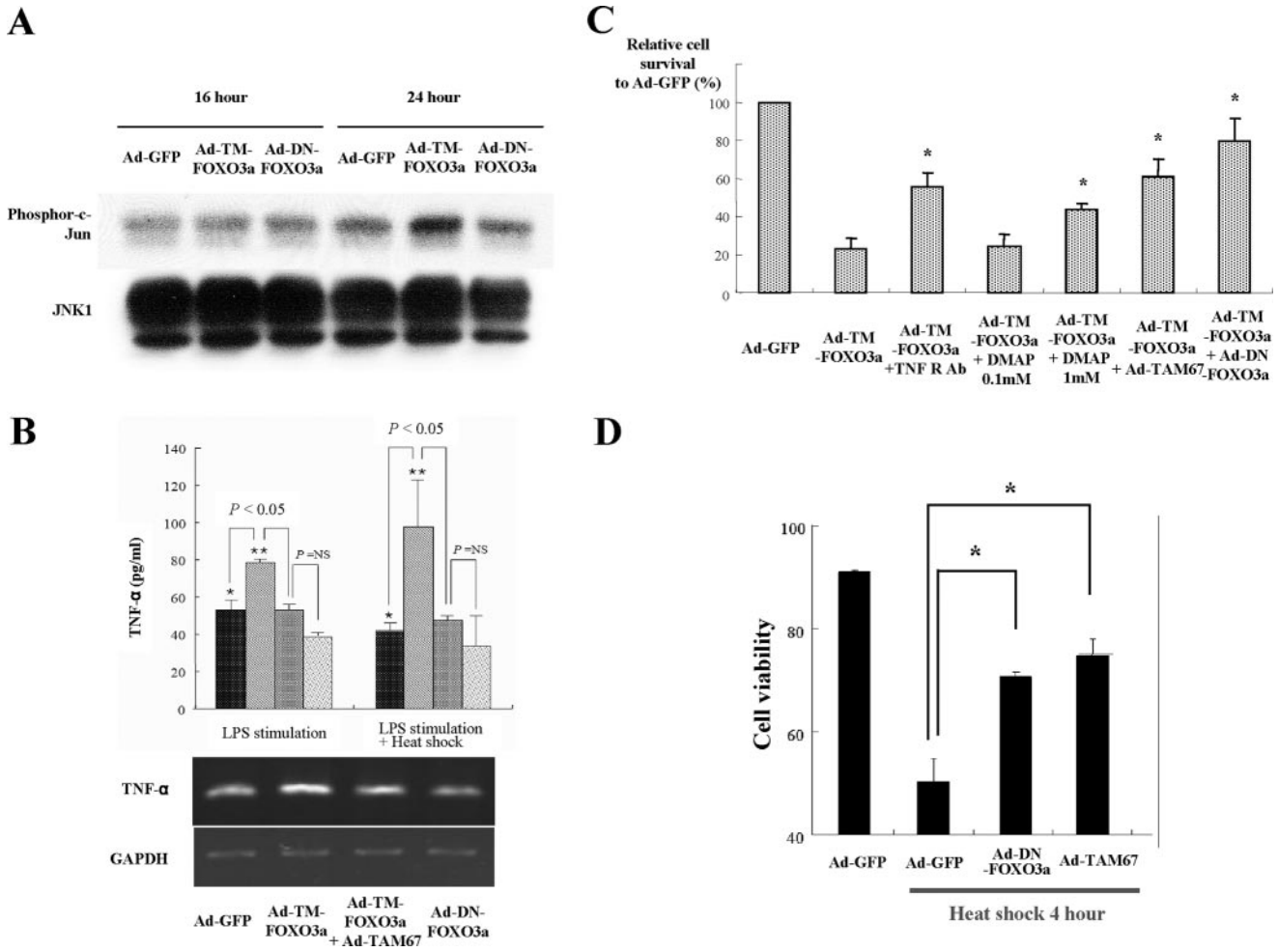

Figure 3. FOXO3a activates JNK, turning TNF receptor pathway toward apoptosis. A, Immunoprecipitation/Kinase assay for JNK activity. c-jun N-terminal phosphorylation activities determined by in vitro kinase assay. B, ELISA in supernatant (upper) and RT-PCR of whole cell lysates for TNF-α (lower). C, A bar graph of apoptotic FACS analysis counting hypodiploid cells. HUVECs were cultured in the presence of serum for 24 hours after transduction with indicated adenoviral vectors or designated blocking agents. TNF-α R Ab, neutralizing antibody of TNF receptor; DMAP, 6-Dimethylaminopurine; Ad-TAM67, adenoviral vector expressing a dominant negative c-Jun mutant. (*$P<0.05$ compared with Ad-GFP, n=3). D, Trypan blue exclusion assay evaluating HUVEC viability after 4 hours of heat incubation with indicative adenoviral transfection. Results are expressed as the mean±SEM of triplicate assay (*$P<0.05$, n=3).

novel findings. First, oligonucleotide microarray showed the change of the expression of several genes, which had not been reported to be regulated by FOXO3a previously. We found that FOXO3a induced TANK, TTRAP, and κB-Ras1, which might be responsible for the activation of JNK as well as suppression of NF-κB. Second, inhibition of TNF receptor–JNK signaling pathway significantly attenuated the apoptosis of EC after FOXO3a activation, suggesting that the TNF receptor signaling lies in downstream of FOXO3a. This study not only shows that JNK activation and NF-κB suppression are novel mechanism of FOXO3a-induced apoptosis in ECs, but also provides new insight into the role of FOXO3a in the TNF receptor signaling.

Finding Putative Genes Mediating FOXO3a-Induced Apoptosis by Oligonucleotide Microarray

By using adenoviral gene transfection and the oligonucleotide microarray, we identified several candidate genes that were regulated by FOXO3a in ECs. Among them, TNF-α, TRAPP, and TANK are proapoptotic molecules,[23–25] of which relation to the forkhead transcription factors has not been previously reported. Another molecule upregulated by FOXO3a was κB-Ras1 (IκB-interacting Ras-like protein-1), known as an inhibitor of NF-κB activation.[20,21] Among them, TANK and κB-Ras1 were found to have the forkhead factor binding motif, WAARYAAAYW (W=A or T, R=A or G, Y=C or T)[26] in their promoter sequences and transcriptionally regulated by FOXO3a. Though TTRAP and TNF-α also have similar motifs in their presumed promoter sequences, their regulation by FOXO3a was not done at transcriptional level. FOXO transcription factors can activate 2 different subsets of genes[27,28]: (1) genes that require FOXO DNA binding, and, intriguingly, (2) genes regulated independently of FOXO DNA binding. For example of first category, TANK and κB-Ras1 gene expression in our study were transcriptionally regulated by direct binding of FOXO3a in its promoter region. For genes of secondary category, FOXO transcription factors can cooperate with other additional transcription factors to activate transcription of genes. This second mech-

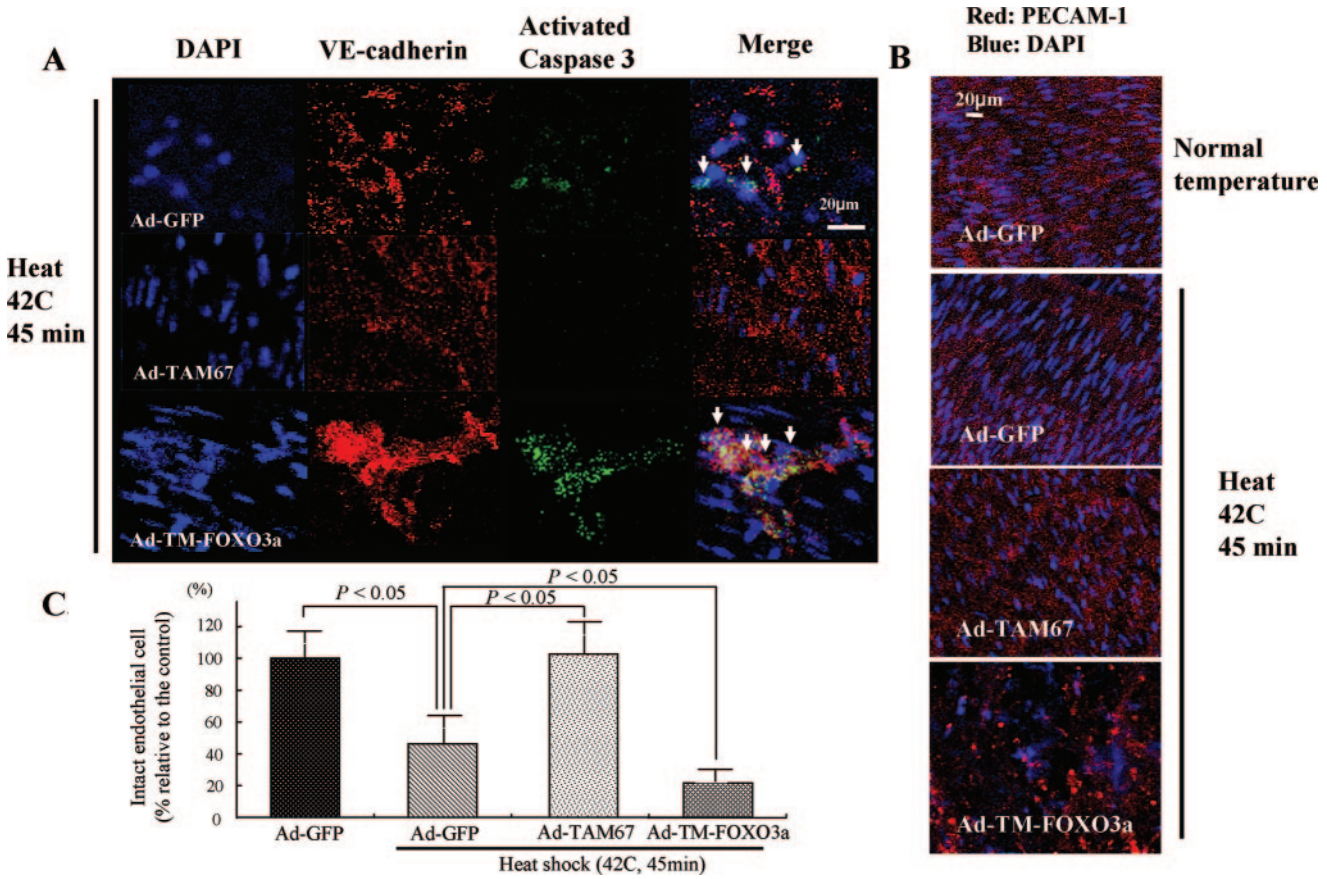

Figure 4. In vivo pathophysiologic significance of FOXO3a in heat shock treated blood vessel. A, Confocal microscopic en face view of luminal surface of blood vessels immunostained with antibodies against VE-cadherin and activated caspase-3 with DAPI. White arrows indicate apoptotic endothelial cells. B, Confocal microscopic en face view of luminal surface of blood vessels immunostained with antibody against PECAM-1 plus DAPI before and after heat shock treatment. Viable ECs are PECAM-1 positive (red) cells with round nucleus. In contrast, vascular smooth muscle cells below endothelium are PECAM-1 negative cells with elongated nucleus. C, Quantitative data of viable ECs on luminal surface of blood vessels.

anism may explain the discrepancy between the increased protein level in immunoblot analysis and absence of FOXO3a-DNA binding in ChIP analysis observed in cases of TTRAP and TNF-α.

Relevance of the TNF Receptor Signaling in FOXO3a-Induced Apoptosis

Because TNF-α, TRAPP, TANK, and κB-Ras1 are all known to be related with either JNK or NF-κB, the 2 main downstream molecules of the TNF receptor pathway, we hypothesized that the TNF receptor pathway might mediate the proapoptotic action of FOXO3a. Indeed, blocking either TNF receptor or JNK significantly reduced apoptosis after FOXO3a activation. Because TNF receptor pathway is reported to be important in inflammation as well as apoptosis,[29] the finding of this study might extend the role of forkhead transcription factor from as a downstream molecule of Akt[6] to as a upstream molecule pivoting TNF receptor signaling. TNF-α does not induce apoptosis unless the expression of cytoprotective genes, especially NF-κB, is blocked, because NF-κB inhibits JNK activation by TNF-α.[30–32] However, when NF-κB is suppressed or JNK is activated, as in the case of FOXO3a activation, the TNF receptor signaling shifts the fate of the cells from survival toward apoptosis. Here, we showed that FOXO3a activation inhibited NF-κB activation while activating JNK.

Suppression of NF-κB by FOXO3a

Inhibition of NF-κB by IκB family proteins is an important mechanism of NF-κB regulation. In the resting state, NF-κB p50/p65 dimers are bound to the inhibitor proteins IκBα and IκBβ, thus remained inactive.[33,34] Among 2 IκB proteins, IκBβ is known to play a critical role isolating NF-κB exclusively in the cytoplasm.[20] κB-Ras proteins have been identified as inhibitors of NF-κB activation by preventing IκBβ degradation while having no effect on IκBα.[21] We found FOXO3a induces κB-Ras1, thus inhibiting NF-κB. Considering our findings with a recent report that IκB kinase conversely inhibits FOXO3a and serves as a negative regulator,[35] it appears that a short negative feedback loop exists between FOXO3a and NF-κB.

Role of FOXO3a in Activation of JNK

Signal transduction in the TNF receptor pathway involves several regulatory factors. Most of these proteins have been identified as TRAF-binding proteins, eg, TTRAP and TANK. Overexpression of these factors either inhibits TRAF-mediated activation of NF-κB or potentiates TRAF-mediated

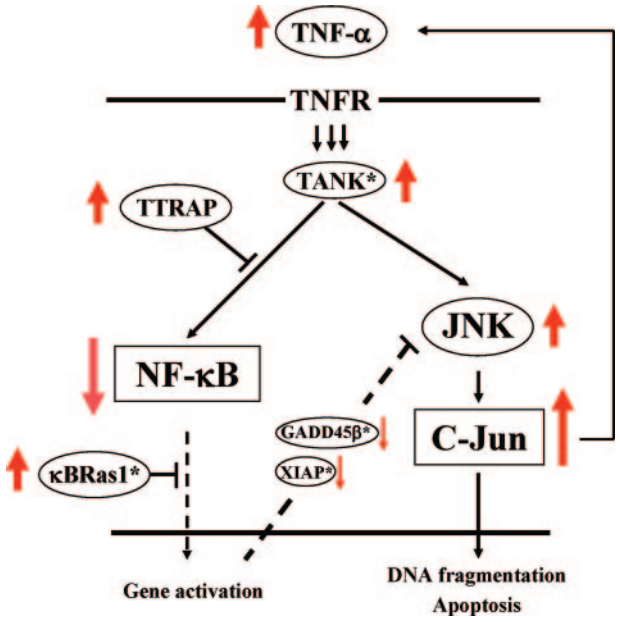

Figure 5. Proposed scheme of EC apoptosis by FOXO3a through pivoting TNF receptor pathway with reciprocal regulation of NF-κB and JNK. Arrow indicates change of expression after FOXO3a activation. Molecules having forkhead responsive motifs in their promoter regions are indicated by the asterisks (*). FOXO3a increased TANK and TTRAP, thus amplifying and turning TNF receptor pathway signals to JNK. In downstream, NF-κB inhibition by FOXO3a led to attenuation of GADD45β and XIAP which are inhibitors of JNK in resting state, thus activating JNK. FOXO3a plays a pivotal role in switching the TNF receptor pathway toward apoptosis by suppressing NF-κB and activating JNK.

activation of JNK. TANK is reported to be a regulatory molecule potentiating both NF-κB and JNK, thus amplifying TNF receptor signaling.[25] On the other hand, TTRAP is known to inhibit NF-κB in a dose-dependent and stimulus-dependent manner,[24] thus turning TNF receptor pathway signals to JNK activation. This fits in with the results of the present study, where both TANK and TTRAP were found to be upregulated by FOXO3a activation. Taken together, expression of not only TANK but also TTRAP after FOXO3a activation may not only potentiate but also turn TNF receptor pathway signals toward JNK. Another regulating mechanism of JNK by NF-κB is through GADD45β and XIAP.[22] In our study, GADD45β and XIAP expression was found to be decreased after FOXO3a activation, which could also result in JNK activation. We do not make any conclusion on whether induction of TANK and TTRAP or attenuation of GADD45β and XIAP plays a major role in activation of JNK by FOXO3a. However, we think it is important that activation of FOXO3a results, at the end, in an overall increase in JNK activity, resulting in EC apoptosis. To the best of our knowledge, activation of JNK by forkhead transcription factors is a novel finding which has not been previously reported.

In Vivo Pathophysiologic Significance of FOXO3a-Induced Endothelial Damage

Using in vivo animal experiment, we confirmed in vivo pathophysiologic significance of FOXO3a-induced JNK-medicated endothelial damage in heat shock–treated rat carotid artery. In this experiment, we demonstrated that heat shock, an activator of endogenous FOXO3a, significantly induced endothelial damage, and that such damage was completely prevented by pretreatment of Ad-TAM67 (dominant-negative c-jun). These findings suggest that pathophysiologic stress like heat shock may induce FOXO3a and JNK, leading to endothelial damage and vasculopathy.

Conclusion

As summarized in Figure 5, our study demonstrates that FOXO3a plays a pivotal role in switching the TNF receptor pathway toward apoptosis by suppressing NF-κB and activating c-Jun. This novel finding provides insight into endothelial signaling of TNF receptor pathway regulated by FOXO3a.

Acknowledgments

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Disclosures

None.

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