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Original article

FoxO3a suppresses the senescence of cardiac microvascular endothelial cells by regulating the ROS-mediated cell cycle

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ABSTRACT

FoxO3a plays an important role in the aging process and decreases with age. However, the potential regulatory roles of FoxO3a in processes involved in cardiac microvascular endothelial cell (CMEC) senescence, and its underlying molecular mechanisms have not been elucidated. This study demonstrates that FoxO3a is deactivated in senescent CMECs together with the inhibition of proliferation and tube formation. Furthermore, the activation of the antioxidant enzymes catalase and SOD, downstream FoxO3a targets, was significantly decreased, thereby leading to cell cycle arrest in G1-phase by increased ROS generation and subsequently the activation of the $p27^{Kip1}$ pathway. However, FoxO3a overexpression in primary low-passage CMECs not only significantly suppressed the senescence process by increasing the activation of catalase and SOD but also markedly inhibited ROS generation and $p27^{Kip1}$ activation, although it failed to reverse cellular senescence. Moreover, both cell viability and tube formation were greatly increased by FoxO3a overexpression in primary CMECs during continuous passage. In addition, FoxO3a, deficiency in low-passage CMECs, accelerated the senescence process. Collectively, our data suggest that FoxO3a suppresses the senescence process in CMECs by regulating the antioxidant/ROS/$p27^{Kip1}$ pathways, although it fails to reverse the cellular senescent phenotype.

1. Introduction

FoxO3a (also referred to as FKHRL-1) belongs to the O subclass of the forkhead family of transcription factors, which are characterized by a fork head DNA binding domain. The phosphorylation of FoxO3a by Akt leads to its association with 14-3-3 proteins and subsequent nuclear exclusion, retention, and degradation in the cytoplasm, which deactivates the FoxO3a pathway [1,2]. Conversely, the inhibition of Akt phosphorylation, which results in the dephosphorylation, nuclear translocation, and activation of FoxO3a, leads to the expression of target genes associated with aging [3], apoptosis [4], oxidative stress [5], and cell cycle progression [6]. Thus, this protein exhibits different functions based on cell type.

Senescence is a biological state in which cells have irreversible proliferative arrest while continuing to be metabolically active. The senescent phenotype is acquired *in vitro* after multiple rounds of cell division (replicative senescence) or upon oncogene activation or oxidative stress [7]. In particular, oxidative stress is considered a major factor contributing to the aging process and leads to aberrant signaling pathways. Aged rats have increased oxidative stress and weakened antioxidative defense systems [3]. Therefore, it is necessary to attenuate oxidative stress and strengthen the antioxidant system to modulate altered signaling pathway molecules to constrain the aging process. It has been suggested that increased FoxO3a activity strengthens antioxidant defense systems that have led to extended longevity in several experimental organisms [8,9]. Previous evidence has demonstrated that FoxO3a directly induces the antioxidant enzymes manganese superoxide dismutase (MnSOD) and catalase due to the presence of FoxO3a binding sites in the promoters of these enzymes [5,10,11].

Healing from cardiovascular diseases, including myocardial infarctions, requires angiogenesis, which involves new capillary blood vessel

Abbreviations: CMECs, cardiac microvascular endothelial cells; SA-β-Gal, senescence-associated β-galactosidase; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; SOD, superoxide dismutase; CAT, catalase.

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formation from pre-existing vessels. Angiogenesis is impaired in aging individuals and results in increased incidence and less favorable clinical outcomes for cardiovascular disease, including myocardial infarctions, compared with younger patients [12–14]. Cardiac microvascular endothelial cells (CMECs) are damaged immediately after cardiac ischemia [15,16]. Together with their angiogenic ability, CMECs play an important role in therapy for cardiovascular diseases, including myocardial infarctions. Furthermore, a previous study has demonstrated age-related impairments in angiogenesis in aging rat CMECs together with the reduced activation of the vascular endothelial growth factor (VEGF) gene [17]. These reports suggest that therapeutic strategies aimed at increasing the angiogenic capacity of senescent CMECs are warranted.

However, to our knowledge, the FoxO3a signaling cascade in senescent CMECs has not been well elucidated, and its potential involvement in regulating the proliferation and angiogenic capacity of senescent CMECs remains unclear. In this study, we investigated molecular events related to FoxO3a activity and its function in senescent rat CMECs. This report demonstrates that the activation of FoxO3a and its downstream genes catalase and SOD is decreased in senescent CMECs together with oxidative stress and cell cycle arrest, which inhibits CMEC proliferation and angiogenesis. Although FoxO3a overexpression in senescent CMECs cannot reverse the senescent phenotype, the overexpression of FoxO3a in primary low-passage CMECs not only suppresses senescence progression by increasing the activation of catalase and SOD but also markedly inhibits ROS generation and $p27^{Kip1}$ activation.

2. Materials and methods

2.1. Reagents

Propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI), 2′,7′-dichlorodihydrofluorescin diacetate (DCFHDA), superoxide dismutase (SOD), and catalase (Cat) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). LY294002 was purchased from Calbiochem (La Jolla, CA, USA). Recombinant rat IGF-1 and anti-SOD antibodies were purchased from Abcam, Inc. (Cambridge, MA, USA). Restriction enzymes were purchased from ThermoFisher (Rockford, IL, USA). The cytochemical staining kit for SA-β-gal (Senescence β-Galactosidase Staining Kit) and cell counting kit-8 (CCK-8 Kit) were obtained from Beyotime Biotechnology (Haimen, China). Antibodies directed

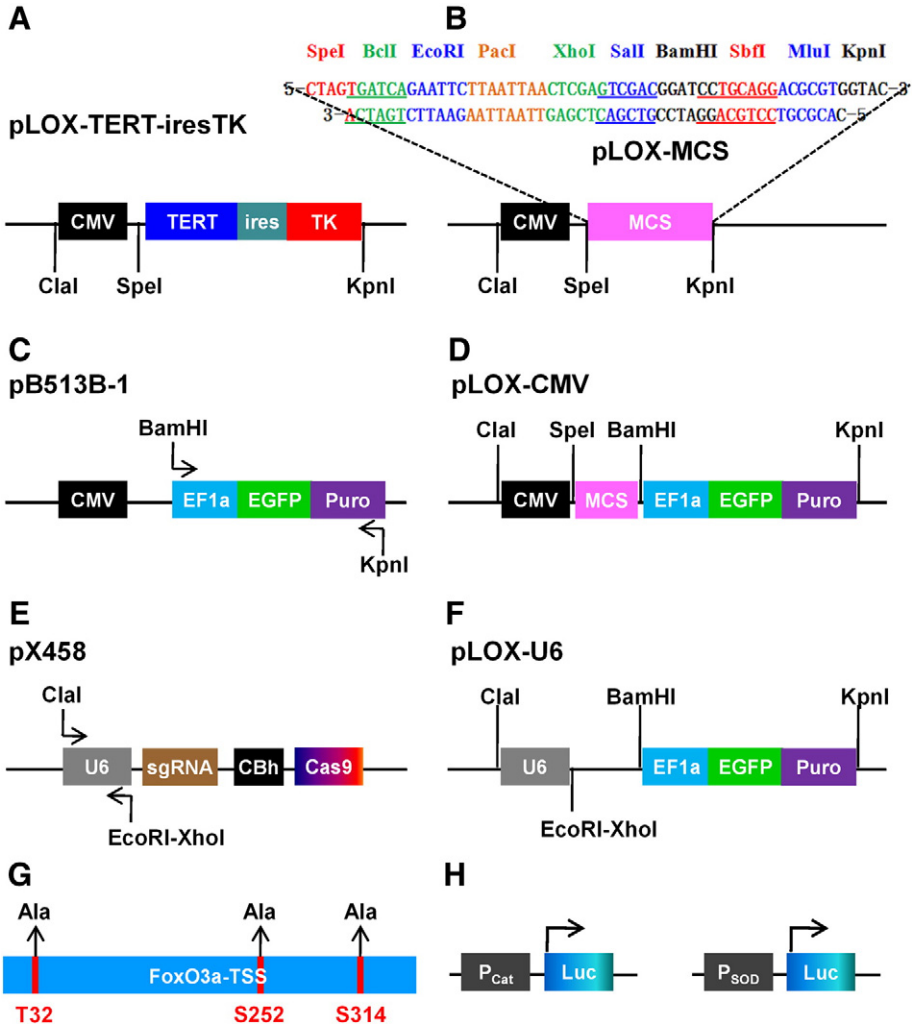

Fig. 1. Schematic representation of the plasmid constructs used in this study. (A) pLOX-TERT-iresTK plasmid construct from Addgene (#12245). (B) pLOX-MCS plasmid construct with a multiple cloning site (MCS) replacing the TERT-iresTK region. (C) The pB513B-1 plasmid (ThermoFisher) was used as a template to amplify the EF1α-EGFP-Puro fragments using primers including *Bam*HI and *Kpn*I restriction sites. (D) pLOX-CMV-E/P plasmid construct with an MCS downstream of the CMV promoter and selective markers (EGFP and Puromycin) driven by the EF1α promoter. (E) The pX458 plasmid (Addgene, #48138) was used as template to amplify the U6 promoter using primers including *Cla*I and EcoRI-*Xho*I restriction sites. (F) pLOX-U6-E/P plasmid construct with a shRNA expression frame (flanked by *Eco*RI and *Xho*I) driven by the U6 promoter and selective markers driven by the EF1α promoter. (G) Mutation of the rat FoxO3a gene with replacements of the T32, S252, and S314 residues with Ala residues. The mutant constructs cannot be phosphorylated by the Akt pathway under physiological conditions. (H) Preparation of Luc reporter plasmids containing the Cat or SOD promoters, including 2 kb upstream of the TSS.