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Role of FoxO3a as a negative regulator of the cardiac myofibroblast conversion induced by TGF-β1

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ABSTRACT

Cardiac fibroblasts (CFs) are necessary to maintain extracellular matrix (ECM) homeostasis in the heart. Normally, CFs are quiescent and secrete small amounts of ECM components, whereas, in pathological conditions, they differentiate into more active cells called cardiac myofibroblasts (CMF). CMF conversion is characteristic of cardiac fibrotic diseases, such as heart failure and diabetic cardiomyopathy. TGF-β1 is a key protein involved in CMF conversion. SMADs are nuclear factor proteins activated by TGF-β1 that need other proteins, such as forkhead box type O (FoxO) family members, to promote CMF conversion. FoxO1, a member of this family protein, is necessary for TGF-β1-induced CMF conversion, whereas the role of FoxO3a, another FoxO family member, is unknown. FoxO3a plays an important role in many fibrotic processes in the kidney and lung. However, the participation of FoxO3a in the conversion of CFs into CMF is not clear. In this paper, we demonstrate that TGF-β1 decreases the activation and expression of FoxO3a in CFs. FoxO3a regulation by TGF-β1 requires activated SMAD3, ERK1/2 and Akt. Furthermore, we show that FoxO1 is crucial in the FoxO3a regulation induced by TGF-β1, as shown by overexpressed FoxO1 enhancing and silenced FoxO1 suppressing the effects of TGF-β1 on FoxO3a. Finally, the regulation of TGF-β1-induced CMF conversion was enhanced by FoxO3a silencing and suppressed by inhibited FoxO3a degradation. Considering these collective findings, we suggest that FoxO3a acts as a negative regulator of the CMF conversion that is induced by TGF-β1.

1. Introduction

Cardiac fibroblasts (CFs) maintain the homeostasis of the extracellular matrix (ECM) by secreting ECM components such as collagen I and fibronectin [1]. In pathological conditions, such as hypertension, myocardial infarction or diabetes, CFs differentiate into more active cells called cardiac myofibroblasts (CMF) [2–4]. CMFs secrete large amounts of profibrotic proteins, such as collagen type I and connective transforming tissue factor (CTGF). CMFs have an important role in cardiac tissue healing, for which they express contractile proteins, such as smooth muscle actin type α (α-SMA) [5]. Normally, CMFs are eliminated by apoptosis after tissue healing occurs. However, under pathological conditions, CMFs remain in the injury zone and cause tissue fibrosis [6]. The determination of the molecular mechanisms and regulators of CMF conversion will enable the identification of new molecular targets against cardiac fibrosis.

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TGF-β1 is a cytokine involved in several fibrotic processes, such as pulmonary, renal and cardiac fibrosis [7]. TGF-β1 is the main protein that regulates CF differentiation into CMF. To realize this function, TGF-β1 actives its specific receptors and induces the formation and activation of the SMAD complex, which localizes to the nucleus and regulates gene transcription [8,9]. SMAD3-regulated TGF-β1 is involved in CMF conversion, requiring the regulation of gene transcription in conjunction with other necessary other proteins, such as the forkhead box type O (FoxO) family [10–12]. The FoxO family is involved in several biological processes, such as apoptosis and cell differentiation [13]. The FoxO family comprises 4 proteins, being FoxO1 and FoxO3a the more studied in heart. Both proteins are involved in physiological and pathological processes, such as aging, cancer and cardiovascular diseases [14]. Further, it has been shown that CFs express FoxO1 and FoxO3a [15,16]. We previously demonstrated that TGF-β1 requires FoxO1 to promote CF differentiation into CMF; however, whether FoxO3a participates in this crucial physiopathological mechanism remains unknown.

The evidence that FoxO3a has a role in CF is limited. FoxO3a regulates the proliferation and the antioxidant response of CFs [16,17]. FoxO3a has a role in fibrotic pathologies. The silencing of FoxO3a affects pulmonary fibroblast differentiation and lung fibrosis [18]. In contrast, FoxO3a is involved in heart pathologies as shown by its necessary inactivation to induce cardiac fibrosis due to pressure overload [19]. These data suggest that FoxO3a would act negatively in cardiac fibrosis; however, the role of FoxO3a in CMF conversion has not been studied.

The TGF-β1-induced regulation of FoxO3a has been studied in several tissues and cells. TGF-β1 induces the inactivation of FoxO3a in pulmonary fibrosis [20], whereas an inhibitor of TGF-β1 prevents kidney fibrosis and FoxO3a inactivation [21]. However, the TGF-β1-induced regulation of FoxO3a in CFs is unknown. The activity of FoxO3a is regulated mainly by posttranslational modifications. The phosphorylation of FoxO3a induces its localization to the cytosol, proteasomal degradation and transcriptional activity suppression [22]. The main proteins that regulate the FoxO3a activity through phosphorylation are ERK1/2 and AKT [22,23], both of which are activated by TGF-β1 and are involved in CMF conversion [24–26]. The activation of FoxO3a increases the expression of catalase and SOD2 [27]. In addition, inhibited FoxO3a expression promotes high levels of ROS in cardiac tissue, whereas ROS is crucial for CMF conversion induced by TGF-β1 [28].

FoxO1 and FoxO3a act together in many situations, whereas under other some conditions, FoxO1 and FoxO3a have contradictory functions [14]. In T-regulatory cells, FoxO1 and FoxO3a are important for the regulation of FoxP3 expression [29]; however, in human endometrial stromal cells, FoxO1 and FoxO3a regulate oxidative stress differently [30]. Cross-talk between FoxO family proteins has not been extensively studied. The nature of the cross-talk between FoxO3a and FoxO1 in CFs and its role in TGF-β1-induced CMF conversion are unknown.

In this work, we hypothesize that TGF-β1 decreases the expression and activity of FoxO3a, requiring SMAD3, ERK1/2, AKT and FoxO1. In addition, we analyzed the role of FoxO3a in the TGF-β1-induced differentiation of CFs into CMFs.

2. Materials and methods

2.1. Materials

TGF-β1 and Accutase® were obtained from Millipore. 488 Alexa Fluor® conjugated secondary antibodies, actinomycin D, cycloheximide, MG132, Bradford solution, propidium iodide, and primary antibodies (against GAPDH, β-tubulin, α-SMA) were obtained from Sigma-Aldrich. Primary antibodies (against p-SMAD3, SMAD3, FoxO1, p-ERK1/2, ERK1/2, p-AKT, AKT, Catalase, SOD2, FoxO3a and p-FoxO3a) were obtained from Cell Signaling Technologies. Primary antibodies (against lamin A/C, pro-collagen I, and CTGF) were obtained from Abcam. Secondary antibodies conjugated with horseradish peroxidase were obtained from Calbiochem. Collagen rat tail solution, RNAse, Trypan blue, lipofectamine, Opti-MEM®, and the siRNAs were obtained from Life Technologies. All adenoviruses used in this work were a kind gift from Dr. Joseph A Hill, University of Texas, Southwestern Medical Center (Dallas, Texas). The ECL Advance Western Blotting Detection kit was obtained from GE Healthcare Europe GmbH.

2.2. Cell culture and treatments

CFs were isolated from the left ventricle (LV) of 3-day-old Sprague-Dawley rats. Briefly, LVs were harvested, cut into small pieces (~1–2 mm), and placed in a culture dish. A total of 10 mL Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics were added to culture dishes. The homogenized cells were maintained at 37 °C in a 5% CO_2humidified incubator until they reached 70% confluence. Adherent cells were cultivated. Cells from passage 1 were used for all experiments. For experimental conditions, the cells were maintained in DMEM-F12 medium for 18 h. CFs were stimulated with TGF-β1 (1–10 μg/mL) for different durations in the experiments (2 to 72 h). For the inhibition experiments, the cells were incubated with actinomycin D (transcription inhibitor, 2 μg/mL), cycloheximide (translation inhibitor, 1 μM), MG132 (proteasome inhibitor, 10 nM), PD98056 (MEK/ERK1/2 signaling inhibitor, 10 μM), or LY294002 (PI3K/AKT signaling inhibitor, 10 μM), AS1842856 (FoxO1 inhibitor, 100 nM) for 1 h before TGF-β1 incubation.

2.3. Western blot analysis

After the respective treatments, the cells were lysed in 50 mM Tris, 300 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 20% glycerol, 1% NP40, 0.5 mM DTT, and inhibitor cocktail. The lysates were vigorously vortexed for 10 s and centrifuged at 10,000 rpm for 10 min, and the total protein content was determined using the Bradford assay. Equivalent amounts of protein (30 μg) were subjected to SDS-PAGE. Western blotting was performed by transferring proteins to nitrocellulose membranes, which were subsequently blocked with 5% fat-free milk (w/v) in TBS-Tween for 1 h at room temperature. The membranes were probed with the appropriate primary antibody overnight at 4 °C and then with a peroxidase-conjugated secondary antibody for 2 h at room temperature. Finally, the ECL Advance Western Blotting Detection kit was used for immunodetection in a blot scanner (Li-COR). The protein levels were determined by densitometric analysis using ImageJ software (NIH, Bethesda, MD, USA) and normalized to the corresponding GAPDH level.

2.4. Immunofluorescence assay

A total of 150,000 cells was fixed in 4% paraformaldehyde solution for 20 min at room temperature and permeabilized in 0.1% TritonX-100 for 10 min at room temperature. Nonspecific proteins were blocked with 3% bovine serum albumin solution for 30 min at room temperature. Cells on the coverslips were incubated with the appropriate primary antibody overnight at 4 °C and then with an appropriate fluorophore-conjugated secondary antibody for 2 h at room temperature. Images were obtained using a spinning-disk microscope (Olympus BX43).

2.5. Subcellular fractionation

A total of 500,000 cells were lysed in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.05% NP40 (pH 7.9), kept on ice for 10 min, and centrifuged at 300 rpm for 10 min at 4 °C. The supernatants corresponded to the cytosol samples. The pellets were resuspended in

5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 26% glycerol (v/v) (pH 7.9). NaCl was added to obtain a 300 mM NaCl final concentration; the mixture was maintained on ice for 30 min and then centrifuged at 24,000*g* for 20 min at 4 °C. The pellets were discarded, and the supernatants corresponded to nuclear extracts. Protein content was analyzed using the Bradford assay. The purity of the subcellular fractions was assessed using β-tubulin for the cytosol and lamin A/C for the nucleus.

2.6. Cell proliferation

CF proliferation was induced with FBS at 10% in DMEM-F12 medium for 24 h. To evaluate cell proliferation, the CFs were detached from culture dishes with Accutase®. After detachment, 100% FBS was added to ensure cell integrity and inhibit further Accutase® action. The cell number was determined by the trypan blue exclusion method with a Neubauer chamber. The cells were counted in quadruplicate.

2.7. Cell cycle analysis

The cells detached by Trypsin-EDTA 1× were centrifuged at 2000 rpm for 5 min at 4 °C, resuspended in cold methanol, and stored at −20 °C overnight. The cells were centrifuged at 2000 rpm for 5 min at 4 °C, and the pellet was resuspended in PBS solution containing RNAse (1 μg/mL) and kept at room temperature for 2 h. The cells were incubated with propidium iodide solution (10 μg/mL) for 1 min at room temperature. Finally, the cell cycle was analyzed in a flow cytometer (Becton Dickinson). Data were expressed as the percentage of cells in G0/G1 and S/G2/M.

2.8. FoxO1 and FoxO3a silencing

CFs were seeded in 35 mm dishes at 80% confluence and serum-deprived overnight. Lipofectamine and Opti-MEM® (100 μL) and specific siRNA and Opti-MEM® (100 μL) were prepared, incubated for 15 min at room temperature, mixed, and then incubated for an additional 15 min. Opti-MEM 800 μL was added and the mixture added to the cells. After incubation for 16 h at 37 °C, the medium was changed to DMEM-F12 for an additional 24 h. Scramble siRNA (Silencer® negative control) was used as a control.

2.9. Adenoviral transduction

CFs were plated at 70% confluence and deprived of serum overnight. Three different adenoviruses were used: wild-type FoxO1 (Ad-FoxO1-wt), constitutively active FoxO1 (Ad-FoxO1-ca), and adenovirus green fluorescent protein (Ad-GFP) as a control. Adenoviruses at 50 and 100 multiplicity of infection (MOI) were added and incubated for 24 h.

2.10. Collagen gel contraction assay

The 24-well plates were coated with 3% BSA solution and maintained overnight. Then, in each well, 400 μL of collagen gel solution (1 mg/mL in PBS) and 100 μL of cell suspension (50,000 cells per well) were added, gently homogenized, and maintained at 37 °C for 2 h. Gels were detached from the edge of the well with a sterile pipette tip, and 500 μL of DMEM-F12 was added. Gel contraction was determined after 48 h and expressed as the ratio between the initial and final area of collagen gel.

2.11. Measurement for gene expressions

Briefly, Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA), followed by DNAse treatment and purification using RNAmini column kit (Thermofisher, PureLink RNA Mini Kit). cDNA was synthesized using miScript PCR Starter Kit (Qiagen, Valencia, CA) in a 20 μL reaction mixture. qPCR was performed on the iQ5 Gradient Real Time PCR system (Bio-Rad) using the protocol of miScript PCR Starter Kit (Qiagen, Valencia, CA). The primers for qPCR were synthesized as follows: catalase I forward primer: 5′-CCCAGAAGCCTAAGAATG CAA-3′; reverse primer: 5′-TCCCTTGGCAGCTATGTGAGA-3′; SOD2 forward primer: 5′-CTGGCCAAGGGAGATGT-3′, reverse primer: 5′-GGCCTGTGGTTCCTTGC-3′ and GAPDH forward primer: 5′-TCTGA CGTGCCGCCTGGAGA-3′, reverse primer: 5′-TGGGCCCTCAGATGCCT GCT-3′. The relative expression level between treatments was then calculated using the following equation: relative gene expression $= 2 - (\Delta Ct,sample - \Delta Ct,control)$.

2.12. Statistical analysis

Data are presented as the mean ± SEM from at least four independent experiments. Statistical analysis was performed using one-way ANOVA and, for multiple comparisons, Tukey's test with GraphPad Prism 5.0 software. $p < 0.05$ was considered statistically significant. Shapiro-Wilk test was performed to verify the normal distribution of data.

3. Results

3.1. TGF-β1 decreases FoxO3a expression

First, we studied the effects of TGF-β1 on FoxO3a expression. TGF-β1 decreased FoxO3a expression in a concentration-dependent manner, which was statistically significant at a TGF-β1 concentration of 5 ng/mL at 48 h (Fig. 1A). Additionally, using a single concentration of TGF-β1 (10 ng/mL) at different times in the experiment, we observed a time-dependent decrease in FoxO3a protein levels. The effects of TGF-β1 were significant starting at 24 h and reaching a maximum effect at 48 h (Fig. 1B). The TGF-β1-induced decrease in FoxO3a expression was corroborated by immunofluorescence staining against FoxO3a (Fig. 1C), for which similar results were observed. To evaluate the mechanism involved in the TGF-β1 regulation of FoxO3a, CFs were preincubated for 1 h with inhibitors of transcription (actinomycin D), translation (cycloheximide) and proteasomal degradation (MG132). MG132 blocked the decrease of FoxO3a expression induced by TGF-β1, whereas actinomycin D and cycloheximide enhanced TGF-β1 effects (Fig. 1D). These results suggest that TGF-β1 decreases the protein level of FoxO3a through the proteasomal degradation mechanism.

3.2. TGF-β1 decreases FoxO3a activity

The phosphorylation of FoxO3a at serine 253 promotes its inactivation and degradation through the proteasome system [22]. The stimulation of CFs with TGF-β1 at 10 ng/mL increased the phosphorylation of FoxO3a in a time-dependent manner, being significant at 4 h (Fig. 2A). The phosphorylation of FoxO3a promotes its recognition by 14-3-3 proteins, which maintain FoxO3a in the cytosol and outside the nucleus [22]. Therefore, we sought to evaluate the intracellular localization of FoxO3a by subcellular fractionation. TGF-β1 induced increase of FoxO3a protein expression in the cytosol and decreased it in the nucleus (Fig. 2B), a finding that was corroborated by immunofluorescence. The images show that, under basal conditions, FoxO3a is localized mainly in the nucleus, whereas TGF-β1 induced its translocation into the cytosol (Fig. 2C). Once in the nucleus, FoxO3a induces the regulation of several genes. A specific transcriptional targets are catalase and SOD2 [31]. TGF-β1 decreased catalase and SOD2 expression, both mRNA (Fig. 2D) and protein (Fig. 2E), in a manner parallel to the decrease in FoxO3a nuclear localization. These results suggest that TGF-β1 decreases the transcriptional activity of FoxO3a in CFs.

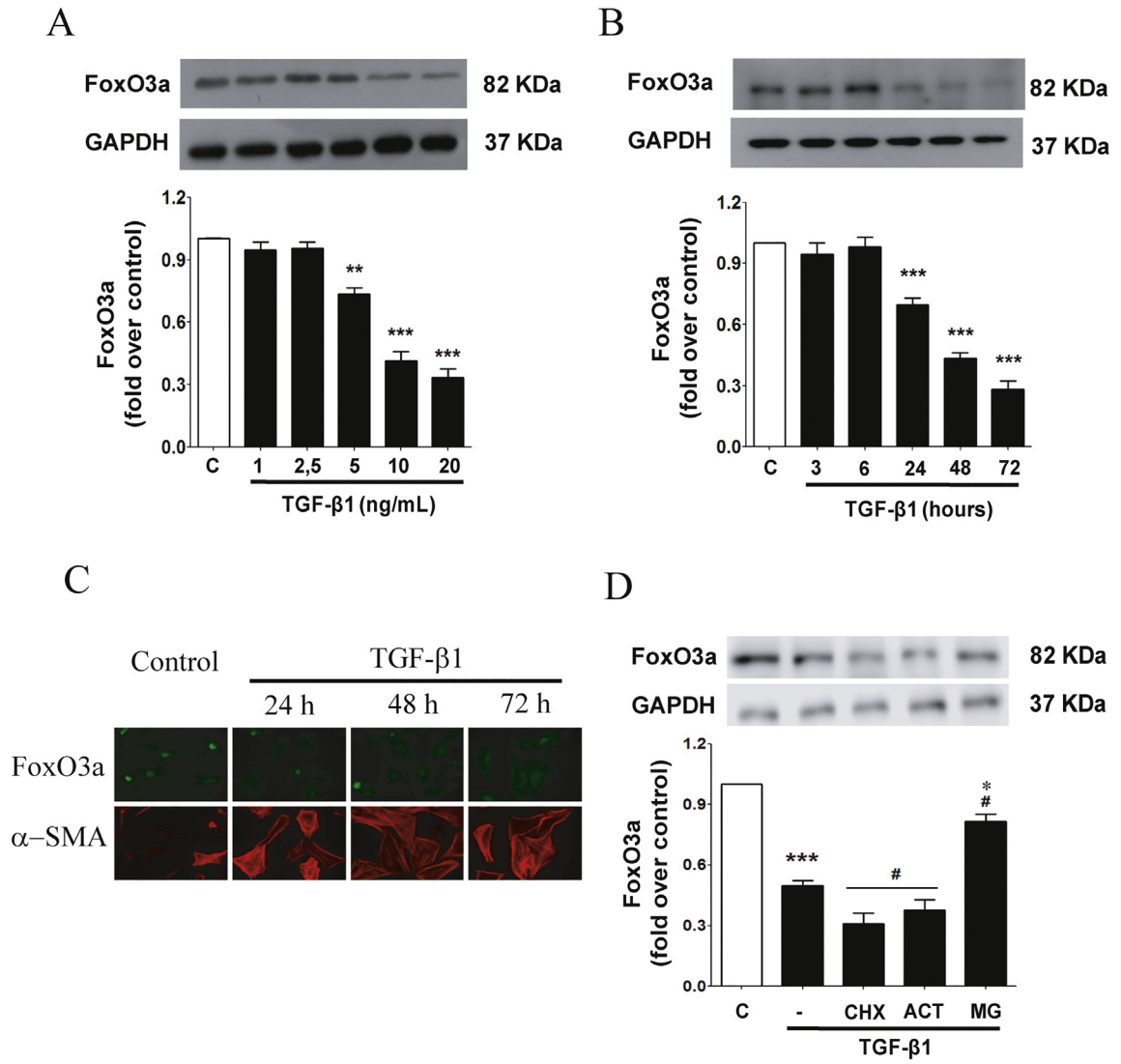

Fig. 1. TGF-β1 decreases FoxO3a expression. FoxO3a level protein was analyzed by western blotting. Cardiac fibroblasts were stimulated with (A) TGF-β1 in a concentration-dependent manner for 48 h and (B) with TGF-β1 10 ng/mL in a time-dependent manner. (C) Immunofluorescence against FoxO3a in cardiac fibroblasts incubated with TGF-β1 10 ng/mL in a time-dependent manner. FoxO3a was evaluated by labeling cells with anti-FoxO3a antibodies and then with Alexa Fluor 488-coniugated secondary antibodies (green staining) and cells was incubated with anti-α-SMA antibody and then with Alexa Fluor 566-coniugated secondary antibodies (red staining). (D) Cardiac fibroblast were incubated with actinomycin D (ACT) 2 μg/mL, cycloheximide (CHX) 10 μM or MG132 (MG) 10 nM for 1 h, then the cells were incubated with TGF-β1 10 ng/mL for 24 h. FoxO3a level protein was analyzed by western blotting. *p < 0,05, **p < 0,01 and ***p < 0,001 vs C; # p < 0,05 vs TGF-β1. All of these results represent the average of four different experiments.

3.3. TGF-β1 requires the activation of SMAD3, AKT and ERK1/2 to regulate FoxO3a

It is well known that ERK1/2 and AKT regulate the activation and expression of FoxO3a [32], whereas SMAD3 role is unknown. To model this fact in our study, we analyzed the participation of these proteins in the TGF-β1-induced regulation of FoxO3a. The phosphorylation (activation) of SMAD3, ERK1/2 and AKT increased with TGF-β1 stimulation (Fig. 3A). To demonstrate the importance of SMAD3, ERK1/2 and AKT activation in the TGF-β1-induced regulation of FoxO3a activity and expression in the CFs, SMAD3, ERK1/2 and AKT expression was inhibited with SIS3, PD98059 and LY294002, respectively, for 48 h. The decrease in FoxO3a expression was prevented by SIS3, PD98059 and LY294002 (Fig. 3B). On the contrary, the phosphorylation and cytosolic relocation of FoxO3a induced by TGF-β1 were only prevented by the addition of PD98059 and LY294002, whereas SIS3 not affected this effect induced by TGF-β1 (Fig. 3C–D). Finally, the TGF-β1-induced decrease in catalase and SOD2 expression, used as markers of FoxO3a transcriptional activity, was suppressed by SMAD3, ERK1/2 and AKT inactivation (Fig. 3E). Collectively, these results suggest that TGF-β1 regulates the activation and expression of FoxO3a through the activation of SMAD3, ERK1/2 and AKT.

3.4. TGF-β1-induced FoxO3a downregulation requires FoxO1

We previously showed that FoxO1 is crucial to the CMF conversion induced by TGF-β1 [15], whereas the role of FoxO1 in decreasing TGF-β1-induced FoxO3a expression is unknown. To elucidate this mechanism, we used AS1842856, a specific inhibitor of the FoxO1 transcriptional activity, to determine whether FoxO1 regulates FoxO3a levels and activity. Fig. 4A shows that AS1842856 prevented the increase of FoxO3a phosphorylation and decrease in FoxO3a, catalase and SOD2 expression levels, whereas inhibited FoxO1 suppressed the nuclear localization of FoxO3a induced by TGF-β1 (Fig. 4B). To complement these results, CFs were transfected with FoxO1 siRNA (siFoxO1) or FoxO3a siRNA (siFoxO3a). The TGF-β1-induced decrease in FoxO3a

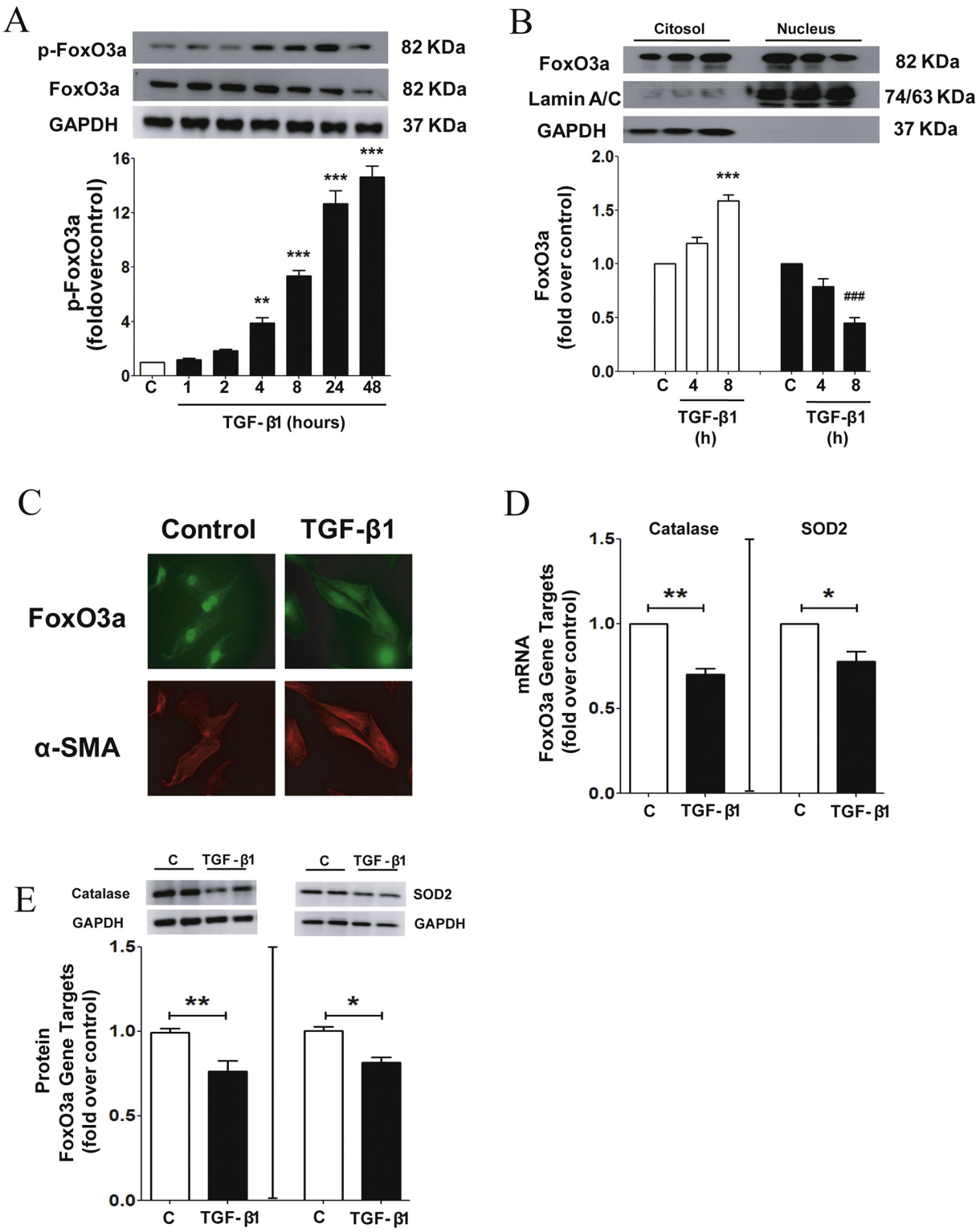

Fig. 2. TGF-β1 decreases FoxO3a activity. A) Cardiac fibroblasts were incubated with TGF-β1 10 ng/mL in a time-dependent manner. p-FoxO3a and FoxO3a level protein was analyzed by western blotting. Bar graph shows level protein obtained from the ratio between the phosphorylated and total FoxO3a and reported as fold over the control. GAPDH was used as a load control. B) Cardiac fibroblasts were stimulated with TGF-β1 10 ng/mL in a time-dependent manner. FoxO3a level protein in nuclei was determined by western blotting. Lamin A/C was used as a nucleus control and GAPDH as a cytosol control. C) Cardiac fibroblast were seeded on coverslips and then stimulated with TGF-β1 10 ng/mL for 8 h. Nuclear localization of FoxO3a was evaluated by labeling the cells with anti-FoxO3a antibodies and then with Alexa Fluor 488-coniugated secondary antibodies (green staining). Cardiac fibroblast shape was evaluated by labeling the cells with anti-α-SMA antibody and then with Alexa Fluor 566-coniugated secondary antibodies (red staining). D) Cardiac fibroblast was incubated with TGF-β1 10 ng/mL for 48 h and catalase and SOD2 mRNA was analyzed by RT-qPCR. GAPDH was used as a load control. E) Cardiac fibroblast was incubated with TGF-β1 10 ng/mL for 48 h and catalase and SOD2 protein was analyzed by western blot. GAPDH was used as a load control. *p < 0,01, **p < 0,01 and ***p < 0,001 vs C; ###p < 0,001 vs C (nucleus). All of these results represent the average of four different experiments.

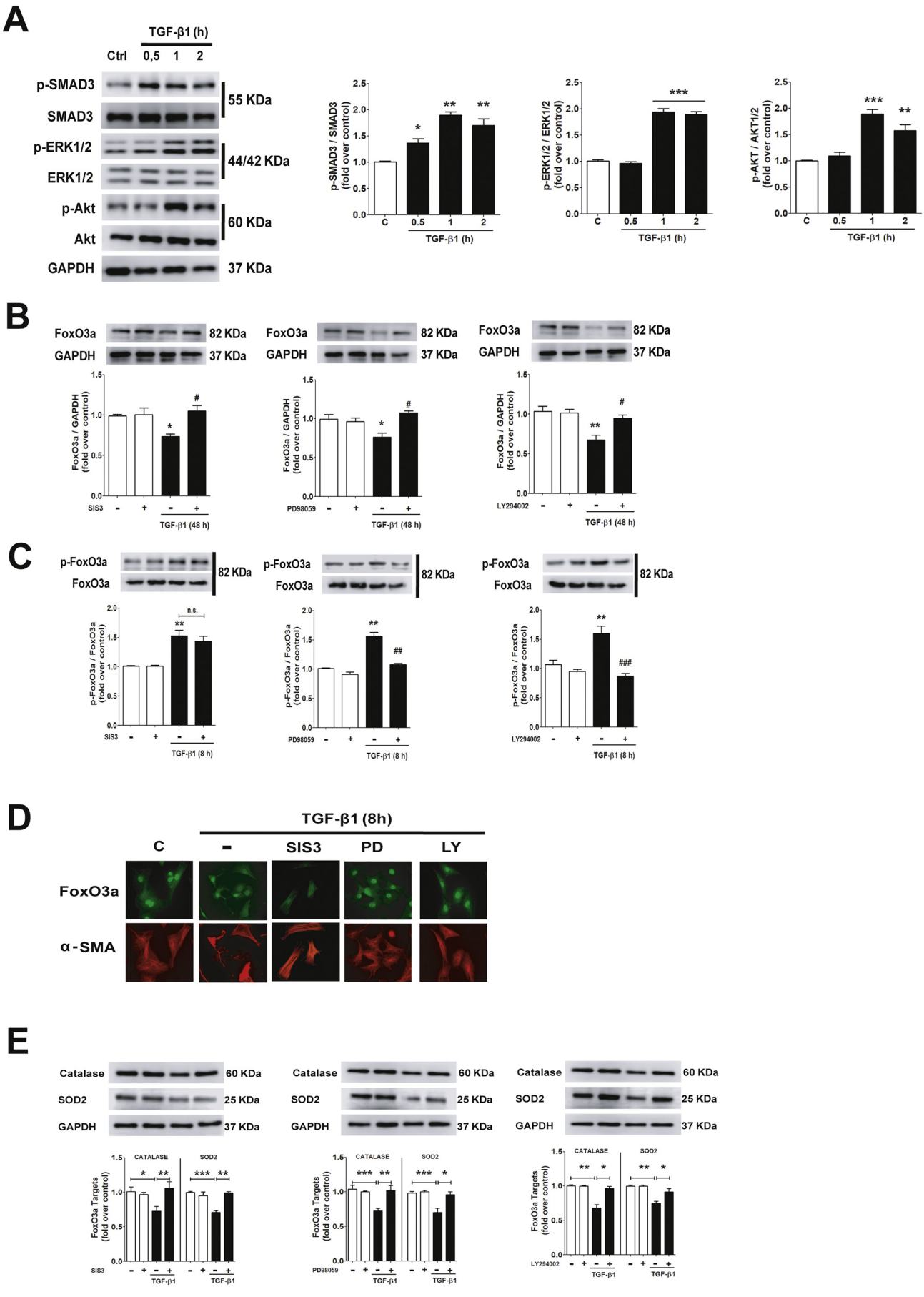

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Fig. 3. TGF-β1 requires the activation of SMAD3, ERK1/2 and AKT to regulate FoxO3a. A) Cardiac fibroblasts were stimulated with TGF-β1 10 ng/mL for 0,5, 1 and 2 h and p-SMAD3, SMAD3, p-ERK1/2, ERK1/2, p-AKT and AKT were analyzed by western blotting. GAPDH was used as a control. B) Cardiac fibroblasts were stimulated with TGF-β1 10 ng/mL for 48 h in presence or absence of SIS3, PD98056 (PD) or LY294002 (LY) all of them at 10 μM and FoxO3a was analyzed by western blotting. GAPDH was used as a load control. C) Cardiac fibroblast were stimulated with TGF-β1 10 ng/mL for 8 h in presence and absence of SIS3, PD98056 (PD) or LY294002 (LY) all of them at 10 μM. p-FoxO3a and FoxO3a were analyzed by western blotting. D) Cardiac fibroblast were seeded on coverslips and then stimulated with TGF-β1 10 ng/mL for 8 h in presence and absence of SIS3, PD98056 (PD) or LY294002 (LY) all of them at 10 μM. Nuclear localization of FoxO3a was evaluated by labeling the cells with anti-FoxO3a antibodies and then with Alexa Fluor 488-coniugated secondary antibodies (green staining). Cardiac fibroblast shape was evaluated by labeling the cells with anti-α-SMA antibody and then with Alexa Fluor 566-coniugated secondary antibodies (red staining). E) Cardiac fibroblast were stimulated with TGF-β1 10 ng/mL for 48 h in presence and absence of SIS3, PD98056 (PD) or LY294002 (LY) all of them at 10 μM. Catalase and SOD2 protein were analyzed by western blot. GAPDH was used as a load control. *$p < 0,05$, **$p < 0,01$ and ***$p < 0,001$ vs C; #$p < 0,05$, ##$p < 0,01$ and ###$p < 0,001$ vs TGF-β1. All of these results represent the average of four different experiments.

expression was prevented by siFoxO1, whereas FoxO3a silencing enhanced the effects of TGF-β1 on FoxO3a expression (Fig. 4C). Additionally, wild-type FoxO1 adenoviruses or constitutively active forms of adenoviruses were used to overexpress FoxO1. Overexpressed FoxO1 enhanced the FoxO3a decrease induced by TGF-β1, and the differences in the ability of the adenoviruses to induce overexpression were not significantly different (Fig. 4D). Collectively, these results suggest that FoxO1 is necessary for FoxO3a downregulation induced by TGF-β1.

3.5. FoxO3a is a negative regulator of cardiac myofibroblast conversion induced by TGF-β1

Given that TGF-β1 is crucial to CMF conversion and that we demonstrated that TGF-β1 regulates FoxO3a, we sought to analyze the role of FoxO3 in the CMF conversion induced by TGF-β1. Procollagen I, α-SMA and CTGF expression levels and total protein content were used as markers of CMF conversion. It has been demonstrated that these markers are increased when CFs differentiate into CMFs. These results were complemented with an immunofluorescence against α-SMA. We demonstrated that the TGF-β1-induced increase in procollagen I, α-SMA and CTGF expression levels, the total protein content and CFs size was enhanced by FoxO3a silencing. In contrast, preventing FoxO3a downregulation using FoxO1 siRNA prevented the CF differentiation induced by TGF-β1 (Fig. 5A–C). In order to determinate the mechanism of TGF-β1 inhibition induced by FoxO3a, we evaluated the activation of SMAD3. Fig. 5D shows that TGF-β1 increased the phosphorylation of SMAD3, whereas the activation of this protein was enhanced by FoxO3a silencing and was decreased by FoxO3a downregulation prevent. We obtained similar results with an immunofluorescence against SMAD3. TGF-β1 increased SMAD3 nuclear localization and FoxO3a regulate negatively this effect promoted by TGF-β1 (Fig. 5E). These results suggest that FoxO3a acts as a negative regulator of the CMF conversion induced by TGF-β1, at least in part, decreasing the activation of SMAD3.

3.6. The negative role of FoxO3a in the antiproliferative and contractile effects promoted by TGF-β1 in cardiac fibroblasts

TGF-β1 induces CMF conversion, which increases the contractile capacity and decreases the proliferative capacity of CF [15]. However, the participation of FoxO3a in these cellular effects promoted by TGF-β1 is unknown. First, we demonstrated that TGF-β1 increased the contractile capacity of CFs by analyzing the results of a contraction collagen gel assay. TGF-β1 decreased the collagen gel area compared with that of the control (Fig. 6A). In addition, we analyzed the proliferative capacity of CFs by counting cells and determining the population of cells in G0/G1 and G2/S stages. Fetal bovine serum (FBS) was used as an inductor of proliferation. TGF-β1 prevented the increase in total cell number and the number of cells in the G2/S stage, whereas it decreased the number of FBS-treated cells in the G0/G1 stage (Fig. 6B–D). The effects induced by TGF-β1 on contractile and proliferative capacities of CFs were enhanced by FoxO3a silencing. The opposite result was observed when FoxO3a downregulation was prevented (Fig. 6A–D). These results suggest that FoxO3a is a crucial component in the contractile and proliferative capacity of the CFs regulated by TGF-β1.

4. Discussion

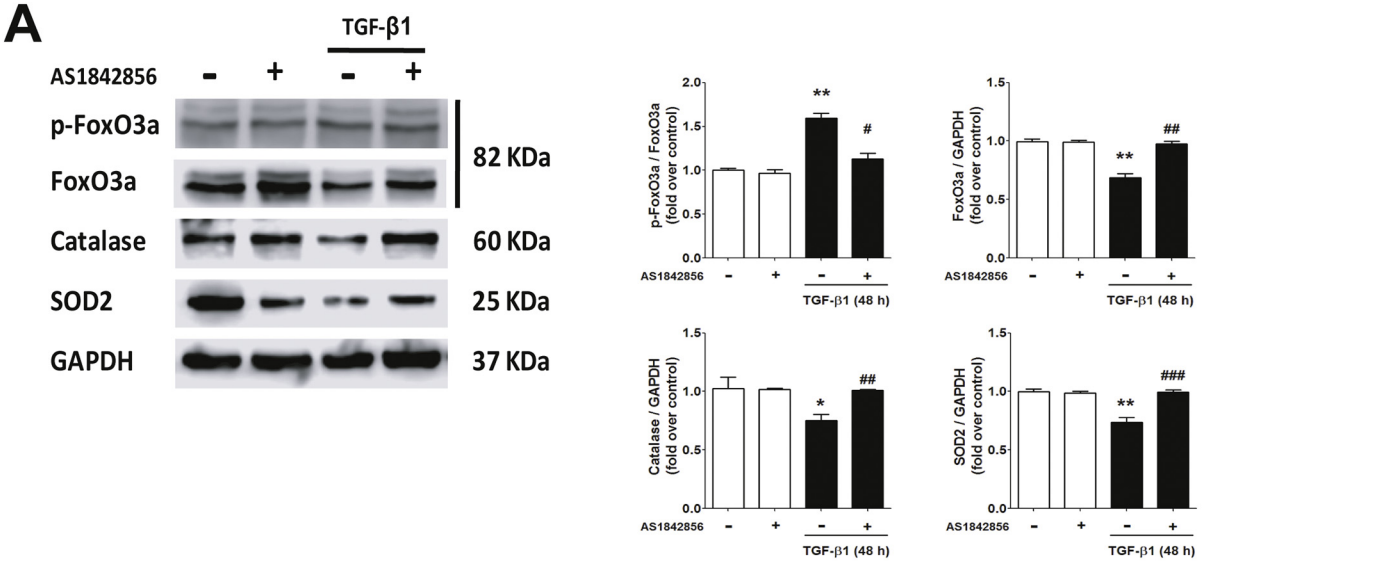
Taken together, our results suggest that TGF-β1 requires FoxO1, SMAD3, ERK1/2 and AKT to decrease the expression and activity of FoxO3a, whereas FoxO3a has a negative role in the CMF conversion induced by TGF-β1.

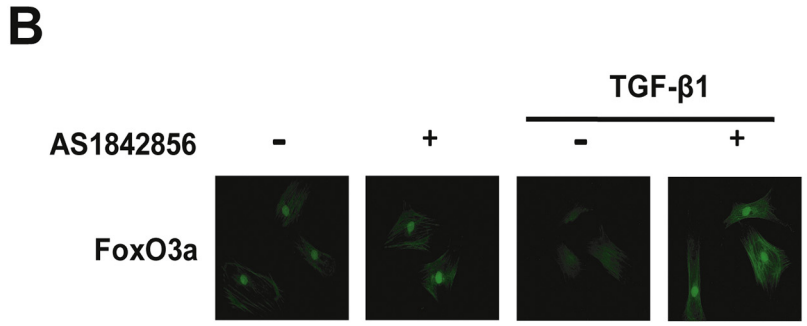
TGF-β1 regulates the expression of FoxO family members. We have previously demonstrated that TGF-β1 increases the expression of FoxO1 in CFs [15], whereas data regarding the regulation of FoxO3a by TGF-β1 are contradictory. Our results indicate that TGF-β1 induced the degradation of FoxO3a through the proteasome mechanism. Similar results have been shown in mesangial cells obtained from diabetic rats, where TGF-β1 decreased the expression of FoxO3a [33]; however, in patients with liver fibrosis induced by the hepatitis C virus, TGF-β1 was shown to increase the expression of FoxO3a [34]. Therefore, the regulation and degradation of FoxO3a induced by TGF-β1 appear to be dependent on both the species and the tissue analyzed.

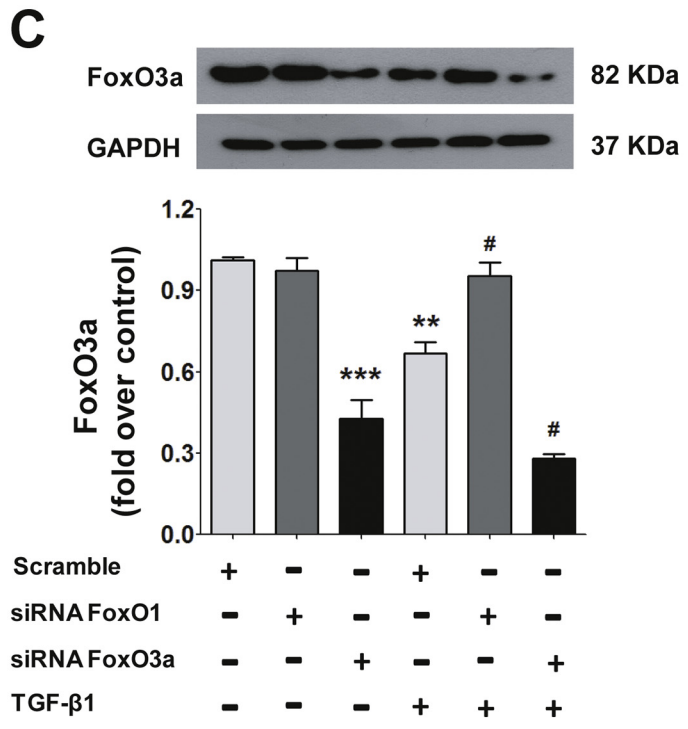
The mechanism by which TGF-β1 induces a decrease in the FoxO3a protein is unclear. There is evidence that shows that FoxO3a degradation is induced by regulation of its expression, whereas other results indicate that the decrease in FoxO3a expression is the result of proteasome complex activation [22]. It has been described that the degradation of FoxO3a requires the activation of ERK1/2, AKT and the proteasomal system [35,36]. In this regard, the results obtained in this work indicate that the TGF-β1 regulation of FoxO3a levels depends on both mechanisms. MG132, an inhibitor of the proteasome pathway, blocked the degradation of FoxO3a induced by TGF-β1, whereas the inhibition of transcription with actinomycin and inhibition of translation with cycloheximide enhanced the TGF-β1-promoted effects on FoxO3a, suggesting that TGF-β1 regulates FoxO3a expression.

TGF-β1 is crucial to CMF conversion and cardiac fibrosis and evidence show that SMAD3 is crucial to these effects of TGF-β1 [37]. However data regard FoxO3a regulation by TGF-β1 is unclear. Our results demonstrate that TGF-β1 decreases FoxO3a expression and the inhibition of SMAD3, using SIS3, prevents FoxO3a knockdown induced by TGF-β1. On the contrary, Bollinger et al. showed that FoxO3a and SMAD3 collaborate in the expression of MURF1 in human embryonic kidney cells [38]. Nevertheless data demonstrate that FoxO3a has an antifibrotic role, whereas TGF-β1 regulates in a negative manner to FoxO3a [33], however these authors do not show the mechanism of FoxO3a regulation by TGF-β1.

Evidence obtained from rats with diabetic nephropathy and isolated mesangial cells showed that TGF-β1 increased the phosphorylation and cytosolic localization of FoxO3a in a finding that correlated with decreased FoxO3a transcriptional activity [33]. In contrast, in hepatic cells obtained from rat hepatoma, it has been shown that TGF-β1 increases FoxO3a transcriptional activity [39]. This latest evidence suggests that the TGF-β1 effects on FoxO3a regulation could be tissue-dependent. Our results indicate that TGF-β1 induces FoxO3a phosphorylation and that this posttranscriptional modification promotes the cytosolic localization of FoxO3a, which is correlated with a decrease in







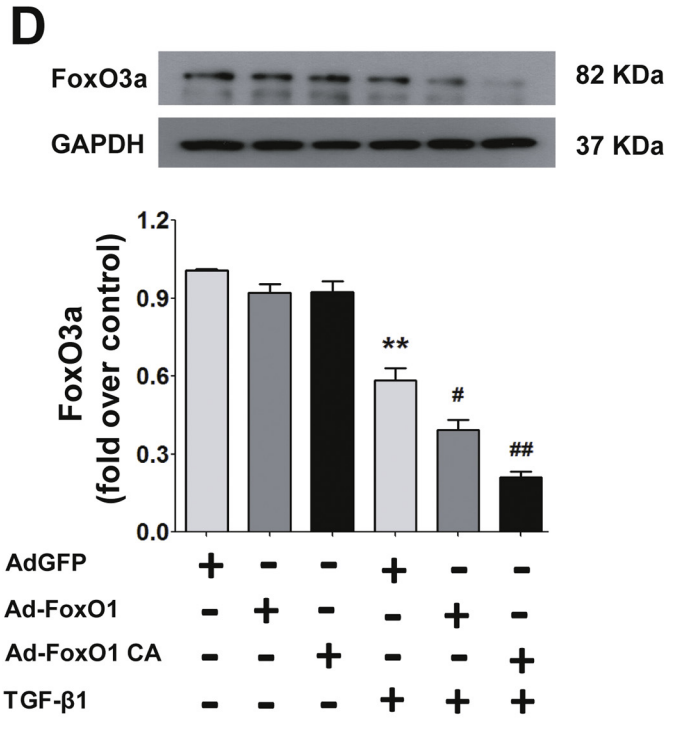


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Fig. 4. TGF-β1-induced FoxO3a down-regulation requires FoxO1. Cardiac fibroblasts were incubated with TGF-β1 10 ng/mL in presence or absence of AS1842856 (FoxO1 inhibitor) 100 nM for 48 h. A) p-FoxO3a, FoxO3a, catalase and SOD2 protein were analyzed by western blot. GAPDH was used as a load control. B) Cardiac fibroblast were seeded on coverslips and then stimulated with TGF-β1 10 ng/mL for 48 h. FoxO3a expression and nuclear localization were evaluated by labeling the cells with anti-FoxO3a antibodies and then with Alexa Fluor 488-coniugated secondary antibodies (green staining). C) Cardiac fibroblasts were incubated with FoxO3a or FoxO1 siRNA 10 nM for 16 h, then cells were stimulated with TGF-β1 10 ng/mL for 48 h. FoxO3a was evaluated by western blot using GAPDH as a load control. A siRNA scramble was used as a transfection control. D) Wild-type FoxO1 adenoviruses or constitutively active forms of adenoviruses were used to overexpress FoxO1. Adenoviruses was used at MOI 100 for 24 h, then cells were stimulated with TGF-β1 10 ng/mL for 48 h. FoxO3a protein was evaluated by western blot using GAPDH as a load control. An adenovirus that expresses GFP (Ad-GFP) was used as a transduction control. *p < 0,05, **p < 0,01 and ***p < 0,001 vs Control (−), SCR or Ad-GFP; #p < 0,05, ##p < 0,01 and ###p < 0,001 vs TGF-β1, SCR + TGF-β1 or Ad-GFP + TGF-β1. All of these results represent the average of four different experiments.

FoxO3a transcriptional activity. This last result is confirmed by the reduced expression of catalase and SOD2 mRNA, specific targets of FoxO3a. The transcriptional activity of FoxO3a is regulated mainly through phosphorylation, in which ERK1/2 and AKT are crucial. In HeLa cells, IGF-1 increases AKT activation, FoxO3a phosphorylation and FoxO3a degradation [40], whereas, in human tenocytes, dexamethasone stimulates increased FoxO3a expression and activity, effects prevented by insulin through the activation of ERK1/2 and AKT [41]. Our data demonstrate that the enzymatic inhibition of AKT and ERK1/2 prevents the phosphorylation and cytosolic localization of FoxO3a and the TGF-β1-induced decrease in catalase and SOD2 expression. These results suggest that TGF-β1 decreases the activity and expression of FoxO3a by regulating the phosphorylation induced by ERK1/2 and AKT.

FoxO1 and FoxO3a act together in several cellular processes, such as apoptosis and antioxidant defense, suggesting that they have overlapping functions in the cellular regulation. However, Zhu et al. demonstrated that, in human neoplastic cells, FoxO3a promotes a decrease in FoxO1 expression [42], whereas Essaguir et al. showed that FoxO3a induces the FoxO1 expression in human fibroblasts [43]. However, whether FoxO3a expression is regulated by FoxO1 remains unknown. Our results suggest that FoxO1 is necessary for the regulation of FoxO3a by TGF-β1, as indicated by overexpressed FoxO1 enhancing and silenced FoxO1 preventing the TGF-β1-induced decrease in FoxO3a in CFs. However, FoxO3a is not involved in the TGF-β1-induced regulation of FoxO1 in CFs. We speculate that FoxO1 activated by TGF-β1 localizes to the FoxO3a promoter and inhibits the expression of FoxO3a. In this regard, it has been described that the promoter of FoxO3a has sites of recognition for FoxO family members, called FHRE (ForkHead Box Element Response) [43]. However, whether FoxO1 localizes to the FoxO3a promoter in CFs is unknown. To our knowledge, this is the first time it has been shown that FoxO1 regulates FoxO3a in a negative manner and that TGF-β1 induces cross-talk between these two members of the FoxO family.

TGF-β1 induces fibroblast differentiation into myofibroblasts in many tissues in correlation with fibrotic diseases [44]. In this regard, our data show that TGF-β1 promotes CMF conversion, as demonstrated by the total protein content; the level of α-SMA, procollagen I and CTGF proteins; the increase of CFs size; and cellular contractile ability. In this work, we suggest that FoxO3a acts as an inhibitor of the CF differentiation induced by TGF-β1 because the silencing of FoxO3a enhances the effects of TGF-β1, whereas the blocking FoxO3a degradation by silencing FoxO1 inhibits the CMF conversion that is induced by TGF-β1. To our knowledge, this is the first time that the role FoxO3a in CMF conversion has been demonstrated. In addition, FoxO3a has been shown to have antifibrotic effects in several models. In this respect, Altamari et al. showed that fibroblasts in fibrotic lungs isolated from rats that had been treated with bleomycin have decreased FoxO3a expression [18], whereas Nho et al. demonstrated that FoxO3a inactivation induces the progression of idiopathic pulmonary fibrosis disease [45]. In addition, in a model of kidney fibrosis induced by ischemia-reperfusion injury, cyclic helix B peptide prevented FoxO3a degradation in a process parallel with the decrease in α-SMA expression [46], suggesting that FoxO3a inhibits the epithelial-mesenchymal transition in the kidney. Nevertheless evidence about FoxO3a role in cardiac fibrosis is scarce. To this regard, Skurk et al. showed that transaortic constriction performed on mice increased cardiomyocyte size and decreased FoxO3a activity, whereas the gene transfer of FOXO3a leads to reduction in cardiac cell size in mice [47]. In addition, Chang et al. demonstrated that the cardiac injury and heart fibrosis induced by paraquat was enhanced by FoxO3a knockdown, which suggest the importance of FoxO3a as a protector of heart [48]. Collectively, these data suggest that FoxO3a is an antifibrotic protein in the heart, lung and kidney that is being recognized as an important regulator of cardiac and pulmonary fibrosis. Moreover, our data indicate a possible molecular mechanism explaining how FoxO3a could be regulating in a negative manner the fibrosis process. In this regard, our results show that FoxO3a decreases the activation of SMAD3 induce by TGF-β1, ameliorating the phosphorylation and nuclear localization of FoxO3a induced by TGF-β1. The silencing of FoxO3a enhanced the activation of SMAD3 induced by TGF-β1, whereas the FoxO3a degradation prevention decreased the activation of SMAD3. The molecular mechanism of this regulation is unclear. The phosphatase PP2a is the main protein that regulates the activation of SMAD3 [49], whereas evidence show that PP2a increase the activity of FoxO3a [50]. However, if FoxO3a regulates the expression or activity of PP2a is unknown.

It has been demonstrated that FoxO3a decreases cellular proliferation and promotes apoptosis. In rat pulmonary fibroblasts, FoxO3a decreases cellular proliferation by inducing proteins that inhibit the cell cycle [45], whereas in human neoplastic cells, FoxO3a induces apoptosis by increasing proapoptotic proteins, such as Bim [51]. Our results showed that TGF-β1 inhibits the proliferation of CF induced by FBS, which was enhanced by the silencing of FoxO3a, suggesting that FoxO3a regulates the proliferation of CFs. In this regard, Hui et al. showed that chronic activation of FoxO3a induced by doxorubicin is required for the acquisition of drug resistance and the proliferation of leukemic cells [52]. Thus, in this work, we suggest that FoxO3a regulates the cytostatic effect of TGF-β1 in CFs.

5. Conclusion

TGF-β1 decreases the activity and expression of FoxO3a by activating SMAD3, ERK1/2 and AKT. In addition, we showed that FoxO1 is necessary for the FoxO3a expression decrease induced by TGF-β1. Finally, the silencing of FoxO3a enhances the effects of TGF-β1 in CFs, which suggests that FoxO3a negatively regulates the differentiation of CFs into CMFs that is induced by TGF-β1.

Transparency document

The Transparency document associated this article can be found, in online version.

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

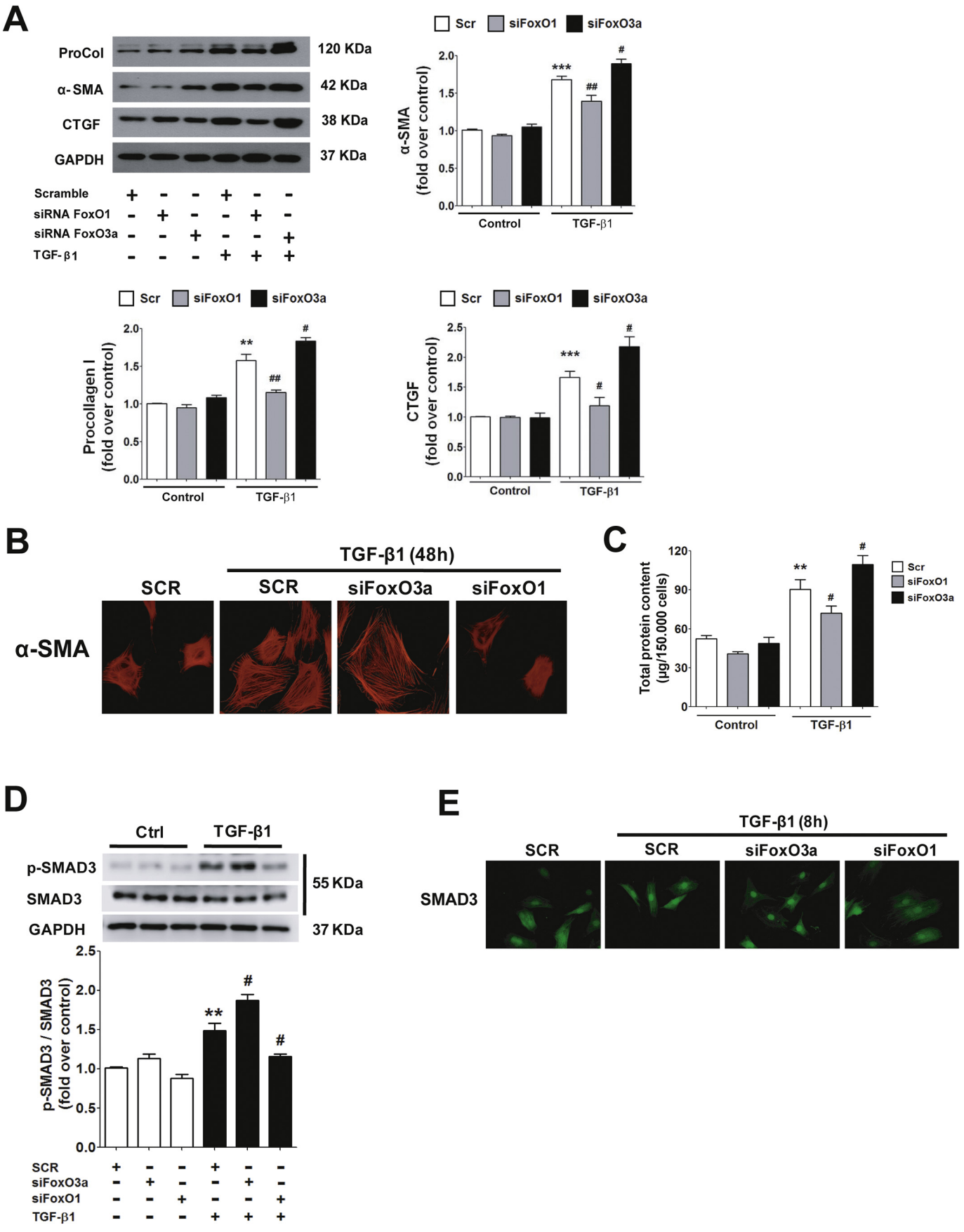

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Fig. 5. FoxO3a is a negative regulator of cardia myofibroblast conversion induced by TGF-β1. Cardiac fibroblasts were incubated with FoxO3a or FoxO1 siRNA 10 nM for 16 h, then cells were stimulated with TGF-β1 10 ng/mL for 48 h. A) Procollagen I (Procol), CTGF and α-SMA protein were evaluated by western blot using GAPDH as a load control. A siRNA scramble was used as a transfection control. B) An immunofluorescence against α-SMA was performed to evaluate cell size and stress fibers. C) Total protein content was evaluated by Bradford protocol assay. D) p-SMAD3 and SMAD3 protein were analyzed by western blot, using GAPDH as a load control. E) An immunofluorescence against SMAD3 was performed to evaluate SMAD3 nuclear localization. **p < 0,01 and ***p < 0,001 vs SCR; #p < 0,05 and ##p < 0,01 vs SCR + TGF-β1. All of these results represent the average of four different experiments.

Availability of data and materials

Not applicable. Our manuscript did not contain any data.

Authors' contributions

R.V. designed the study, analyzed the data, and wrote the manuscript. R.V., R.A., S.B., M.C., C.H. and G.D.A. performed the experimental work. J.H. performed the adenoviruses synthesis. G.D.A. and S.L. contributed to project development and data interpretation. We

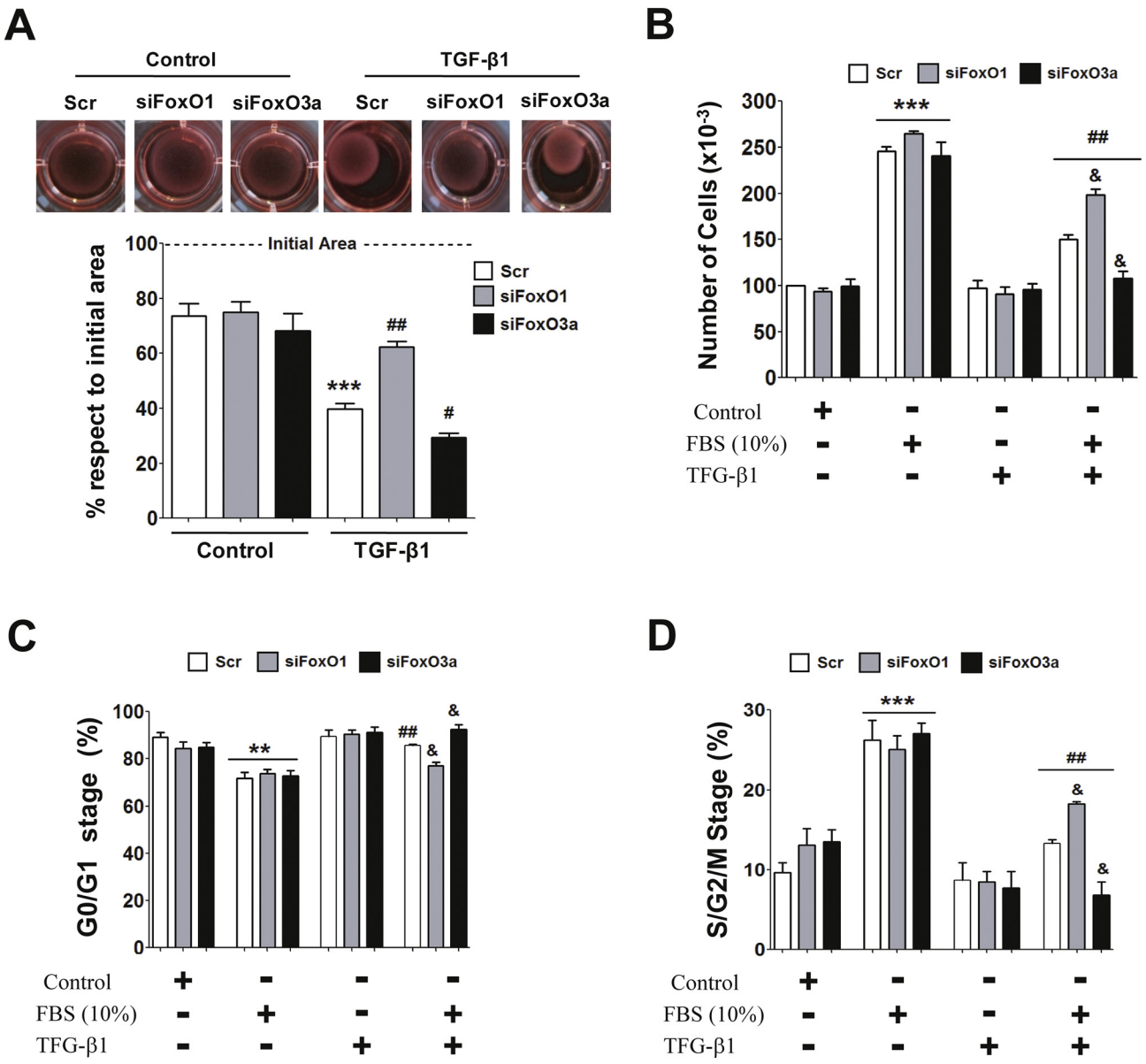

Fig. 6. The negative role of FoxO3a in the antiproliferative and contractile effects promoted by TGF-β1 in cardiac fibroblasts. (A) Cardiac fibroblasts were incubated with FoxO3a or FoxO1 siRNA 10 nM for 16 h and then stimulated with TGF-β1 10 ng/mL for 48 h. Cardiac fibroblast contractile ability was evaluated by collagen gel contraction assay for 24 h. Representatives images of collagen gel contraction and bar graph shows the percentage of gel area reduction respect gel area initial. To evaluate proliferation cardiac fibroblasts were incubated with FoxO3a or FoxO1 siRNA 10 nM for 16 h, then cells were stimulated with TGF-β1 10 ng/mL for 48 h. Cardiac fibroblasts were stimulated with FBS 10% in DMEM-F12 for 24 h to induce proliferation. (B) Cell count was evaluated using trypan blue dye cell exclusion in triplicate. Cardiac fibroblast cellular cycle was evaluated by flow cytometry. Flow cytometer histograms analysis shows percentage of cardiac fibroblast distribution in G0/G1 phase (C) and S/M/G2 phase (D). **p < 0,01 and ***p < 0,001 vs Scr; ##p < 0,01 vs SCR-TGF-β1 (A) or SCR + FBS (B–D); &p < 0,05 vs SCr + FBS + TGF-β1. All of these results represent the average of four different experiments.

declare that all authors have read and approved the manuscript.

CRediT authorship contribution statement

Raúl Vivar: Methodology, Formal analysis, Writing - original draft, Investigation. **Claudio Humeres**: Investigation. **Renatto Anfossi**: Investigation. **Samir Bolivar**: Investigation. **Mabel Catalán**: Investigation. **Guillermo Diaz-Araya**: Writing - review & editing. **Sergio Lavandero**: Resources. **Joseph Hill**: Resources.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2020.118695.

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