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EMBRYO BIOLOGY

FoxO transcription factors 1 regulate mouse preimplantation embryo development

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Abstract

Purpose The aim of the present study is to investigate role of FoxO transcription factors in preimplantation embryo development by knocking down FoxO1, FoxO3, and FoxO4 genes and also to assess cell cycle arrest related proteins, p53 and p21, and apoptosis-related proteins, fas ligand (FASL), and cleaved caspase 3.

Methods Knockdown of FoxOs using siRNA was confirmed utilizing RT-PCR and qRT-PCR in gene level and using immunofluorescence in protein level. Following knockdown of FoxO1, FoxO3, and FoxO4 in two-cell mouse embryos with or without resveratrol treatment; developmental competence of embryos and expression patterns of SIRT1, p53, p21, FASL, and CLEAVED CASPASE 3 proteins in embryos by immunofluorescence were assessed after 48 h. ROS levels were measured in knockdown embryos. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay was used to determine resveratrol dose.

Results Successful knockdown of FoxO genes in mouse embryos utilizing a non-invasive siRNA method was achieved. Significantly, knockdown of FoxO genes impaired preimplantation embryo development which cannot be prevented by resveratrol treatment. Immunofluorescence results showed that resveratrol could protect embryos from cell cycle arrest and apoptosis. FOXO proteins regulate apoptosis and cell cycle related proteins in mouse preimplantation embryos. Moreover, there might be an autofeedback mechanism where FOXO1, FOXO3, and FOXO4 regulate SIRT1 protein expression.

Conclusions These results suggest that FOXO transcription factors could contribute to mouse preimplantation embryo development, and it remains to investigate whether they have crucial roles in human preimplantation embryo and infertility.

Keywords FoxO · Preimplantation embryo · Sirtuin1 · Resveratrol · Oxidative stress · Apoptosis

Introduction

The forkhead box (Fox) transcription factor family consists of 19 subclasses of Fox genes, FoxA–FoxS [44, 45]. FOXO family comprises four members as FOXO1, FOXO3, FOXO4, and FOXO6 in mammals, and they are characterized by a highly conserved forkhead domain termed the "forkhead box" or a "winged helix" [46]. *FoxO* transcription factors regulate multiple cellular functions including cell differentiation, growth, survival, cell cycle, metabolism, stress, and apoptosis in order to coordinate cellular responses up against changing environmental conditions [46]. Because of the multitasks of FOXO proteins, they are considered to be new therapeutic targets for several diseases including cancer, diabetes, aging, and infertility [29]. Members of FOXO superfamily are downstream targets of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [31, 36]. Presence and function of the PI3K/Akt pathway have been shown in mammalian preimplantation embryos previously [38]. Although expression pattern of various FOXO proteins has been identified in many different tissues including embryos, there is a gap in their functional significance. Our previous study has demonstrated that FOXO1, FOXO3, and FOXO4 proteins are differentially expressed in prophase I, metaphase I, metaphase II oocytes, as

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well as during in vivo preimplantation development in mice [19]. FOXOs translocate to nucleus in embryos with developmental delay [19]. Our findings provided a fundamental knowledge that FOXOs may regulate in vitro embryo development which needs to be clarified.

FoxO transcription factors work mostly as transcriptional activators, and their activity is inhibited by PI3K pathway by phosphorylation and eventually they move to cytoplasm from nucleus [13]. Under stress conditions, deacetylation of FOXO proteins by sirtuins activates their target genes which regulate many cellular processes including resistance to oxidative stress [3, 18, 22, 48]. It is known that in vitro embryo culture conditions induce many cellular stressors, which consequently lead to growth and survival factor deprivation [34, 35], metabolic and substrate imbalances [24, 25], oxidative stress [32], and genotoxic damage [8]. Because oocytes and embryos are negatively affected by oxidative stress in in vitro culture systems, various antioxidants have been added to culture systems to improve the maturation of oocytes and the developmental competence of preimplantation embryos. However, the underlying mechanisms that regulate oxidative stress and/or apoptotic mechanisms have not been fully clarified yet [49].

Resveratrol is a natural polyphenolic compound found in several plants and foods and have many beneficial effects on cancer, diabetic, and cardiovascular diseases [49]. Beneficial effects of resveratrol on oocyte maturation and subsequent embryonic development after in vitro fertilization have been reported in various studies [15, 27, 49]. Resveratrol regulates sirtuin molecules, which are NAD+-dependent deacetylase enzymes that comprise seven proteins (SIRT1–7), and activates especially SIRT1 [9, 49]. Proteins that are deacetylated by sirtuin regulate cellular metabolism, stress responses, and aging processes [9]. SIRT1 plays a role in cell survival and decreases reactive oxygen species (ROS) levels under stress conditions. Moreover, SIRT1 regulates p53 and FoxO transcription factors that are both vital molecules to protect the balance between cell survival and death [14].

Based on the recent findings regarding the differential expression of *FoxO* transcription factors in preimplantation mouse embryos and their possible roles in regulating cell cycle arrest and apoptosis related to oxidative stress, the aim of the present study is to evaluate functional significance of *FoxO* transcription factors in preimplantation embryo development by knocking down *FoxO1*, *FoxO3*, and *FoxO4* genes and also to assess cell cycle arrest and apoptosis-related proteins.

Materials and methods

Animals

BALB/c female mice at 4–6 weeks of age and BALB/c male mice at 8–10 weeks of age were obtained from Akdeniz University Animal Research Unit, Antalya, Turkey, and the experimental protocol was approved by the local ethical committee (number of the ethical approval 2014.04.04). Mice were housed in a controlled environment with a cycle of 12 L:12 D (12 h light: 12 h darkness) with ad libitum access to food.

Collection of mouse embryos and in vitro culture

To induce superovulation, female mice were treated through intraperitoneal injection with 10 IU pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet) followed by 10 IU human chorionic gonadotropin (hCG) (Chorulon, Intervet) 48 h intervals. For embryo collection, females were mated with males of proven fertility. Successful mating was determined the next morning (which was considered to be E0.5 of development) by the presence of a vaginal plug. Two-cell embryos were collected at 42 h following hCG injection by punching reproductive tracts under the stereomicroscope (Zeiss). Embryos were immediately placed in morpholinepropanesulfonic acid (MOPS)-buffered medium (G-MOPSTM) (Vitrolife) during collection. Embryos were cultured in potassium-supplemented simplex optimized medium (SOM) (KSOM) media containing 4 mg/ml bovine serum albumin (BSA) (Sigma A8412) as 50 μl volumes of culture drops in 35 mm culture dish (Corning 430165) that overlaid by approximately 3 ml of heavy paraffin oil (Sigma M5310). Resveratrol (Sigma R5010) was dissolved in 0.3% dimethyl sulfoxide (DMSO) to establish resveratrol treatment groups. Two-cell embryos (0 h) were cultured to blastocyst stage (48 h) at 37 °C in 5% CO_2. For each group, at least 100 embryos were evaluated and all experiments were performed at least in three replicates. Our experimental protocol is summarized in Fig. 1.

FoxO1, FoxO3, and FoxO4 genes silencing in embryos by siRNA

Two-cell embryos were transfected with FoxO1, FoxO3, and FoxO4-spesific small interfering RNA (siRNA) duplexes vs non-targeting control siRNA duplexes at 100 mM for 48 h using DharmaFECT (Dharmacon, T-2001-03) as a transfection reagent. siRNA duplexes (Life Sciences) that specifically targeted FoxO1, FoxO3, and FoxO4 are given in Supplement Figure 1. Non-targeting siRNA duplex (Silencer Select Negative Control 1 siRNA; Ambion) served as the negative control. Forty nanomolars of siRNA duplexes were used. Embryos were collected for RNA extraction for RT-PCR and qRT-PCR analysis, and immunofluorescence analysis was performed after 48 h culture. Embryos were co-transfected with 1 nM siGLO red transfection indicator (Dharmacon) to track successful transfection [12, 42]. The siGLO red transfection

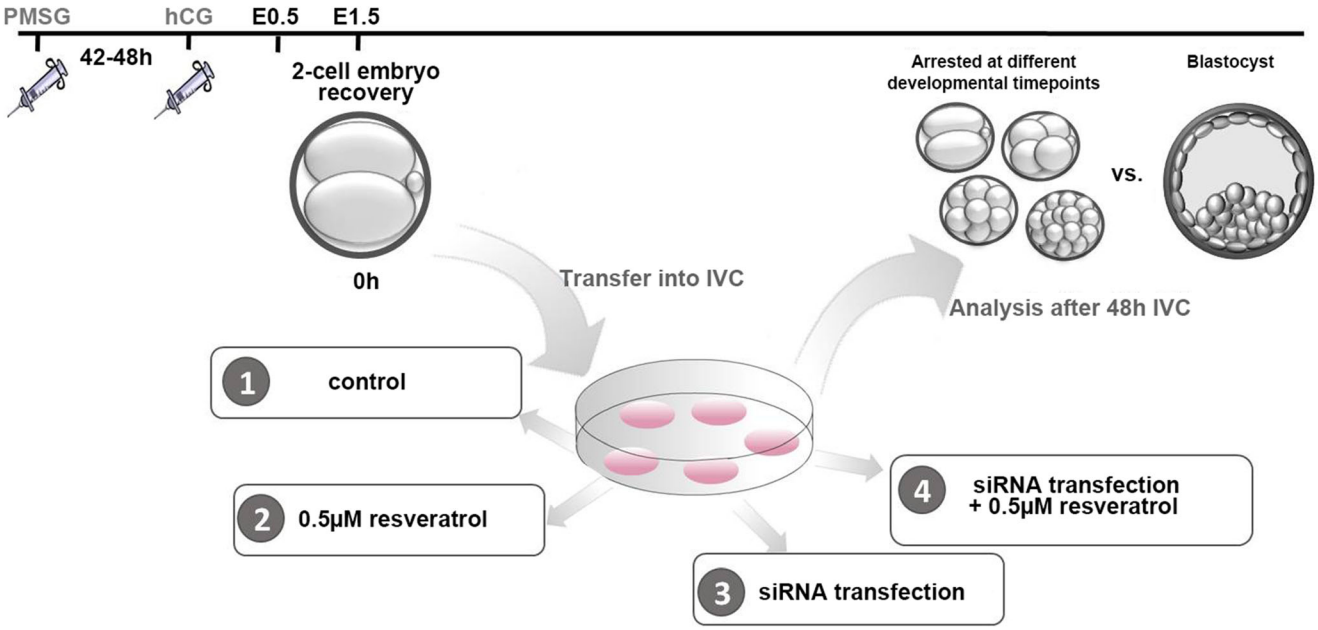

Fig. 1 A schematic diagram summarizing our experimental setup. Female mice were superovulated with an intraperitoneal (ip) injection of 10 IU/animal equine PMSG and ip injection of 10 IU/animal hCG 48-h intervals. Two-cell embryos were collected at 42 h following hCG injection and were cultured in KSOM media contained 4 mg/ml BSA. Four main groups were established: control group, resveratrol-treated group, siRNA transfection group, and resveratrol-treated siRNA transfection group. Two-cell embryos (0 h) were cultured to blastocyst stage (48 h), and analyses were performed at the end of the culture

indicator (absorbance/emission max is 557/570 nm, Dharmacon) is used in experiments to determine optimal siRNA transfection conditions for tracking individually transfected cells and for monitoring relative efficiency of delivery when co- transfected with siRNA.

Cell counting and TUNEL assay

At the end of 48 h culture, embryos were collected to perform terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for determining dose for resveratrol. TUNEL assay was performed by in Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Briefly, embryos were fixed with 4% paraformaldehyde (Sigma) solution in phosphate-buffered saline (PBS) for 20 min at room temperature and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution for 15 min at 4 °C. Embryos were washed three times in PBS. Fixed embryos were incubated with TUNEL reaction mixture, and for negative control, embryos were incubated with TUNEL label solution (Roche) only for 1 h at 37 °C in the dark. After the reaction was stopped, embryos were washed and transferred to 1 mg/mL DAPI solution (Sigma) for 3 min at room temperature. Embryos were mounted onto glass slides in 10 μl of a glycerol:PBS (1:1) mixture and analyzed. Total number of nuclei and number of TUNEL-labeled nuclei were determined under fluorescence microscope (Olympus BX61) for each embryo. Ratio of TUNEL-positive cells to total number of cells was defined as TUNEL-stained nuclei. TUNEL staining for each group was performed at least three times.

Evaluation of developmental competence of mouse embryos

To determine the developmental competence of embryos, control or FoxO knockdown embryos were monitored for specific stages of development using an inverted phase-contrasted microscope (Olympus IX71) and harvested for follow-up detecting assays as planned.

RT-PCR and qRT-PCR analyses

Total cellular RNA extraction was performed by RNA queous-Micro kit (Ambion), and complementary DNA (cDNA) reaction was carried out using the RETROscript kit (Ambion) according to the manufacturer's instructions. Specific primer pairs used for RT-PCR are presented in Supplement Figure 2. For RT-PCR, all PCR products were separated on 1.5% agarose/TBE gels and visualized by ethidium bromide. qRT-PCR reaction was set up in a volume of 25 μl containing 12.5 μl of 2X SYBR green supermix (Qiagen) and was performed on a Rotor-Gene (Corbett Research). Relative gene expressions of *FoxO1*, *FoxO3*, and *FoxO4* transcripts were calculated by using $2^{-\Delta\Delta Ct}$ (cycle threshold) method and reported as fold changes in embryos. Specificity of qRT-PCR was confirmed by melting curve analysis for each reaction.

Immunofluorescence staining

Immunofluorescence staining was performed in order to determine the presence and localization of FOXO1, FOXO3, FOXO4, SIRT1, p53, p21, FASL, and C-CASPASE 3 proteins in blastocysts and embryos with developmental delay and also in in vivo collected 2-cell embryos. All embryos were fixed in 3% paraformaldehyde (Sigma) for 20 min and permeabilized with 0.25% Tween-20 (Sigma) for 15 min at room temperature. Fixed embryos were then washed three times in PBS-BSA for 10 min each and incubated overnight at 4 °C with rabbit FOXO1 (Cell Signaling Technology, #2880S, dilution 1:50), rabbit FOXO3 (Cell Signaling Technology, #2497S, dilution 1:50), and rabbit FOXO4 (Santa Cruz, #sc-25359, dilution 1:50), rabbit SIRTUIN1 (Cell Signaling Technology, #9475S, dilution 1:100), rabbit p53 (Santa Cruz, #sc-6243, dilution 1:200), rabbit p21 (Santa Cruz, #sc-756, dilution 1:200), rabbit Fas Ligand (FASL) (Santa Cruz, #sc-834, dilution 1:200), and rabbit CLEAVED CASPASE 3 (Cell Signaling Technology, #9661S, dilution 1:200). All stainings were performed using mini well trays (VWR-Thermo Scientific) in a humidified chamber at room temperature in the dark. After washing with PBS-BSA, an anti-rabbit Alexa 488 secondary antibody was added to the trays at a concentration of 1:300 (Invitrogen, #A11008) for 60 min at room temperature. After washing with PBS-BSA, embryos were counterstained with DAPI (Sigma, D8417) at a dilution of 1:500 for 1 min at room temperature. In all the stainings, negative controls were incubated with equivalent protein concentrations of rabbit control immunoglobulin. Finally, embryos were washed three times for 10 min each in PBS-BSA and mounted in mounting solution including PBS and glycerol (1:1 v/v) using coverslips. Fluorescence signals were detected with Olympus BX61 (Zeiss) motorized fluorescence microscope at × 400 magnification. All experiments were performed at least three times, and representative images were presented. ImageJ (NIH, Bethsada, MD) software was used to quantify the extent of immunofluorescence.

ROS measurement by DCFDA fluorescence

In order to determine the quantity of cellular ROS produced by the embryos, 2,7-dichlorodihydrofluorescein diacetate (DCFDA), cellular ROS detection assay kit (Abcam, ab113851) was used according to the manufacturer's protocol. DCFDA was prepared in DMSO just before the start of each experiment and kept in the dark. Embryos washed with PBS and incubated with DCFDA at 37 °C for 30 min in the dark and then completely washed with 1× buffer. Reactive oxygen species levels produced by embryos were measured by microplate reader (Biotek) with excitation wavelength at 485 nm and emission wavelength at 530 nm. Reactive oxygen species content was calculated based on the fluorescence intensity of the reaction product of DCFDA and ROS. The extent of ROS formation was expressed in arbitrary fluorescence unit.

Statistical analysis

Experiments were performed at least three times for each group. Data were reported as mean ± SEM. Comparison of protein quantifications in control and resveratrol-treated blastocysts was performed utilizing *t* test. Group comparisons for (i) percentage of blastocyst development, (ii) ROS analysis, and (iii) protein quantification in 2-cell embryos and arrested knockdown embryos were performed by one-way ANOVA followed by Holm-Sidak post hoc test for parametric data utilizing Sigma Stat v3.0 (Jandel Scientific Corp.) software. p values < 0.05 among different groups were considered statistically significant.

Results

Silencing of FoxO transcription factors has been performed successfully with siRNA without zona removal

FoxO1, *FoxO3*, and *FoxO4* genes were knocked down successfully, and it has been proven at gene and protein level. Immunofluorescence staining showed that FOXO1, FOXO3, and FOXO4 protein expressions were present in the cytoplasm of developing blastocysts in control siRNA groups, whereas their expression levels decreased in *FoxO1*, *FoxO3*, and *FoxO4* knockdown groups (Fig. 2a). Relative mRNA levels of all *FoxO* genes decreased significantly after *FoxO1*, *FoxO3*, and *FoxO4* gene knockdowns which have been further proven by real-time qRT-PCR with an efficacy of approximately 90% when compared to their control siRNA transfected counterparts (Fig. 2b).

Our transfection and knockdown success results are as follows: when a two-cell embryo in *FoxO* siRNA transfection group can develop to the blastocyst stage, no siGLO signal was present in its blastomeres (Supplement 3). More importantly, these blastocysts expressed all FoxO proteins confirming that transfection did not occur. When a two-cell embryo in *FoxO* siRNA transfection group arrested, clear siGLO signal was present in its blastomeres present as small red dots indicating transfection success (Supplement 3). Additionally, these arrested embryos did not express (very low expression) all FoxO proteins confirming that the transfection did occur. Then, we quantified the success of transfection rate and our results are as follows: in FoxO1 knockdown group, 78% of embryos arrested and had siGLO red signal in their blastomeres while 22% of embryos developed to the

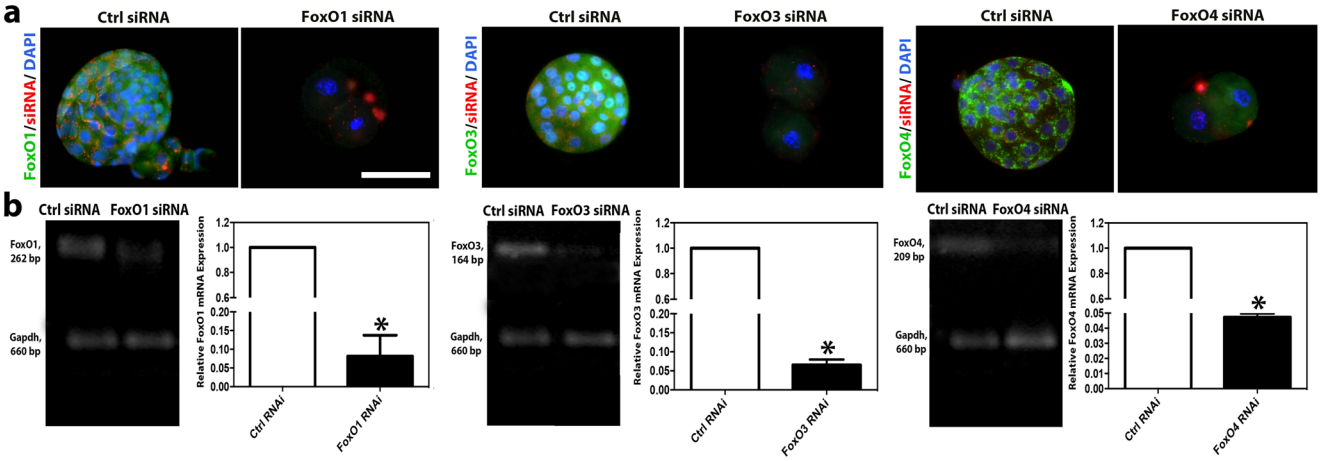

Fig. 2 The expression of FOXO1, FOXO3, and FOXO4 in mice preimplantation embryos after *FoxO1*, *FoxO3*, and *FoxO4* siRNA transfection. **a** Immunofluorescence of FOXO1, FOXO3, and FOXO4 in mice preimplantation embryos obtained 48 h after transfection. Blue signal represents DAPI staining which was used to stain the nucleus; red signal represents siGLO red transfection indicator, illustrating successful transfection; and green signals shows target protein expression. **b** RT-PCR and qRT-PCR analyses of *FoxO1*mRNA, *FoxO3*mRNA, and *FoxO4*mRNA expressions in mice embryos obtained 48 h after transfection. GAPDH served as controls. Asterisk refers to $p < 0.05$. Scale bar represents 50 μm. The same magnification was used in all images

blastocyst stage and did not have siGLO signal in their blastomeres. In FoxO3 knockdown group, 69% of embryos arrested and had siGLO red signal in their blastomeres while 31% of embryos developed to the blastocyst stage and did not have siGLO signal in their blastomeres. In FoxO4 knockdown group, 76% of embryos arrested and had siGLO red signal in their blastomeres while 24% of embryos developed to the blastocyst stage and did not have siGLO signal in their blastomeres. In summary, the transfection success ratio was 78%, 69%, and 76% for FoxO1, FoxO3, and FoxO4 siRNA knockdown, respectively. Importantly, if embryos had siGLO signal in their blastomeres, their development arrested which indicates that when transfection was successful, embryo developmental arrest was 100%.

Knockdown of FoxO1, FoxO3, and FoxO4 genes impairs preimplantation embryo development and increases ROS levels

To further check whether *FoxO1*, *FoxO3*, and *FoxO4* play important roles in preimplantation embryo development, their development was evaluated at different time points (Fig. 3a). Blastocyst development percentage of *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos decreased significantly when compared to their control siRNA counterparts, indicating that silencing of *FoxO* transcription factors impairs embryo development ($p < 0.001$) (Fig. 3b). Blastocyst development percentage in *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos was determined as follows: 22%, 31%, and 24%, respectively (Fig. 3b).

In order to determine the distribution of developmental stage of *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos, we evaluated the number of embryos at each developmental stage. Strikingly, more than 50% of all FoxO silenced embryos arrested at 2-cell at the end of 48 h culture. Percentage of embryos arrested at 2-cell stage was 59%, 52%, and 62% in *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively (Fig. 3c). Percentage of embryos arrested at 4-cell stage was 10%, 7%, and 8% in *FoxO1*, *FoxO3*, and *FoxO4* embryos, respectively (Fig. 3c). Percentage of embryos arrested at morula stage was 9%, 10%, and 6% in *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively (Fig. 3c). Taken together, silencing of *FoxO* genes lead to impairment of mouse preimplantation embryo development by causing mostly 2-cell stage arrest.

Moreover, in order to assess the reactive oxygen species (ROS) levels in *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos, ROS analysis was performed. Reactive oxygen species levels increased significantly in all *FoxO* knockdown embryos when compared to control siRNA treated embryos ($p < 0.001$) (Fig. 3d).

Embryo development arrest after knockdown of FoxO1, FoxO3, and FoxO4 genes cannot be prevented by resveratrol treatment

Since we showed that knockdown of *FoxO*s leads to 2-cell stage embryo arrest and oxidative stress increases in these embryos, we then determined whether resveratrol treatment could prevent embryo developmental impairment. Dose of resveratrol used in the present study was chosen based on previously published data [4, 16]. In order to determine appropriate dose of resveratrol, we performed TUNEL analysis in blastocyst (Supplement Figure 4). Two-cell stage embryos treated with resveratrol at concentrations of 0.5 μM, 1 μM, or 2 μM were evaluated after 48 h culture by counting number of

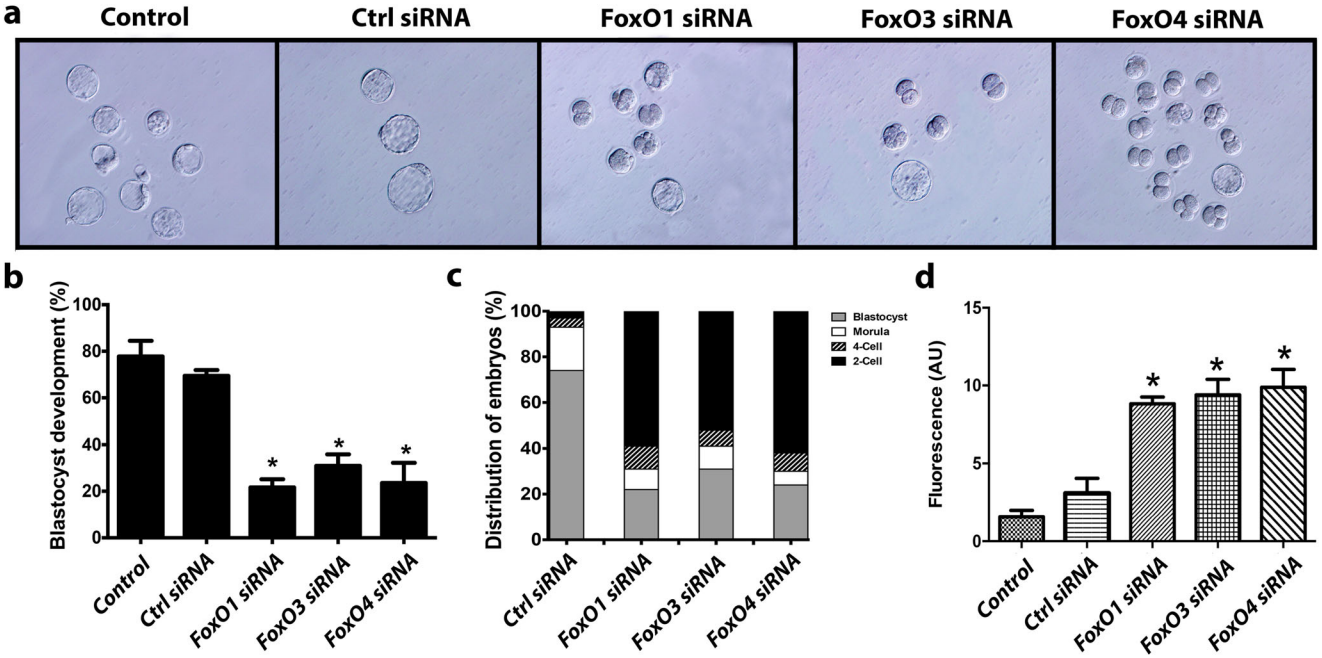

Fig. 3 The development of mice preimplantation embryos and ROS levels after *FoxO1* siRNA, *FoxO3* siRNA, and *FoxO4* siRNA transfections. Control refers non-treated embryos and control (Ctrl) siRNA refers to the embryos injected with the control-siRNA corresponding to *FoxO*s. **a** Representative pictures showing different developmental stages of the embryos. **b** Percentage of blastocyst development potential. **c** Percentage of embryo distribution in preimplantation distinct developmental stages. **d** Reactive oxygen species levels of embryos. Asterisk refers to $p < 0.05$

total cells and determining TUNEL-positive cell ratio in embryos. Highest total cell number and lowest TUNEL-positive cell percentage were present in 0.5 μM resveratrol; therefore, this dose was selected as the appropriate concentration (Supplement Fig. 4).

To check whether resveratrol can prevent embryo development arrest *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos, their development was evaluated at different time points (Fig. 4a). Blastocyst development percentage of resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos was similar to resveratrol non-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos (Fig. 4b). Blastocyst development percentage in resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos was determined as follows: 22%, 32%, and 24%, respectively (Fig. 4b).

In order to determine the distribution of developmental stage of resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos, we evaluated the number of embryos at each developmental stage. More than 45% of all resveratrol-treated *FoxO*-silenced embryos arrested at 2-cell stage at the end of 48 h culture. Percentage of embryos arrested at 2-cell stage was 46%, 41%, and 52% in resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively (Fig. 4c). Percentage of embryos arrested at 4-cell stage was 16%, 11%, and 14% in resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively (Fig. 4c). Percentage of embryos arrested at morula stage was 15%, 16%, and 9% in resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively (Fig. 4c). Taken together, resveratrol treatment does not prevent embryo development impairment in *FoxO* knockdown embryos.

Resveratrol treatment increases SIRT1 expression and regulates FOXO1, FOXO3, and FOXO4 expressions

In order to evaluate the effect of resveratrol on expressions of SIRT1 and FOXOs in blastocysts and arrested embryos, resveratrol, a sirtuin activator, treatment was performed for 48 h during embryo culture. Resveratrol increased nuclear expression of SIRT1 in normal developed blastocysts confirming that SIRT1 is activated by resveratrol (Fig. 5), whereas in arrested embryos, SIRT1 expression was similar in resveratrol treated group when compared to control group (Fig. 5). Resveratrol increased both cytoplasmic and nuclear expression of FOXO1 and FOXO3 in normal developed blastocysts, indicating that FOXO1 and FOXO3 are regulated by resveratrol (Fig. 5). FOXO4 expression did not change by resveratrol treatment in normal developed blastocysts (Fig. 5). In arrested embryos, expression of all FOXOs was translocated to nucleus and this nuclear localization of FOXOs was not present in resveratrol treated arrested embryos, indicating that resveratrol cannot activate nuclear FOXO expression in arrested embryos (Fig. 5).

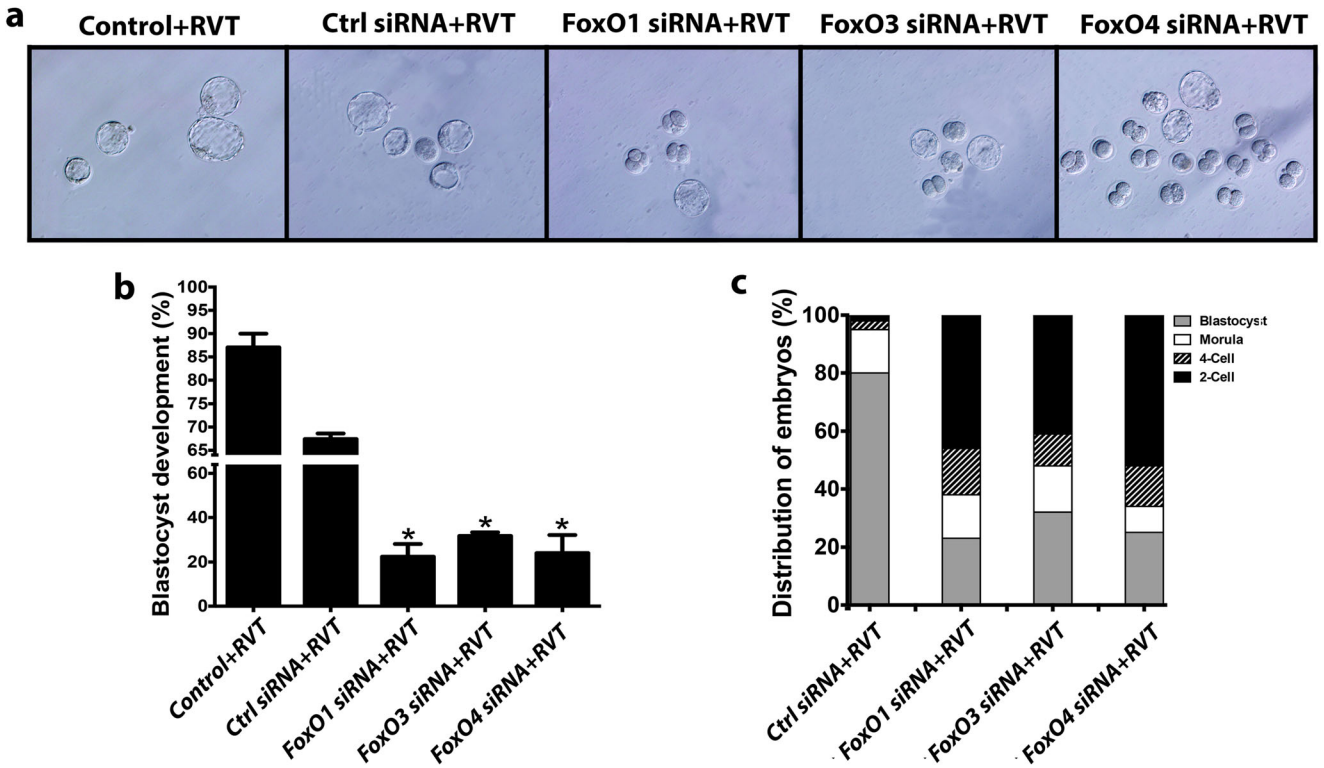

Fig. 4 The development of mice preimplantation embryos after *FoxO1* siRNA, *FoxO3* siRNA, and *FoxO4* siRNA transfections with resveratrol treatment. Control refers non-treated embryos and control (Ctrl) siRNA refers to the embryos injected with the control-siRNA corresponding to *FoxO*s. **a** Representative pictures showing different developmental stages of the embryos. **b** Percentage of blastocyst development potential. **c** Percentage of embryo distribution in preimplantation distinct developmental stages. Asterisk refers to $p < 0.05$

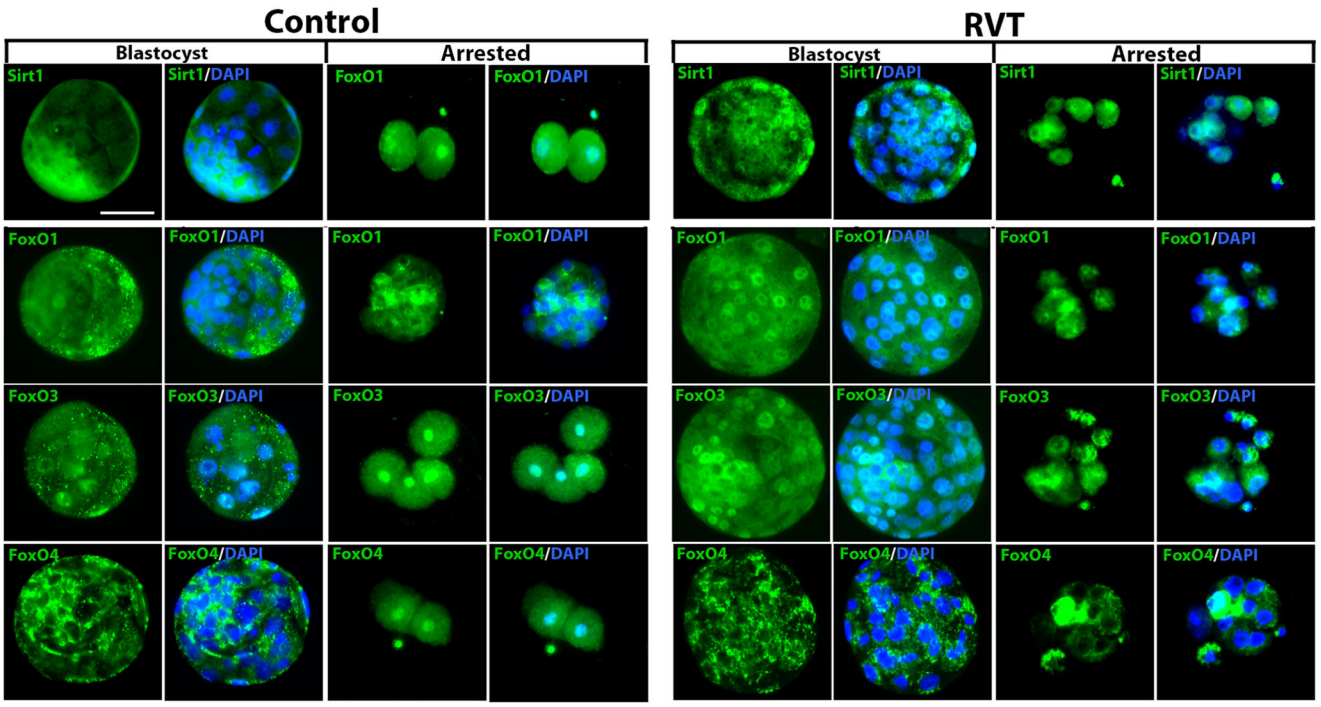

Fig. 5 SIRT1, FOXO1, FOXO3, and FOXO4 expressions after resveratrol (RVT) treatment. Green signal indicates immunopositive staining for the respective primary antibody, and blue signal (DAPI) indicates cell nuclei. Scale bar represents 50 μm. The same magnification was used in all images

Resveratrol treatment decreases p53, p21, and FASL expressions and has no effect on CLEAVED CASPASE 3

In order to evaluate the effect of resveratrol on expressions of p53, p21, FASL, and CLEAVED CASPASE 3 in blastocysts and arrested embryos, resveratrol treatment was performed for 48 h during embryo culture. Resveratrol decreased expression of p53, p21, and FASL in normal developed blastocysts, indicating that resveratrol may prevent embryos from cell cycle arrest and apoptosis (Fig. 6a). Expression of CLEAVED CASPASE 3 did not change with resveratrol treatment (Fig. 6a). In arrested embryos, expressions of p53, p21, FASL, and CLEAVED CASPASE 3 were not different after resveratrol treatment when compared to control group (Fig. 6a). Comparison of the expression levels of p53, p21, FASL, and CLEAVED CASPASE 3 between control and resveratrol treated blastocysts indicated that resveratrol treatment significantly decreases p53 ($p = < 0.001$), p21 ($p = < 0.001$), and FASL ($p = < 0.001$) expressions while it has no effect on CLEAVED CASPASE 3 ($p = 0.088$) expression (Fig. 6b). Taken together, our results suggest that resveratrol could protect embryos from cell cycle arrest and apoptosis.

Knockdown of FoxO1, FoxO3, and FoxO4 upregulates FASL, CLEAVED CASPASE 3, p53, and p21 expressions in preimplantation embryos

In order to determine whether *FoxO*s regulate cell cycle arrest and apoptosis during preimplantation embryo development, we next investigated the effect of *FoxO1*, *FoxO3*, and *FoxO4* knockdown on protein expressions of SIRT1, p53, p21, FASL, and CLEAVED CASPASE-3 in arrested embryos after 48 h culture when compared to in vivo 2-cell embryos (Fig. 7). Cytoplasmic expression of SIRT1 in in vivo 2-cell embryos decreased in *FoxO1* knockdown embryos, indicating that FOXO1 may regulate SIRT1 expression (Fig. 7a). Moreover, cytoplasmic expression of SIRT1 in in vivo 2-cell embryos was nuclear in *FoxO3* and *FoxO4* knockdown embryos, indicating that FOXO3 and FOXO4 may also regulate SIRT1 expression (Fig. 7a). Expression of p53 and p21 increased in all *FoxO* knockdown embryos when compared to in vivo 2-cell embryos, indicating that expression of p53 and p21 is regulated by FoxOs (Fig. 7a, b). Expression of FASL and CLEAVED CASPASE 3 increased in all *FoxO* knockdown embryos when compared to in vivo 2-cell embryos, indicating that their expressions are regulated by FOXOs (Fig. 7a, b). Comparison of the expression levels of p53, p21, FASL, and CLEAVED CASPASE 3 between in vivo 2-cell embryos and *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos showed that knockdown of *FoxO1*, *FoxO3*, and *FoxO4* significantly upregulates p53 ($p = < 0.001$), p21 ($p = < 0.001$), FASL ($p = < 0.001$), and CLEAVED CASPASE 3 ($p = < 0.001$) expressions in preimplantation embryos (Fig. 7b). Taken together, our results suggest that FOXO proteins regulate cell cycle and apoptosis in mouse preimplantation embryos. Two-cell embryos transfected with control-siRNA developed to blastocyst stage and express SIRT1, p53, p21, FASL, and CLEAVED CASPASE-3 (Fig. 7).

Discussion

FoxO transcription factors have been identified as important regulators of many crucial cellular process including oxidative stress resistance, cell cycle arrest, and apoptosis [10, 15, 21]. FOXO1, FOXO3, and FOXO4 proteins are differentially expressed during in vivo preimplantation embryo development in mice, and they translocate to nucleus in embryos with developmental delay, indicating that FoxOs may regulate in vitro embryo development. Although a distinct relationship between FoxOs and their cellular functions has been identified in many cells and tissues, their functional significance is still

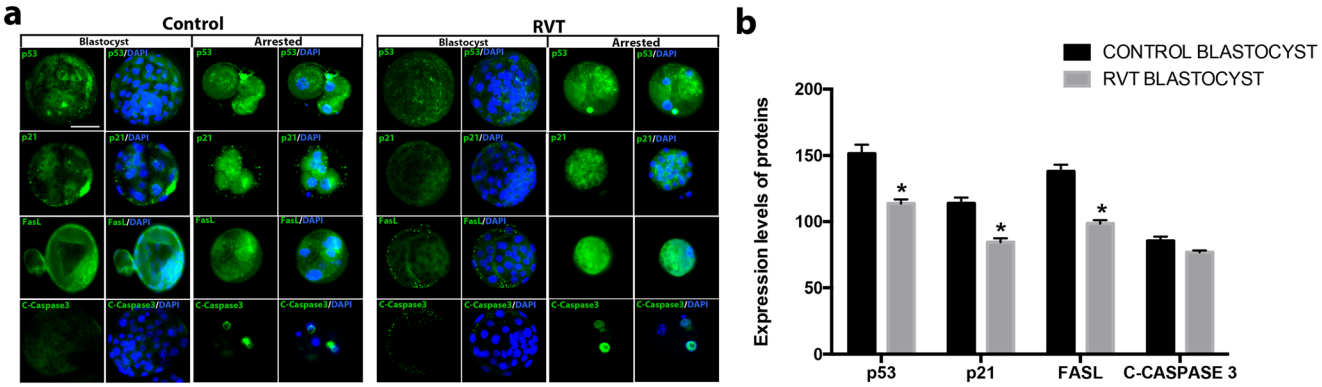

Fig. 6 p53, p21, FASL, and CLEAVED CASPASE 3 expressions after resveratrol (RVT) treatment. **a** Representative pictures showing protein expressions in blastocysts and arrested embryos for control and resveratrol treated groups. Green signal indicates immunopositive staining for the respective primary antibody, and blue signal (DAPI) indicates cell nuclei. Scale bar represents 50 μm. The same magnification was used in all images. **b** Quantification of the expression levels of p53, p21, FASL, and CLEAVED CASPASE 3 proteins in control and resveratrol treated blastocysts. Asterisk refers to $p < 0.05$ when compared to control for each protein

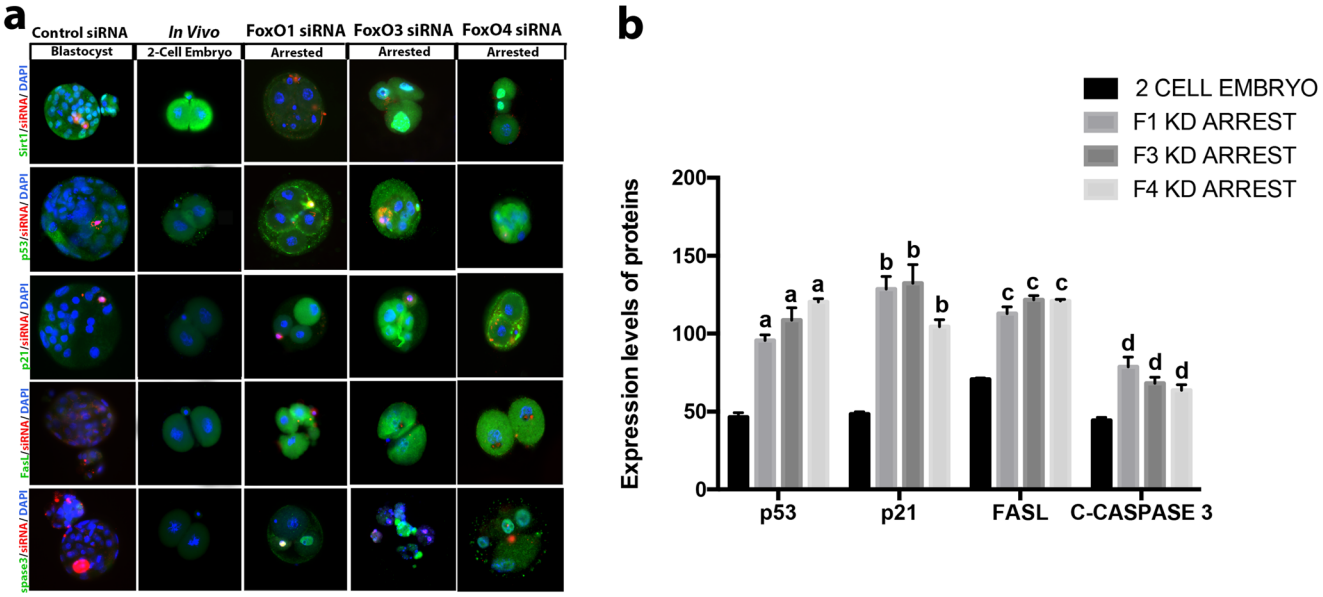

Fig. 7 SIRT1, p53, p21, FASL, and CLEAVED CASPASE 3 expressions after *FoxO1* siRNA, *FoxO3* siRNA, and *FoxO4* siRNA transfections. **a** Representative pictures showing protein expressions in blastocysts and arrested embryos for control and resveratrol treated groups. Control (Ctrl) siRNA refers to the embryos transfected with the control-siRNA corresponding to *FoxO*s. Green signal indicates immunopositive staining for the respective primary antibody, blue signal (DAPI) indicates cell nuclei, and red signal represents siGLO red transfection indicator, illustrating successful transfection. Scale bar represents 50 μm. The same magnification was used in all images represented here. **b** Quantification of the expression levels of p53, p21, FASL, and CLEAVED CASPASE 3 proteins in control and resveratrol treated blastocysts. a, b, c, and d refer to $p < 0.05$ when compared to 2-cell embryo for p53, p21, FASL, and CLEAVED CASPASE 3 proteins, respectively

not completely understood during preimplantation embryo development [19]. Based on this knowledge, in the present study, we evaluated the functional significance of FoxO transcription factors in preimplantation embryo development by knocking down FoxO1, FoxO3, and FoxO4 genes and also assessed cell cycle arrest and apoptosis-related proteins.

For this purpose, we first confirmed successful knockdown of *FoxO* genes with siRNA without removing their zona pellucida. Two-cell stage mouse embryos were co-transfected with siGLO, a red transfection indicator, to track successful transfection. To perform knockdown studies with preimplantation embryos, either their zona pellucida should be removed or direct siRNA injection could be done to transfer silencing mRNA to the cells which are both effective but also invasive interventions [5, 17, 33]. A study compared gene silencing effectiveness in bovine zygotes when siRNAs were introduced into bovine zygotes by microinjection or lipid-based transfection with zona removal and found that there was no significant difference between the two siRNA delivery techniques on mRNA gene silencing efficiency [33]. It is of interest to note that our lipid-based transfection RNA silencing non-invasive novel method without zona removal was 90% successful and thus can be used for knockdown experiments in preimplantation embryos in further studies.

To explore the roles of FoxO genes in preimplantation embryo development, we performed knockdown of *FoxO1*, *FoxO3*, and *FoxO4*. Our results showed that knockdown of these *FoxO* genes impaired preimplantation embryo development where blastocyst development percentage in *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos was 22%, 31%, and 24%, respectively. Our study provides the first evidence that *FoxO1*, *FoxO3*, and *FoxO4* are required to complete embryo development successfully. More than 50% of all FoxO silenced embryos arrested at 2-cell stage at the end of 48 h culture. Percentage of embryos arrested at 2-cell stage was 59%, 52%, and 62% in *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos, respectively. Taken together, silencing of *FoxO* genes leads to impairment of mouse preimplantation embryo development by causing mostly 2-cell stage arrest. Since two- to four-cell embryos characterized by permanent arrest show high intracellular ROS levels [2] and FOXO-mediated defense against oxidative stress by detoxification of ROS by catalase and MnSOD is a well-known mechanism [26, 30], we assessed ROS levels in *FoxO1*, *FoxO3*, and *FoxO4* knockdown embryos. ROS levels increased significantly in all *FoxO* knockdown embryos which indicate FOXO-mediated defense against oxidative stress is also present in preimplantation embryos and could lead to early embryo arrest that needs further evaluation. Our result correlates well with previous studies which showed that conditional deletion of *FoxO1*, *FoxO3*, and *FoxO4* could lead to an increase in ROS levels in mouse hematopoietic stem cells [43] and gene silencing of FOXO3A protein resulted in generation of ROS in human dermal fibroblasts [20]. As SIRT1 plays a role in cell survival and

decreases ROS levels and resveratrol is a SIRT1 activator, we next evaluated whether resveratrol could prevent embryo development arrest caused by knockdown of *FoxO1*, *FoxO3*, and *FoxO4*. Blastocyst development percentage in resveratrol-treated *FoxO1*, *FoxO3*, and *FoxO4* silenced embryos was 22%, 32%, and 24%, respectively, indicating that embryo development impairment due to *FoxO* knockdown cannot be prevented by resveratrol treatment. Altogether, our findings suggest that FOXO signaling may be one of the underlying mechanisms of early embryo arrest caused by increased ROS levels.

Since in our study, majority of embryos arrested at 2-cell stage after knockdown of *FoxO*s, *FoxO* transcription factors might be also important for mouse embryo zygotic genome activation (ZGA) which occurs at 2-cell stage and maternal transcription factors play an important role [23]. It has already been demonstrated that *FoxO1*, *FoxO3*, and *FoxO4* transcription factors are differentially expressed in prophase I, metaphase I, and metaphase II oocytes [19]. It is important to note that microRNAs (miRNAs) are already known to be required for direct clearance of maternal RNAs and inhibition of maternal transcription factor activity thus for zygotic genome activation [23]. It has been shown that *FoxO* transcription factor activity is regulated by various miRNA's, and also, there are also some evidence that miRNA clusters can be regulated by *FoxO* transcription factors [6, 28, 41, 47]. Due to this reciprocal regulation of *FoxO*s and miRNAs, it is possible that knockdown of *FoxO*'s may cause embryo development impairment via miRNA dysregulation that may disrupt ZGA. Future studies should be performed in order to verify whether this relationship exists.

Sirtuins regulate *FoxO* transcription factors, and resveratrol is a known Sirt1 activator [40, 53]. SIRT1 expression has been shown in mouse eggs and early embryos, and after the first cleavage, its expression was downregulated [17]. In order to evaluate the relationship between SIRT1 activity and *FoxO* transcription factors, we evaluated the effect of resveratrol treatment on expression of SIRT1 and FOXOs in blastocyst and arrested embryos. We found that resveratrol increased nuclear expression of SIRT1 in normal developed blastocysts confirming that SIRT1 is activated by resveratrol in mouse embryos. Resveratrol also increased both cytoplasmic and nuclear expression of FOXO1 and FOXO3 in normal developed blastocysts, indicating that FOXO1 and FOXO3 are regulated by resveratrol. Altogether, our results suggest that *FoxO* transcription factor activity in mouse blastocysts is regulated probably in a manner dependent on SIRT1 expression which is activated by resveratrol treatment.

FoxO transcription factors control cell proliferation and survival by regulating the expression of genes involved in cell cycle progression and apoptosis including Fas ligand [10]. Moreover, p53 induces DNA damage repair, reversible or irreversible cell cycle arrest in G1 phase, induction of apoptosis if the DNA damage is irreversible, and inhibition of mitogenic metabolic pathways through activation of PTEN and FOXOs [50]. p53-dependent cell cycle arrest induces p21 expression which is responsible for cell cycle arrest in response to a variety of stress stimuli [39]. In order to evaluate the effect of resveratrol on expressions of p53, p21, FASL, and CLEAVED CASPASE 3 in blastocysts and arrested embryos, resveratrol treatment was performed. Resveratrol decreased expression of p53, p21, and FASL in normal developed blastocysts, indicating that resveratrol may prevent embryos from cell cycle arrest and apoptosis. Expression of CLEAVED CASPASE 3 did not change with resveratrol treatment. Taken together, our results suggest that resveratrol could protect embryos from p53 and p21 related cell cycle arrest and FASL-related apoptosis.

Furthermore, we searched whether *FoxO*s regulate cell cycle arrest and apoptosis during preimplantation embryo development; we evaluated protein expressions of SIRT1, p53, p21, FASL, and CLEAVED CASPASE-3 in in *FoxO1*, *FoxO3*, and *FoxO4* knockdown models. Interestingly, cytoplasmic expression of SIRT1 in in vivo 2-cell embryos decreased in *FoxO1* knockdown embryos, whereas its cytoplasmic expression is translocated to nucleus in *FoxO3 and FoxO4* knockdown embryos. Altogether, these results suggest that FOXOs may also regulate SIRT1 expression in a reciprocal manner. There are few new data regarding this possible reciprocal regulation in the literature. It is already known that SIRT1 has been shown to interact physically with FOXO1 to regulate its transcriptional activity through SIRT1-mediated deacetylation [1, 7, 11, 37]. Moreover, an autofeedback loop regulation has been shown where *FoxO1* depletion by siRNA decreases SIRT1 expression in human embryonic kidney 293 cells and vascular smooth muscle cells [51]. Although there is limited knowledge in the literature regarding the regulation of SIRT1 by FOXOs, our results for the first time suggest that there might be an autofeedback mechanism where *FoxOs* regulate SIRT1 protein expression in mouse embryos. On the other hand, expression of p53 and p21 increased in all *FoxO* knockdown embryos when compared to in vivo 2-cell embryos, indicating that cell cycle arrest is regulated by *FoxO*s. When compared to in vivo 2-cell embryos, expression of FASL and CLEAVED CASPASE 3 increased in all *FoxO* knockdown embryos, indicating that apoptosis is regulated by *FoxO*s. Taken together, our results suggest that *FoxO1*, *FoxO3*, and *FoxO4* regulate cell cycle arrest and apoptosis in mouse preimplantation embryos. Our results are summarized in schematic drawing in Fig. 8.

In conclusion, the work presented here demonstrates that *FoxO* transcription factors are crucial for completion of successful mouse preimplantation development. We used a non-invasive lipid-based siRNA transfection method which was efficient for mRNA downregulation and can be used for future embryo knockdown studies. Knockdown of *FoxO1*, *FoxO3*, and *FoxO4* genes causes mostly 2-cell stage arrest and

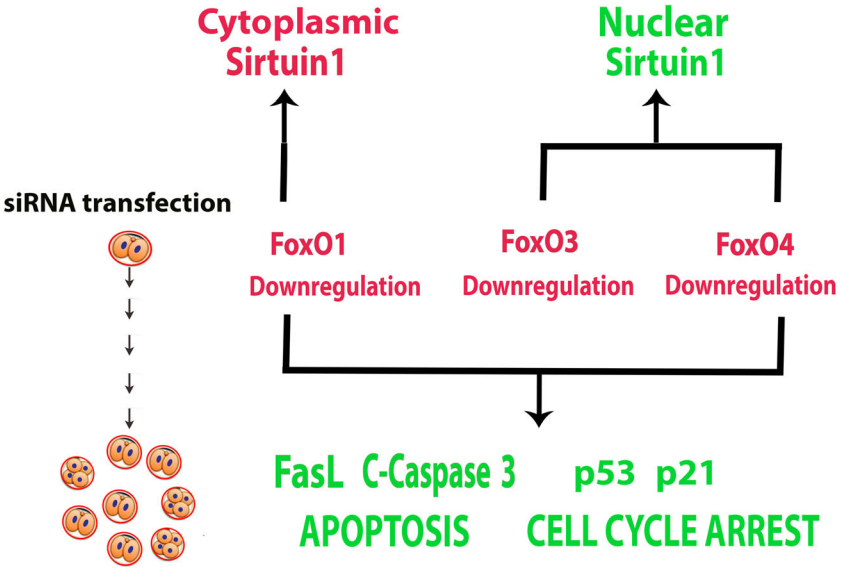

Fig. 8 A schematic diagram summarizing the possible molecular pathway regulated by *FoxO* transcription factors in arrested (*FoxO1*, *FoxO3*, and *FoxO4 knock down*) mouse preimplantation embryo development. Knockdown of *FoxO* transcription factors leads to upregulation of apoptosis (FASL and CLEAVED CASPASE 3) and cell cycle arrest (p53 and p21) in arrested preimplantation embryos. Moreover, there seems to be an autofeedback mechanism where FOXOs regulate SIRT1 protein expression. *FoxO1* downregulation leads to decreased cytoplasmic expression of SIRT1 while *FoxO3* and *FoxO4* downregulations lead to increased nuclear expression of SIRT1. Green color represents increased expression, and red color represents decreased expression

increases ROS levels in these embryos; however, embryonic arrest cannot be prevented by resveratrol treatment. On the other hand, resveratrol treatment increases SIRT1 expression and regulates FOXO1, FOXO3, and FOXO4 expression while it decreases p53, p21, and FASL expressions and has no effect on CLEAVED CASPASE 3 expression. Moreover, knockdown of *FoxO1*, *FoxO3*, and *FoxO4* upregulates p21, p53, FASL, and CLEAVED CASPASE 3 expressions in mouse preimplantation embryos. Our results suggest new insights for literature regarding possible functions of FOXO proteins regarding cell cycle arrest and apoptosis in mouse preimplantation embryo development. On the other hand, it is known that FoxOs are pleiotropic genes and their knockdown could affect a multitude of cellular pathways; therefore, further studies such as gene sequencing after knockdown of these genes are needed to clarify which pathways are affected and thus necessary for preimplantation embryo development. Dynamic changes occur in the transcriptional architecture during very complex preimplantation embryo development process; therefore, it may be more informative to perform comprehensive transcriptome dynamics analysis in the embryos using single-cell RNA sequencing (scRNA-seq) technique which provides novel opportunity to study gene regulation in embryos under high resolution [52, 54]. Because single-cell high-throughput sequencing has been widely used in investigations of transcriptional regulation during embryonic development, the underlying mechanism of early embryo development arrest caused by gene silencing of FoxOs can help to reveal the transcriptional regulation related to FoxO genes in preimplantation embryos by analyzing the data from scRNA-seq in the further studies. Although this point could be a limitation for the present manuscript, we believe that our results are fundamental for the possible roles of FoXO transcription factors in mouse preimplantation embryo development and will lead to perform further studies related to the topic. Moreover, our results point out that it still remains to investigate whether *FoxO* transcription factors have also crucial roles in human preimplantation embryo development and infertility.

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Compliance with ethical standards

The experimental protocol was approved by the local ethical committee (number of the ethical approval 2014.04.04)

Conflict of interest The authors declare that they have no conflict of interest.

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