Effects of *NRF1* on steroidogenesis and apoptosis in goat luteinized granulosa cells

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Abstract

During goat follicular development, abnormal expression of nuclear respiratory factor 1 (*NRF1*) in granulosa cells may drive follicular atresia with unknown regulatory mechanisms. In this study, we investigated the effects of *NRF1* on steroidogenesis and cell apoptosis by overexpressing or silencing it in goat luteinized granulosa cells (LGCs). Results showed that knockdown of *NRF1* expression significantly inhibited the expression of *STAR* and *CYP19A1*, which are involved in sex steroid hormones synthesis, and led to lower estrogen levels. Knockdown of *NRF1* resulted in an increased percentage of apoptosis, probably due to the release of cytochrome c from mitochondria, accompanied by upregulating mRNA and protein levels of apoptosis-related markers BAX, caspase 3 and caspase 9. These data indicate that *NRF1* might be related with steroidogenesis and cell apoptosis. Furthermore, *NRF1* silence reduced mitochondrial transcription factor A (*TFAM*) transcription activity, mtDNA copy number and ATP level. Simultaneously, knockdown of *NRF1* suppressed the transcription and translation levels of SOD, GPx and CAT, decreased glutathione level and increased 8-OHdG level. However, the overexpression of *NRF1* in LGCs or gain of *TFAM* in *NRF1* silenced LGCs increased the expression of genes involved in mitochondrial function and biogenesis, and elevated the antioxidant stress system and steroids synthesis. Taken together, aberrant expression of *NRF1* could induce mitochondrial dysfunction and disturb the cellular redox balance, which lead to disturbance of steroid hormone synthesis, and trigger LGC apoptosis through the mitochondria-dependent pathway. These findings will be helpful for understanding the role of *NRF1* in goat ovarian follicular development and atresia.

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Introduction

In mammals, only a few follicles will ovulate, and more than 99% of follicles will undergo atresia during follicular growth and development (Asselin *et al.* 2000). Follicular atresia is normally considered to be a hormonally controlled apoptotic process (Kaipia & Hsueh 1997), which was regulated by endocrine and paracrine changes. Among which granulosa cell (GC) apoptosis is considered as a main mechanism for follicular atresia (Asselin *et al.* 2000). GC apoptosis is a morphologically and biochemically distinct process induced and executed via several signaling pathways (Lin *et al.* 2012). Some factors known to regulate GC apoptosis include reproductive hormones, cytokines and apoptotic related factors (Matsuda *et al.* 2012), while the intracellular regulation of goat GC apoptosis is unclear.

Mitochondria play central roles in the initiation of apoptosis triggered by intrinsic death signals (Zamzami & Kroemer 2001). It has been reported that mitochondrial dysfunction in GCs results in follicular atresia (Glister *et al.* 2014). Nuclear respiratory factor 1

(*NRF1*), as a major transcription factor, is involved in mitochondrial biogenesis, signal transduction and protein synthesis (Zhang & Manning 2015). Downregulation of *NRF1* contributes to mitochondrial dysfunction (Wang *et al.* 2006), while overexpression of *NRF1* could protect neuronal cells from MPP⁺-induced mitochondrial dysfunction (Piao *et al.* 2012). Moreover, our previous study has shown that goat GCs have a reduced *NRF1* expression in atretic follicles compared with healthy follicles (Zhang *et al.* 2015). Despite these findings, the regulation of *NRF1* in GC apoptosis and follicular atresia remains to be elucidated, especially in goat.

GCs constitute the vast majority of follicular cells in mammalian ovary, and function as steroid synthesis during follicular development (Sanchez *et al.* 2016). Sex steroid hormones progesterone (P₄) and estradiol (E₂) participate in regulating ovarian function (Drummond 2006), and act as antioxidant protecting follicles from oxidative stress and atresia (Jain *et al.* 2013, Mahmoodi *et al.* 2015). In addition, E₂-induced DNA synthesis in MCF-7 breast cancer cells depends on

mitochondrial oxidant signaling to *NRF1* (Okoh *et al.* 2015), knockdown of *NRF1* blocks E_2 stimulation of mitochondrial biogenesis and activity (Mattingly *et al.* 2008). These studies indicate *NRF1* may be involved in the regulation of steroidogenesis, oxidative stress and cell apoptosis.

To provide insights into the role of *NRF1* in regulating goat follicular atresia, we investigated the effects of *NRF1* on steroidogenesis, mitochondrial function and cell apoptosis through overexpressing or silencing it in luteinized granulosa cells (LGCs). Gain-of-function assay was further performed by overexpression of mitochondrial transcription factor A (*TFAM*, a downstream target of *NRF1* gene (Piantadosi & Suliman 2006)) in *NRF1* knockdown goat LGCs. The results indicate that *NRF1* plays a critical role in regulating goat LGC steroidogenesis and apoptosis, which is of great significance to understand the role of *NRF1* in follicular development and atresia.

Materials and methods

Cell culture

Goat GCs were isolated from healthy follicles (2–5 mm), and cell survival rate was measured by the trypan blue exclusion test ($81.6 \pm 5.9\%$). GCs were plated on T-25 culture flasks at 5×10^4 cells/cm² with cell culture medium (DMEM/F12 with 10% fetal bovine serum, 2-mM L-glutamine, 100 IU/mL of penicillin and 100 µg/mL of streptomycin), and incubated under a humidified atmosphere containing 5% CO₂ at 37°C. Non-adherent cells were gently removed after 2 days by changing medium. Even if the cells collected were GCs, after 48 h of culture with serum, the cells probably became LGCs (Tosca *et al.* 2010). Unless otherwise indicated, all chemicals used in this study were purchased from Sigma-Aldrich Company and the media from Life Technologies. This study was approved by the Animal Ethical Committee of Nanjing Agricultural University.

Cell transfection and infection

The sequence of *NRF1*, *TFAM* and pEX-4 vector was provided in [Supplementary data 1](#) (see section on [Supplementary data](#) given at the end of this article). The efficiency of the constructed vectors used in this study was provided in [Supplementary Fig. 1](#). For *NRF1* overexpression, goat LGCs were transfected with pEX-4-*NRF1* vector (provided by GenePharma, Shanghai, China, pEX-4-*NRF1* group) or pEX-4 empty vector (pEX-4-control group) using Lipofectamine 2000 (Life Technologies). For RNA interference experiments, lentiviral supernatants were produced by co-transfection of psPAX2 and pMD2.G with shRNA-3-*NRF1* (details were provided in [Supplementary Fig. 1 and Supplementary Table 1](#), sh*NRF1* group) or scramble shRNA vector (negative control) into 293T cells. Then goat LGCs were infected with lentivirus in the presence of 8 µg/mL Polybrene for 24 h. To perform gain-of-function assay, *NRF1* silenced LGCs were transfected with pEX-4-*TFAM* vector (provided

by GenePharma, Shanghai, China, sh*NRF1* + pEX-4-*TFAM* group) using Lipofectamine 2000. *NRF1*-knockdown LGCs were transfected with pEX-4 used as control (sh*NRF1* + pEX-4-control group). The transfected and infected LGCs were cultured for another 48 h before analysis.

Steroid assay

The concentrations of P_4 and E_2 in culture medium were measured by radioimmunoassay (RIA) using commercial kits (Iodine [¹²⁵I]-Prog RIA Kit, No. RG5-04; Iodine [¹²⁵I]- E_2 RIA Kit, No. RG6-04; Jiuding Biotechniques, Tianjin, China). Before analysis, each sample was 2-, 10-, 25- and 50-fold diluted with Dulbecco's phosphate-buffered saline (DPBS) and 100 µL of the diluted fluid was analyzed. The sensitivity of the assays for P_4 and E_2 was 0.03 ng/mL and 2.1 pg/mL respectively. The intra- and inter-assay coefficients of variation for P_4 and E_2 were 7.2% and 8.9%, and 7.7% and 8.9% respectively.

Assessment of ATP levels, ATPase and CCO enzyme activity

ATP levels were measured by a bioluminescence assay employing the luciferase enzyme using the ATP Determination kit (No. A22066, Invitrogen) according to the procedures described previously (Bacsi *et al.* 2007). Standard curves for quantification were generated using known amounts of an ATP standard. The ATP content of the samples was determined by comparison with a standard curve, and total ATP levels were expressed as normalized luminance in nmol/mg protein. The protein concentration was determined by using bicinchoninic acid (BCA) Protein Assay kit (No. P0012S, Beyotime Biotechnology, Nantong, China).

ATPase activity was determined by measuring the formation rate of phosphoric acid from ATP according to the manufacturer's instruction (Na⁺-K⁺-ATPase assay kit, No. A070-2; Ca²⁺-Mg²⁺-ATPase assay kit, No. A070-3; Jiancheng, Nanjing, China). ATPase activity was calculated based on the formula of the kit instructions and expressed as µmol Pi/h/mg protein. The protein concentration was determined by using BCA method.

Cytochrome c oxidase (CCO) activity as a biomarker of mitochondrial function was determined by the Cytochrome c Oxidase Assay kit (No. CYTOCOX1, Sigma) according to the manufacturer's instruction. CCO activity was calculated by the changes in the maximal rate of oxidation of reduced cytochrome c (CYCS) in absorbance at 550 nm, and expressed as µmol/min/mg protein. The protein content was quantified by Bradford Protein Assay kit (No. P0006, Beyotime Biotechnology).

Measurement of 8-OHdG level and antioxidant enzyme activities

Cells were homogenized in lysis buffer and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was immediately collected and used for the measurement of oxidative stress parameters. The concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was measured by an 8-hydroxy-2'-deoxyguanosine ELISA kit (No. K4160-100, BioVision)

Table 1 Details of primer sequences, expected product sizes and GenBank accession numbers of genes used for qPCR.

Target gene	Primer sequence (5'-3')	Product size (bp)	Accession number
<i>NRF1</i>	F: 5'-AGGCTGGGGCAAAGAAAG-3' R: 5'-CCAACCTGGATAACGAGAC-3'	303	AY368269.1
<i>STAR</i>	F: 5'-GGTCCCGAGACTTTGTGAG-3' R: 5'-AATCCACTTGGGTCTGCGAG-3'	262	XM_013975437.1
<i>3BHSD</i>	F: 5'-AGACCAGAAAGTTCGGGAGGAA-3' R: 5'-TCTCCCTGTAGGAGTTGGGC-3'	292	NM_001285716.1
<i>CYP19A1</i>	F: 5'-CAGCATGGTGTCCGAAGTTG-3' R: 5'-GGGCCCAATTCCCAGAAAGT-3'	133	NM_001285747.1
<i>TFAM</i>	F: 5'-CCAAGCTGTGGAGGGAACTT-3' R: 5'-GCTGACCGAGGTCTTTTGG-3'	229	XM_005699371
<i>SOD2</i>	F: 5'-GTGAACAACCTCAACGTCGC-3' R: 5'-GCGTCCCTGCTCCTTATTGA-3'	300	XM_013966636
<i>CAT</i>	F: 5'-CACTCAGGTGCGGATTTCT-3' R: 5'-ATGCGGGAGCCATATTCAGG-3'	159	GQ204786.1
<i>GPX1</i>	F: 5'-ACATTGAAACCCTGCTGTCC-3' R: 5'-TCATGAGGAGCTGTGGTCTG-3'	216	XM_005695962.2
<i>BAX</i>	F: 5'-GCATCCACCAAGAAGCTGAG-3' R: 5'-CCGCCACTCGGAAAAAGAC-3'	130	XM_002701934
<i>BCL2</i>	F: 5'-ATGTGTGTGGAGAGCGTCA-3' R: 5'-AGAGACAGCCAGGAGAAATC-3'	182	NM_001166486.1
<i>CASP3</i>	F: 5'-GCTCGAGTCATGCACATTC-3' R: 5'-CCATTGGGCACTTGGCATAC-3'	197	NM_001286089.1
<i>CASP9</i>	F: 5'-AGTCAGGCCCTTCCTTTGTT-3' R: 5'-ATGGGTCTGCTTCATCACT-3'	193	XM_005690814
<i>GAPDH</i>	F: 5'-CGACTTCAACAGCGACACTCAC-3' R: 5'-CCCTGTTGCTGTAGCCGAATTC-3'	119	NM_001034034.1

3BHSD, 3 beta-hydroxysteroid dehydrogenase/isomerase; *BAX*, BCL2 associated X, apoptosis regulator; *BCL2*, BCL2, apoptosis regulator; *CASP3*, caspase 3; *CASP9*, caspase 9; *CAT*, catalase; *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *GPX1*, glutathione peroxidase 1; *NRF1*, nuclear respiratory factor 1; *STAR*, steroidogenic acute regulatory protein, mitochondrial; *SOD2*, superoxide dismutase 2; *TFAM*, transcription factor A, mitochondrial.

using a standard protocol in the manual described previously (Chiang *et al.* 2013). The concentration of 8-OHdG was calculated by comparison with predetermined 8-OHdG standard curve, and expressed as $\mu\text{mol Pi/h/mg protein}$. The protein concentration was determined by using BCA method. The sensitivity of 8-OHdG was 0.94 ng/mL, and the coefficients of variation for intra- and inter-assay were 5.5% and 6.1% respectively.

Enzyme activities of superoxide dismutase (SOD) were detected using the SOD Assay Kit (No. S311-10, Dojindo Molecular Technologies, Tokyo, Japan) as described previously (Grasselli *et al.* 2010). Catalase (CAT), glutathione peroxidase (GPx), intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by using commercially available assay kits from Beyotime

Biotechnology (Catalase Assay Kit, No. S0051; Cellular Glutathione Peroxidase Assay kit, No. S0056; GSH and GSSG Assay Kit, No. S0053; Nantong, China) by strictly following the manufacturer's instruction. The antioxidant enzyme activities were expressed as U/mg of protein, and the levels of GSH and GSSG were expressed as nmol/mg protein. The protein concentration was determined by using BCA method.

mtDNA copy number quantification

Mitochondrial DNA (mtDNA) copy number quantification was performed by quantitative real-time reverse transcription polymerase chain reaction (qPCR) as previously described (Zhang *et al.* 2016).

Table 2 Details of antibodies used for Western blot in this study.

Antibodies	Cat No.	Source	Dilution of Western blot
STAR	sc-25806	Santa Cruz	1:400
3BHSD	NB110-78644	Novus (CO, USA)	1:500
CYP19A1	ab18995	Abcam	1:1000
BAX	sc-526	Santa Cruz	1:200
BCL2	BA0412	Boster (Wuhan, China)	1:200
CYCS	ab16076	Abcam	1:1000
ACTB	AA128	Beyotime (Haimen, China)	1:200

3BHSD, 3 beta-hydroxysteroid dehydrogenase/isomerase; *ACTB*, actin beta; *BAX*, BCL2 associated X, apoptosis regulator; *BCL2*, BCL2, apoptosis regulator; *CYCS*, cytochrome c, somatic; *CYP19A1*, cytochrome P450 family 19 subfamily A member 1; *STAR*, steroidogenic acute regulatory protein.

Figure 1 *NRF1* regulates P₄ and E₂ synthesis in goat LGCs. (A and B) Concentrations of progesterone (P₄) and estradiol (E₂) in *NRF1* overexpressed or silenced LGCs culture medium were detected by RIA. (C, D and E) The mRNA levels of genes (*STAR*, *3BHSD* and *CYP19A1*) associated with steroid hormone synthesis in *NRF1* overexpressed or suppressed LGCs were analyzed by qPCR. The relative expression levels were normalized to the expression amount of *GAPDH*. (F) Protein levels of *STAR*, *3BHSD* and *CYP19A1* in *NRF1* overexpressed or silenced LGCs were immunoblotted and quantified. ACTB served as an internal control. Data are expressed as mean $\pm$ s.e.m. (Student's *t*-test, *n* = 4), **P* < 0.05.

Gene expression analysis

Total RNA was extracted using a commercial RNA isolation kit (No. 74104, Qiagen) and reverse-transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (No. RR047A, Takara) according to the manufacturer's instructions. qPCR was performed on a Step One Plus Real-Time PCR System (Applied Biosystems) using FastStart Universal SYBR Green Master kit (No. 04913850001, Roche). Primer sequences were provided in Table 1. Melting curves were analyzed to verify amplification specificity. Gene expression levels were quantified using the form of $2^{-\Delta\Delta Ct}$ and normalized to *GAPDH* mRNA expression.

Cell apoptosis analysis

Cell apoptosis was analyzed using the Annexin V-APC and 7-ADD apoptosis detection kit (No. KGA1025, KeyGEN,

Nanjing, China) according to the manufacturer's instructions. Briefly, the cells about 90% confluency were detached and resuspended in DPBS, then incubated with 5 μ L of APC-labeled Annexin V for 5 min, followed by an additional 5 μ L of 7-ADD for 15 min in darkness. The fluorescence of the cells was determined by a flow cytometer (BD Biosciences, NJ, USA).

Caspase activity

Caspase 3 (CASP3) and caspase 9 (CASP9) activities were determined by using commercially available assay kits from Beyotime Biotechnology (Caspase 3 Activity Assay Kit, No. C1116; Caspase 9 Activity Assay Kit, No. C1158) by following the manufacturer's instruction. The CASP3 and CASP9 enzyme activities

Figure 2 *NRF1* silence induces goat LGC apoptosis. The percentage of apoptotic cells was analyzed by flow-cytometry using Annexin V-APC/7-ADD staining in *NRF1* overexpressed or suppressed LGCs. Quadrants: Left, live cells; Right, apoptotic cells. Data are expressed as mean $\pm$ s.e.m. (Student's *t*-test), **P* < 0.05.

Figure 3 Effects of *NRF1* on apoptosis-related genes *BAX* and *BCL2* expression in goat LGCs. (A, B and C) Relative expression levels of *BAX* and *BCL2*, and the ratio of *BAX/BCL2* in *NRF1* overexpressed or silenced LGCs were analyzed by qPCR. The relative expression levels were normalized to the expression amount of *GAPDH*. (D) *BAX* and *BCL2* protein levels in *NRF1* overexpressed or silenced LGCs were immunoblotted and quantified. ACTB served as an internal control. Data are expressed as mean $\pm$ S.E.M. (Student's *t*-test, *n* = 4), **P* < 0.05.

were calculated with reference to a standard curve, and the data were represented as U/mg of protein. The protein content was determined with Bradford method.

Mitochondrial and cytosolic fractionation

LGC fractionation (mitochondria and cytosol) protein extracts obtained from different treatment conditions were subjected to Western blot for CYCS protein analysis. The Cell Mitochondria

Figure 4 Effects of *NRF1* on CYCS release, *CASP3* and *CASP9* expression in goat LGCs. (A) Western blotting analysis for detecting the CYCS distribution and quantification in mitochondria and cytosol of *NRF1* overexpressed or silenced LGCs. ACTB served as an internal control. (B and C) The expression of apoptosis-related genes *CASP3* and *CASP9* in *NRF1* overexpressed or silenced LGCs was analyzed by qPCR. The relative expression levels were normalized to the expression amount of *GAPDH*. Data are expressed as mean $\pm$ S.E.M. (Student's *t*-test, *n* = 4), **P* < 0.05.

Table 3 Effects of *NRF1* on caspase activity in goat LGCs.

Group	CASP3 (U/mg pro)	CASP9 (U/mg pro)
pEX-4-control	582.4 ± 49.1 ^a	273.9 ± 17.3 ^a
pEX-4-NRF1	397.1 ± 19.3 ^b	198.4 ± 11.9 ^b
Scramble	543.7 ± 27.6	295.1 ± 16.4
shNRF1	729.8 ± 34.6*	329.7 ± 13.8*

Data are presented as mean ± S.E.M. (Student's *t*-test, *n* = 4). In the same parameter, ^{a,b} values mean significant difference between pEX-4-NRF1 (*NRF1* overexpression) and pEX-4-control groups (*P* < 0.05); * values mean significant difference between shNRF1 (*NRF1* silence) and scramble groups (*P* < 0.05). CASP3, caspase 3; CASP9, caspase 9; pro, protein.

Isolation kit (No. C3601, Beyotime Biotechnology) was used to perform mitochondria and cytosol isolation following the manufacturer's protocol.

Western blot analysis

Total protein was extracted from the cells with radio immunoprecipitation (RIPA) lysis buffer (No. P0013B, Beyotime Biotechnology) and quantified by BCA method. Protein samples (30 µg) were diluted in gel-loading buffer, boiled for 10 min, separated in an 8–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% (w/v) bovine serum albumin for 1 h at room temperature (RT), followed by incubation with a primary antibody overnight at 4°C. Details of the primary antibodies were provided in Table 2. After washing, the proteins of interest were incubated with secondary antibodies (No. A0216 for CYCS protein, 1:1000 dilution; No. A0208 for other target proteins, 1:1000 dilution; Beyotime Biotechnology) at RT for 1 h. After washing, the immunoreactive bands were visualized using Image Quant LAS 4000 (Fujifilm, Tokyo, Japan) with the Western Bright ECL kit (No. K-12045-D20, Advanta, CA, USA). Band intensities were estimated by densitometry and

Figure 5 *NRF1* silence is involved in mitochondrial dysfunction in goat LGCs. (A) qPCR was used to detect mRNA level of *TFAM* in *NRF1* overexpressed or silenced LGCs. The relative expression levels were normalized to the expression amount of *GAPDH*. (B) The mtDNA copy number in *NRF1* overexpressed or silenced LGCs was determined by qPCR. The ratio of mtDNA to nuclear DNA reflects the relative mtDNA copy number. Data are expressed as mean ± S.E.M. (Student's *t*-test, *n* = 4), **P* < 0.05.

normalized to actin beta (ACTB). Bands were quantified using Image J software (Wayne Rasband, Maryland, USA).

Statistical analysis

All data were analyzed with statistical product and service solutions (SPSS, version 19.0) (IBM, Chicago, IL, USA), presented as mean values ± standard error of the mean (S.E.M.), and calculated from at least three independent experiments (*n* > 3). Student's *t*-test was performed to calculate *P* value, where *P* < 0.05 was considered a statistically significant difference.

Results

NRF1 promotes *P*₄ and *E*₂ synthesis in goat LGCs

To characterize *NRF1* in steroidogenesis of goat LGCs *in vitro*, we first examined its effect on *P*₄ and *E*₂ production. The concentrations of *P*₄ and *E*₂ were significantly increased with the overexpression of *NRF1* (*P* < 0.05, Fig. 1A and B), while the level of *E*₂ was significantly decreased by *NRF1* silencing (*P* < 0.05, Fig. 1B). The expression levels of steroidogenic acute regulatory protein (*STAR*), 3 beta-hydroxysteroid dehydrogenase/isomerase (*3HSD*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) – crucial genes of steroidogenesis – were significantly increased with the overexpression of *NRF1* (*P* < 0.05, Fig. 1C, D, E and F). Accordingly, knockdown of *NRF1* in LGCs significantly decreased the expression levels of *STAR* and *CYP19A1* (*P* < 0.05, Fig. 1C, D, E and F). These results support the assumption that *NRF1* may be critical for goat LGC steroidogenesis.

Suppression of *NRF1* induces goat LGC apoptosis

To investigate whether *NRF1* plays a role in goat LGC apoptosis, Annexin V-APC and 7-ADD staining were used to examine the effects of *NRF1* overexpression or suppression on LGC apoptosis. The results showed that *NRF1* silencing significantly increased the percentage of cell apoptosis (28.92% ± 3.16%) compared with scramble group (5.15% ± 1.82%, *P* < 0.05, Fig. 2). qPCR and Western blot assays also confirmed the enhanced expression of BCL2 associated X (*BAX*) and reduced BCL2 level in *NRF1* suppressed LGCs, which was supported by the increased ratio of *BAX/BCL2* (*P* < 0.05, Fig. 3A, B, C and D). In contrast, the overexpression of *NRF1* significantly decreased the ratio of *BAX/BCL2* in LGCs (*P* < 0.05, Fig. 3C). These data suggest reduced expression of *NRF1* might contribute to the apoptosis of goat LGCs.

Furthermore, we observed a significant release of CYCS from mitochondria to cytosol with *NRF1* suppression (*P* < 0.05, Fig. 4A). Concomitantly, the mRNA levels of *CASP3* and *CASP9* were significantly increased in *NRF1* suppressed LGCs (*P* < 0.05, Fig. 4B and C), with enhanced cleavage activity (*P* < 0.05,

Table 4 Effects of *NRF1* on ATP and CCO content, and ATPase activity in goat LGCs.

Group	ATP (nmol/mg pro)	CCO (μmol/min/mg pro)	Na ⁺ -K ⁺ -ATPase (μmol Pi/h/mg pro)	Ca ²⁺ -Mg ²⁺ -ATPase (μmol Pi/h/mg pro)
pEX-4-control	28.43 ± 3.25 ^a	2.09 ± 0.46 ^a	68.27 ± 1.83 ^a	15.41 ± 2.06 ^a
pEX-4-NRF1	37.61 ± 2.38 ^b	4.23 ± 0.28 ^b	92.4 ± 2.36 ^b	21.29 ± 1.47 ^b
Scramble	25.72 ± 1.98	2.18 ± 0.32	61.5 ± 2.43	16.03 ± 1.28
shNRF1	18.29 ± 2.64*	1.61 ± 0.25*	41.25 ± 1.84*	12.47 ± 1.82

All values are mean ± S.E.M. (Student's *t*-test, *n* = 4).

In the same parameter, ^{a,b}Values mean significant difference between pEX-4-NRF1 (*NRF1* overexpression) and pEX-4-control groups (*P* < 0.05);

*values mean significant difference between shNRF1 (*NRF1* silence) and Scramble groups (*P* < 0.05).

ATP, adenosine triphosphate; CCO, cytochrome c oxidase; pro, protein.

Table 3). However, the expression (mRNA and cleavage activity) of CASP3 and CASP9 was significantly decreased by the overexpression of *NRF1* (*P* < 0.05, Fig. 4B and C, and Table 3). These data suggest *NRF1* silencing impairs cell survival probably through mitochondria-mediated apoptosis pathway.

NRF1 regulates mitochondrial function in goat LGCs

To investigate the mechanism how *NRF1* regulates steroidogenesis and cell survival, we aim to identify the responsible downstream targets of *NRF1* in goat LGCs. It has been reported that *NRF1* functions as a major transcription factor that activates mitochondrial function. The effects of *NRF1* on mitochondrial biogenesis, energy metabolism and redox states were investigated. By using qPCR, we found that mitochondrial biogenesis marker *TFAM* and mtDNA copy number were significantly decreased by the knockdown of *NRF1*, while their levels were significantly increased by the overexpression of *NRF1* (*P* < 0.05, Fig. 5). In addition, the overexpression of *NRF1* in LGCs significantly increased the ATP and CCO levels, and enhanced the activities of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase (*P* < 0.05, Table 4). On the contrary, the ATP and CCO levels, and the activity of Na⁺-K⁺-ATPase were significantly reduced with *NRF1* silencing in goat LGCs (*P* < 0.05, Table 4).

The redox state assay was performed by evaluating the 8-OHdG level, the activities of antioxidant enzymes SOD, CAT and GPx, as well as the ratio of GSH/GSSG. As shown in Table 5, silencing of *NRF1* in goat LGCs significantly increased 8-OHdG level (*P* < 0.05). The mRNA levels and activities of SOD, GPx and CAT

were both significantly reduced by the silencing of *NRF1* (*P* < 0.05, Fig. 6 and Table 5). In contrast, the activities of SOD, CAT, GPx and GSH were significantly upregulated in *NRF1* overexpressed LGCs, followed by the increased ratio of GSH/GSSG (*P* < 0.05, Table 5). Accordingly, the mRNA levels of superoxide dismutase 2 (*SOD2*) and *CAT* were significantly elevated with *NRF1* overexpression (*P* < 0.05, Fig. 6). Surprisingly, GSSG levels were not significantly affected by *NRF1* overexpression or silencing (Table 5). These data indicate that *NRF1* plays an important role in mitochondrial function, suggesting *NRF1* regulates steroidogenesis and cell survival probably through the regulation of mitochondria.

NRF1 regulates steroidogenesis and cell survival by targeting *TFAM* in goat LGCs

We further identified the downstream target genes of *NRF1* in LGC regulation. It has been reported that the activation of proximal *TFAM* promoter is highly dependent on *NRF1* and decreased *TFAM* represses mitochondrial function (Choi et al. 2004). By using gain-of-function assay, the results showed that the gain of *TFAM* compensated the abnormal E₂ expression, and recovered the decreased expression of STAR, 3BHSD and CYP19A1 in *NRF1* silenced LGCs (*P* < 0.05, Fig. 7A, B and E). In addition, the overexpression of *TFAM* could significantly reduce the percentage of cell apoptosis and decrease the expression ratio of BAX to BCL2 in *NRF1* suppressed LGCs, along with the increased expression of CASP9 (*P* < 0.05, Fig. 7C, D and E, and Table 6). The disturbed mitochondrial function and the redox balance in *NRF1* knockdown LGCs could

Table 5 Effects of *NRF1* on oxidative stress in goat LGCs.

Group	8-OHdG (μmol Pi/h/mg pro)	SOD (U/mg pro)	CAT (U/mg pro)	GPx (U/mg pro)	GSH (nmol/mg pro)	GSSG (nmol/mg pro)	GSH/GSSG
pEX-4-control	1.18 ± 0.13	11.84 ± 2.47 ^a	6.49 ± 1.52 ^a	30.26 ± 5.41 ^a	17.47 ± 2.16 ^a	1.03 ± 0.32	17.36 ± 2.47 ^a
pEX-4-NRF1	0.89 ± 0.21	18.76 ± 1.91 ^b	11.52 ± 1.74 ^b	42.63 ± 3.85 ^b	23.81 ± 2.35 ^b	0.73 ± 0.29	30.58 ± 4.83 ^b
Scramble	1.21 ± 0.19	12.35 ± 2.23	5.81 ± 0.89	27.58 ± 2.92	16.15 ± 1.92	1.19 ± 0.21	13.64 ± 1.89
shNRF1	4.17 ± 0.26*	8.51 ± 1.46*	3.76 ± 1.21*	18.37 ± 3.26*	11.49 ± 1.68*	1.37 ± 0.16	9.06 ± 2.18*

Data are presented as mean ± S.E.M. (Student's *t*-test, *n* = 4).

In the same parameter, ^{a,b}Values represent significant difference between pEX-4-NRF1 (*NRF1* overexpression) and pEX-4-control groups

(*P* < 0.05); *values represent significant difference between shNRF1 (*NRF1* silence) and Scramble groups (*P* < 0.05).

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; pro, protein; SOD, superoxide dismutase.

Figure 6 *NRF1* regulates oxidative stress in goat LGCs. (A, B and C) Antioxidant related genes *SOD2*, *CAT* and *GPX1* expression levels in *NRF1* overexpressed or silenced LGCs were analyzed by qPCR. The relative expression levels were normalized to the expression amount of *GAPDH*. Data are expressed as mean $\pm$ S.E.M. (Student's *t*-test, $n=4$), $*P<0.05$.

be recovered by overexpression of *TFAM* ($P<0.05$, Fig. 8, Tables 7 and 8). These data further indicate *NRF1* regulates steroidogenesis and cell apoptosis by targeting the mitochondrial regulator *TFAM*.

Discussion

Here, we identify that *NRF1* is required for goat LGC steroidogenesis and cell survival. *NRF1* regulates goat LGC identity through mitochondria-dependent pathway, and mitochondrial transcription factor *TFAM* may participate in this regulation process. These findings

underscore the importance of *NRF1* regulation in goat LGC steroid synthesis and apoptosis.

STAR participates in the initial and rate-limiting step in steroidogenesis, which mediates the transport of cholesterol from the outer to the inner mitochondrial membrane (Arakane *et al.* 1998). The cholesterol converted to P_4 and E_2 is catalyzed by the key enzymes of cytochrome P450 family 11 subfamily A member 1 (CYP11A1), 3BHSD and CYP19A1 (Ke *et al.* 2004, Sahmi *et al.* 2004). The expression levels of these enzymes could reflect the production of P_4 and E_2 (Mizutani *et al.* 2015). In the present study, the overexpression of *NRF1* in goat LGCs significantly increased the mRNA and

Figure 7 Gain of *TFAM* rescues steroidogenesis and cell apoptosis in *NRF1* silenced goat LGCs. (A and B) Gain of *TFAM* recovered the P_4 and E_2 synthesis and steroidogenesis related genes (*STAR*, *3BHSD* and *CYP19A1*) expression in *NRF1* silenced LGCs. The relative expression levels were normalized to the expression amount of *GAPDH*. (C) Overexpression of *TFAM* in *NRF1* silenced LGCs decreased cell apoptosis percentage. (D) Gain of *TFAM* rescued the expression of apoptosis-related genes in *NRF1* silenced LGCs. The relative expression levels were normalized to the expression amount of *GAPDH*. (E) The levels of steroidogenesis and cell apoptosis-related proteins were restored by overexpression of *TFAM* in *NRF1* silenced LGCs. ACTB served as an internal control. Data are expressed as mean $\pm$ S.E.M. (Student's *t*-test, $n=4$), $*P<0.05$.

Table 6 Effects of *TFAM* on caspase activity in *NRF1* suppressed goat LGCs.

Group	CASP3 (U/mg pro)	CASP9 (U/mg pro)
shNRF1 + pEX-4-control	691.3 ± 42.8	342.1 ± 20.6
shNRF1 + pEX-4- <i>TFAM</i>	581.3 ± 28.1	219.8 ± 17.6*

Data are presented as mean ± S.E.M. (Student's *t*-test, *n* = 4).

In the same parameter, *Values mean significant difference between shNRF1 + pEX-4-*TFAM* (*TFAM* overexpression) and shNRF1 + pEX-4-control groups (*P* < 0.05).

CASP3, caspase 3; CASP9, caspase 9; pro, protein.

protein levels of STAR, 3BHSD and CYP19A1, as well as the levels of P_4 and E_2 , which was consistent with previous reports (Boruszewska *et al.* 2013). These results indicate that *NRF1* may get involved in the regulation of steroidogenesis in goat LGCs.

It has been reported that mitochondrial dysfunction leads to low levels of testosterone synthesis in Leydig cells (Shabalina *et al.* 2015). Blocked mitochondrial fusion by mitofusin 2 (*Mfn2*) knockdown has a negative impact on steroid synthesis (Duarte *et al.* 2012). Li and coworkers (2016) found that oxidative stress suppressed the activity of STAR and CYP11A1 in mitochondria, and substantially impaired testicular steroidogenesis. In addition, CYCS release was associated with the destruction of mitochondrial structure and steroidogenic function during apoptosis process (Amsterdam *et al.* 2003). In our study, the silencing of *NRF1* significantly decreased the expression levels of STAR, CYP19A1 and E_2 production, but increased the release of CYCS. These results were consistent with the previous report that mitochondrial dysfunction impaired steroidogenesis in human cumulus

cells *in vitro* (Ge *et al.* 2015). These data support that *NRF1* regulates the production of steroid hormones through the regulation of mitochondrial function in goat LGCs.

Extensive researches have revealed that the members of BCL2 family play major roles in modulating apoptosis, and the ratio of BAX (pro-apoptotic protein) to BCL2 (anti-apoptotic protein) is generally regarded as an important indicator of apoptosis (Korsmeyer *et al.* 1993). Our findings show that the expression level of BAX/BCL2 was significantly increased in *NRF1* silenced LGCs. This is consistent with a previous study, which found that *NRF1* silence stimulated MCF-7 breast cancer cell apoptosis (Radde *et al.* 2016). Consequently, the change in mRNA ratio of BAX/BCL2 could destabilize mitochondria, lead to CYCS release from mitochondria to cytoplasm and induce caspases activation (Raisova *et al.* 2001). Caspase activation is regarded as an important step in the execution phase of apoptosis. CASP3 and CASP9 are the most important members of the caspase family. As expected, the release of CYCS, mRNA level and cleavage activity of CASP3 and CASP9 were significantly increased by following *NRF1* silencing, which led to mitochondrial dysfunction and cell apoptosis. These results are consistent with the data in the study by Khalaj and coworkers (2013). Our data suggest that lack of *NRF1* may lead to goat LGC apoptosis through the mitochondria-dependent apoptotic pathway.

NRF1 participates in the regulation of cellular oxidative damage (Ohtsuji *et al.* 2008). High levels of 8-OHdG have been associated with low *TFAM*

Figure 8 Gain of *TFAM* restores mitochondrial function in *NRF1* silenced LGCs. (A) Overexpression of *TFAM* restored *TFAM* expression and mtDNA copy number in *NRF1* silenced LGCs. The ratio of mtDNA to nuclear DNA reflects the relative mtDNA copy number. (B) The CYCS distribution in mitochondria and cytosol was recovered by gain of *TFAM* in *NRF1* silenced LGCs. ACTB served as an internal control. (C) Antioxidant related genes *SOD2*, *CAT* and *GPX1* expression levels were rescued by overexpression of *TFAM* in *NRF1* silenced LGCs. The relative expression levels were normalized to the expression amount of *GAPDH*. Data are expressed as mean ± S.E.M. (Student's *t*-test, *n* = 4), **P* < 0.05.

Table 7 *TFAM* regulates mitochondrial function in *NRF1* suppressed goat LGCs.

Group	ATP (nmol/mg pro)	CCO (μ mol/min/mg pro)	Na ⁺ -K ⁺ -ATPase (μ mol Pi/h/mg pro)	Ca ²⁺ -Mg ²⁺ -ATPase (μ mol Pi/h/mg pro)
shNRF1 + pEX-4-control	19.31 $\pm$ 1.72	1.49 $\pm$ 0.15	45.59 $\pm$ 2.13	11.92 $\pm$ 0.79
shNRF1 + pEX-4-TFAM	30.14 $\pm$ 2.16*	2.48 $\pm$ 0.09*	72.84 $\pm$ 3.93*	14.83 $\pm$ 2.04

Data are expressed as mean $\pm$ s.e.m. (Student's *t*-test, *n* = 4).

In the same parameter, *Values mean significant difference between shNRF1 + pEX-4-TFAM (*TFAM* overexpression) and shNRF1 + pEX-4-control groups (*P* < 0.05).

ATP, adenosine triphosphate; CCO, cytochrome c oxidase; pro, protein.

levels and impaired mtDNA replication (Lee *et al.* 2014). Mitochondrial dysfunction resulted in the loss of CCO, increased 8-OHdG level and, subsequently, induced oxidative stress and cell apoptosis (Yang *et al.* 2016), which are supported by our results. In addition, perturbation of *NRF1* expression would disrupt the cellular redox balance, which was consistent with previous research (Zou *et al.* 2016). Among the antioxidants, GSH is a key player in intracellular redox regulation, and GSH/GSSG ratio is used to evaluate the cellular oxidative stress (Guo *et al.* 2007). Our findings showed that perturbation of *NRF1* expression could disrupt the cellular redox balance, which was consistent with previous research (Zou *et al.* 2016). It has been reported that superoxide is reduced by SOD to H₂O₂, and then reacts with GPx and CAT. These interacting defense mechanisms permit cells to live in an oxidative environment (Devine *et al.* 2012). Sultana and coworkers (2016) found that hyperglycemia induced by streptozotocin led to mitochondrial dysfunction through generating excessive ROS with diminished antioxidant defense system in heart. Banerjee and coworkers (2016) also reported that loss of C/EBP δ increased ionizing radiation and mitochondrial dysfunction, which led to antioxidant defense damage and cell apoptosis. In addition, our previous study showed that abnormal expression of *NRF1* led to mitochondrial dysfunction (Zhang *et al.* 2015). These results suggest that aberrant expression of *NRF1* alters the intracellular redox balance by mitochondrial dysfunction in goat LGCs.

TFAM is a key regulator of mitochondrial transcription and replication, whose transcription is regulated by *NRF1*. The downregulation of *TFAM*

could attenuate the coding capacity of mtDNA and mitochondrial biogenesis (Brenmoehl & Hoeflich 2013). *TFAM* silencing resulted in decreased basal oxygen consumption and mitochondrial ATP synthesis (Tsutsui *et al.* 2009), which is consistent with our study that gain of *TFAM* could rescue the reduced ATP levels and activity of CCO in *NRF1* silenced LGCs. Interestingly, it has been reported that *TFAM* overexpression successfully increased the ATP content and suppressed cell apoptosis by protecting mtDNA against oxidative damage (Xu *et al.* 2009). In this study, we also found that the ectopic expression of *TFAM* in *NRF1* silenced LGCs could alleviate the apoptosis and oxidative stress of goat LGCs. Moreover, Xu and coworkers (2012) found that the overexpression of *TFAM* could reverse the mitochondrial dysfunction and inhibit mitochondrial oxidative stress. In this study, we provided further evidences that the overexpression of *TFAM* in *NRF1* silenced goat LGCs could rescue mitochondrial dysfunction induced by *NRF1* knockdown. These studies indicate that *NRF1* may regulate mitochondrial function via controlling *TFAM* transcription in goat LGCs, while further studies are needed to elucidate this hypothesis.

In conclusion, this study demonstrates that attenuated expression of *NRF1* leads to mitochondrial dysfunction, disrupts the cellular redox balance, impairs steroid synthesis and finally results in GC apoptosis through the mitochondria-dependent pathway. In addition, overexpression of *TFAM* could restore the steroid synthesis and anti-apoptosis capacity in *NRF1* suppressed goat LGCs. These findings provide novel insights into the mechanisms behind mitochondria-dependent GC identity in goat ovaries during follicular atresia.

Table 8 Effects of *TFAM* on oxidative stress in *NRF1* suppressed goat LGCs.

Group	8-OHdG (μ mol Pi/h/mg pro)	SOD (U/mg pro)	CAT (U/mg pro)	GPx (U/mg pro)	GSH (nmol/mg pro)	GSSG (nmol/mg pro)	GSH/GSSG
shNRF1 + pEX-4-control	3.94 $\pm$ 0.47	8.79 $\pm$ 1.86	4.25 $\pm$ 0.89	16.92 $\pm$ 2.85	13.21 $\pm$ 1.48	1.18 $\pm$ 0.29	11.19 $\pm$ 3.08
shNRF1 + pEX-4-TFAM	1.89 $\pm$ 0.39*	12.09 $\pm$ 2.09	9.13 $\pm$ 2.07*	35.84 $\pm$ 3.17*	19.81 $\pm$ 1.72*	0.97 $\pm$ 0.31	20.42 $\pm$ 2.86*

Data are presented as mean $\pm$ s.e.m. (Student's *t*-test, *n* = 4).

In the same parameter, *Values mean significant difference between shNRF1 + pEX-4-TFAM (*TFAM* overexpression) and shNRF1 + pEX-4-control groups (*P* < 0.05).

8-OHdG, 8-hydroxy-2'-deoxyguanosine; CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; pro, protein; SOD, superoxide dismutase.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0583>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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