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

Guo-Min Zhang1,2, Ming-Tian Deng2, Zhi-Hai Lei1, Yong-Jie Wan2, Hai-Tao Nie2, Zi-Yu Wang2, Yi-Xuan Fan2, Feng Wang2 and Yan-Li Zhang2

1College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China and 2Jiangsu Livestock Embryo Engineering Laboratory, Nanjing Agricultural University, Nanjing, China

Correspondence should be addressed to Y-L Zhang; Email: zhangyanli@njau.edu.cn

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.

Reproduction (2017) 154 111–122

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 inducted 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 (Glisteter 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 estriadiol (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 E2 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 (Plantadosi & 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±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, shNRF1+pEX-4-TFAM group) using Lipofectamine 2000. NRF1-knockdown LGCs were transfected with pEX-4 used as control (shNRF1 +pEX-4-control group). The transfected and infected LGCs were cultured for another 48 h before analysis.

Steroid assay

The concentrations of P₄ and E₂ in culture medium were measured by radioimmunnoassay (RIA) using commercial kits (Iodine [¹²⁵I]-Prog RIA Kit, No. RG5-04; Iodine [¹²³I]-E₂ RIA Kit, No. RG6-04; Jiding 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 P₄ and E₂ was 0.03 ng/mL and 2.1 pg/mL respectively. The intra- and inter-assay coefficients of variation for P₄ and E₂ 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 (Basci 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; Jiacheng, 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 12,000g 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)
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 μmol/Pr/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), and oxidized glutathione (GSSG) were determined 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 1
Details of primer sequences, expected product sizes and GenBank accession numbers of genes used for qPCR.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5′→3′)</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF1</td>
<td>F: 5′-AGGCTGGGCAAAAGAAG-3′&lt;br&gt;R: 5′-CCACCTGTGAAAGCCGAC-3′</td>
<td>303</td>
<td>AY368269.1</td>
</tr>
<tr>
<td>STAR</td>
<td>F: 5′-GGTCCCGAGACTTTGAG-3′&lt;br&gt;R: 5′-AATCCACTTGTTGCTGGC-3′</td>
<td>262</td>
<td>NM_013975437.1</td>
</tr>
<tr>
<td>3BHSD</td>
<td>F: 5′-AGACCAAGTCCGAGGGA-3′&lt;br&gt;R: 5′-TCTCTCTTGATGTTGCGG-3′</td>
<td>292</td>
<td>NM_001285747.1</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>F: 5′-CAAGATGATGTCGGACT-3′&lt;br&gt;R: 5′-GGGCCCCTATTCGAGAAG-3′</td>
<td>133</td>
<td>NM_001285747.1</td>
</tr>
<tr>
<td>TFAM</td>
<td>F: 5′-CCAAGCTGGGAAGCCTT-3′&lt;br&gt;R: 5′-GCTGAGGAGCATTTTGC-3′</td>
<td>229</td>
<td>XM_005699371</td>
</tr>
<tr>
<td>SOD2</td>
<td>F: 5′-GTGAACCAACACGCTTGC-3′&lt;br&gt;R: 5′-GGCTCCCCTGTCATITGA-3′</td>
<td>300</td>
<td>XM_013966636</td>
</tr>
<tr>
<td>CAT</td>
<td>F: 5′-CCTCAGGTCGGAGATCT-3′&lt;br&gt;R: 5′-ATGGTGATGGGAGTTTGC-3′</td>
<td>159</td>
<td>GQ204786.1</td>
</tr>
<tr>
<td>GPX1</td>
<td>F: 5′-ACATTTAACCCCTGCTTCC-3′&lt;br&gt;R: 5′-TCATAGGAGGCTGTGTCG-3′</td>
<td>216</td>
<td>XM_005695962.2</td>
</tr>
<tr>
<td>BAX</td>
<td>F: 5′-GCACCCACTGTCACGGTC-3′&lt;br&gt;R: 5′-CCGGCCATCGGAAAGAC-3′</td>
<td>182</td>
<td>NM_001166486.1</td>
</tr>
<tr>
<td>BCL2</td>
<td>F: 5′-ATGGTGATGGGAGTTTGC-3′&lt;br&gt;R: 5′-AGAAGACCGGCAAGAATC-3′</td>
<td>197</td>
<td>NM_001286089.1</td>
</tr>
<tr>
<td>CASP3</td>
<td>F: 5′-GCACCCACTGTCACGGTC-3′&lt;br&gt;R: 5′-CCGGCCATCGGAAAGAC-3′</td>
<td>190</td>
<td>NM_001286089.1</td>
</tr>
<tr>
<td>CASP9</td>
<td>F: 5′-AGTACGGCCCTTCTTGTGTT-3′&lt;br&gt;R: 5′-AGTGTTCCGGCTTCTTCTAC-3′</td>
<td>193</td>
<td>XM_005690814</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′-GGACCTTACACCGGACTCACC-3′&lt;br&gt;R: 5′-CCCTGCTGCCGTAAGCGA-3′</td>
<td>119</td>
<td>NM_001034034.1</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Cat No.</th>
<th>Source</th>
<th>Dilution of Western blot</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAR</td>
<td>sc-25806</td>
<td>Santa Cruz</td>
<td>1:400</td>
</tr>
<tr>
<td>3BHSD</td>
<td>NB110-7864</td>
<td>Novus (CO, USA)</td>
<td>1:500</td>
</tr>
<tr>
<td>CYP19A1</td>
<td>ab18995</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>BAX</td>
<td>sc-526</td>
<td>Santa Cruz</td>
<td>1:200</td>
</tr>
<tr>
<td>BCL2</td>
<td>BA0412</td>
<td>Boster (Wuhan, China)</td>
<td>1:200</td>
</tr>
<tr>
<td>CYCS</td>
<td>ab16076</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>ACTB</td>
<td>AA128</td>
<td>Beyotime (Haimen, China)</td>
<td>1:200</td>
</tr>
</tbody>
</table>

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; mtDNA, mitochondrial DNA; GFP, green fluorescent protein; qPCR, quantitative real-time polymerase chain reaction; BCA, bicinchoninic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, catalase; GPX1, glutathione peroxidase 1; SOD2, superoxide dismutase 2; TFAM, transcription factor A, mitochondrial.
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 $2^{-\Delta\Delta C_{T}}$ 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% confluence were detached and resuspended in DPBS, then incubated with 5 μL of APC-labeled Annexin V for 5 min, followed by an additional 5 μL 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 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 ± s.e.m. (Student's t-test, n = 4), *P < 0.05.

Figure 2 NRF1 silence induces goat LGC apoptosis. The percentage of apoptotic cells was analyzed by flow-cytometry using Annexin V-APC/7-AAD staining in NRF1 overexpressed or suppressed LGCs. Quadrants: Left, live cells; Right, apoptotic cells. Data are expressed as mean ± s.e.m. (Student's t-test), *P < 0.05.
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 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 ± s.e.m. (Student’s t-test, n=4), *P<0.05.

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 ± s.e.m. (Student’s t-test, n=4), *P<0.05.
In the same parameter, *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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>CASP3 (U/mg pro)</th>
<th>CASP9 (U/mg pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEX-4-control</td>
<td>582.4 ± 49.1*</td>
<td>273.9 ± 17.3*</td>
</tr>
<tr>
<td>pEX-4-NRF1</td>
<td>397.1 ± 19.3h</td>
<td>198.4 ± 11.9h</td>
</tr>
<tr>
<td>Scramble</td>
<td>543.7 ± 27.6</td>
<td>295.1 ± 16.4</td>
</tr>
<tr>
<td>shNRF1</td>
<td>729.8 ± 34.6*</td>
<td>329.7 ± 13.8*</td>
</tr>
</tbody>
</table>

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


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, Advansta, CA, USA). Band intensities were estimated by densitometry and 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 P₄ and E₂ synthesis in goat LGCs**

To characterize NRF1 in steroidogenesis of goat LGCs in vitro, we first examined its effect on P₄ and E₂ production. The concentrations of P₄ and E₂ were significantly increased with the overexpression of NRF1 (P < 0.05, Fig. 1A and B), while the level of E₂ 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 (3BHSD) 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 (Fig. 3A and B). 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 cyt c 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, Fig. 4D).
in LGCs significantly increased the ATP and CCO silencing impairs ATP plays an important role in mitochondrial
92.4 17.47 Ca CCO 18.29 23.81 and decreased 1.61 P overexpression (13.64 18.37 Na silenced LGCs (0.73 on oxidative stress in goat LGCs.
1.18 SOD2 silence) and Scramble groups (P <0.05). ATP, adenosine triphosphate; CCO, cytochrome c oxidase; pro, protein.
NRF1 involves in regulation of goat LGCs
Table 5 Effects of NRF1 on oxidative stress in goat LGCs.

<table>
<thead>
<tr>
<th>Group</th>
<th>8-OHdG (μmol Pi/h/mg pro)</th>
<th>SOD (U/mg pro)</th>
<th>CAT (U/mg pro)</th>
<th>GPx (U/mg pro)</th>
<th>GSH (nmol/mg pro)</th>
<th>GSSG (nmol/mg pro)</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEX-4-control</td>
<td>1.18 ± 0.13</td>
<td>11.84 ± 2.47a</td>
<td>6.49 ± 1.52a</td>
<td>30.26 ± 5.41a</td>
<td>17.47 ± 2.16b</td>
<td>1.03 ± 0.32</td>
<td>17.36 ± 2.47a</td>
</tr>
<tr>
<td>pEX-4-NRF1</td>
<td>0.89 ± 0.21</td>
<td>18.76 ± 1.91b</td>
<td>11.52 ± 1.74b</td>
<td>24.63 ± 3.85b</td>
<td>23.81 ± 2.35b</td>
<td>0.73 ± 0.29</td>
<td>30.58 ± 4.83b</td>
</tr>
<tr>
<td>Scramble</td>
<td>1.21 ± 0.19</td>
<td>13.25 ± 2.23</td>
<td>5.81 ± 0.89</td>
<td>27.58 ± 2.92</td>
<td>16.15 ± 1.92</td>
<td>1.19 ± 0.21</td>
<td>13.64 ± 1.89</td>
</tr>
<tr>
<td>shNRF1</td>
<td>4.17 ± 0.26a*</td>
<td>8.51 ± 1.46a</td>
<td>3.76 ± 1.21a</td>
<td>18.37 ± 3.26a</td>
<td>11.49 ± 1.68a</td>
<td>1.37 ± 0.16</td>
<td>9.06 ± 2.18a*</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.E.M. (Student’s t-test, n=4).
In the same parameter, a,bValues 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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP (nmol/mg pro)</th>
<th>CCO (μmol/min/mg pro)</th>
<th>Na⁺-K⁺-ATPase (μmol Pi/h/mg pro)</th>
<th>Ca²⁺-Mg²⁺-ATPase (μmol Pi/h/mg pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEX-4-control</td>
<td>28.43 ± 3.25a</td>
<td>2.09 ± 0.46a</td>
<td>68.27 ± 1.83a</td>
<td>15.41 ± 2.06a</td>
</tr>
<tr>
<td>pEX-4-NRF1</td>
<td>37.61 ± 2.38b</td>
<td>4.23 ± 0.28b</td>
<td>92.4 ± 2.36b</td>
<td>21.29 ± 1.47b</td>
</tr>
<tr>
<td>Scramble</td>
<td>25.72 ± 1.98</td>
<td>2.18 ± 0.32</td>
<td>61.5 ± 2.43</td>
<td>16.03 ± 1.28</td>
</tr>
<tr>
<td>shNRF1</td>
<td>18.29 ± 2.64a</td>
<td>1.61 ± 0.25a</td>
<td>41.25 ± 1.84*</td>
<td>12.47 ± 1.82</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (Student’s t-test, n=6).
In the same parameter, a,bValues 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 though 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...
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 P₄ and E₂ 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 P₄ and E₂ (Mizutani et al. 2015). In the present study, the overexpression of NRF1 in goat LGCs significantly increased the mRNA and

---

**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 ± s.e.m. (Student’s t-test, $n=4$), *$P < 0.05$.

**Figure 7** Gain of TFAM rescues steroidogenesis and cell apoptosis in NRF1 silenced goat LGCs. (A and B) Gain of TFAM recovered the P₄ and E₂ 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 ± s.e.m. (Student’s t-test, $n=4$), *$P < 0.05$.
protein levels of STAR, 3BHSD and CYP19A1, as well as the levels of P4 and E2, 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 E2 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 (Radda 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

---

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

<table>
<thead>
<tr>
<th>Group</th>
<th>CASP3 (U/mg pro)</th>
<th>CASP9 (U/mg pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shNRF1+pEX-4-control</td>
<td>691.3 ± 42.8</td>
<td>342.1 ± 20.6</td>
</tr>
<tr>
<td>shNRF1+pEX-4-TFAM</td>
<td>581.3 ± 28.1</td>
<td>219.8 ± 17.6</td>
</tr>
</tbody>
</table>

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).

---

**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. (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.
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/EBPβ 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 (Brennoebl & 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 7 TFAM regulates mitochondrial function in NRF1 suppressed goat LGCs.

<table>
<thead>
<tr>
<th>Group</th>
<th>ATP (nmol/mg pro)</th>
<th>CCO (μmol/min/mg pro)</th>
<th>Na⁺-K⁺-ATPase (μmol Pi/h/mg pro)</th>
<th>Ca²⁺-Mg²⁺-ATPase (μmol Pi/h/mg pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>shNRF1+pEX-4-control</td>
<td>19.31±1.72</td>
<td>1.49±0.15</td>
<td>45.59±2.13</td>
<td>11.92±0.79</td>
</tr>
<tr>
<td>shNRF1+pEX-4-TFAM</td>
<td>30.14±2.16*</td>
<td>2.48±0.09*</td>
<td>72.84±3.93*</td>
<td>14.83±2.04</td>
</tr>
</tbody>
</table>

Data are expressed as mean±s.e.m. (Student’s t-test, n=4).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>8-OHdG (μmol Pi/h/mg pro)</th>
<th>SOD (U/mg pro)</th>
<th>CAT (U/mg pro)</th>
<th>G Px (U/mg pro)</th>
<th>G SH (nmol/mg pro)</th>
<th>G SS (nmol/mg pro)</th>
<th>G SH/G SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>shNRF1+pEX-4-control</td>
<td>3.94±0.47</td>
<td>8.79±1.86</td>
<td>4.25±0.89</td>
<td>16.92±2.85</td>
<td>13.21±1.48</td>
<td>1.18±0.29</td>
<td>11.19±3.08</td>
</tr>
<tr>
<td>shNRF1+pEX-4-TFAM</td>
<td>1.89±0.39*</td>
<td>12.09±2.09</td>
<td>9.13±2.07*</td>
<td>35.84±3.17*</td>
<td>19.81±1.72*</td>
<td>0.97±0.31</td>
<td>20.42±2.86*</td>
</tr>
</tbody>
</table>

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).

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.

Funding
This study was financially supported by the National Nature Science Foundation of China (No. 31272443), the China Postdoctoral Science Foundation (No. 80252115) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (280100745113).

Acknowledgements
The authors thank all the members of Feng Wang’s laboratory who contributed to sample collection, and doctor Hong-Yan Sun from UCAS for her technical assistance.

References


www.reproduction-online.org

Reproduction (2017) 154 111–122
2006 Mitochondrial transcription factor
2015 mTORC1 signaling activates NRF1
2016 Effect of PGC-1
2001 The mitochondrion in apoptosis: how
493–507.
465–473.
43–54.
449–456.
577–585.
2010 Metformin
2012 Overexpression of TFAM, NRF-1
11567–11572.
324–333.
2016 Endometriosis as a detrimental condition for granulosa
2004 Expression of 17beta-
2009 Mitochondrial oxidative
222–231.
35–46.
3800–3809.
2016 Garlic activates SIRT-3 to prevent cardiac oxidative stress and premature aging mice. 
2015 Expression of mitochondria-associated genes (PPARGC1A, NRF-1, BCL-2 and BAX) in follicular development and atresia of goat ovaries. 
2015 Leydig cell pregnancy reduces fetal skeletal muscle mitochondrial biogenesis in the pigs. 
2016 Moderately decreased maternal dietary energy intake during pregnancy reduces fetal skeletal muscle mitochondrial biogenesis in the pigs. 

Received 29 October 2016
First decision 16 November 2016
Revised manuscript received 12 May 2017
Accepted 19 May 2017