Molecular and Cellular Endocrinology 452 (2017) 138–147

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

MicroRNA-145 protects follicular granulosa cells against oxidative stress-induced apoptosis by targeting Krüppel-like factor 4

Lu Xu [a, 1], Haixiang Sun [b, 1], Mei Zhang [b, 1], Yue Jiang [b], Chunxue Zhang [b], Jianjun Zhou [b], Lijun Ding [b], Yali Hu [a, *], Guijun Yan [b, **]

[a] Reproductive Medicine Center, Drum Tower Clinic Medical College of Nanjing Medical University, Nanjing, Jiangsu, China
[b] Reproductive Medicine Center, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nanjing, Jiangsu, China

ARTICLE INFO

Article history:
Received 22 January 2017
Received in revised form
16 April 2017
Accepted 25 May 2017
Available online 28 May 2017

Keywords:
miR-145
Granulosa cell
Oxidative stress
Apoptosis
KLF4

ABSTRACT

Oxidative stress-induced follicular granulosa cell (GC) apoptosis plays an essential role in abnormal follicular atresia, which may trigger ovarian dysfunction. To investigate the role of microRNA (miR)-145 in the regulation of GC apoptosis and modulation of the apoptotic pathway in the setting of oxidative stress, we employed an H_2O_2-induced *in vitro* model and a 3-nitropropionic acid (NP)-induced *in vivo* model of ovarian oxidative stress. We demonstrated *in vitro* that miR-145 expression was significantly down-regulated in KGN cells and mouse granulosa cells (mGCs) treated with H_2O_2, whereas miR-145 over-expression attenuated H_2O_2-induced apoptosis in GCs. Moreover, miR-145 protected GCs against H_2O_2-induced apoptosis by targeting KLF4, which promoted H_2O_2-induced GC apoptosis via the BAX/BCL-2 pathway. Importantly, decreased miR-145 expression in the *in vivo* ovarian oxidative stress model promoted apoptosis by up-regulating KLF4 expression, whereas GC-specific miR-145 over-expression attenuated apoptosis by targeting KLF4. In conclusion, miR-145 protects GCs against oxidative stress-induced apoptosis by targeting KLF4.

1. Introduction

The follicle is the major endocrine and reproductive compartment of the ovary and consists of a central oocyte surrounded by one or more layers of somatic granulosa cells (GCs). Although numerous follicles are present in the ovaries, most undergo degeneration during growth and development in a process known as follicular atresia. Abnormal follicular atresia may accelerate follicular depletion, leading to ovarian dysfunction. Recent studies have suggested that GC apoptosis is the main cause of follicular atresia (Asselin et al., 2000; Tilly et al., 1991; Valdez et al., 2005). Oxidative stress, which arises as a consequence of the excessive production of reactive oxygen species (ROS) and/or an impaired antioxidant defence mechanism, is believed to be the major cause of GC apoptosis (Agarwal et al., 2012). Female homozygous mutant mice lacking the important endogenous antioxidant enzyme copper/zinc superoxide dismutase 1 (SOD1) have many primary follicles but few antral follicles and corpora and are subfertile, indicating abnormal follicular atresia (Matzuk et al., 1998). Under physiological conditions, age-associated ROS accumulation drives GCs to apoptosis (Broekmans et al., 2007; Tatone et al., 2008). Additionally, ROS accumulation generated by intracellular sources, such as mitochondrial mutations, nutrient deprivation and ischemia, as well as external factors, including smoking, air pollution, exposure to xenobiotics and electromagnetic radiation, accelerates GC apoptosis and leads to ovarian dysfunction, such as

Abbreviations: GCs, granulosa cells; ROS, reactive oxygen species; SOD1, superoxide dismutase 1; H_2O_2, hydrogen peroxide; KLF, Krüppel-like factor; LH, luteinizing hormone; IGF1, insulin-like growth factor 1; LDLR, low-density lipoprotein receptor; StAR, steroidogenic acute regulatory protein; CYP11A, P-450 cholesterol side-chain cleavage complex; PCOS, polycystic ovary syndrome; BAX, Bcl-2-associated X; BCL-2, B cell leukemia/lymphoma-2; 3-NP, 3-nitropropionic acid; cKI, conditional knockin; PBS, phosphate buffered saline; FBS, fetal bovine serum; RNA-seq, RNA-sequencing; qRT-PCR, quantitative real-time PCR; NF-κB, nuclear factor-κB; SD, standard deviation; PDTC, pyrrolidine dithiocarbamate; BBC3, Bcl2-binding component 3; MAP2K4, mitogen-activated protein kinase kinase 4; GADD45A, growth arrest and DNA damage inducible alpha.

* Corresponding author. Reproductive Medicine Center, Drum Tower Clinic Medical College of Nanjing Medical University, 321 Zhongshan Road, Nanjing 210008, China.

** Corresponding author. Reproductive Medicine Center, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, China.

E-mail addresses: dtylhu@126.com (Y. Hu), yanguijun55@163.com (G. Yan).

[1] Lu Xu, Haixiang Sun and Mei Zhang contributed equally to this work.

http://dx.doi.org/10.1016/j.mce.2017.05.030
0303-7207/

premature ovarian insufficiency (Agarwal et al., 2003; Kumar et al., 2010; Venkatesh et al., 2010).

MicroRNAs (miRNAs) are a class of small, non-encoding RNAs that transcriptionally or post-transcriptionally modulate the expression of their target genes. Conditional inactivation of Dicer1 in follicular GCs leads to increased degenerate follicles, suggesting an essential role for miRNAs in the regulation of GC functions (Donadeu et al., 2012; Hong et al., 2008; Lei et al., 2010). Previously, we reported that miR-145 protected cardiomyocytes against oxidative stress-induced apoptosis by targeting the mitochondrial apoptotic pathway (Li et al., 2012). Moreover, aberrant miR-145 expression is associated with the response of vascular smooth muscle cells to hydrogen peroxide (H_2O_2)-induced oxidative stress (Lin et al., 2009). Together, these investigations indicate that miR-145 may participate in the regulation of the oxidative stress-induced apoptotic pathway. However, whether miR-145 is associated with GC apoptosis under oxidative stress or how this association interferes with the apoptotic pathway is mostly unknown.

The Krüppel-like factor (KLF) family of transcription factors regulates diverse cellular processes (Knoedler and Denver, 2014; McConnell and Yang, 2010). DNA microarray technology and other exploratory analyses have identified multiple *Klf* genes (*Klf*2, *Klf*4, *Klf*5, *Klf*6, *Klf*9, *Klf*13, *Klf*15 and *Klf*17) in the mammalian ovary, and accumulating evidence has implied their regulatory roles in the apoptosis, proliferation and differentiation of ovarian cells (Jo et al., 2004; Kezele et al., 2005; Liu et al., 2001; Simmen et al., 2015). In follicular GCs, *Klf*4 and *Klf*13 mRNA expression is regulated by luteinizing hormone (LH) and/or insulin-like growth factor 1 (IGF1) (Natesampillai et al., 2008). Dysregulated KLF2 and KLF4 expression has been identified by microarray analysis in polycystic ovary syndrome (PCOS) patients compared with normal ovaries (Jansen et al., 2004). Aberrant KLF2, KLF4, KLF5, KLF6 and KLF9 expression has been validated in ovarian cancer cells (Chen et al., 2014; DiFeo et al., 2006; Dong et al., 2013; Wang et al., 2005; Zhang et al., 2015). In particular, KLF4 is the most highly implicated protein in apoptosis among the seventeen members of the mammalian KLF family (McConnell and Yang, 2010). As a transcription factor, KLF4 induces cell apoptosis by activating the transcription of pro-apoptotic Bcl-2-associated X (BAX) while repressing the transcription of anti-apoptotic B cell leukemia/lymphoma-2 (BCL-2) (Hu et al., 2015; Li et al., 2010, 2015; Ohnishi et al., 2003).

H_2O_2 is widely used as a classic reagent to trigger oxidative stress in cultured cells and therefore may serve as a reliable *in vitro* model of oxidative stress-induced apoptosis (Kaczara et al., 2010). 3-Nitropropionic acid (3-NP) is a toxic substance that irreversibly inhibits the succinic dehydrogenase complex (Complex II), which is an important link between the tricarboxylic acid cycle and respiratory chain in the inner mitochondrial membrane. The inhibition of Complex II interferes with the electron transport cascade and interrupts oxidative phosphorylation, resulting in ATP reduction and oxidative stress (Alston et al., 1977). For instance, elevated ovarian ROS levels were detected in mice injected intraperitoneally with 3-NP (Shen et al., 2012). Herein, we investigated the role of miR-145 in the regulation of GC apoptosis in the setting of oxidative stress. Employing the H_2O_2-induced *in vitro* model and the 3-NP-induced *in vivo* model of ovarian oxidative stress, we demonstrate that miR-145 protects follicular GCs against oxidative stress-induced apoptosis by targeting KLF4.

2. Materials and methods

2.1. Mouse strains and breeding schemes to generate miR-145 conditional knockin (miR-145 cKI) female mice

Rosa26-LoxP.GFP.Stop.LoxP-miR-145 mice were purchased from the Model Animal Research Centre of Nanjing University (Nanjing, China). Briefly, CAG-Loxp-Stop-Loxp-miR-145 cassettes were inserted into the Rosa26 locus (Fig. S4A). Cyp19-Cre transgenic mice were generated by oocyte microinjection of a DNA fragment in which the Cyp19 promoter (304 bp) was ligated to the iCre cDNA. To generate female mice with miR-145 over-expression in GCs, miR-145$^{fl/fl}$ mice were bred with Cyp19-Cre mice to obtain miR-145$^{fl/fl}$; Cyp19-Cre females (miR-145 cKI) (Fig. S4B). The animals were housed under a 12-h light/12-h dark schedule and provided food and water ad libitum. All animal experiments were conducted according to the guidelines of the Experimental Animal Management Committee (Jiangsu Province, China) and were approved by the Ethics Review Board for Animal Studies of the Drum Tower Hospital Affiliated to Nanjing Medical University.

2.2. Establishment of the ovarian oxidative stress mouse model

The ovarian oxidative stress mouse model was established as previously described (Shen et al., 2012). Briefly, female 4-week-old C57BL/6 mice were used in the experiments. 3-NP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) to a concentration of 10 mg/ml (pH 7.4) and administered by intraperitoneal injection at a dose of 50 mg/kg twice daily for 5 days at 12 h intervals (8:00 a.m. and 8:00 p.m.). At 12 h after the last injection, the mice were euthanized and their ovaries were dissected. The left ovary of each mouse was used to isolate GCs to assess the mRNA and protein levels, and the right ovary of each mouse was used for immunohistochemical analysis.

2.3. Cell lines

The KGN cell line (a generous gift from Dr. Yiming Mu at the General Hospital of the People's Liberation Army, Beijing, China), which was established from a human ovarian granulosa-like tumour and expressed typical GC markers, was cultured as previously described (Nishi et al., 2001). Human embryonic kidney (HEK293A) cells were maintained in DMEM/F12 medium (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin; Gibco BRL/Invitrogen) at 37 °C in a humidified environment with 5% CO_2.

2.4. Isolation and culture of primary mouse granulosa cells (mGCs)

mGCs were collected from the ovaries of 21-day-old immature C57BL/6 mice using the follicular puncture method as previously described (Zhang et al., 2013). The mGCs were cultured in DMEM/F12 medium (Gibco BRL/Invitrogen) containing 10% FBS (HyClone), 1 mM sodium pyruvate (HyClone), 2 mM glutamine (HyClone) and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin; Gibco BRL/Invitrogen) at 37 °C in a humidified environment with 5% CO_2.

2.5. Recombinant adenovirus construction

Adenoviruses harbouring a 441-bp DNA fragment encompassing the hsa-miR-145 gene (Ad-miR-145) were generated using the AdMax (Microbix Biosystems, Inc., Toronto, ON, Canada) system according to the manufacturer's instructions. Adenoviruses harbouring His-tagged human *Klf4* (Ad-his-h*Klf4*) and His-tagged mouse *Klf4* (Ad-his-m*Klf4*) were purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). The adenovirus bearing LacZ (Ad-LacZ) was obtained from Clontech (Mountain View, CA, USA). The viruses were packaged and amplified in HEK293A cells and purified using CsCl banding followed by dialysis against 10 mM