Research paper

Cyclophosphamide promotes the proliferation inhibition of mouse ovarian granulosa cells and premature ovarian failure by activating the lncRNA-Meg3-p53-p66Shc pathway

Ying Xiong [a], Te Liu [b,d,e,*], Suwei Wang [b], Huiying Chi [b], Chuan Chen [b,**], Jin Zheng [c,**]

[a] Department of Gynaecology and Obstetrics, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200092, China
[b] Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200031, China
[c] Gynecology of Traditional Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200031, China
[d] Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China
[e] Department of Pathology, Yale University School of Medicine, CT 06520, USA

A B S T R A C T

The dysfunction of ovarian granulosa cells (OGCs) directly affects the premature ovarian failure (POF). In vivo experiments showed that cyclophosphamide significantly induced mouse ovarian atrophy and proliferation inhibition of OGCs. The expressions of p53, p66Shc and p16 were significantly higher in OGCs of the cyclophosphamide treatment group. MTT assay showed that cyclophosphamide effectively inhibited the proliferation of OGCs in vitro. SA-β-Gal staining showed that the OGCs in the cyclophosphamide treatment group had many senescent cells. And, the expression of p53, p66Shc, p16 and cleaved caspase-3 in the OGCs of the cyclophosphamide treatment group significant increases. The Northern blot showed that the intensity of the lncRNA-Meg3 hybridization signal of the OGCs in the cyclophosphamide treatment group was significantly higher than that in the control group. ChIP results confirmed the significant increase in the obtained p66Shc promoter DNA fragment, which was enriched on p53 protein, in the OGCs treated with cyclophosphamide. When cyclophosphamide treatment was conducted after siRNA-Meg3 was used, the expression of endogenous lncRNA-Meg3, p53, p66Shc, p16 and cleaved caspase-3 was significantly lower than that in the siRNA-Mock control group. In summary, cyclophosphamide promotes the proliferation inhibition of mouse OGCs and premature ovarian failure by activating the lncRNA-Meg3-p53-p66Shc pathway.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Premature ovarian failure (POF) refers to a disease in women who suffer from amenorrhea, infertility, a low oestrogen level, an excess of gonadotropin, a lack of mature follicles and other symptoms before the age of 40, and it is one of the common causes of female infertility (Beck-Peccoz and Persani, 2006; Liu et al., 2012; Liu et al., 2013; Liu et al., 2014b; Chapman et al., 2015; Liu et al., 2015; Liu et al., 2016).

The occurrence of POF is closely associated with the condition and quality of ovarian granulosa cells (OGCs) (Beck-Peccoz and Persani, 2006; Liu et al., 2012; Liu et al., 2013; Liu et al., 2014b; Chapman et al., 2015; Liu et al., 2015; Liu et al., 2016). The occurrence of POF is largely associated with the sustained reduction in the follicle pool that results in a premature failure of ovarian function, but the mechanism is unclear. The decline in ovarian reserve is closely associated with POF (Beck-Peccoz and Persani, 2006; Liu et al., 2012; Liu et al., 2013; Liu et al., 2014b; Chapman et al., 2015; Liu et al., 2015; Liu et al., 2016). Additionally, the senescence and apoptosis of OGCs are important causes of the decline in ovarian reserve (Beck-Peccoz and Persani, 2006; Liu et al., 2012; Liu et al., 2013; Liu et al., 2014b; Chapman et al., 2015; Liu et al., 2015; Liu et al., 2016).

Long non-coding RNA (lncRNA) is a type of RNA in which the transcript length is over 200 nt and does not encode any protein. lncRNA can regulate gene expression at the epigenetic, transcriptional and post-transcriptional levels, and it is involved in the silencing of the X chromosome, genomic imprinting and chromosome modification, transcription activation and inhibition, intranuclear transport and many

important regulatory processes that are closely associated with the occurrence, development, prevention and treatment of human diseases (Zhou et al., 2007; Liu et al., 2014a; Wu et al., 2014; Fang et al., 2016). The mode of action of lncRNA includes effects on signaling, decoys, guidance and scaffolding, among others (Zhou et al., 2007; Liu et al., 2014a; Wu et al., 2014; Fang et al., 2016). Modern studies have confirmed that approximately 80% of mammalian genomic transcripts are lncRNA (Zhou et al., 2007; Liu et al., 2014a; Wu et al., 2014; Fang et al., 2016). Despite the large quantities of lncRNAs, we still have a poor understanding of their processing and biological regulatory mechanism. Maternally expressed gene 3 (Meg3) is a special lncRNA (Zhou et al., 2007; Mondal et al., 2015; You et al., 2016; Zhuang et al., 2015; Pawar et al., 2016; Zhang et al., 2016) and was the first lncRNA shown to have a tumour-inhibitory function (Zhou et al., 2007; Mondal et al., 2015; You et al., 2016; Zhuang et al., 2015; Pawar et al., 2016; Zhang et al., 2016). lncRNA-Meg3 is a single-copy, imprinted gene consisting of 10 exons, and a total of 12 Meg3 phenotypes have been found from alternative splicings. Each phenotype contains the common exons 1–3 and 8–10, while exons 4–7 have different alternative splicings (Zhou et al., 2007; Mondal et al., 2015; You et al., 2016; Zhuang et al., 2015; Pawar et al., 2016; Zhang et al., 2016). The last few introns of lncRNA-Meg3 gene can encode microRNA-770 (Zhou et al., 2007; Mondal et al., 2015; You et al., 2016; Zhuang et al., 2015; Pawar et al., 2016; Zhang et al., 2016). Functionally, lncRNA-Meg3 can directly or indirectly regulate the expression of cAMP, p53, Gdf15, murine double minute 2 (MDM2) and other proteins (Zhou et al., 2007; Mondal et al., 2015; You et al., 2016; Zhuang et al., 2015; Pawar et al., 2016; Zhang et al., 2016). Studies by Zhou et al. found that overexpression of lncRNA-Meg3 can effectively inhibit the proliferation of tumour cells and promote their apoptosis (Zhou et al., 2007). An in-depth study found that lncRNA-Meg3 can bind to MDM2 (with E3 ubiquitin-protein ligase-mediated degradation function) mRNA through reverse complementary binding to inhibit its expression and eventually reduce the ubiquitin-mediated degradation of the cell cycle regulation factor p53 by MDM2, which allows tumour cells to highly express p53 and inhibit cell cycle progression, ultimately promoting tumour cell apoptosis (Zhou et al., 2007). However, the lncRNA-Meg3 regulation of POF is unclear yet.

p66Shc is an important member of the ShcA gene family, which are important in regulating cell senescence. This protein can promote body aging by inducing apoptosis and necrosis in many types of cells, and its deficiency can enhance the cell resistance to reactive oxygen species (ROS) and thus extend cell lifetime (Migliaccio et al., 1999; Trinei et al., 2002; Napoli et al., 2003; Francia et al., 2004; Yamamori et al., 2005; Camici et al., 2007; Fabbrocini et al., 2010; Cai et al., 2016). Three Shc genes, ShcA, ShcB (Sli) and ShcC (Rai), have been found in mammals The ShcA protein is widely distributed in a variety of tissues except the brain and neurons in human and mouse, and it is related to the signal transduction mechanisms for the growth, differentiation, survival and death of many cells. Multiple studies have confirmed that p66Shc plays an important physiological role in promoting the aging of cells and organs and in the pathogenesis and progression of cardio-vascular diseases (Migliaccio et al., 1999; Trinei et al., 2002; Napoli et al., 2003; Francia et al., 2004; Yamamori et al., 2005; Camici et al., 2007; Fabbrocini et al., 2010; Cai et al., 2016). We also found that oxidative stress can induce high expression of p66Shc in vascular endothelial cells, and this stable expression depends on the methylation modification of its mRNA 3'UTR region by NSun2 (Cai et al., 2016). However, so far, there is no report on whether the mechanism of aging and injury of OGCs is also related to p66Shc during the pathogenesis of POF.

Based on these clues, we intended to establish a mouse POF model, which is caused by the chemotherapy injury induced by cyclophosphamide, to conduct an in-depth investigation of the pathogenesis of POF and the regulatory effect of the endogenous lncRNA-Meg3 on the downstream p53-p66Shc-p16 pathway in OGCs. We hoped to elucidate our hypothesis: cyclophosphamide induces the senescence and proliferation inhibition of OGCs and the pathogenesis of POF by promoting the

expression of endogenous lncRNA-Meg3 and activating the p53-p66Shc-p16 pathway.

2. Materials and methods

2.1. Isolation and culture of mouse ovarian granulosa cells

Ten-week-old female C57BL/6 mice (n = 10) were purchased from the Experimental Animal Centre of Shanghai University of Traditional Chinese Medicine. The mice were sacrificed by cervical dislocation. Ovarian tissues were isolated under sterile conditions and were placed in 4 °C phosphate-buffered saline (PBS). The ovarian tissues were minced, and 2.0 ml of hyaluronidase (0.1%, Sigma-Aldrich, St. Louis, MO, USA) was added to the tissues for 1 min of digestion at 37 °C. The tissue suspension was gently pipetted, and 200 μl of foetal calf serum (Gibco, Gaithersburg, MD, USA) was added to the suspension to terminate the digestion; the suspension was then filtered by a 200-mesh cell strainer. Next, 5.0 ml of PBS was added to the filtrate and mixed well, followed by centrifugation at 1500 r/min for 5 min at 10 °C. The supernatant was discarded, and the pellet was re-suspended in 5.0 ml of PBS, followed by centrifugation at 1500 r/min for 5 min at 10 °C. The supernatant was discarded, and the cell pellet was re-suspended with Dulbecco's Modified Eagle Medium: Ham's F-12 medium (DMEM:F12) (1:1) and mixed well; the medium contained 10% foetal bovine serum, 10 ng/ml basic fibroblast growth factor (bFGF), 10 ng/ml epidermal growth factor (EGF), 2 mM L-glutamine, 10 ng/ml growth hormone (Gh), and 15 ng/ml estradiol (E2) (Gibco, Gaithersburg, MD, USA). The cell suspension was seeded in 6-well cell culture plates, which were then incubated at 37 °C with 5% CO_2 until 80% confluency.

2.2. Preparation of a mouse model of premature ovarian failure

Ten-week-old female C57BL/6 mice (n = 20) were purchased from the Experimental Animal Centre of Shanghai University of Traditional Chinese Medicine. The mice were randomly divided into 2 groups, with 10 mice in each group. Mice in the cyclophosphamide-treated group (POF group) were given an initial intraperitoneal injection of 70 mg/kg cyclophosphamide (Sigma-Aldrich, St. Louis, USA); subsequently, one intraperitoneal injection of 30 mg/kg cyclophosphamide was given every two days for 3 weeks to construct the mouse POF model. In addition, the mice in the control group were given an intraperitoneal injection of an equal amount of saline, and the injection continued for 3 weeks, once every 2 days. This study was approved by the ethics committee of Shanghai Traditional Chinese Medicine Institute of Geriatrics (SHAGESYDW2016018), and all of the experiments followed the Laboratory Animal Regulations stipulated by State Science and Technology Commission of China.

2.3. HE staining

Briefly, all fresh tissues were fixed for 30 min by soaking in 4% para-formaldehyde (Sigma-Aldrich, St. Louis, USA) at room temperature. Tissues were dehydrated using an ethanol gradient, embedded in paraffin, sectioned (thickness of 6 μm), and dewaxed by immersion in xylene. Tissue sections were stained with haematoxylin-eosin (H & E, Sigma-Aldrich, St. Louis, USA), finally permeabilized with xylene (Sigma-Aldrich, St. Louis, USA) and then mounted with neutral resin (Sigma-Aldrich, St. Louis, USA).

2.4. Immunohistochemical staining

Briefly, all fresh tissues were fixed for 30 min by soaking in 4% para-formaldehyde (Sigma-Aldrich, St. Louis, USA) at room temperature. Tissues were dehydrated using an ethanol gradient, embedded in paraffin, sectioned (thickness of 6 μm), and dewaxed by immersion in xylene. Tissue sections were blocked with immunohistochemistry-grade