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# Long noncoding RNA growth arrest-specific 5 promotes proliferation and survival of female germline stem cells in vitro

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ABSTRACT

Female germline stem cells (FGSCs) are proposed to be a key factor for ameliorating female infertility. Previously we have shown that neonatal and adult FGSCs could be isolated and purified from mouse ovarian tissues. The long noncoding (lnc) RNA growth arrest-specific 5 sequence (GAS5) transcribed from mammalian genomes plays important regulatory roles in various developmental processes. However, there is no study on the relationship between GAS5 and FGSC development in vitro. In this study, we showed that GAS5 was highly expressed in the neonatal mouse ovary and was located in both FGSCs and oocytes. GAS5 facilitated FGSC proliferation and promoted their survival in vitro. Moreover, GAS5 also inhibited apoptosis of cultured FGSCs. These findings indicate that GAS5 is a crucial regulator of FGSC development. This might serve as a foundation for a strategy of lncRNA-directed diagnosis or treatment of female infertility.

## 1. Introduction

Fertility is a critical component of reproductive health. In female mammals, the quantity and quality of eggs are two key factors affecting fertility. It was believed that the number of oocytes in the ovary had been thought to cease during the perinatal period (Mandl and Zuckerman, 1951; Borum, 1961; McLaren, 1984; Anderson and Hirshfield, 1992), until female germline stem cells (FGSCs) have been discovered. The FGSCs carry the capacity of producing functional oocytes and fertile offspring after transplantation into ovaries (Zou et al., 2009), which are a potential source of oocytes.

The ability of stem cells to proliferate and produce a large number of differentiated progeny cells is critical for development in mammals (Kubota et al., 2004; Liu et al., 2010). Nevertheless, the processes and mechanisms underlying FGSC proliferation remain largely unknown.

Long noncoding RNAs (lncRNAs), from regions of the transcriptome that do not encode for proteins, are important regulators of cellular development, metabolism, differentiation and transcription or post-transcriptional regulator of gene expression (Costa, 2010). They can recruit transcription factors to DNA, segregating micro-RNAs (miRNAs) and destabilizing messenger (m) RNA (Gibb et al., 2011; Wapinski and Chang, 2011). LncRNAs have potential roles during the development of FGSCs in vivo (Li et al., 2017; Wu et al., 2017). Growth arrest-specific 5 (GAS5), which is encoded at 1q25, is a long noncoding RNA, a chromosomal locus that was originally isolated from NIH 3 T3 cells using subtraction hybridization (Schneider et al., 1988; Smith and Steitz, 1998). It was ubiquitously expressed in mouse tissues and detected in FGSCs (Li et al., 2017). Several studies have shown that lncRNAs act as mammalian transcriptional regulators in response to developmental signals (Costa, 2010; Derrien et al., 2012). During embryo development, the GAS5 is regarded as a vital transcriptional factor for the regulation of growth, differentiation and development (Almendral et al., 1988; Coccia et al., 1992; Friedel et al., 2009; Tani et al., 2012;). LncRNAs are also considered to play key regulatory roles in stem cells (Worringer et al., 2014). Xu et al. (2016) showed that GAS5 gene knockdown or overexpression significantly affected human embryonic stem cell (hESC) proliferation. However, whether GAS5 affects FGSC proliferation is still unclear.

Female germline stem cells (FGSCs) could be successfully isolated and purified from ovarian tissues of neonatal and adult mice (Zou et al.,

*Abbreviations:* GAS5, Growth arrest-specific 5; FGSCs, Female germ stem cells; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling; EdU, 5-Ethynyl-2′-deoxyuridine; MVH, Mouse vasa homologue; GV, Germinal vesicle; RT-PCR, Reverse transcription-PCR; qPCR, Quantitative Real-Time PCR

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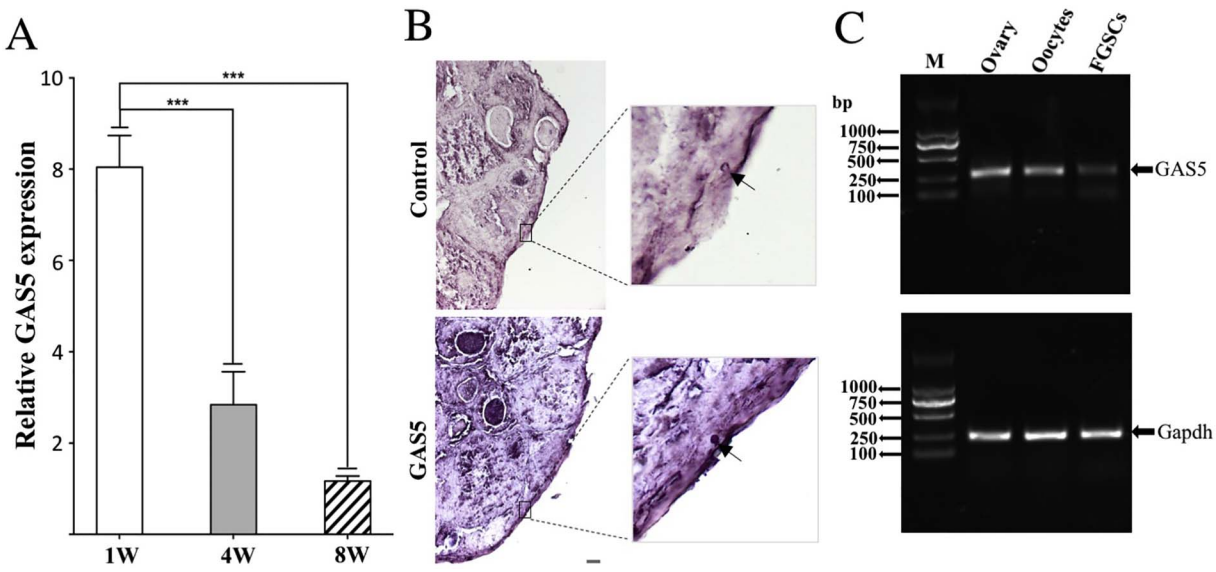


**Fig. 1.** GAS5 is highly expressed in the neonatal mouse ovary and presumptive germline stem cells. (A) The expression levels of GAS5 in ovaries of mice at different ages are shown by qRT–PCR. Neonatal, adolescent and adult mice were used at 1, 4, and 8 weeks of age, respectively. Error bars represent the SEM; $n = 3$; ***$P < 0.001$. (B) A representative image of in situ hybridization of GAS5 in the adolescent mouse ovary; the right diagram has been magnified three times. Scale bar = 50μm. (C) RT–PCR analysis of ovary, oocytes, and FGSCs. Gapdh was used as a sample loading control gene. M, DNA markers.

2009), postnatal rats and pigs, and healthy reproductive-age women (Pacchiarotti et al., 2010; Bai et al., 2013; Xie et al., 2014; Zhou et al., 2014). In this study, we investigated the role of GAS5 in FGSC development in vitro.

## 2. Materials and methods

### 2.1. Animals

One-week-old mice, 4-week-old mice and 8-week-old CD-1 strain female mice were used in these studies. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Shanghai University, and were conducted in accordance with our National Research Council Guide for Care and Use of Laboratory Animals.

### 2.2. Culture of FGSCs

An FGSC line obtained from our previous study was maintained as described with modifications (Wang et al., 2013). FGSCs were cultured on SIM mouse embryo-derived thioguanine- and ouabain-resistant (STO) feeder cells in 500 μl/well FGSC culture medium. This consisted of minimum essential medium alpha medium, 1 mM nonessential amino acids, 2 M/ml-glutamine, 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA), 10 ng/ml mouse leukemia inhibitory factor (LIF; Santa Cruz Biochemicals Inc., Dallas, TX, USA), 10 ng/ml mouse epidermal growth factor (Sigma-Aldrich), 40 ng/ml human glial cell line-derived neurotrophic factor (R&D Systems, Minneapolis, MN, USA), 10 ng/ml human basic fibroblast growth factor (bFGF; BD Biosciences, Franklin Lakes, NJ, USA), 10% fetal bovine serum (FBS), and 15 mg/ml penicillin. The medium was changed every 2 days. All cultures were maintained at 37 °C under 5% $CO_2$ in humidified air.

### 2.3. Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using Trizol reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Approximately 500 ng RNA was used to generate cDNA using M-MLV reverse transcriptase. PCR analyses were performed with Taq DNA polymerase. The amount of product was determined relative to the internal control gene for glyceraldehyde 3 phosphate dehydrogenase (Gapdh). The final PCR reaction volume of 20 μl contained 10 μl SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 1 μl cDNA template, 2 μl primer mixture and 7 μl water. The PCR thermal cycling started with denaturation at 95 °C for 10 min, followed by 30 two-step cycles: 5 s at 95 °C, 30 s at 60 °C, and 12 s at 72 °C. Amplification data were collected using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers used for qPCR were as follows: 5′–GGCAAAGGAG GATGAAGGCT–3′ (forward primer for GAS5), 5′–TGTCCACTTGTCAC AGGAGC–3′ (reverse primer for GAS5), 5′–GTATGACTCCACTCACGG CAAA–3′ (forward primer for Gapdh), 5′–GGTCTCGCTCCTGGAAG ATG–3′ (reverse primer for Gapdh). Melting curves showed that PCR yielded a single product. The Ct values for each sample were normalized against that of input DNA and percentage recovery was plotted. Results were analyzed by applying the $2 - \Delta\Delta Ct$ method.

### 2.4. Preparation of tissue sections

Ovarian tissues were rapidly rinsed in diethylpyrocarbonate (DEPC)- treated phosphate-buffered saline (PBS), then fixed overnight in 4% paraformaldehyde (PFA) in PBS and dehydrated through successive baths of ethanol (70%, 85%, 95%, and 100%) and xylol (2 × 30 min each), and embedded in three successive baths of Paraplast. After paraffin wax solidification at room temperature, sections were cut (6 μm thick, room temperature), mounted on poly-L-lysine-coated slides, air dried, and stored at 4 °C in a dry atmosphere until used for in situ hybridization (ISH) experiments. The sections were rehydrated through successive baths of xylol and ethanol (100%, 95%, 85% and 70%, respectively), DEPC-treated water, and DEPC-treated PBS (2 × 5 min each). They were then post-fixed for 10 min in 4% PFA-PBS and processed for ISH.

### 2.5. In situ hybridization

After post-fixation in 4% PFA-PBS, sections were incubated for 2 × 15 min in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5 × saline-sodium citrate (SSC) buffer. The sections were then prehybridized for 4 h at 58 °C in the hybridization mix. Probe primers (Roche) were added to the hybridization mix primer (forward