Gene 626 (2017) 158–162

Contents lists available at ScienceDirect

Gene

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

Short communication

Disruption of *NNAT*, *NAP1L5* and *MKRN3* DNA methylation and transcription in rabbit parthenogenetic fetuses

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ARTICLE INFO

Keywords:
Imprinted gene
NNAT
NAP1L5
MKRN3
Parthenogenetic
Rabbit

ABSTRACT

Parthenogenetically activated oocytes cannot develop to term in mammals due to lack of paternal gene expression. Disruption of imprinted gene expression and DNA methylation status in parthenogenetic fetuses has been reported in mice and pigs, but not in rabbits. In this study, the genomic imprinting status of the paternally expressed genes Neuronatin (*NNAT*), Nucleosome assembly protein 1-like 5 (*NAP1L5*), and Makorin ring finger protein 3 (*MKRN3*) was compared between rabbit parthenogenetic (PA) and normally fertilized fetuses (Con) using quantitative real-time PCR (qRT-PCR) and bisulfite sequencing PCR (BSP). The results revealed a significantly reduced expression of *NNAT*, *NAP1L5*, and *MKRN3* in rabbit PA fetuses compared with Con fetuses ($p < 0.05$). In addition, the BSP results demonstrated hypermethylation in the differentially methylated regions (DMRs) of *NNAT*, *NAP1L5,* and *MKRN3* in rabbit PA fetuses. Taken together, these results suggest that hypermethylation of DMRs is associated with decreased *NNAT*, *NAP1L5,* and *MKRN3* expression, which may be responsible for developmental failure of rabbit PA fetuses.

1. Introduction

Parthenogenetic (PA) animal models have been extensively used to study epigenetic profiles or to reveal the expression patterns of imprinted genes in human diseases (Liu et al., 2008; Szabo et al., 1998; Epsztejn-Litman et al., 2015). However, due to a lack of paternal gene expression, PA embryos cannot develop to term in mammals. Previous studies have demonstrated that paternally expressed genes, such as *IGF2*, *H19* and *XIST*, were abnormally expressed in PA embryos (Wang et al., 2014; Chen et al., 2014). These reports suggest that the inappropriate expression of paternally expressed genes is responsible for the developmental failure and/or abortion of mammalian parthenogenetic fetuses.

Previous studies have revealed that aberrant expression of imprinted genes is controlled by differentially methylated regions (DMRs) in parthenogenesis (Park et al., 2011; Han et al., 2013). Aberrant methylation patterns of the DMRs are epigenetically regulated by DNA methylation modifications (Oswald et al., 2000; Xu et al., 2013). This evidence was based on studies in mice (Horii et al., 2008), pigs (Park et al., 2009) and cows (Zhang et al., 2016), but limited studies have been performed in rabbits. In order to ensure epigenetic regulation of embryo development, the paternal genome undergoes active, replication independent demethylation, while the maternal genome undergoes passive, cell division dependent diffusion of methylation (Marcho et al., 2015).

Although the role of imprinted genes in embryonic development and disease syndromes has been reported, the expression patterns of paternally expressed imprinted genes in rabbit PA fetuses have not been well studied. In the present study, the DNA methylation and expression patterns of three paternally expressed genes (*NNAT*, *NAP1L5*, and *MKRN3*) in rabbit PA and normal control fetuses were determined using quantitative real-time PCR (qRT-PCR) and bisulfite sequencing PCR (BSP).

Abbreviations: BSP, bisulfite sequencing PCR; DMR, differentially methylated regions; DNA, deoxyribonucleic acid; FSH, follicle-stimulating hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hCG, human chorionic gonadotropin; MKRN3, Makorin ring finger protein 3; NNAT, Neuronatin; NAP1L5, nucleosome assembly protein 1-like 5; PA, parthenogenetic; qRT-PCR, quantitative real-time PCR

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http://dx.doi.org/10.1016/j.gene.2017.05.035
Received 17 February 2017; Received in revised form 6 May 2017; Accepted 15 May 2017
Available online 16 May 2017

Table 1
In vivo development of PA rabbit embryos.

Recipients	Embryos transferred	Pregnancy	Fetuses collected
1	53	Yes	12
2	59	Yes	9
3	46	No	0
4	48	No	0
Total	206		21

2. Materials and methods

2.1. Ethics statement

Female New Zealand white rabbits (aged 6 month, weight about 3 kg) were used in this study. Animals were cared for under standard laboratory conditions with free access to water and a standard laboratory pellet diet. All rabbit experiments were carried out in accordance with the guidelines on animal care and the use of animals in research, which were approved by the Animal Care and Use Committee of Jilin University, Changchun, China.

2.2. Production of rabbit PA fetuses

The production of rabbit PA fetuses was performed as previously described (Meng et al., 2009; Naturil-Alfonso et al., 2012). Briefly, superovulation was induced in the rabbits with follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG). After the final injection, the rabbits were euthanized and oviducts were collected. The matured oocytes were washed with activation medium (0.3 M mannitol supplemented with 100 mM $MgSO_4$ and 100 mM $CaCl_2$) and activated by 2 DC pulses of 3.2 kV/cm for 20 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA). The rabbit PA embryos were then cultured in EBSS medium supplemented with 5 mg/mL of cycloheximide and 2 mM of 6-DMAP for 1 h at 38.5 °C with 5% carbon dioxide. Approximately 30–50 PA embryos were transferred into the oviduct of the recipients. The PA and the Con (normal fertilization) fetuses were collected from each uterine horn at day 13 of gestation.

2.3. Gene expression analysis

Quantitative RT-PCR (qRT-PCR) was performed as previously described (Duan et al., 2015; Yuan et al., 2016). Briefly, total RNA was isolated from PA (n = 3) and Con (n = 3) fetuses using the TRNzol reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. qRT-PCR was performed to determine the gene expression levels of *NNAT*, *NAP1L5* and *MARN3*. The primer sequences used in this study are listed in Table S1. The $2^{-\Delta\Delta CT}$ formula was used to determine relative gene expression, which was normalized to the amount of *GAPDH* mRNA. All experiments were performed three times for each gene. Data are expressed as the mean ± SEM.

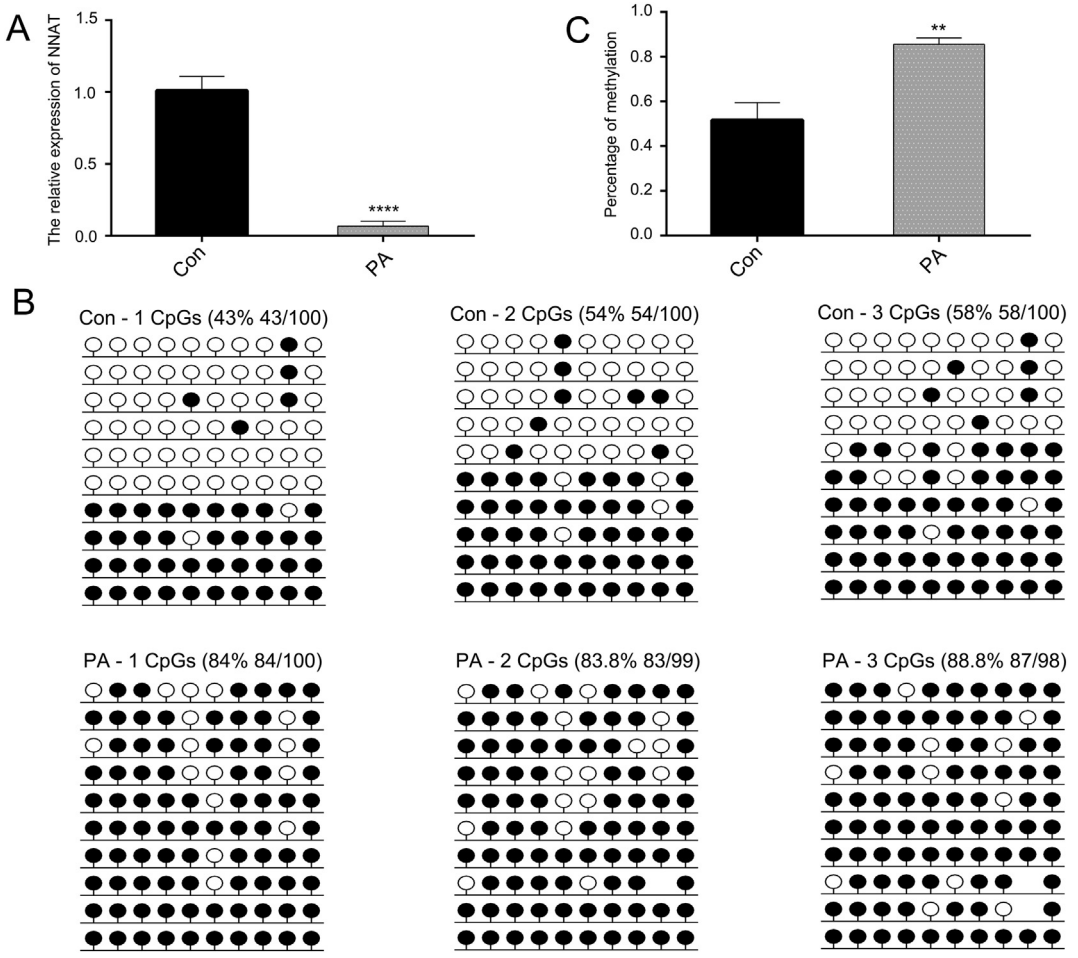

Fig. 1. The expression pattern and methylation status of *NNAT*. A, Relative expression levels of *NNAT*. B, Statistical analysis of methylated CpG sites of *NNAT* DMR. C, CpG methylation profiles of *NNAT* DMR in Con and PA fetuses. The black and white circles indicate methylated and unmethylated CpGs, respectively. The numbers indicate the proportion of methylated CpG sites relative to all counted CpG sites. The data are represented as the mean ± SEM (n = 3). $^{**}p < 0.01$, $^{****}p < 0.001$.