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Expression spectrum of new circFBN1 in various tissues and follicles of Taihang chicken

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CircRNA is a type of circular non-coding RNA that undergoes reverse splicing after 3′ end and 5′ end covalent binding (Yu *et al.* 2019), and some circRNAs may function in follicular development. Nevertheless, it is still uncertain what function circRNA performs in chicken follicles. Therefore, a new circFBN1 derived from the FBN1 gene was predicted, and RNase R digestion, qRT-PCR, and CCK-8 experiments were used to identify circFBN1 and analyze the expression spectrum and the effect on follicle granulosa cells (GCs) in Taihang chickens (a domestic breed in China) to reveal a new mechanism of regulation of follicular development in chickens.

The experimental chickens were selected by Hebei Tiankai Food Co., Ltd. and slaughtered at the age of 43 weeks. Samples of the heart, liver, spleen, lung, kidney, ovary and different grades of follicles of five chickens were collected and stored at –80°C in freezer.

Total RNA was taken out of every tissue using an EasySpin plus tissue cell RNA rapid extraction kit (Aidlab, Beijing, China). Reverse transcription was performed with the HiScript III 1st Strand cDNA Synthesis Kit (+ gDNA wiper) (Vazyme, Nanjing, China). 3 µL RNase R was used for the digestion of total RNA of small yellow follicle (SYF) at 37°C for 15 min. qRT-PCR was used with SYBR® qPCR Master mix to check the circFBN1 and FBN1 expression change between RNase R-treated RNA and untreated RNA. The 18S rRNA and β-actin genes were used as internal references. The primers are given in Table 1.

Previous work from our lab revealed that circFBN1 may be generated from exons 3, 4, 5 and 6 of the FBN1 gene. FBN1, a 350 kD glycoprotein, regulates TGF-β signaling in the extracellular matrix (Neptune *et al.* 2003). Emerging evidence suggests that FBN1 is associated with cell proliferation and apoptosis (Zhai *et al.* 2013). The gene structure of chicken FBN1 and how circFBN1 was derived, was investigated (Fig 1A) and the PCR product's reverse splicing sites were evaluated (Figs. 1A, 1B). RNase R digestion had no impact on circFBN1. Gene expression of FBN1 and β-actin was decreased significantly ($P < 0.01$) (Fig. 1C). This proves that chicken follicles did contain circFBN1. Thus, the possible coordination and compensation mechanism between circFBN1 and FBN1 mRNA was inferred, suggesting that circFBN1 is closely related to chicken cell activity.

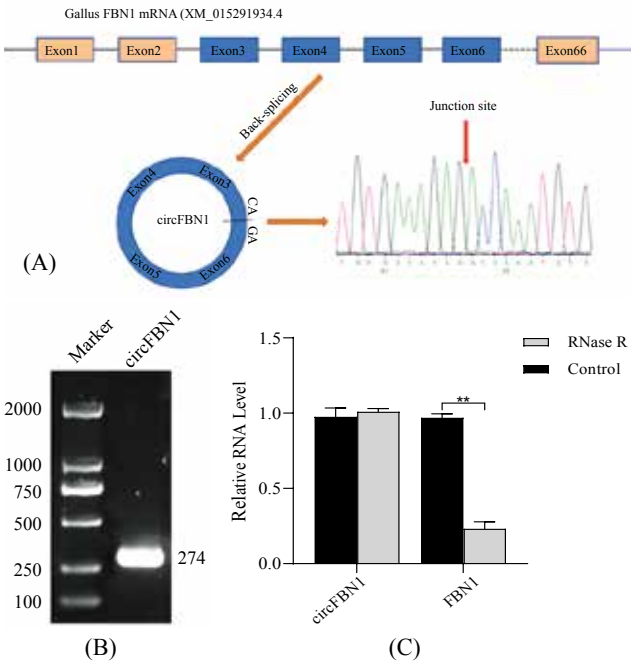

Fig. 1. Validation of chicken circFBN1: (A) CircFBN1 pattern maps and conjugated sequence; (B) Agarose gel electrophoresis of the circFBN1 conjugated sequence; (C) Relative expression of circFBN1 and FBN1 in the RNase R treatment or control group. (mean ± SEM). *$P < 0.05$, **$P < 0.01$.

The wide tissue expression of circFBN1, along with the significantly variable expression levels in each tissue, revealed that circFBN1 showed tissue expression selectivity (Fig. 2). Notably, the expression of circFBN1 in large yellow follicle (LYF) was approximately twice that

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Table 1. qRT-PCR primers

Primer	Sequence (5′→3′)	Annealing Temp (°C)	Product Size (bp)
circFBN1	F: GAACGCATTGTGGACAGCCT R: GGCTATCTGACCATTTGGGC	60	274
FBN1	F: GAAGGTGGCTATGTCTGTGGATGTC R: GGGCAGGAAGCAGTTTCAGGATG	60	352
18s rRNA	F: TAGTTGGTGGAGCGATTTGTCT R: CGGACATCTAAGGGCATCACA	60	169
β-actin	F: CTGTGCCCATCTATGAAGGCTA R: ATTTCTCTCTCGGCTGTGGTG	60	139

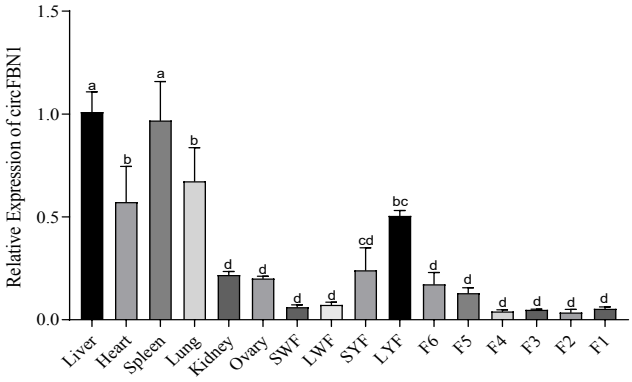

Fig. 2. Expression of circFBN1 in different chicken tissues (mean ± SEM). Different letters indicate significant differences ($P < 0.05$).

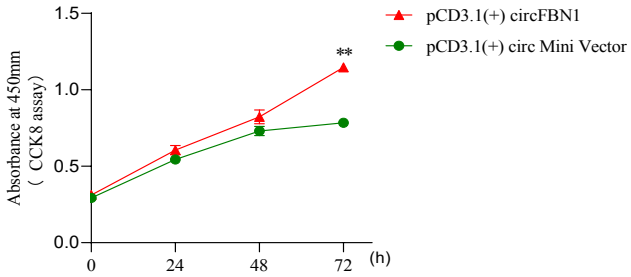

Fig. 3. CircFBN1 induces chicken GC proliferation. Follicle GC absorbance changes were detected at 0, 24, 48 and 72 h after transfection with pCD3.1(+) circFBN1 or control plasmid (mean ± SEM). *$P < 0.05$, **$P < 0.01$.

in SYF. In chickens, the ovary is a dynamic component of the female reproductive system (Etches and Petitte 1990). Follicle selection refers to the daily selection of one SYF to develop into a LYF from the SYF pool (Johnson 2015). The circFBN1 expression pattern in chickens revealed that it might be involved in follicle selection. The spatiotemporal expression profile of circRNA in Jinghai yellow chicken GCs was fully described by previous authors, and the data revealed that circRNAs are often expressed in GCs and do so in a tissue- and stage-specific way (Shen *et al.* 2019). Our study is consistent with previous studies (Zhu *et al.* 2019), which provides strong evidence that circRNA might regulate chicken reproduction.

The chicken abdominal cavity was opened aseptically. The ovaries were removed and washed with PBS, and the SYF (6-8 mm) was isolated from the ovaries. GCs were gathered on the basis of Gilbert *et al.* (1977). In a CO_2 incubator at 37°C, GCs were grown in M199 medium with 10% fetal calf serum.

The synthetic full-length circFBN1 linear sequence was cloned into the pCD3.1(+) circ Mini Vector applying the EcoRI and EcoRV restriction enzymes. GCs were seeded into 96-well plates at a suitable density, and lipofectamine 3000 was employed to transiently transfect plasmids into cells. It was divided into two groups: the pCD3.1(+) circFBN1 treatment group and the control plasmid treatment group, with six independent replicates in each treatment group. After transfection for 24, 48, and 72 h, 10 µL of CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The absorbance at 450 nm was tested with a microplate reader.

The results indicated that at 24 and 48 h, the absorbance was not significantly different. At 72 h, the absorbance of GCs transfected with pCD3.1(+) circFBN1 was extremely higher than that of GCs transfected with empty pCD3.1(+) circ Mini Vector ($P < 0.01$) (Fig. 3), indicating that GC proliferation could be induced. A review of the literature revealed that chicken circGHR positively regulates hepatocyte and myoblast proliferation (Xu *et al.* 2021), and by binding to miR-204, circFNDC3AL increases the expression of BCL9 and encourages the growth and differentiation of SMSCS in chickens (Wei *et al.* 2021). Moreover, circRNAs in follicular GCs are widely distributed at various developmental stages (Shen *et al.* 2019). These findings, which corroborate our findings, strongly imply that circRNAs have a significant function in chicken cell proliferation and could influence follicular development.

SUMMARY

In this study, the new circFBN1 derived from the FBN1 gene was identified. The expression spectrum of circFBN1 in various tissues and follicles of Taihang chickens was verified and analyzed. The effects on proliferation of GCs were checked. The results revealed that circFBN1 is indeed present and was differentially expressed in tissues and follicles and significantly promoted cell proliferation. In conclusion, our results suggested that circFBN1 could affect chicken follicular development by regulating the proliferation of GCs. These results will enable us recognize the molecular mechanisms in animal reproductive regulation.

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