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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A NOVEL CIRCULAR TRANSCRIPT OF THE *TC2N* GENE FROM RAT MAMMARY GLAND

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Abstract: The regulatory mechanism of mammary gland development and lactation is important, as lactation not only provides healthy milk for infants, but also provides health benefits to mothers. Circular RNAs (circRNAs) have recently received much attention, but only a few have been functionally characterized. In the present study, the structure and expression profile of a rat *Tc2n*-derived circular RNA (cTc2n) was investigated by several protocols. The cTc2n comprised exons 10 and 11 of the *Tc2n* gene and had no poly(A) structure. The expression of cTc2n was significantly higher in the mammary gland of rat than in other tissues, including the heart, liver, spleen, lung, kidney, uterus and ovary. The abundance of cTc2n was the highest on day 1, and significantly declined on day 7, slightly increasing on day 21 postpartum. This finding indicates that cTc2n might play an important role in lactation.

Key words: circular RNA; *Tc2n*; mammary gland; lactation; expression profile

INTRODUCTION

Mammary gland development and lactation are regulated by hormones and related metabolic pathways, the detailed mechanisms of which are still not fully investigated. The gene regulatory networks in lactation have been preliminarily revealed by high throughput sequencing techniques and bioinformatics methods in the past decade [1-2]. Interestingly, noncoding RNAs, such as microRNA and long noncoding RNA, have been recently reported to play key roles in mammary gland function [3].

Circular RNAs (circRNAs), a novel class of noncoding RNAs, have recently received much attention from molecular biologists as they are very abundant and are differentially expressed [4-5]. More than 10000 circRNAs have been predicted in human H19 cells [6], nearly 2000 circRNAs have been predicted in mouse sequences and over 700 in *Caenorhabditis elegans* [7]. Drosophila has RNA sequencing evidence for over 800 scrambled exon spliced junctions [8]. Additionally, circRNAs have been recently reported in plants, yeast and protists [9]. Although two circRNAs (CDR1as/ciRS-7 and Sry) have been evidenced as functioning as miRNA sponges [8,10], the function of most of the newly identified circRNAs remains unknown [8-11]. Thus, the current work on circular RNA is focused on elucidating their function.

In our previous study, we also found a large number of circRNA candidates in lactating rats by high throughput sequencing technology (GEO access number GSE60539). A circRNA candidate derived from nuclear tandem C2 domains gene (*Tc2n*) exons is one of the highly expressed circRNAs (cTc2n). Interestingly, it is differentially expressed in lactation stages. In the present study, we employed several procedures to confirm the circular structure of cTc2n and to analyze its expression profile in rat tissues. Moreover, the sequence character of cTc2n was also analyzed to predict its biological functions.

MATERIALS AND METHODS

RNA extraction from tissues

Sprague-Dawley rats (*Rattus norvegicus*) at the fifth gestation were obtained from Vital River Laboratories (Beijing, China). Lactating mammary glands were harvested at days 1, 7 and 21 postpartum. Other tissues, including heart, liver, spleen, kidney, ovary and uterus, were also collected from the rats on day 21 postpartum. Five rats, designated as biological replicates, were used per time point. The tissues were rinsed with sterilized saline and frozen in liquid nitrogen within 30 s after tissue dissection. Frozen samples were stored at -80°C until further analysis. The Review Committee for the Use of Animal Subjects of Jiangsu Normal University approved all the animal protocols. Total RNA was extracted from samples using RNAiso Plus (Takara) according to the standard procedures. Purity and concentration were detected by NanoDrop 2000 (Thermo, USA) before being stored at -80°C.

RNase R digestion treatment

RNA (5 μg) was incubated with or without 20 U of RNase R (Epicentre Biotechnologies) for 0.5 h at 37°C. The resulting RNA was purified by ethanol precipitation and quantified. Equal amounts of RNA with or without RNase R treatment were subjected to reverse transcription using random primers.

Reverse transcription by random primers or oligo(dT) primer

RNA (1 μg) was reverse-transcribed *in vitro* into cDNA with a PrimeScript™ RT reagent Kit with a gDNA Eraser (Takara) using random primers (Promega, Madison, MI, USA) and an oligo(dT) primer (Takara, Dalian, China), respectively.

Real time PCR

Expression of the transcript was detected using SYBR Green-based real-time PCR, which was performed using the Step One plus Real-Time PCR System (Applied Biosystems, USA) with SYBR Green I PCR premix (Takara, Dalian, China). The optimized reaction was performed in a 20-μL final reaction volume containing 10 μL of kit-supplied SYBR® Premix Ex Taq™ II, 1 μL ROX Reference Dye (50×), 0.5 μL of both the forward and reverse primers (each 10 μM), 1 μL cDNA template and 7 μL distilled water, giving a final volume of 20 μL. The outward-facing primers were used for cTc2n quantification, and inward-facing primers were used for canonical *Tc2n* quantification (Table 1). The equation $2^{-\Delta Ct}$ was used to measure the change of the novel transcript expression after RNase R digestion.

Table 1. The primer pairs for reverse transcription-PCR and real time-PCR.

Primer pairs	Accession number	Sequence	Amplicon size	Tm
**Tc2n*	KU240047	F1: AAGAAGACACGCTTACTGAAGG R1: TGAACTTGGAAGGTATTGTGCC	246	60°C
Tc2n	XM_008764840	F2: GCATCAGCCTCAAATAGCCAAA R2: GCAGATCCTTCCAGTTCATCCA	215	60°C
Gapdh	NM_017008	F3: CAGGGCTGCCTTCTCTTGTG R3: TGGTGATGGGTTTCCCGTTG	170	60°C
Rps9	NM_031108	F4: ACCCTTCGAGAAATCGCGTC R4: CTTCTCGTCCAGCGTCAACA	145	60°C
Hprt1	NM_012583	F5: CAGCGAAAGTGGAAAAGCCAA R5: AAAAGGGACGCAGCAACAGA	198	60°C

*This is an outward-facing primer pair for circular RNA amplification.

Sequencing of back-splice site

PCR was performed with outward-facing primers (Table 1). Amplification conditions were as follows: denaturation at 94°C for 4 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. Each 20 μL of PCR reaction contained 1 μL cDNA template, 10 μL of 2× Taq PCR MasterMix (Tiangen, Beijing, China), 1 μL forward primer and 1 μL reverse primer. Aliquots (140 μL) of the amplified PCR products were loaded onto 2% agarose gels for electrophoresis and visualization with ethidium bromide. The band was cut from the gel and purified using the Gel Extraction Kit (Sangon Biotech, Shanghai, China). The purified DNA fragment was subcloned to a pMD 19-T Vector and transformed into JM109 cells (Takara, Dalian, China) according to the manufacturer's instructions.

Plasmid DNA was extracted using a Plasmid DNA Extract kit and sequenced by Sanger sequencing (Sangon Biotech, Shanghai, China). The sequences were aligned with the rat *Tc2n* mRNA sequence by BLAST. The RNA secondary structures were predicted by RNAfold software [12,20].

Statistical analysis

Values were presented as means±SD. Statistical analysis was done using SPSS 19.0 with one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Identification and validation of cTc2n

RNA-seq analysis indicated that rat cTc2n is comprised of the exons 10 and 11 of the Tc2n gene. If this transcript is circular, a junction (back-splice site) between the 5' end of exon 10 and 3' end of exon 11 of *Tc2n* should exist in the cTc2n. We performed PCR with total RNA from rat mammary glands using a pair of outward-facing primers from *Tc2n* exon 11 (Fig. 1A). The PCR products were subcloned and sequenced. Fragments were observed in all samples at 246 bp (Fig. 1B). The sequence (KU240047) was aligned with *Tc2n* canonical mRNA (XM_008764840), and the back-splice site was detected. The back-splice site linked the 5' end of exon 10 and 3' end of exon 11 of *Tc2n* (Fig. 1C). The cTc2n was derived from the back-spliced exons 10 and 11 of *Tc2n*.

To further verify the circularization of the non-canonical transcript, we determined its resistance to RNA exonuclease R (RNase R). RNase R specifically digests linear RNA, both structured and non-structured, but does not digest circular or lariat RNA [13-14]. Real-time PCR was performed to detect the change in its expression after RNase R digestion using cDNAs of five rat mammary glands. The results showed a significant enrichment in the circular transcript (Fig. 2A). The result of calculation using the

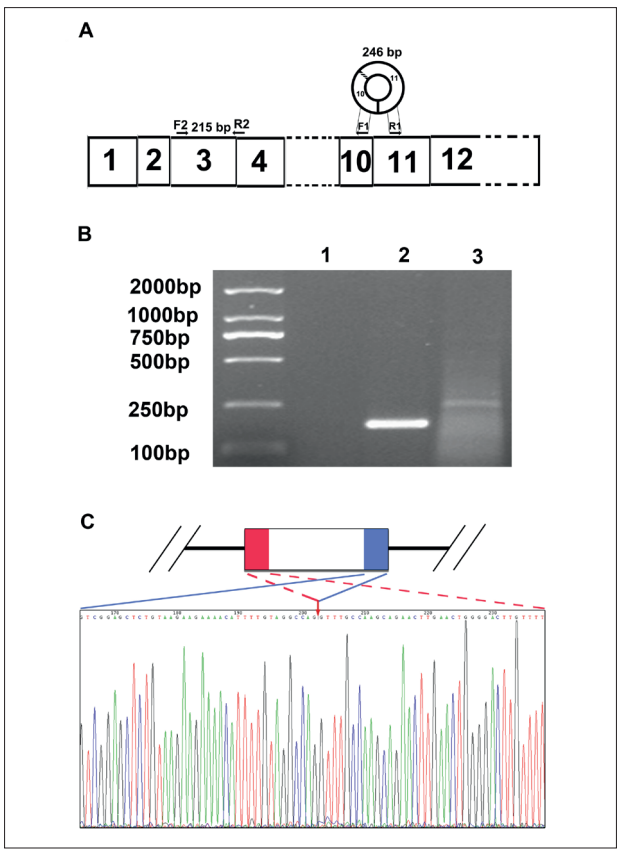

Fig. 1. Sequence structure of circular *Tc2n* (cTc2n). A – Scheme of *Tc2n*. F1/R1 primer pair is an outfacing primer used for cTc2n amplication; F2/R2 primer pair is a standard primer for linear *Tc2n* amplication. B – electrophoreses of linear (lane 2), circular (lane 3) *Tc2n*, and genomic DNA amplication control (lane 1). C – Partial sequence of cTc2n that includes the back-splice site, marked by an arrow.

equation 2-ΔCt showed there was on average an 8-fold increase in cTc2n, but a 16-fold decrease in canonical *Tc2n*, which confirmed that cTc2n was circular.

Circular RNAs lack a polyadenylated tail [15]. To investigate whether cTc2n is without a polyadenylated tail, total RNA was reverse-transcribed with either random primers or oligo(dT) primers into cDNAs. Canonical transcripts were amplified from both kinds of cDNAs; cTc2n was only amplified from random priming cDNAs (Fig. 2B). This further confirmed the circularization of cTc2n. The results also indicated that cTc2n is abundant, when compared with canonical *Tc2n*.

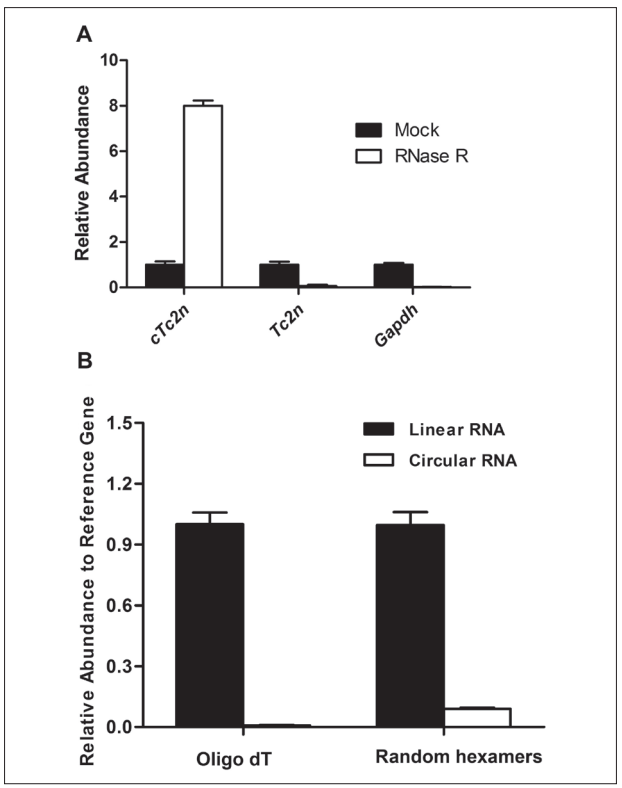

Fig. 2. Validation of the circular structure of cTc2n. A – Enrichment of cTc2n by RNase R digestion. B – Relative abundance of cTc2n to canonical *Tc2n* in cDNAs reverse-transcripted by oligo dT or random hexamers primer.

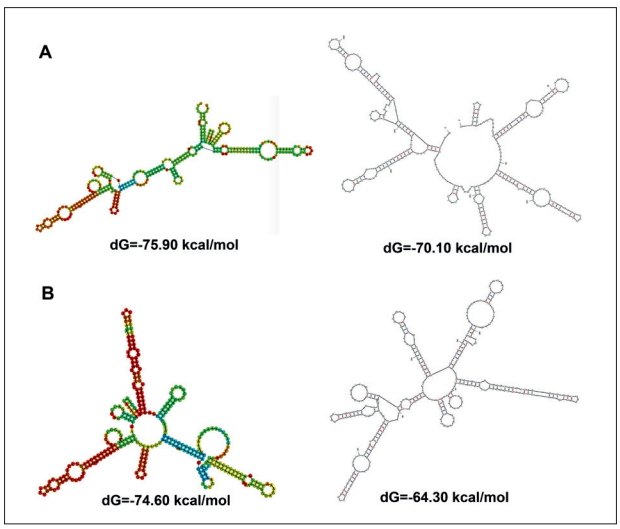

Fig. 3. Putative secondary structure of cTc2n and corresponding linear region of *Tc2n*. A – cTc2n; B – linear cTc2n. The left structure was predicted by *RNAfold*, and the right structure was predicted by *mfold* software.

Putative second structure of cTc2n

Circular RNAs do not in general act by encoding a protein [7,9], and a handful studies have indicated that they may belong to a new class of regulatory RNAs [7,11]. We compared the putative second structure of cTc2n with that of the corresponding region of *Tc2n* mRNA. The cTc2n had more stem-loop structure with higher minimum free energy than that of the corresponding region of *Tc2n* mRNA (Fig. 3).

Expression profile of cTc2n

Our previous study found that cTc2n was one of the most highly expressed circular RNAs in lactating mammary glands [16]. Therefore, we investigated the expression profile of cTc2n during lactation. The abundance of cTc2n was highest on day 1, significantly declined on day 7, and then slightly increased in day 21 postpartum (Fig. 4A). As the triacylglycerol concentration in rat milk decreased significantly during the first 5 days postpartum and then remained constant for the remaining lactation stages [17], cTc2n might be involved in milk lipid synthesis. To further confirm the importance of cTc2n in the mammary gland, we analyzed its expression in different tissues, including the heart, liver, spleen, lung, kidney, uterus and ovary in five rats on lactation day 21. Results show that the expression of cTc2n was significantly higher in the mammary gland than in the other seven tissues (Fig. 4B). Thus, the results indicated that cTc2n might play an important role in the mammary gland, which needs further study.

We browsed the circBase (http://www.circbase.org) for circular RNAs in mouse and human *Tc2n*, but we could not find a homolog of rat circular *Tc2n*, which suggested it was species-specific.

Function of cTc2n

The function of most circRNAs remains largely unclear. Two circRNA, ciRS-7/CDR1as and Sry, have been demonstrated to function as miRNA sponges

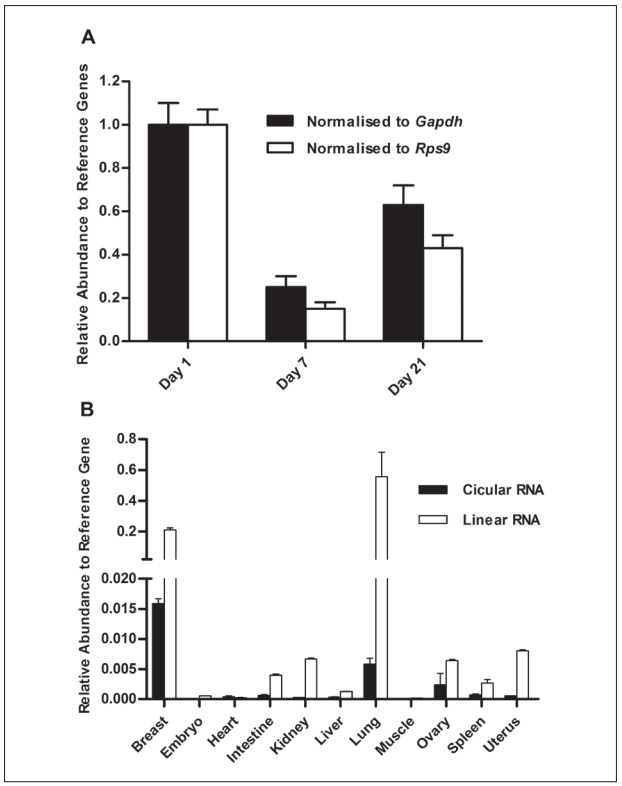

Fig. 4. Expression profile of cTc2n. A – Relative expression level of cTc2n on day 1, day 7 and day 21 postpartum. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein S9 (*Rps9*) gene was used as reference genes. B – Relative expression level of cTc2n in different tissues at day 21 postpartum. The hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) gene served as a reference gene.

[8,11]. We analyzed the putative miRNA target sites in cTc2n by BLAST software. No specific miRNA target sites were predicted to be densely distributed in cTc2n, which indicated that cTc2n does not function to modulate miRNA activity. This is in agreement with a previous report that the enrichment of microRNA binding sites was not a global feature under selection [9].

A recent work showed that circRNA biogenesis *per se* can regulate mRNA production; for example, circRNAs could compete with the linear splicing of pre-mRNA [18] and that circularization of exons correlated with exon skipping [19]. Interestingly, in the present study the expression level of *Tc2n* mRNA in rat mammary glands increased from lactation day 1 to day 7. Conversely, the expression level of cTc2n declined significantly from lactation day 1 to day 7. Furthermore, cTc2n comprised exons 10 and 11 of *Tc2n*. one of two conserved domains in the Tc2n protein, suggesting that cTc2n might suppress *Tc2n* splicing. However, this needs further confirmation.

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Authors' contributions: ShiQi Zhu, YanHong Wang, YuLong Zhao, Jia Chen, Chen Chen, YaMei Jiang and Jing Pang performed the experiments; XingTang Fang and Hong Chen analyzed the results; ChunLei Zhang designed the experiment.

Conflict of interest: The authors declare that they have no competing interests.

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