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*BMP*2 gene is a direct target gene of miR-378 in cattle

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MicroRNAs (miRNAs), small single-stranded non-coding endogenous RNA, regulate gene expression through targeting its 5'-UTR, ORF or 3'-UTR (Bartel 2004, Ma *et al.* 2016). miR-378 is differentially expressed in bovine corpus lutuem and follicle. The objective of this study was to find the target gene of miR-378 and explore the cell signal pathway of miRNA regulation in bovine ovary. Firefly-Renilla luciferase reporter assay is the most applicable method for validation of miRNA direct target gene (Alvarez 2014). In our study, the dual luciferase reporter assay was employed to test whether the *BMP2* gene is a direct target gene of miR-378 in cattle.

BMP2 gene was predicted as a target gene of bovine miR-378 (Fig. 1). The 3'-UTR sequence of *BMP2* gene was amplified from the genomic DNA with primers P1 (5'GCGCTCGAGGCCCC AGCACATGAAGTAT3') and P2 (5'AATGCGGCCGCTAGGAAAGAACAACAAACC AT 3'), and then the fragment was cloned into pmiR-RB-REPORT vector at the Xho and Not restriction sites. The miR-378 target site in *BMP2* gene was mutated from AGTCCAG to GTAAATC using the primer P3 (5'AAGGTCACAAGTTCAGTAAATCGAAAAAA AAAAAAAGTGG3') and P4 (5' CCACTTTTTTTTTT TTTCGATTTACTGAACTTGTGACCTT 3') and site-directed gene mutagenesis kit (Biyuntian, Jiangsu, China). All the constructs were sequenced in Shenggong Biotech, Shanghai, China.

For transfection, 293T cells were cultured with basic condition. miR-378 mimics or negative control RNA were ordered from Ribio Biotech, Guangzhou, China. miR-378 mimics or negative control RNA at a final concentration of 50 nM was co-transfected with 250 ng target gene wild construct or mutant using lipofectamine 2000 according to the manufacturer's instructions.

Firefly and Renilla luciferase activities were tested using the dual-luciferase reporter assay system (Promega) and read by luminometer (Mithras LB960; Berthold Technologies, Bad Wilbad, Germany). The ratio of Renilla luciferase to firefly luciferase was calculated for each well. All luciferase assays were repeated a minimum of three independent experiments with triplicate wells in each group and analyzed by Student's *t*-test.

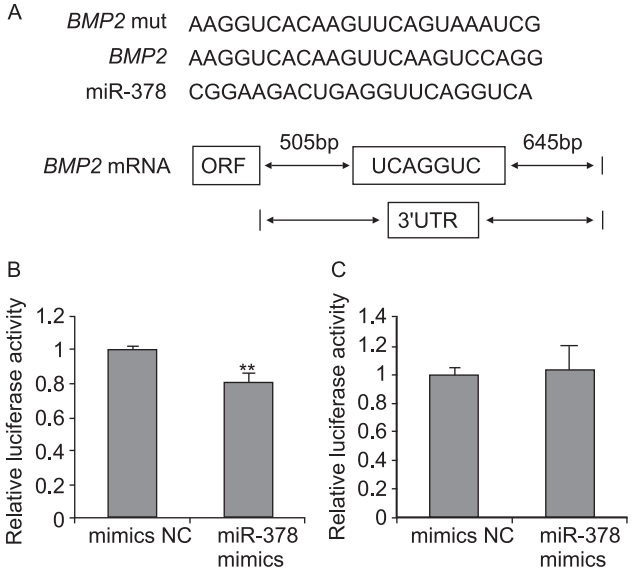

Fig. 1. miR-378 regulates expression of *BMP2* gene. (A) The putative miR-378-binding site and mutated site in the bovine *BMP2* 3'-UTR were labeled with underline (B).The relative luciferase activity assays of 293T cells co-transfected with the pmiR-RB-REPORT-*BMP2*-3'UTR construct and miR-378 mimic or NC (C) . The relative luciferase activity assays of 293T cells co-transfected with the pmiR-RB-REPORT- *BMP2*-3'UTR mutant construct and miR-378 mimic or NC. Data were represented as mean ± s.d. (n = 3). *P<0.05, **P<0.01.

In this study, miR-378 mimics had extremely significant effects on the luciferase activity of vector expressed *BMP2* 3'-UTR (Fig. 1), which suggested that *BMP2* 3'-UTR may include the binding site of miR-378. To double check this conclusion, miR-378 mimics or mimics NC was co-transfected with this mutant construct. The result indicated that miR-378 mimics had no effect on the luciferase activity (Fig.1C), which confirmed that *BMP2* is a direct target gene of miR-378.

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Bone morphogenetic proteins (BMPs) which belongs to the transforming growth factor β (TGFβ) superfamily participate in the autocrine/paracrine regulation of follicular granulosa and theca cells to affect the follicle growth and development (Gregson *et al.* 2016, Poole *et al.* 2016). In cattle, *BMP2* may play a regulatory role in development of bovine follicles to the preovulatory stage (Selvaraju *et al.* 2013). miR-378 may target the *BMP2* gene and then function in bovine follicle development, which need further investigation.

In conclusion, this study showed that the *BMP*2 is a direct target gene of bovine miR-378. Thus, miR-378 may regulate *BMP*2 gene and affect the BMP2-derived pathway in bovine follicle development.

SUMMARY

In this study, dual luciferase assay was emplyed to validate whether the *BMP2* is a direct target gene of bovine miR-378. miR-378 mimics had extremely significant effects on the luciferase activity of wild *BMP2* 3'-UTR, but had no effect on the mutated *BMP2* 3'-UTR. Our result indicated *BMP2* is a direct target gene of bovine miR-378.

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