Long Non-Coding RNA MPNCR is a Negative Regulator of Bovine Mammary Epithelial Cell Proliferation

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Abstract

Background

Numerous studies have reported that many long non-coding RNAs (lncRNAs) may play multiple essential roles during mammary gland development; however, little is known about their functions and regulatory mechanism.

Results

In this study, we confirmed a novel lncRNA, MPNCR, that was highly expressed during lactation compared to the dry period in dairy cattle. The potential biological and regulatory functions of MPNCR were explored in bovine mammary epithelial cells (BMECs). The MTT and EdU assays revealed that lncRNA MPNCR significantly suppressed cell viability and proliferation of BMECs. The subcellular localization indicated that MPNCR had a potential function as a competing endogenous RNA (ceRNA) to regulate the proliferation of BMECs. Bioinformatics analysis showed that lncRNA MPNCR had two binding sites for miR-31, further confirmed by the dual-luciferase report assay. The RT-qPCR result showed that the expression of miR-31 was downregulated following the overexpression of MPNCR in BMECs. Furthermore, the expression of MPNCR was significantly inhibited by miR-31 overexpression and upregulated by miR-31 inhibition. Cell viability and proliferation were significantly suppressed or promoted with the transfection of miR-31 inhibitor or miR-31 mimic into BMECs, respectively, whereas rescue with MPNCR reduced the proliferation of BMECs. The dual-luciferase report assay showed an interaction between miR-31 and the 3' UTR of the target gene *CAMK2D*. RT-qPCR and western blot further showed that the expression of *CAMK2D* was significantly increased with the overexpression of miR-31, while its expression was significantly suppressed with the inhibition of miR-31, indicating *CAMK2D* was a target of miR-31.

Conclusion

Taken together, these results demonstrate the function and regulatory mechanism of lncRNA MPNCR on the proliferation of BMECs, and that its regulation may contribute to bovine mammary cell population management.

Background

The development of the mammary gland undergoes periodic changes during adult life, such as growth and degeneration associated with mammary epithelial cell proliferation, differentiation, and apoptosis due to the change of age, reproductive stage, and lactation status [1–3]. In mammary gland tissue, only mature mammary epithelial cells can synthesize and secrete milk proteins, and the number of cells and secretion ability of each cell determine the milking capacity of dairy cow [4, 5]. Mammary epithelial cells maintain lactation by increasing proliferation and reducing apoptosis, which retains a dynamic equilibrium during a lactation cycle in the mammary gland [6].

Increasing evidence suggests that long non-coding RNAs (lncRNAs) play essential roles in mammary gland development and breast cancer biology [7, 8]. LncRNAs are defined as transcribed RNAs with the length of more than 200 nucleotides that lack an open reading frame and have limited protein-coding capacity; they can modulate numerous processes at the epigenetic, transcriptional, and post-transcriptional levels [9, 10]. LncRNAs play crucial roles in cell proliferation, differentiation, apoptosis, and invasion [11–13]. To date, various mechanisms have been proposed to illustrate how lncRNAs regulate gene expression. The best-characterized mechanism is lncRNAs mediating gene expression by acting as scaffolds [14], decoys [15], and signaling molecules [16]. The lncRNA AK023948 is a positive regulator for AKT in breast cancer, operating through a functional interaction with DHX9 and p85 [8]. Mouse pregnancy-induced non-coding RNA (mPINC) and Zfas1 (Znfx1 antisense 1) suppress the growth of mammary epithelial cells [17, 18]. LncRNA-HAL, which is a nuclear lncRNA, inhibits the proliferation of mammary epithelial cells at the chromatin level and through transcriptional regulation by binding to histones and hnRNPs, respectively [19]. The intergenic lncRNA EPR (epithelial cell program regulator) inhibits mammary epithelial cell proliferation by modulating gene expression in response to TGF-β [20].

One of the regulatory mechanisms of lncRNA is acting as competing endogenous RNAs (ceRNAs) by binding miRNA to modulate the expression of the sponged miRNA target [21–23]. For example, lncRNA TTN-AS1 regulates the expression of *ZEB1* through competing with miR-139-5p to enhance the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of breast cancer cells [24]. Long non-coding RNA XIST promotes chemoresistance of breast cancer cells to doxorubicin by sponging miR-200c-3p to upregulate the expression of *ANLN*, which is a downstream target gene of miR-200c-3p [25]. LncRNA-PNUTS serves as a competitive sponge for miR-205 during EMT, and its expression correlates with *ZEB* mRNA level [26].

Numerous studies have identified many differentially expressed lncRNAs in different types or phases of bovine mammary gland tissues, reflecting their potential roles in bovine mammary inflammation, immune response, and lactation based on bioinformatics and integrative analysis [27–31]. However, relatively few lncRNA has been explored for their functions in the mammary gland.

The lncRNA MPNCR (mammary proliferation-associated long non-coding RNA) has been reported to be highly expressed in the lactation period compared to the dry period of Chinese Holstein mammary tissues, and the bioinformatics analysis implied that MPNCR had a potential ceRNA regulatory mechanism in lactation [32]. Given the critical role of MPNCR in bovine lactation, we aimed to investigate its function and molecular mechanism using the bovine mammary epithelial cells (BMEC) model, which could help decipher the regulatory machinery of lactation and contribute to further improvement in milk yield in dairy cattle.

Methods

RNA isolation and RT-qPCR

Mammary gland biopsies were harvested from eight healthy Chinese Holstein dairy cows as described by Yang et al [32]. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA USA) according to the instructions and quantified by a NanoDrop 2000c Spectrophotometer (Thermo, Waltham, MA, USA). Then, 1 μg RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). MiR-31 was reverse-transcribed through a specific stem-loop RT primer using the PrimerScript RT reagent kit. RT-qPCR was performed using a Bio-Rad CFX96 Touch Real-time PCR detection system (BioRad, Hercules, CA, USA), and the relative abundance of the mRNAs was determined using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) according to the manufacturer's instructions. The following thermal settings were used for lncRNA and coding gene: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 54 °C for 30 s. The RT-qPCR conditions for miR-31 were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 12 s and 60 °C for 34 s. The primers used for RT-qPCR are listed in Table 1.

Table 1
Real-time quantitative PCR primer information

gene	Sequence (5' ~3'; F: forward, R: reverse)	Product size (bp)
lncRNA MPNCR	F: tattgagtgactttcatcgta	192
	R: tagagtttgagatttcttgga	
CAMK2D	F: cattgacacctaaatggcatag	125
	R: tctccttggacttctatggc	
GAPDH	F: agatggtgaaggtcggagtg	189
	R: cgttctctgccttgactgtg	
miR-31 RT-primer	ctcaactggtgtcgtggagtcggcaattcagttgagagctat	
U6	F: ctcgcttcggcagcaca	94
	R: aacgcttcacgaatttgcgt	
miR-31	F: tgcccgaggcaagatgctgg	57
	R: gtgtcgtggagtcggcaa	

Plasmid construction

The pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) was used to construct lncRNA MPNCR overexpression plasmid to explore its function in BMECs. The full-length complementary sequence of MPNCR was amplified by PCR from the BMECs genome. Then, the PCR product was digested with *XhoI* and *BamHI* restriction enzymes and cloned into the pcDNA3.1(+) vector. The constructed overexpression vector was named pcDNA3.1(+)-MPNCR. The potential binding sites between MPNCR and miR-31 were predicted by RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). The potential target genes *MAPK6*, *MAPK14*, and *CAMK2D* of miR-31 were predicted by miRBase

(http://www.mirbase.org/) and TargetScan (http://www.targetscan.org/vert_71/). The target sequences of lncRNA MPNCR and the 3'-UTRs of *MAPK6*, *MAPK14*, and *CAMK2D* containing the wild binding sites of miR-31 were amplified by PCR, and the 3'-UTRs of these three genes containing the mutant binding sites of miR-31 were obtained by overlap PCR. Then, the PCR products were cloned into the downstream of the Renilla gene of the psiCHECK-2 vector (Promega, Madison, WI, USA), which was digested by the restriction enzymes *Xho*I and *Not*I (NEB, New England) for the dual-luciferase assay. The PCR products' sizes and overlap primer information are listed in Table 2. The miR-31 mimic, mimic-NC, inhibitor, and inhibitor-NC were synthesized by GenePharma Co., Ltd. (Shanghai, China).

Table 2
Vector construction primer information

gene	Sequence (5' ~3'; F: forward, R: reverse)	Product size (bp)
lncRNA MPNCR	F: CGggatcctcacatattgctgaagcctggc	1176
	R: CCGctcgagctggaaacctcacacgcagc	
lncRNA MPNCR-3'UTR	F: CCGctcgaggtcagtcagtcatctgactc	569
	R: ATTTgcggccgcgcttcctgtaaaagggcag	
MAPK6-3'UTR	F1: CCGctcgagctggcatgtcgtttgcac	396
	R1: ctcactgtgtaaatttcatccgttctttttgtttcctgtaaaac	
	F2: gttttacaggaaacaaaaagaacggatgaaatttacacagtgag	
	R2: ATTTgcggccgccgctggctaaatagaagtgc	
MAPK14-3'UTR	F1: CCGctcgaggaccttccgggtagttcagaac	300
	R1: agtttgttgaaaaccagatccgttctatggaaatgctatacatcc	
	F2: ggatgtatagcatttccatagaacggatctggttttcaacaaact	
	R2: ATTTgcggccgcagcattggttactgggagtgaag	
CAMK2D-3'UTR	F1: CCGctcgagcactttaagcatgtggtgt	252
	R1: atcagtcatttaccattatccgttctaatatatacagtgtcccatg	
	F2: catgggacactgtatatattagaacggataatggtaaatgactgat	
	R2: ATTTgcggccgctcagcagaaatcacaaatttat	

Note: The nucleotides in bold and underline indicate the restriction enzyme sites. "ATTT", "CG" and "CCG" represent the protective nucleotides. The nucleotides in italic and bold represent the mutant binding sites of miR-31.

Cell culture and transfection

The bovine mammary epithelial cell line was used to explore the function and regulatory mechanism of lncRNA and miRNA. It was obtained from the primary bovine mammary alveolar cells by stable transfection with SV-40 T antigen [33] and cultured in a 1:1 mixture of Ham's F-12 and Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin according to Jiao et al [34]. LncRNA MPNCR overexpression plasmid vector, miR-31 mimic or inhibitor were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions when the cells were at 60–80% confluence.

The luciferase assay was used to test possible interactions of miR-31 with its target genes and with lncRNA MPNCR using HEK293T cell transfections. HEK293T cells were cultured in DMEM and supplemented with 10% FBS and 100 U/mL penicillin-streptomycin at 37 °C with 5% CO_2, and 600 ng/mL wild (or mutant) dual-luciferase reporter vector. Then, and 20 nM miR-31 mimic (or mimic-NC) per well were co-transfected into HEK293T cells using Lipofectamine 2000. The transfected HEK293T cells were lysed with passive lysis buffer, and the cell lysate (10 μL) was added into a 96-well enzyme label plate. Then, the optical density (OD) was measured using a 30 μL luciferase assay reagent on a M200 Pro (Tecan, Mannedorf, Switzerland). The data were presented as normalized relative luciferase activity (Renilla/Firefly, OD/OD). Each experiment was performed in triplicate.

MTT assay and EdU staining

To assess the effect of MPNCR on BMEC proliferation, the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) and 5-Ethynyl-2'-deoxyuridine (EdU) assays were performed. The bovine mammary epithelial cells were seeded in a 96-well plate. When the confluence reached 1×10^4 cells per well, 100 ng pcDNA3.1(+)-MPNCR vector, 20 nM miR-31 mimic (and mimic-NC) or 40 nM miR-31 inhibitor (and inhibitor-NC) per well were transfected into BMECs by lipofectamine 2000 according to the manufacturer's protocol with six replicates. The MTT treatment and absorbance measurements were performed, as described in Zhang et al [35].

EdU staining was performed when BMECs reached a confluence of 1×10^4 cells per well in 96-well plates with three replicates per condition. PcDNA3.1(+)-MPNCR (100 ng per well), miR-31 mimic (20 nM), or miR-31 inhibitor (40 nM) were transfected into BMECs. After 36 h, cells were stained with 50 μM Edu (Ribobio, Guangzhou, China) for 2 h according to the manufacturer's protocol. The cells were visualized under a fluorescence microscope with 50 μm scale. The ratio of EdU positive cells was calculated according to the formula (EdU staining cells/the total of cells) × 100%.

Nuclear and cytoplasmic RNA fraction

To determine the location of MPNCR in BMECs, a PARIS kit (Life Technologies, Shanghai, China) was used to extract nuclear and cytoplasmic RNA according to the manufacturer's instruction. The RNA was then transcribed into complementary DNA, and the location of MPNCR was analyzed by the PCR method.

Western blot analysis

To detect the effects of MPNCR and miR-31 on their target genes at the protein level, a Western blot assay was performed. The total protein was extracted from BMECs transfected with the overexpression vector pcDNA3.1(+)-MPNCR (or pcDNA3.1(+)) (600 ng/mL), miR-31 mimic (or mimic-NC) or miR-31 inhibitor (or inhibitor-NC) (20 nM) for 72 h, using radioimmunoprecipitation assay (RIPA) cell lysis solution (Applygen, Beijing, China). The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred onto a poly (vinylidene difluoride) (PVDF) membrane (Millipore Corporation, MA, USA). Then, the membranes were sealed with 5% skimmed milk solution for 1 h at room temperature and incubated at 4 °C for 12 h with the primary antibody against *CAMK2D* (Abcam, Cambridge, UK). Then the PVDF membrane was incubated with an HRP conjugated secondary antibody goat-anti-rabbit IgG (Zhongshan-Bio, Beijing, China) for 2 h. After washing three times with PBS, the protein bands were observed with a Super ECL Plus detection kit (Applygen, Beijing, China) using a Mini Chemiluminescent Imaging and Analysis System (Beijing Sage Creation Science Co., Beijing, China). Densitometry analysis was performed using Sage Capture software (Beijing Sage Creation Science Co.) using GAPDH (Abcam) as a control.

Results

LncRNA MPNCR inhibits BMECs proliferation

Figure 1a shows that the expression of lncRNA MPNCR has an over eightfold difference in the lactation period compared to that in the dry period ($P < 0.001$) using RT-qPCR. This result confirms our previous observations of the transcriptome sequencing analysis [32].

To determine the role of lncRNA MPNCR in mammary epithelial proliferation, the overexpression vector of MPNCR was transfected into BMECs. The RT-qPCR results of MPNCR showed over 20 fold difference in expression in cells transfected with pcDNA3.1(+)-MPNCR compared to the control group transfected with the pcDNA3.1(+) vector (P < 0.001) (Fig. 1b). Following the overexpression of MPNCR, the MTT and EdU assays were performed. The MTT result showed that the overexpression of MPNCR inhibited the cell viability of BMECs compared with the control group ($P < 0.001$) (Fig. 1c). EdU staining showed that the number of EdU positive staining cells was significantly decreased in the MPNCR overexpression group compared to the control group (P < 0.01) (Figs. 1d and 1e). Taken together, these results demonstrated that lncRNA MPNCR inhibited the proliferation of BMECs.

LncRNA MPNCR acts as a molecular sponge for miR-31

LncRNA can act as a ceRNA by sponging miRNA to reduce the regulatory effect of miRNA on its target [22, 25]. To explore the regulatory mechanisms by which lncRNA MPNCR affects the proliferation of BMECs, we determined its subcellular localization using the PCR method. The result showed that lncRNA MPNCR was localized to the cytoplasm and nucleus (Fig. 2a). Bioinformatics analysis revealed two seed region binding sites between MPNCR and miR-31 (Fig. 2b). To examine possible interactions between MPNCR and miR-31, the dual-luciferase reporter assay was performed. The result revealed that overexpression of miR-31 could significantly reduce the relative luciferase activity of MPNCR compared

with the control group ($P< 0.05$) (Fig. 2c), indicating an interaction between MPNCR and miR-31. Then, the expression level of miR-31 was measured after transfecting vectors pcDNA3.1(+)-MPNCR and pcDNA3.1(+)into BMECs. The RT-qPCR result showed an over two-fold downregulation in the expression of miR-31 with the overexpression of MPNCR ($P< 0.001$) (Fig. 2d). To identify the optimal overexpression or inhibition effects, different concentrations of miR-31 mimic and inhibitor were transfected into BMECs, respectively. The RT-qPCR results showed that a 20 nM of miR-31 mimic could dramatically increase the expression of miR-31 more than 20 times ($P< 0.001$), and a 40 nM miR-31 inhibitor could significantly suppress its expression about 10 times ($P< 0.001$) (Fig. 2e). LncRNA MPNCR was significantly downregulated when miR-31 was overexpressed ($P< 0.01$), and its expression was upregulated when miR-31 was inhibited compared to their control groups ($P< 0.05$) (Fig. 2f). These data demonstrated that MPNCR contained the functional binding sites of miR-31.

To further investigate the function of miR-31 in BMEC proliferation, MTT and EdU assays were performed after transfecting miR-31 mimic, inhibitor and their controls into BMECs. The MTT results showed that BMEC viability was significantly increased with the overexpression of miR-31 compared with the mimic-NC group ($P< 0.01$) (Fig. 2g). Cell viability was dramatically decreased with the inhibition of miR-31 ($P< 0.05$) compared with its control (Fig. 2g). EdU assay showed that the EdU positive staining cell number was significantly higher with the overexpression of miR-31(Fig. 2h), indicating that the proliferation of BMECs was increased, while the result was reversed with the suppression of miR-31. The result of the EdU assay was consistent with that of the MTT assay result ($P< 0.01$) (Fig. 2i). Thus, it could be deduced that lncRNA MPNCR and miR-31 have converse effects on BMEC proliferation.

CAMK2D is the target of miR-31

To explore how miR-31 regulates BMEC proliferation, the targets of miR-31 were predicted by miRBase (http://www.mibase.org) and TargetScan (http://www.targetscan.org/vert_71). Given their roles in mammary gland development, the *MAPK6*, *MAPK14*, and *CAMK2D* genes were selected as the potential targets of miR-31. The constructed dual-luciferase reporter vectors were co-transfected into HEK293T cells with miR-31 mimic, mimic-NC, miR-31 inhibitor, or inhibitor-NC. The renilla/firefly luciferase ratio showed that only the luciferase activity of the wild type 3' UTR of *CAMK2D* was significantly reduced upon miR-31 overexpression, while the mutant 3' UTR was not changed (Fig. 3a, 3b, and 3c) ($P< 0.01$), which demonstrated that miR-31 could bind to the 3' UTR of the *CAMK2D* gene. Then RT-qPCR and western blot were used to detect the expressions of *CAMK2D* at mRNA and protein levels with miR-31 overexpression and inhibition. The RT-qPCR results showed that the expression of *CAMK2D* was significantly decreased with the transfection of miR-31 mimic compared to the mimic-NC group (Fig. 3d) ($P< 0.01$), while its expression was significantly increased with the transfection of miR-31 inhibitor compared to the inhibitor-NC group (Fig. 3d) ($P< 0.01$). Western blot results further revealed that the overexpression of miR-31 inhibited CAMK2D expression at the protein level ($P< 0.01$), while the inhibition of miR-31 could increase CAMK2D protein expression ($P< 0.05$) (Fig. 3e and 3f). These results demonstrated that *CMAK2D* was a target gene of miR-31.

LncRNA MPNCR modulates CAMK2D by functioning as a ceRNA

We investigated whether MPNCR functions as a molecular sponge of miR-31 for the regulation of *CAMK2D*. The expression of *CAMK2D* was quantified by RT-qPCR and western blot following transfection with pcDNA3.1(+)-MPNCR and pcDNA3.1(+) vectors into BMECs. The RT-qPCR results showed that the *CAMK2D* mRNA expression was dramatically upregulated with the overexpression of MPNCR ($P < 0.001$) (Fig. 4a). The western blot analysis further demonstrated that the overexpression of MPNCR could significantly suppress the expression of CAMK2D at the protein level ($P < 0.01$) (Figs. 4b and 4c). Moreover, the rescue experiment was performed with the co-transfection of miR-31 mimic and pcDNA3.1(+) vector, miR-31 mimic and pcDNA3.1(+)-MPNCR vector, or pcDNA3.1(+)-MPNCR vector and mimic-NC into BMECs. The MTT assay showed that the promoting effect of miR-31 on BMEC cell viability was significantly inhibited with the co-transfection of MPNCR overexpression vector and miR-31 mimic compared to the control group ($P < 0.05$) (Fig. 4d). These results further confirmed that lncRNA MPNCR interacts with miR-31 and, as a ceRNA, inhibits the function of miR-31.

Discussion

The objective of this study was to explore the potential regulatory and biological functions of the lncRNA MPNCR in the epithelial cells of the mammary gland. The number of mammary epithelial cells determines the milk production and lactation curve of dairy cows [36]. Therefore, the proliferation of BMECs is significant for lactation. Non-coding RNAs are untranslated RNA molecules that function to regulate gene expression in multiple biological processes [37]. LncRNAs lack an open reading frame, and they can act as a decoy for miRNAs resulting in release of miRNA-targeted mRNAs from silencing and thereby affect the expression of mRNA, protein, and the further function of targeted-genes [38]. Although several lncRNAs have been associated with lactation in cattle, little is known about their function and regulatory mechanism [31, 32, 39]. In a previous study using high-throughput sequencing analysis, we found that lncRNA MPNCR was highly expressed in lactating cows versus dry cows [32]. RT-qPCR in the current study verified this expression pattern. However, the function and regulatory mechanism of MPNCR in lactation are unknown. Therefore, BMECs were used in this study as an *in vitro* model to understand the function of MPNCR in bovine lactation. In exploration of the regulatory mechanisms of MPNCR, the MTT and EdU assays revealed that MPNCR was a negative regulator during bovine mammary epithelial cell proliferation.

In recent years, increasing evidence has suggested that lncRNAs contain binding sites for miRNA and function as molecular sponges to reduce the effect of miRNA on target mRNA [40–42]. The miR-31 has been found to promote mouse mammary epithelial cell proliferation and mammary stem cell expansion [43]. Interestingly, miR-31 had a higher expression in bovine mammary gland tissues in early lactation than in the dry period [44]. Bioinformatics analysis showed that lncRNA MPNCR had potential binding sites with miR-31. Indeed, the dual-luciferase reporter assay used in this study showed that MPNCR does

interact with miR-31. Overexpression of miR-31 and MPNCR further confirmed that MPNCR contained the functional binding sites of miR-31. These results identified MPNCR as a lncRNA negative regulator of bovine mammary gland development that could inhibit the proliferation of BMECs through regulating the expression of miR-31.

MiRNA can directly bind to the 3' UTR of target mRNA to induce the target's degradation or inhibit its translation. The Ca^{2+}/calmodulin dependent protein kinase IIδ (*CAMK2D*) gene was found to be a target of miR-31. The Ca^{2+}/calmodulin-dependent protein kinase (CAMK) is an extensively expressed, multifunctional Ser/Thr protein kinases, which function through Ca^{2+} signaling to regulate the β cells development [45]. The CAMKs family includes CAMKⅠ, CAMKⅡ, and CAMKⅣ, and each has many isoforms [46]. CAMKII is encoded by four genes (α,βⅠγⅠδ)Ⅰwhere δ isoforms are widely distributed in almost all tissues [47, 48]. The CAMKⅡ isoform participates in multiple cell proliferation. The *CAMK2g* is an inhibitor of myeloid leukemia cell proliferation and negatively regulates vascular smooth muscle cell proliferation [49, 50]. In myogenesis, a miR-31 overlapping lncRNA transcript controls myoblast differentiation [51]. Previous studies have demonstrated that *CAMK2D* was associated with vascular cell proliferation, differentiation, and apoptosis [52–54]. It has been reported that retinoic acid induces miR-31-5p expression and inhibits the proliferation and differentiation of C2C12 cells by suppressing *CamkIIδ* expression [55]. Based on bioinformatics analysis and its role in cell proliferation, in this study, *CAMK2D* was identified and verified as a target of miR-31, which was consistent with the above studies. The expression of *CAMK2D* was upregulated at the mRNA and protein levels when lncRNA MPNCR was overexpressed, while the expression of miR-31 was decreased (Fig. 5).

Conclusions

This study revealed the function and regulatory mechanism of lncRNA MPNCR in BMECs. LncRNA MPNCR inhibits the proliferation of BMECs by acting as a ceRNA that sponges miR-31 to upregulate the expression of *CAMK2D* (Fig. 5).

Abbreviations

lncRNA MPNCR: mammary proliferation-associated long noncoding RNA;

BMECs: bovine mammary epithelial cells;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

EdU: 5-ethynyl-2'-deoxyuridine (EdU);

RT-qPCR: real-time quantitative PCR;

DE: differentially expressed;

FBS: fetal bovine serum;

3'-UTR: 3'-untranslated region;

CAMK2D: calcium/calmodulin dependent protein kinase II delta;

MAPK6: mitogen-activated protein kinase 6;

MAPK14: mitogen-activated protein kinase 14.

EQUATIONS AND FORMULAS

1. ratio of EdU positive cells = (EdU staining cells/the total cells) ×100%.

2. relative luciferase activity = OD490 of Renilla/OD490 of Firefly

Declarations

Author's contributions

LX.W. performed the MTT, EdU, vector construction, RT-qPCR, western blot and wrote the draft. YL.Z. analyzed the data. LH.L., F.L. XL.Z. and WD.Z. cultured cells and performed western blot. H. K. participated in the experimental design and manuscript writing. X.W. designed the experiment.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the experimental procedures involving cows were reviewed and approved by the Experimental Animal Management Committee of Northwest A&F University (2011-31101684).

Consent for publication

Not applicable.

Competing interests

The authors declare tht they have no competing interests.

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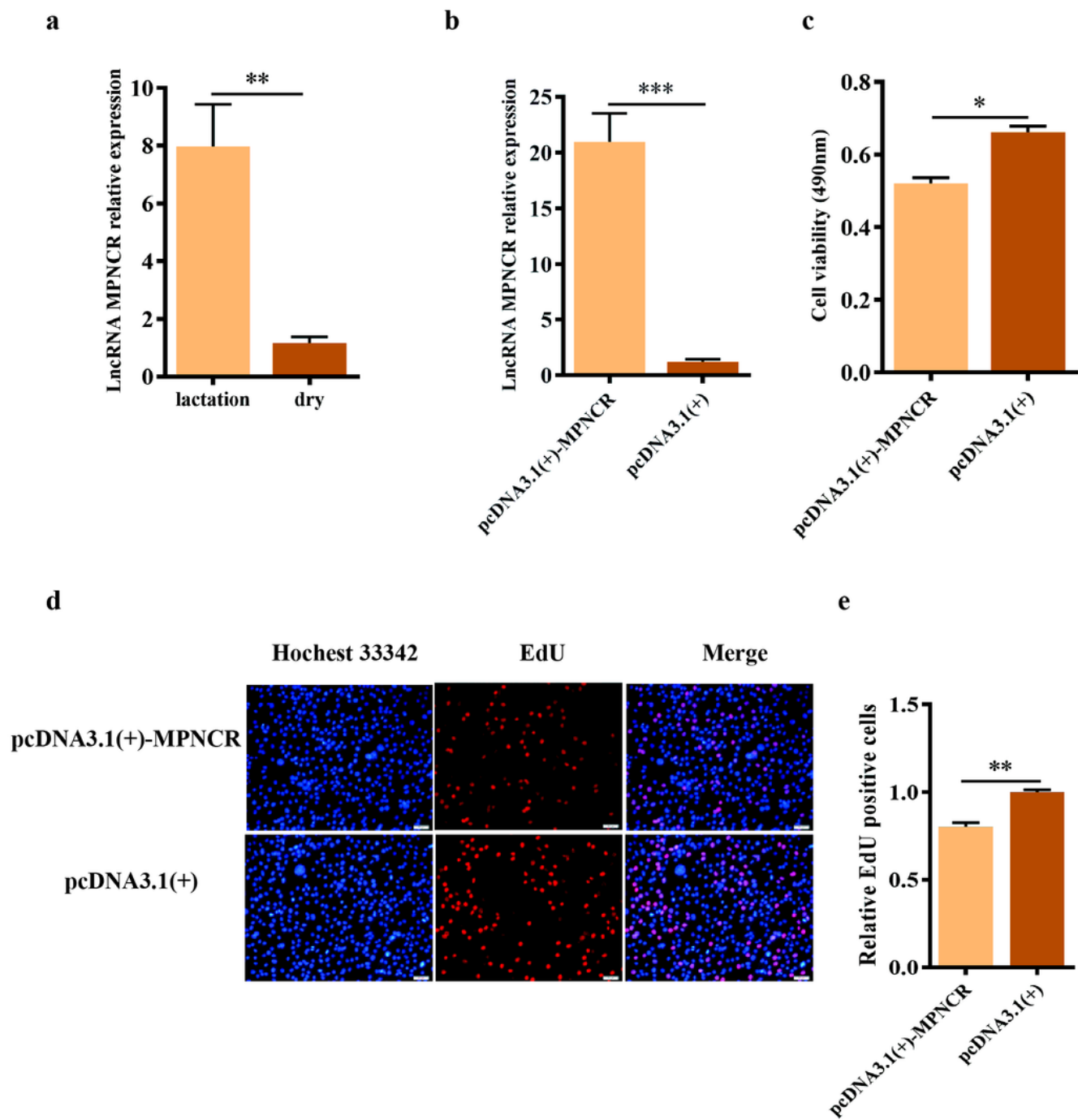
Figures

Figure 1

LncRNA MPNCR inhibited BMEC proliferation. (a and b) RT-qPCR analysis of the expression of MPNCR in lactation and dry period of bovine mammary gland tissues (a) and in BMECs after lncRNA MPNCR overexpression (b). (c) MTT assay demonstrated the effect of lncRNA MPNCR on BMEC viability. (d and e) EdU staining showed that MPNCR inhibited BMEC proliferation by fluorescence (d) (scale bar is 50

µm.) and quantified positive cells (e). All data were presented as mean±SEM (unpaired t-test; n = 3); **: P < 0.01, ***: P < 0.001.
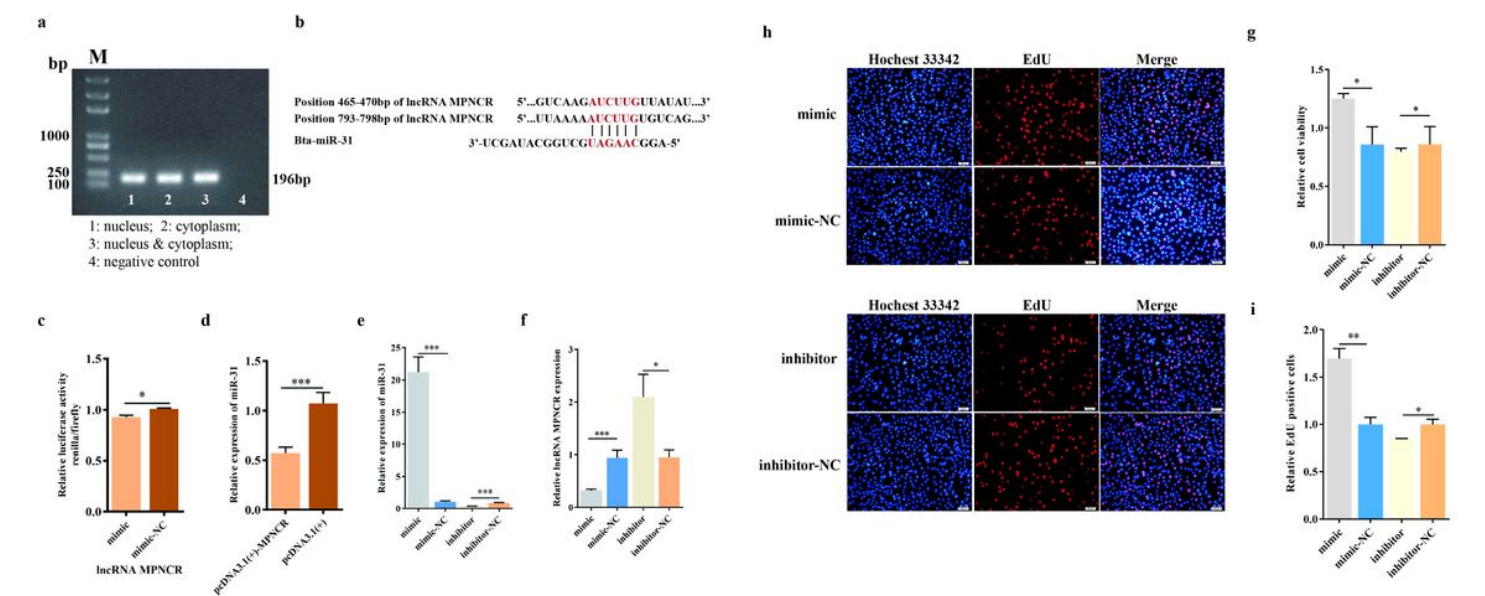
Figure 2

The interaction between lncRNA MPNCR and miR-31. (a) lncRNA MPNCR was located in both the nucleus and cytoplasm. (b) Predicted binding sites of lncRNA MPNCR and miR-31. (c-f) lncRNA MPNCR binds to miR-31 and regulates its activity. (c) The relative luciferase activity detection. (d)The expression of miR-31 was inhibited by lncRNA MPNCR. (e) Detection of miR-31 overexpression efficiency. (f) The expression of lncRNA MPNCR after miR-31 overexpressed or inhibited in BMECs for 48h. (g) MTT assay detected the effect of miR-31 on BMECs viability. (h) EdU staining examined BMEC proliferation after miR-31 overexpressed or inhibited. Scale bar is 50 µm. (i) Percentage of EdU positive cells rate. Data were presented as means ± SEM (n=3); *: P<0.05, **: P<0.01, ***: P<0.001.
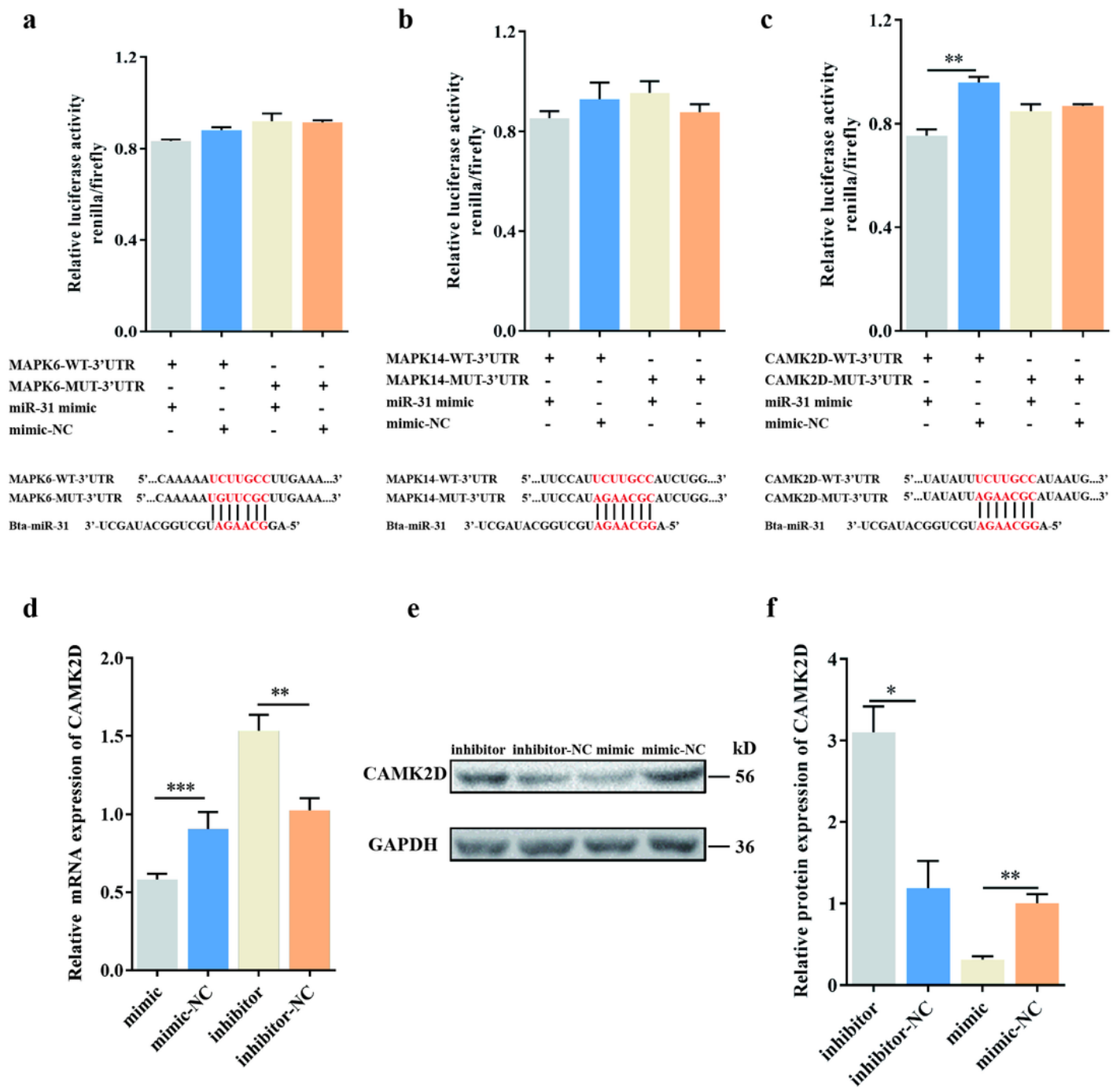
Figure 3

CAMK2D was the target of miR-31. (a-c) The relative luciferase activity between MAPK6, MAPK14 , CAMK2D and miR-31. (d) Relative mRNA expression of CAMK2D transfected with miR-31 mimic and inhibitor into BMECs. (e,f) Western blot and gray intensity analysis of CAMK2D after transfection with miR-31 mimic and inhibitor into BMECs. GAPDH was taken as a control. Data were presented as means ±SEM (n = 3); *: P < 0.05, **: P < 0.01.
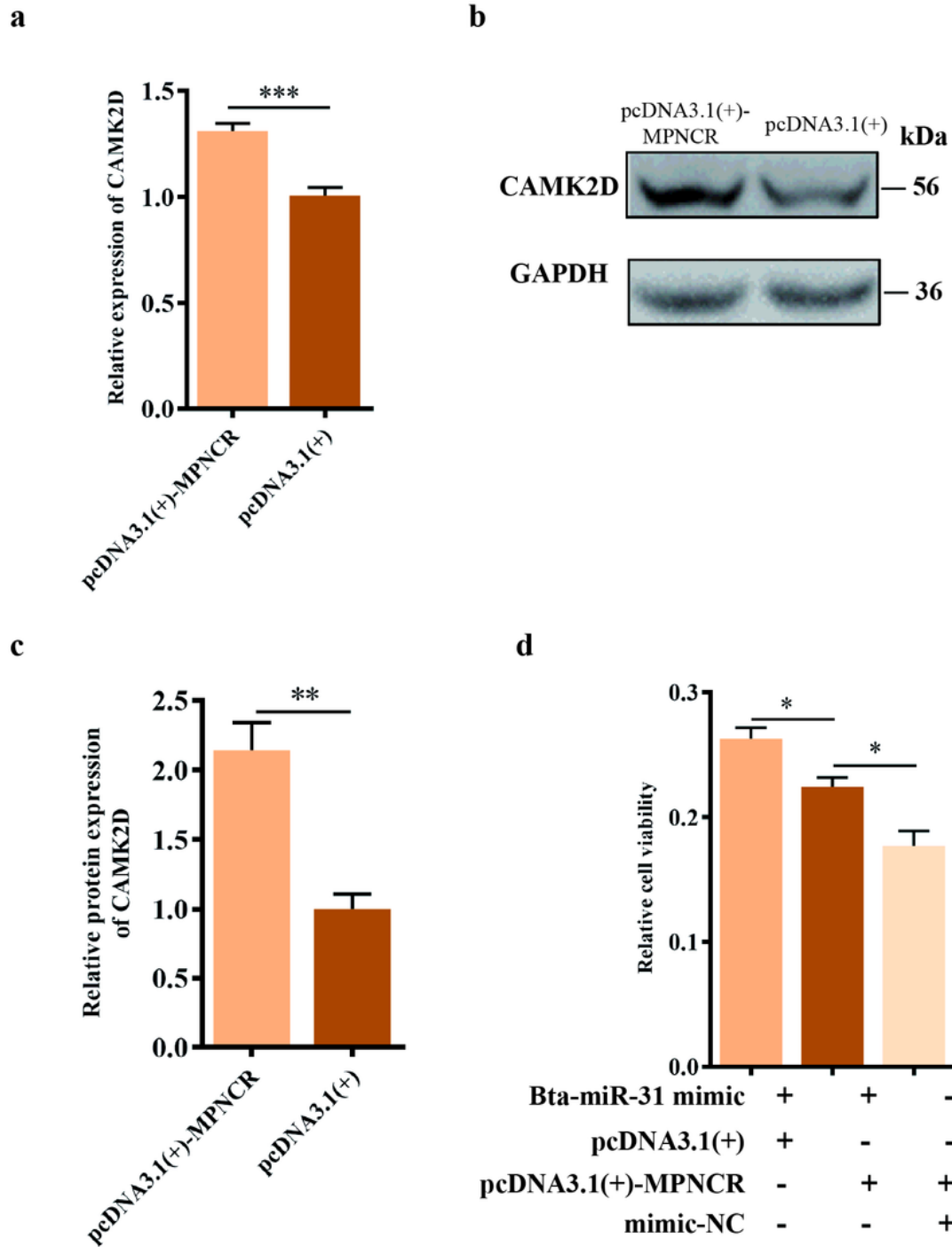
Figure 4

LncRNA MPNCR upregulated the expression of CAMK2D as a ceRNA. (a) Relative mRNA expression of CAMK2D after the overexpression of MPNCR in BMECs. (b and c) Western blot and gray intensity analysis demonstrated that MPNCR upregulated the expression of CAMK2D. (d) MTT assay determined BMEC viability. Data were presented as means ±SEM (n = 3); *: P < 0.05, **: P < 0.01, ***: P < 0.001.
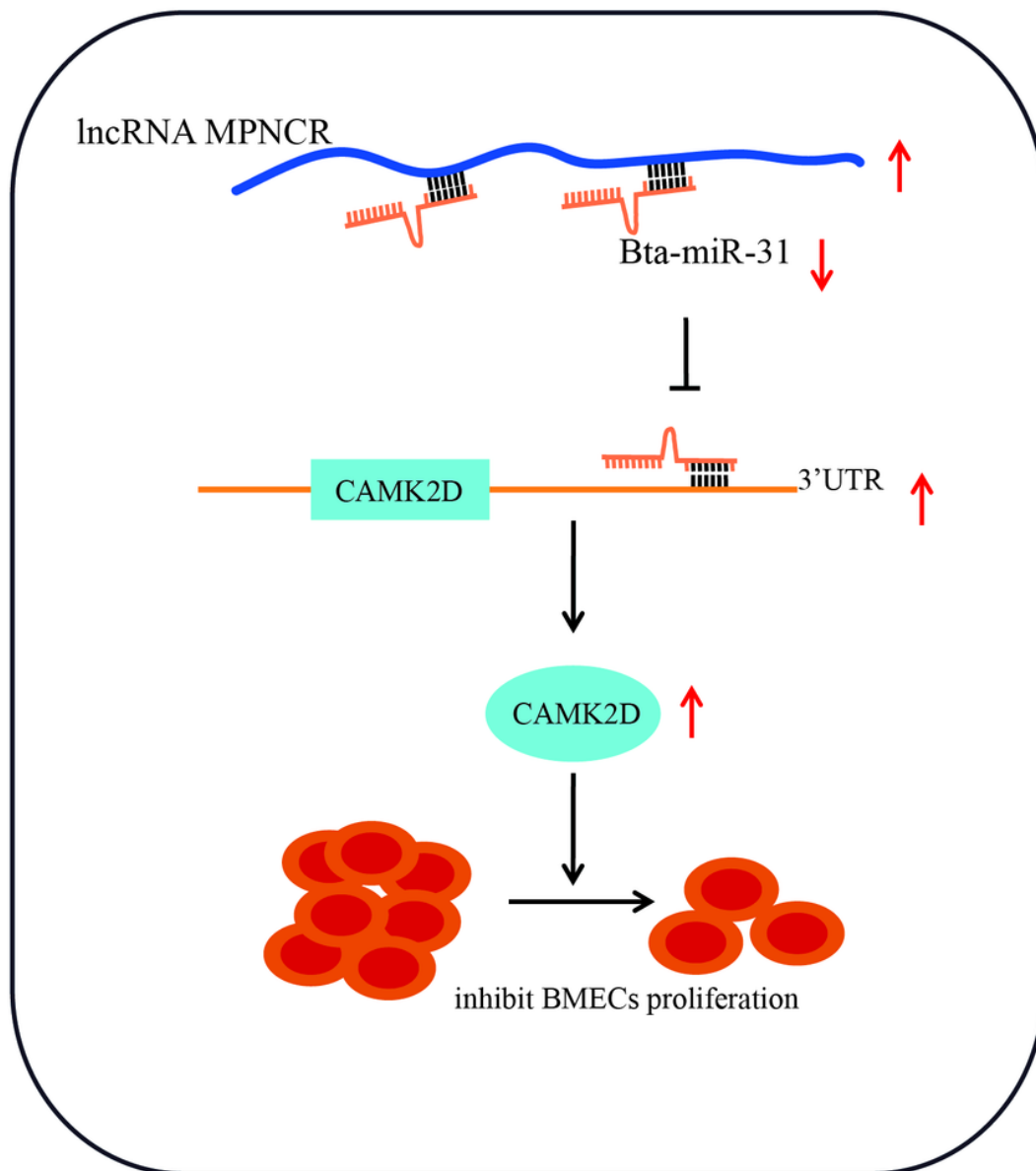
Figure 5

Graphical abstract of lncRNA MPNCR regulatory mechanism. LncRNA MPNCR inhibits BMECSs proliferation. MPNCR sponges miR-31 as ceRNA, while miR-31 binds to the 3' UTR of CAMK2D gene and regulates the gene expression at mRNA and protein levels.