ORIGINAL ARTICLE

WILEY   Journal of Pineal Research
Molecular, Biological, Physiological and
Clinical Aspects of Melatonin

An *AANAT/ASMT* transgenic animal model constructed with CRISPR/Cas9 system serving as the mammary gland bioreactor to produce melatonin-enriched milk in sheep

Teng Ma[1] | Jingli Tao[1] | Minghui Yang[1] | Changjiu He[1] | Xiuzhi Tian[1] |
Xiaosheng Zhang[2] | Jinlong Zhang[2] | Shoulong Deng[3] | Jianzhong Feng[2] |
Zhenzhen Zhang[1] | Jing Wang[1] | Pengyun Ji[1] | Yukun Song[1] | Pingli He[4] |
Hongbing Han[1] | Juncai Fu[1] | Zhengxing Lian[1] | Guoshi Liu[1] (iD)

[1]National Engineering Laboratory for
Animal Breeding, Key Laboratory
of Animal Genetics and Breeding of
the Ministry of Agriculture, Beijing
Key Laboratory for Animal Genetic
Improvement, College of Animal Science
and Technology, China Agricultural
University, Beijing, China

[2]Institute of Animal Husbandry and
Veterinary, Academy of Agricultural
Sciences of Tianjin, Tianjin, China

[3]State Key Laboratory of Stem Cell
and Reproductive Biology, Institute of
Zoology, Chinese Academy of Sciences,
Beijing, China

[4]State Key Laboratory of Animal
Nutrition, College of Animal Science and
Technology, China Agricultural University,
Beijing, China

Correspondence
Guoshi Liu, College of Animal Science and
Technology, China Agricultural University,
Beijing, China.
Email: gshliu@cau.edu.cn

Abstract

Melatonin as a potent antioxidant exhibits important nutritional and medicinal values. To produce melatonin-enriched milk will benefit the consumers. In this study, a sheep bioreactor which generates melatonin-enriched milk has been successfully developed by the technology that combined CRISPR/Cas9 system and microinjection. The *AANAT* and *ASMT* were cloned from pineal gland of Dorper sheep (*Ovis aries*). The in vitro studies found that *AANAT* and *ASMT* were successfully transferred to the mammary epithelial cell lines and significantly increased melatonin production in the culture medium compared to the nontransgenic cell lines. In addition, the Cas9 mRNA, sgRNA, and the linearized vectors pBC1-*AANAT* and pBC1-*ASMT* were co-injected into the cytoplasm of pronuclear embryos which were implanted into ewes by oviducts transferring. Thirty-four transgenic sheep were generated with the transgenic positive rate being roughly 35% which were identified by Southern blot and sequencing. Seven carried transgenic *AANAT,* two carried *ASMT,* and 25 carried both of *AANAT* and *ASMT* genes. RT-PCR and Western blot demonstrated that the lambs expressed these genes in their mammary epithelial cells and these animals produced melatonin-enriched milk. This is the first report to show a functional *AANAT* and *ASMT* transgenic animal model which produce significantly high levels of melatonin milk compared to their wild-type counterparts. The advanced technologies used in the study laid a foundation for generating large transgenic livestock, for example, the cows, which can produce high level of melatonin milk.

KEYWORDS

AANAT, *ASMT*, CRISPR/Cas9, mammary gland bioreactor, melatonin, pronuclear microinjection, sheep

---

Teng Ma and Jingli Tao contributed equally to this work.

1 | INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine), a circadian rhythm regulatory hormone,[1,2] regulates the expression of clock genes at the central and peripheral tissue levels in organisms.[2-4] Thus, it regulates many physiological functions which relate to circadian alterations including promoting sleep in diurnal animals.[5-8] Melatonin is also a potent free radical scavenger and antioxidant.[9] It not only directly interacts with reactive oxygen species (ROS) and reactive nitrogen species (RNS) but also upregulates the activities of antioxidant enzymes.[10-12] Through its antioxidative cascade reaction, melatonin suppresses the apoptotic process in a variety of species.[13-16] Melatonin as an amphiphilic molecule readily enters all subcellular compartments including membrane, cytosol, nucleus, and the mitochondrion.[17,18] Its production decreases with aging.[19] The low melatonin levels in aged population were associated with insomnia, hypertension, heart disease, and neurodegenerative diseases.[19-21] Many studies showed that melatonin supplementation retarded the processes of these diseases in animal models and in clinical trials.[19-21] Melatonin application advanced the endogenous circadian rhythm in humans[22] and enhanced the timing of physiological activities including sleep, core body temperature, cortisol level, and immunological function.[23-25] For example, in the infants, they lack melatonin production and rhythmicity until 4 months after delivery.[26,27] Consequently, the synchronization of their physiological functions majorly depends on melatonin rhythm in mother's milk. It was also reported that the melatonin in milk provided a better nocturnal sleep in newborns and would help their immune system maturation and their early circadian rhythm establishment.[28-31]

Melatonin can be synthesized by various organs, tissues, and cells including the gut, liver, kidneys, ovary and testis, placentas, endometrium, oocytes, sperms, and mammary gland in mammals.[18,32-36] Arylalkylamine N-acetyltransferase [(AANAT or serotonin N-acetyltransferase (SANT)] is considered to be the rate-limiting enzyme for melatonin synthesis.[32] This conclusion was based on the observations that the activities of AANAT showed circadian rhythm which was synchronized with melatonin serum levels[33] and also this enzyme might catalyze the last step of melatonin formation.[34] During scotophase, *AANAT* gene expression in the pineal gland is significantly up-regulated and this likely results in the large rise in melatonin production.[35] Other studies found that the increased efficiency of AANAT during night did not occur at the level of transcription but was due to the post-translational modification by phosphorylation of this enzyme.[36,37] Recent studies have suggested that acetylserotonin methyltransferase (*ASMT*) might also participate in the rate-limiting control of melatonin synthesis.[21,33,38] The transgenic model with melatonin production was first developed in plants. Transgenic plants had a high level of melatonin by overexpressing homologous of sheep *AANAT* and *ASMT* genes and the rising melatonin renders the plants being better resistance to many abiotic and biotic stressors.[39,40] The transgenic animals with enriched melatonin production are not available currently. The purpose of this study was to use advanced technology to develop a transgenic animal model which can produce melatonin-enriched milk.

The type II bacterial CRISPR/Cas9, a RNA-guided nuclease system, has recently been demonstrated to be a robust tool for genome engineering in many cell lines and organisms.[41-46] It demonstrated that using the CRISPR/Cas9 system, long DNA fragment via homology-independent DNA reparation can be achieved.[47,48] In previous study, we successfully integrated the exogenous genes into the sheep genome using the same CRISPR/Cas9 system.[49] Thus, CRISPR/Cas9 system was applied in current study to develop a mammary gland bioreactor which can generate melatonin-enriched milk in sheep.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Follicle-stimulating hormone (FSH), pregnant mare serum gonadotropin (PMSG), and human chorionic gonadotropin (hCG) were purchased from Ningbo Hormone Products Co. Ltd. (Zhejiang, China). Melatonin and other reagents, unless specified, were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2 | Animals

This study was conducted using adult Dorper (*Ovis aries*) fed with a live weight maintenance ration at the experimental facilities of the Institute of Animal Husbandry and Veterinary, Academy of Agricultural Sciences of Tianjin, China. Superovulation, artificial insemination, intradermic injection, and animal operations were performed at the experimental station of the China Agricultural University. The study was carried out in strict accordance with the protocol approved by the Animal Welfare Committee of China Agricultural University (Permit Number: XK662).

2.3 | Expression vector constructions of *AANAT* and *ASMT* genes

Total RNA was extracted from the pineal gland of Dorper sheep using the TRIzol Reagent (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer's instructions and the extracted RNA was immediately used for cDNA synthesis with a first-strand cDNA synthesis kit (TaKaRa Bio Inc., Tokyo, Japan) according to the manufacturer's instructions. The complete open reading frames (ORFs) and partial up-/downstream noncoding regions of *AANAT* and *ASMT* genes were, respectively, amplified using Trans Start FastPfu DNA

Polymerase (Trans Gen Biotech, Beijing, China). The specific primers, which were designed by Primer Premier 5.0 software, were added the same restriction sites XhoI according to the sequences of *AANAT* (mRNA KC290949.1, protein AGG68821.1) and *ASMT* (mRNA KC290950.1 protein AGG68822.1) in *Ovis aries* obtained from National Center for Biotechnology Information (NCBI). The primers contained Xho I restriction enzyme site.

The mammalian expression vector pBC1 (Invitrogen Inc.) was used as a backbone for preparation of gene constructs. *AANAT* and *ASMT* which checked by sequencing was exited from the T-vector by Xho I and subsequently cloned into pBC1. The recombinant vector was referred to as pBC1-*AANAT* and pBC1-*ASMT*. The linearized DNA was extracted from gel and purified by DNA Purification Kit (Tiangen, Beijing, China).

Neomycin-resistant gene (Neo) was amplified with restriction site NotI, using plasmid pcDNA3.1-myc-his. The PCR products were digested with NotI and cloned, respectively, into pIRES2-EGFP-*AANAT* and pIRES2-EGFP-*ASMT* for expression in vitro (Table 1).

2.4 | Preparation of Cas9 mRNA and sgRNA

Cas9 and sgRNA plasmids have been constructed in our laboratory, and the method has been published previously.[50] Cas9 and sgRNA coding regions containing T7 promoter were amplified using PCR by Trans Start FastPfu DNA Polymerase (Trans Gen Biotech) from each plasmid. The T7-Cas9 PCR products were purified and used as the template for in vitro transcription (IVT) using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Life Technologies, Inc., Grand Island, NY, USA). The poly (A) tailing reaction was performed after the completion of capping using Poly (A) Tailing Kit (Life Technologies, Inc.) according to the manufacturer's instruction. After the IVT, the sgRNA and

TABLE 1 Primer for vectors' construction

Primer	Sequences (5'-3')
C-AANAT	
Forward	CCGCTCGAGCCACCATGTCCACGCCAAGC
Reverse	CCGCTCGAGCCACCTCAGCGGTCACTGTT
C-ASMT	
Forward	CCGCTCGAGCCACCATGTGCTCCCAGGAG
Reverse	CCGCTCGAGCCACCTCACTTTCTGGCCAA
C-Neo	
Forward	AAGGAAAAAAGCGGCCGCGGTGTGGAAAGTCCCCAGG
Reverse	AAGGAAAAAAGCGGCCGCTGCTTTATTTGTAACCATT

Cas9-encoding mRNA was purified by ethanol and lithium chloride (LiCl) precipitation, separately, re-dissolved in RNase-free water, and stored at −80°C until to use.

2.5 | Mammary gland epithelial cell culture and DNA transfection

Mammary gland tissues were taken by aseptic operation from transgenic ewe aged 14 months. Mammary tissue was cut into fining pieces and cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 50 μg/mL (v/v) penicillin G and streptomycin sulfate (Solarbio Inc., Beijing, China), 5 μg/mL insulin, and 1 μg/mL hydrocortisone at 38°C in 5% CO_2. When 80% of bottom of culture dish (6 mm diameter) were covered, the monolayer cells were digested with 0.05% trypsin-EDTA (Gibco). Using twice digestion method fibroblast cells was removed, and then, the purified mammary gland epithelial cells were selected for further culture. The confluence mammary gland epithelial cells at passages of four were used for in vitro study. The expression vector was linearized by AseI restriction enzyme digestion. The Lipofectamine 2000 reagent (Invitrogen) was used for transfection. The pure population of transfected cells were obtained after 3 weeks of G418 (500 μg/mL, Gibco) selection. The serotonin (Sigma-Aldrich, St. Louis, MO, USA) was added in culture medium as substrate for melatonin synthesis. The cells and culture solutions were collected at 0, 24, and 48 hours after serotonin ($1×10^{-4}$ mol/L), and then stored in liquid nitrogen for future use.

2.6 | Generation of transgenic animals

The animals were treated with controlled internal drug release (CIDR) device (EAZI-BREED®CIDR® Sheep Device Pfizer Animal Health, New Zealand) for synchronization of their estrus. Superovulation and endoscopic-assisted insemination method were described by Zhang et al.[51] The zygotes were collected from donors using superovulation and collected by oviduct flushing, and the collected embryos were microinjected with 2-5 pL TE solution containing 50 ng/μL of sgRNA and 100 ng/μL of Cas9 mRNA was injected into the cytoplasm first. Then linearized DNA solution was injected into the cytoplasm with equal portions of pBC1-*AANAT* and pBC1-*ASMT* at concentrations of 10 ng/μL, in volumes of 5 pL each. Then, the well-fertilized embryos were transplanted into the recipient oviducts within 1 hour. Each recipient was transplanted with three to five embryos.

2.7 | Identification of transgenic offspring

Genomic DNA was extracted from ears of the offspring to determine their genotype. The transgenic genes were analyzed

by Genome PCR. The forward primer AA8-F of 520-bp product for *AANAT* and AS4-F of 950-bp product for *ASMT* were located in insertion sequence, and MLT-F was located at 56 bp before enzyme XhoI restriction site. The reverse primer was located in the downstream, including 65-bp vector backbone sequences after XhoI site. The PCR cycles were performed as follows: 95°C for 5 minutes, 35 cycles at 95°C for 20 seconds, 56°C for 20 seconds, 72°C for 60 seconds, and 72°C for 10 minutes. The PCR system (20 μL) contained 10 μL of 2×ES Taq Master Mix, 1 μL of DNA (50 ng/μL), 30 μmol/L forward primer and reverse primer, respectively. Products were sequenced by Sangon biotech, Beijing, and blasted in GenBank database (Table 2).

Southern blotting was performed with the DIG High Prime DNA Labeling and DetectionStarter kit II (Roche, Inc., Pleasanton, CA, USA) under the manufacturer's instruction as further detection. The genomic DNA of 20 μg each was digested by restriction enzyme HindIII. The prospective Southern blotting products were about 5.2 kb (Figure 4). DNA samples from wild-type sheep were used as negative controls.

2.8 | Melatonin analysis in milk and in culture medium of mammary gland epithelial cells

The milk was collected from transgenic and nontransgenic offspring of 14-month-old ewes (four each). The animals were treated with lactagogue protocol (estradiol benzoate 0.25 mg/kg and progesterone 0.6 mg/kg and per intramuscular injection two times a day for 7 days, cloprostenol 0.5 mg injection at the days of 8th, 10th, and 12th, respectively). The animals were given breast massage three times a day with gently warm water from 3rd–12th day to stimulate lactation. Both milk and blood were collected at 4:00 hour (in dark condition) and 16:00 hour, respectively. Blood samples were collected from the jugular vein, allowed to clot for 30 minutes, and then centrifuged at 850 *g* for 10 minutes to obtain serum for melatonin assay. All procedures were performed under ice bath and kept in dark.

After 3 days of sample collection, breast tissues were excised for the gene expression study in the in vitro condition. The mammary gland epithelial cell culture was performed as mentioned above. With additional prolactin treated (5 μg/mL prolactin), the serotonin was added (to a final concentration of 10^{-4} mol/L) at 90% confluency, about 5×10^6 cells, and then culture media were collected in 0, 0.5, 1, 2, and 4 hours, respectively, for melatonin assay. The melatonin assay method was described by Zhao et al.[52]

2.9 | Expression analysis of *AANAT* and *ASMT*

After culture media collection, the mammary gland epithelial cells were used for RT-PCR and Western blotting analysis, respectively. Total RNA was extracted using the TRIzol reagent (Invitrogen) and immediately reverse-transcribed using Prime Script™ RT reagent Kit with g DNA Eraser (TaKaRa). The RT-PCR reactions consisted of 10 μL LightCycler® Multiplex Masters (Roche Molecular Systems, Inc.), 25 μmol/L forward and reverse primers, 2 μL template, and ddH$_2$O were added up to a total volume of 20 μL. The procedure was as follows: 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, and 60°C for 10 seconds, melting curve from 65 to 95°C, increasing in increments of 0.5°C every 5 seconds. Normalization was performed using the housekeeping gene *GAPDH* as a control. Relative mRNA expression was calculated by the $2^{-\triangle\triangle Ct}$ method. Primer sequences are listed in Table 3.

Total proteins were extracted by RIPA lysis buffer (Huaxingbio, Beijing, China) and boiled for 10 minutes. For SDS-PAGE, 10 μg of protein was loaded on 12% (w/v) SDS-PAGE gel, separated, and transferred onto PVDF membrane (Millipore, Bedford, MA, USA) using a semi-dry Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). As primary antibodies against β-actin and serotonin N-acetyltransferase, rabbit Ab from Abcam (Cambridge, UK) were used (1/300 dilution). The secondary antibody was anti-mouse IgG conjugated with Pro-light HRP Chemiluminescence detection reagents A and B (Tiangen) according to the manufacturer's instructions.

TABLE 2 Premiers for transgenic offspring identification

Primer	Sequences (5′-3′)
AA8-F	
Forward	GTCCAGCACTTCCTGACCCT
AS4-F	
Forward	ATACGCTGTTTCCTCATCTTCC
MLT-F	
Forward	GATTGACAAGTAATACGCTGTTTCCTC
MLT-R	
Reverse	CATCAGAAGTTAAACAGCACAGTTAG

TABLE 3 Primer for RT-PCR

Gene	Sequences (5′-3′)
ASMT	
Forward	AGGACGAGCGGATCAGCTTCC
Reverse	AGCACTTGGCATCGGTCCAGTC
AANAT	
Forward	CGTTCATCATCGTCTCCCTGTGG
Reverse	GGGATGGAAGGCAAACCTCTGGT
GADPH	
Forward	GTGTCTGTTGTGGATCTGACCTG
Reverse	AGAAGAGTGAGTGTCGCTGTTGAAGT

2.10 | Statistical analysis

The data are expressed as the mean values ± standard error of the mean (SEM) and analyzed using variance analysis of variance (ANOVA) followed by Duncan's test using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P<.05$ is considered statistically significant.

3 | RESULTS

3.1 | Gene cloning and expression vector construction of *AANAT* and *ASMT*

Total RNA was extracted from pineal gland of Dorper sheep (*Ovis aries*), and the first-strand cDNA was synthesized. The complete open reading frames (ORF) of *AANAT* and *ASMT* as well as some upstream and downstream noncoding regions were amplified, respectively. The same restriction sites of XhoI were added. The fragments were identified and sequenced as 634 bp for *AANAT* and 1038 bp for *ASMT*, respectively (Figure 1A).

The corrected cDNA was exited from the T-vector by XhoI and subsequently cloned into mammary gland-specific expressional vector pBC1 (Invitrogen). The recombinant vectors were referred to as pBC1-*AANAT* and pBC1-*ASMT*, respectively. Both of the genes were driven by β-casein promoter designed to produce the recombinant protein. The

results were showed in Figure 1. Neomycin-resistant gene (Neo) was inserted in plasmid pcDNA3.1-myc-his with restriction site NotIas pIRES2-EGFP-*AANAT*-neo and pIRES2-EGFP-*ASMT*-neo for in vitro expression.
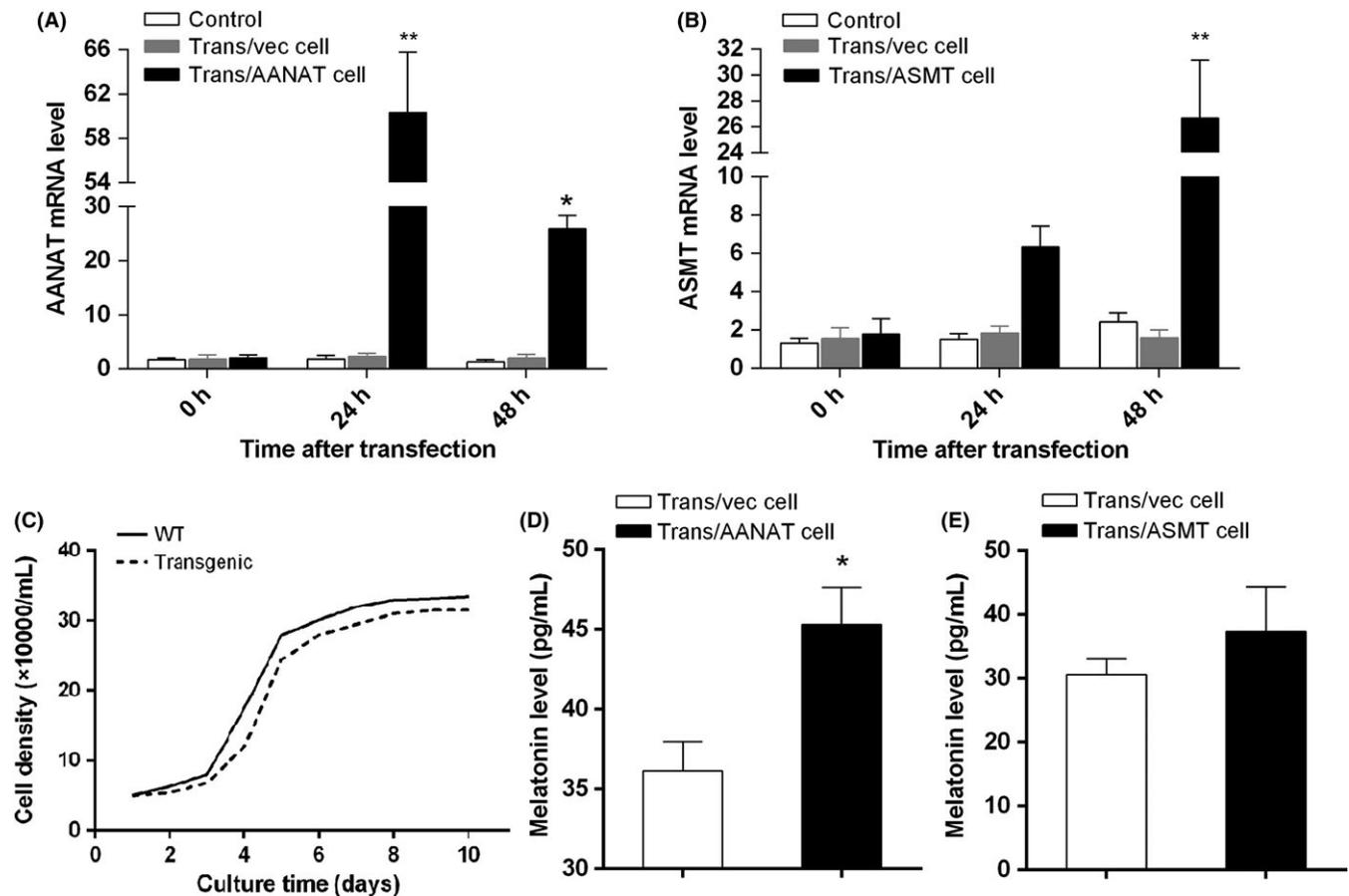
3.2 | Vector transfection, expressions of *AANAT* and *ASMT,* and melatonin production in mammary epithelial cell culture

The mammary epithelial cells of Dorper sheep were collected using adherent tissue culture method combined with trypsin digestion. Expression vectors of pIRES2-EGFP-*AANAT*-neo and pIRES2-EGFP-*ASMT*-neo with antineomycin selection and EGFP markers were transformed by electroporation method. Three independent transgenic lines of overexpressing *AANAT* and four overexpressing *ASMT* were obtained. The cell counting showed that the growth curve of mammary epithelial cells displayed as a "S" curve in 10-day culture (Figure 2C).

The results of RT-PCR showed that the expression of mRNA in the transgenic cell lines was significantly higher than that in the control and empty vector-transfected cells (n=3, $P<.01$, Figure 2A). In the transfected *AANAT* cells, mRNA expression rose sharply around 60-fold within 24 hours after transfection ($P<.01$) and started to reduce at 48 hours but it was still 25-fold higher than the control
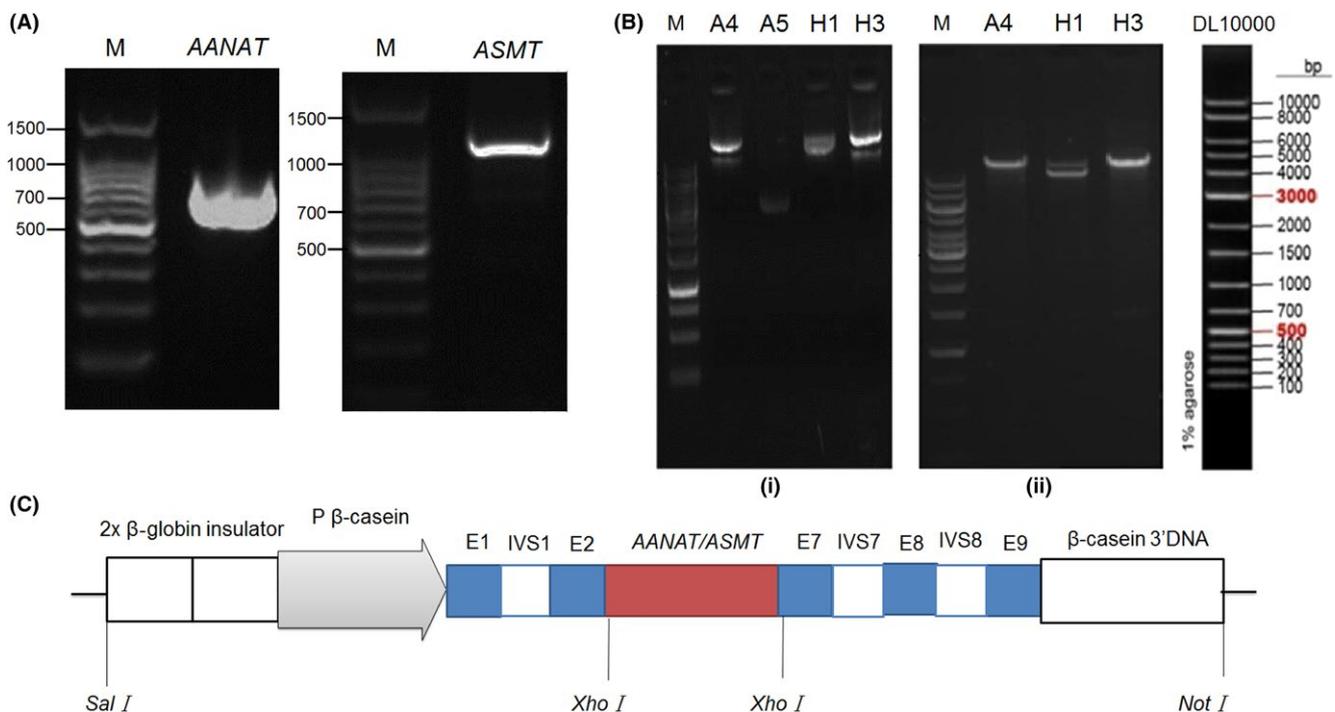
FIGURE 1 The results of gene cloning and expression vector construction of *AANAT* and *ASMT*. (A) Gene fragment amplification of *AANAT* and *ASMT*. Maker: DL1000. Left: *AANAT,* Right: *ASMT.* (B) Identification of recombinant plasmid pBC1-*AANAT/ASMT* by NotI and SalI digesting. Marker DL10000. I: 30 min digestion. II: 60 min digestion. A4 and H3 vectors were constructed successfully. (C) Schematic diagram of expression vector for microinjection containing *AANAT* was 16391 bp, and *ASMT* 16795 bp, β-casein: goat β-casein promoter. E1: exon 1 of goat β-casein, IVS1: intron 1 of goat β-casein

6 of 10 | WILEY— *Journal of* **Pineal Research**
Molecular, Biological, Physiological and
Clinical Aspects of Melatonin

MA ET AL.

FIGURE 2 The expressions of AANAT and *ASMT* after transfection and their relationships with the melatonin production in mammary epithelial cells. (A, B) Relative mRNA expression of *AANAT* and *ASMT* in mammary epithelial cells. (C) The growth curve of wild-type and transfected mammary epithelial cell lines. (D) Melatonin levels in culture medium of *AANAT* transgenic cell lines. (E) Melatonin levels in culture medium of *ASMT* transgenic cell lines. *represents significant differences, $P<.05$, and **represents highly significant differences, $P<.01$

($P<.01$, Figure 2A). The expression of *ASMT* was slightly increased in 24 hours ($P>.05$), and after 48 hours, the expression was around 15-fold higher than that in the control group ($P<.01$, Figure 2B).

Thus, the detection of melatonin biosynthesis in the *AANAT* transgenic lines was performed at 24 hours and in *ASMT* lines was 48 hours after their transfections. Melatonin level in culture medium of transfected *AANAT* cell lines was significantly higher than that in vec cell line (45.3 ± 2.32 vs 36.1 ± 1.82 ng/mL, n=4, $P<.05$, Figure 2D), and the concentration of melatonin in *ASMT* cell line was slightly higher than that in its vec cell lines (37.3 ± 7.02 vs 30.57 ± 2.54 ng/mL); however, this increase failed to reach significant difference (n=4, $P>.05$, Figure 2E).
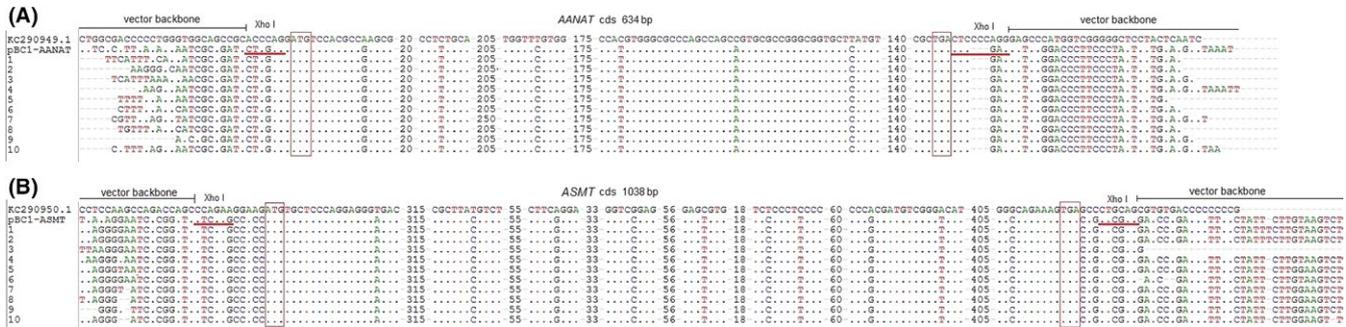
3.3 | Development of transgenic sheep by pronucleus injection

To improve the integration efficiency of CRISPR/Cas9 system, the sgRNA-targeted myostatin (MSTN) gene was designed (Table S1). The Dorper sheep were used as the donors

for superovulation. The mixture of mRNA of Cas9/sgRNA (Table S2) and DNAs of *AANAT/ASMT* was injected into the cytoplasm of pronucleus embryos. A total of 593 transferable embryos were transferred to 150 recipient ewes. Seventy-seven of them had pregnancies evaluated with ultrasonic examination at 21 days after the embryo transfer. The pregnancy rate was 51.33%. Collectively, 98 lambs were obtained including 34 transgenic ones which were identified by sequencing (Figure 3) and Southern blot (Figure 4). Lambing percentage and transgenic positive rate were 65.33% and 34.69%, respectively. There were seven lambs carrying with exogenous *AANAT* gene and two with *ASMT* gene. The rest of 25 lambs carried with both of *AANAT* and *ASMT genes*. The data were listed in Table 4.

3.4 | *AANAT* and *ASMT* expression and melatonin production in transgenic offspring

Different from 3.2, the mammary epithelial cells were harvested from transgenic sheep in which the exogenous *AANAT* and *ASMT* genes were integrated into their DNA. RT-PCR

MA ET AL.

Journal of **Pineal Research**
Molecular, Biological, Physiological and
Clinical Aspects of Melatonin

WILEY

7 of 10

FIGURE 3 Sequences of double-gene transgenic offspring. (A) Genome type of trans-*AANAT* individuals. The wild-type sequence (reference *AANAT* KC290949.1 *ASMT* KC290950.1) and vector sequence were shown at the top. The matched sequences were showed in dots and SNPs in single capital, which were different from references. Vector backbone sequence of pBC1 was showed on both side of start codon ATG and termination codon TGA. Restriction enzyme cutting site of XhoI was underlined between 5′ and 3′ terminal. (B) Genome type of trans-*ASMT* individuals (reference *ASMT* KC290950.1)
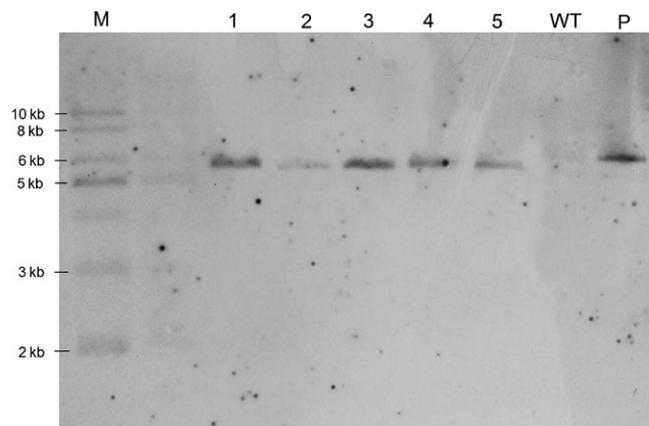
FIGURE 4 Southern blot analysis for *AANAT*-positive offspring with DIG probe. M: molecular weight marker. Band 1 to 5: DNA samples of five individuals of transgenic offspring, respectively (about 5.2 kb). WT: DNA sample of the wild-type sheep. P: the positive band of transfer vector pBC1-*AANAT* digestion by HindIII
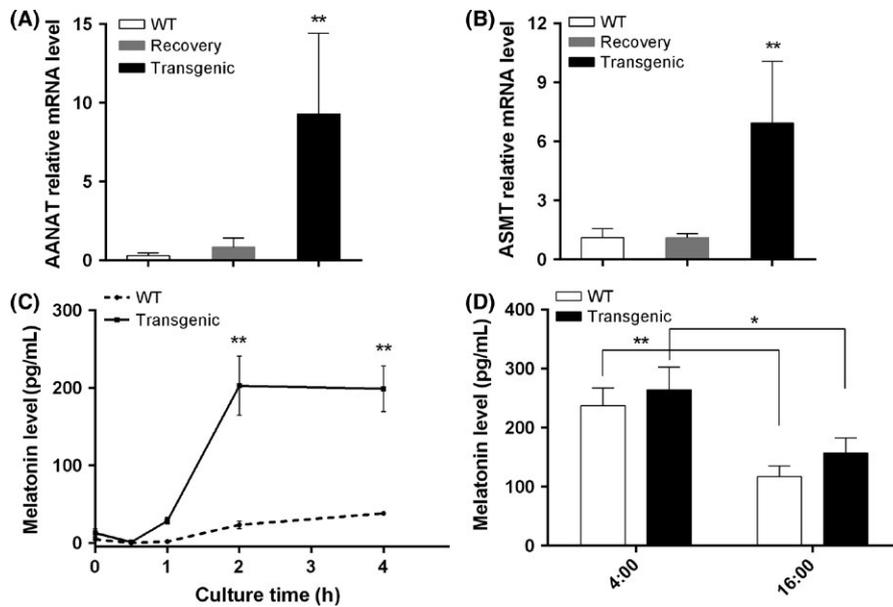
TABLE 4 Results of transgenic procedures

	Total
Donor	62
Total number of embryos	655
Number of transplanted embryos	593
Average viable embryos	9.56
Microinjection surviving rate (%)	90.53
Number of recipients	150
Average number of embryo transplantation/ewe	3.95
Pregnancy number	77
Pregnancy rate (%)	51.33
Number of lambs alive	98
Lambing rate (%)	65.33
Number of transgenic lamb	34
Transgenic rate (%)	34.69

revealed that the mRNA levels of *AANAT* and *ASMT* were around 10 times higher in their mammary epithelial cells that those in the wild types (Figure 5A,B). When the cells were cultivated in prolactin induction medium to activate the gene through β-casein promoter and supplied with serotonin, in accordance with the gene expression levels, their melatonin production was also several folds higher than that in the wild type (Figure 5C). In addition, the serum melatonin level in the transgenic offspring was also significantly higher during the night (4:00) than that of the wild types (Figure 5D).

In addition to the levels of mRNA, the protein of AANAT was significantly elevated compared to the wild type evaluated by Western blot analysis (Figure 6A). Most importantly, melatonin level in the night milk (collected at 4:00 AM) in the transgenic offspring was significantly higher than that in the wild type (40.6±8.35 vs 10.0±3.59 ng/mL). During the day, melatonin levels in the milk of both transgenic and wild type were comparable (Figure 6B).

4 | DISCUSSION

In this study, a marker-free primary transgenic sheep mammary bioreactor was successfully developed via the CRISPR/Cas9 system. The vectors of pBC1-*AANAT* and pBC1-*ASMT*, which integrated in sheep genome, were free of prokaryotic gene fragments. The promoter β-casein was selected in the current study. The purpose of this selection was to ensure a highly specific expression activation of the transfected *AANAT* and *ASMT* genes only occurring in mammary gland tissue. Targeted fragments were inserted inside of exon 7 β-casein genes, located in chromosome 2. This construction kept the mRNA transcription only following casein expression.[53,54] As a result, both *AANAT* and *ASMT* cDNA stably integrated into the genome of transgenic sheep. This was identified by DNA sequencing and Southern blot analyses. The current methods significantly increased the transgenic efficiency.

8 of 10 | **WILEY**— Journal of **Pineal Research**
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

MA ET AL.

FIGURE 5 *AANAT* and *ASMT* expressions and melatonin production in medium and animal serum. (A) The relative *AANAT* mRNA level in mammary epithelial cell culture (n=4). (B) The relative *ASMT* mRNA level in mammary epithelial cell culture (n=4). (C) Melatonin concentration in mammary epithelial cell culture media. The culture time counted form prolactin addition (n=4). (D) Serum melatonin levels of transgenic offspring (n=14). *represents significant differences, $P<.05$, and **represents significant differences, $P<.01$
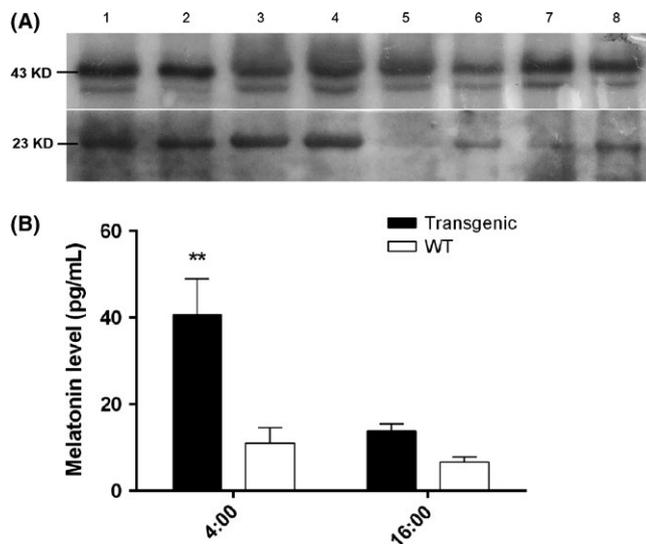
FIGURE 6 The level of AANAT protein and its corresponding melatonin level in transgenic sheep. (A) Western blot analysis of AANAT protein in the mammary gland epithelial cells. The reference protein α-actin about 43KD was detected. AANAT is about 23KD. Bands 1, 2, 3, and 4 showed transgenic offspring; bands 5, 6, 7, and 8 showed wild-type individual. (B) Melatonin level in milk collected at different time points with lactagogue protocol. (n=4) **represents highly significant differences, $P<.01$

For example, the transgenic rate (transgenic offspring/newborn) was 34.69%, or overall rate was 5.73% (transgenic offspring/injected eggs) in this study. These values were several folds higher than those of previously reported transgenic rate of 5%[55] and overall rate of 1.24%.[56] The results confirmed that a direct injection of zygotes with Cas9/gsRNA was an efficient and reliable approach for production of transgenic livestock.[49,57] In addition, all of these 25 double-gene transgenic sheep were healthy and had normal weight at birth. To

date, none of the transgenic sheep have shown any obvious adverse phenotype and all of them have being involved in our breeding programmes.

The expression analysis indicated that the insertion of pBC1 expression vector with exogenous genes of *AANAT* and *ASMT* on board was stably expressed in transcriptional level and the gene location seemed not to impact expression efficiency of vector pBC1-*AANAT* and *pBC1-ASMT*. The targeted genes can stably express in different integration sites in the genome.[58,59] This was confirmed by the significantly high levels of *AANAT* and *ASMT* expressions in transgenic mammary gland epithelial cell lines compared to wild type both in vitro and in vivo conditions.

In transgenic offspring, the upregulated gene expressions of *AANAT* and *ASMT* rendered the elevated melatonin production in milk. However, the serum melatonin levels of transgenic sheep did not exhibit significant difference from nontransgenic ones. The results proved that the transgenic genes introduced by current method specifically expressed in mammary gland and produced the expected biological results.

It was also realized that in the in vitro-transfected mammary epithelial cells, the expression levels of *AANAT* and *ASMT* were not proportional to their melatonin production, that is, the gene expression levels were much higher than those of their melatonin production (Figure 2A,B,D,E). This was not surprising since the post-translational modification of the melatonin synthetic enzymes, particularly the *AANAT*, for example, protein phosphorylation, plays an important role for the enzyme's activity.[33,34] However, this was not the case in the mammary epithelial cells obtained from the transgenic sheep. Their gene expression levels of *AANAT* and *ASMT* were proportionally associated with their melatonin production (Figure 5A-C). The mechanisms of these differences are currently unknown and it will be investigated in the future.

The purpose of this study was to use advanced technologies to develop a transgenic animal model which can produce melatonin-enriched milk. This was achieved by the combination of CRISPR/Cas9 system, constructed marker-free mammary gland-specific expression vectors of *AANAT* and *ASMT,* and β-casein promoter. This combination drives the bioreactor efficiently to express melatonin synthetic enzymes. This process not only increased the melatonin levels in milk of the animals but also reduced the health risks of the transgenic animals due to the overexpression of exogenous genes.[60] The results demonstrated that the direct injection of Cas9/sgRNA into zygotes was a reliable method to generate transgenic domestic animal, for example, cows, to produce high level of natural melatonin milk (vs the artificially fortified melatonin milk).

DISCLOSURE

The authors of this article declare that they do not have any conflict of interest.

REFERENCES

1. Arendt J. Melatonin and pineal gland: influence on mammalian seasonal and circadian physiology. *Rev Reprod*. 1998;3:13-22.
2. Vriend J, Reiter RJ. Melatonin feedback on clock genes: a theory involving the proteasome. *J Pineal Res*. 2015;58:1-11.
3. Farias TSM, Chimin P, Oliveira AC, et al. Pinealectomy interferes with the circadian clock genes expression in white adipose tissue. *J Pineal Res*. 2015;58:251-261.
4. Alonso-Vale M, Anderotti S, Mukai PY, et al. Melatonin and the circadian entrainment of metabolic and hormonal activities in primary isolated adipocytes. *J Pineal Res*. 2008;45:422-429.
5. Zhdanova IV. Melatonin as a hypnotic: pro. *Sleep Med Rev*. 2005;9:51-65.
6. Dijk DJ, Cajochen C. Melatonin and the circadian regulation of sleep initiation, consolidation, structure, and the sleep EEG. *J Biol Rhythms*. 1997;12:627-635.
7. Zhdanova IV, Tucci V. Melatonin, circadian rhythms, and sleep. *Curr Treat Options Neurol*. 2003;5:225-229.
8. Pandi-Perumal SR, Zisapel N, Srinivasan V, Cardinali DP. Melatonin and sleep in aging population. *Exp Gerontol*. 2005;40:911-925.
9. Reiter RJ, Mayo JC, Tan DX, et al. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res*. 2016;61:253-278.
10. Galano A, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. *J Pineal Res*. 2011;51:1-16.
11. Manchester LC, Coto-Montes A, Boga JA, et al. Melatonin: an ancient molecule that makes oxygen metabolically tolerable. *J Pineal Res*. 2015;59:403-419.
12. Ding K, Wang H, Xu J, et al. Melatonin stimulates antioxidant enzymes and reduces oxidative stress in experimental traumatic brain injury: the Nrf2-ARE signaling pathway as a potential mechanism. *Free Radic Biol Med*. 2014;73:1-11.
13. Galano A, Tan DX, Reiter RJ. On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. *J Pineal Res*. 2013;54:245-257.
14. Srinivasan V, Pandi-Perumal SR, Maestroni GJ, et al. Role of melatonin in neurodegenerative diseases. *Neurotox Res*. 2005;7:293-318.
15. Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. *Endocrine*. 2005;27:189-200.
16. Miller SC, Pandi PSR, Esquifino AI, Cardinali DP, Maestroni GJM. The role of melatonin in immunoenhancement: potential application in cancer. *Int J Exp Pathol*. 2006;87:81-87.
17. Reiter RJ, Guerrero JM, Escames G, et al. Prophylactic actions of melatonin in oxidative neurotoxicity. *Ann N Y Acad Sci*. 1997;825:70-78.
18. Acuña-Castroviejo D, Escames G, Venegas C, et al. Extrapineal melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci*. 2014;71:2997-3025.
19. Hardeland R. Melatonin and the theories of aging: a critical appraisal of melatonin's role in antiaging mechanisms. *J Pineal Res*. 2013;55:325-356.
20. Cardinali DP, Srinivasan V, Brzezinski A. Melatonin and its analogs in insomnia and depression. *J Pineal Res*. 2012;52:365-375.
21. Simko F, Paulis L. Antifibrotic effect of melatonin–perspective protection in hypertensive heart disease. *Int J Cardiol*. 2013;168:2876-2877.
22. Arendt J, Bojkowski C, Folkard S, et al. Some effects of melatonin and the control of its secretion in humans. *Ciba Found Symp*. 1985;117:266-283.
23. Arendt J, Skene DJ. Melatonin as a chronobiotic. *Sleep Med Rev*. 2005;9:25-39.
24. Garcia-Maurino S, Gonzalez-Haba MG, Calvo JR, et al. Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating CD4$^+$ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. *J Immunol*. 1997;159:574-581.
25. Maestroni GJ, Conti A, Lissoni P. Colony-stimulating activity and hematopoietic rescue from cancer chemotherapy compounds are induced by melatonin via endogenous interleukin 4. *Cancer Res*. 1994;54:4740-4743.
26. Jaldo-Alba F, Muñoz-Hoyos A, Molina-Carballo A, et al. Light deprivation increases plasma levels of melatonin during the first 72 h of life in human infants. *Acta Endocrinol*. 1993;129:442-445.
27. Jan JE, Wasdell MB, Freeman RD, et al. Evidence supporting the use of melatonin in short gestation infants. *J Pineal Res*. 2007;42:22-27.
28. Arslanoglu S, Bertino E, Nicocia M, et al. WAPM working group on nutrition: potential chronobiotic role of human milk in sleep regulation. *J Perinat Med*. 2012;40:1-8.

29. Bruni O, Alonso-Alconada D, Besag F, et al. Current role of melatonin in pediatric neurology: clinical recommendations. *Eur J Paediatr Neurol*. 2015;19:489-490.

30. Currier NL, Sun LZY, Miller SC. Exogenous melatonin: quantitative enhancement in vivo of cells mediating non-specific immunity. *J Neuroimmunol*. 2000;104:101-108.

31. Çakir ZÜ, Demirel C, Kilciksiz SC, et al. Melatonin can ameliorate radiation-induced oxidative stress and inflammation-related deterioration of bone quality in rat femur. *Inflammation*. 2016;39:1134-1140.

32. Foulkes NS, Borjigin J, Snyder SH, et al. Rhythmic transcription: the molecular basis of circadian melatonin synthesis. *Trends Neurosci*. 1997;20:487-492.

33. Kim TD, Woo KC, Cho S, et al. Rhythmic control of AANAT translation by hnRNP Q in circadian melatonin production. *Genes Dev*. 2007;21:797-810.

34. Carrillo-Vico A, Calvo JR, Abreu P, et al. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. *FASEB J*. 2004;18:537-539.

35. He C, Wang J, Zhang Z, et al. Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions. *Int J Mol Sci*. 2016;17:E939. doi:10.3390/ijms17060939.

36. Ganguly S, Gastel JA, Weller JL, et al. Role of a pineal cAMP-operated arylalkylamine *N*-acetyltransferase/14-3-3-binding switch in melatonin synthesis. *Proc Natl Acad Sci USA*. 2001;98:8083-8088.

37. Szewczuk LM, Tarrant MK, Sample V, et al. Analysis of serotonin *N*-acetyltransferase regulation *in vitro* and in live cells using protein semisynthesis. *Biochemistry*. 2008;47:10407-10419.

38. Ceinos RM, Chansard M, Revel F, et al. Analysis of adrenergic regulation of melatonin synthesis in siberian hamster pineal emphasizes the role of HIOMT. *Neurosignals*. 2004;13:308-317.

39. Li H, He J, Yang X, et al. Glutathione-dependent induction of local and systemic defense against oxidative stress by exogenous melatonin in cucumber (*Cucumis sativus* L). *J Pineal Res*. 2016;60:206-216.

40. Byeon Y, Back K. Low melatonin production by suppression of either serotonin N-acetyltransferase or N-acetylserotonin methyltransferase in rice causes seedling growth retardation with yield penalty, abiotic stress susceptibility, and enhanced coleoptile growth under anoxic conditions. *J Pineal Res*. 2016;60:348-359.

41. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823-826.

42. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339:819-823.

43. Hwang WY, Fu Y, Reyon D, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 2013;31:227-229.

44. Wang H, Yang H, Shivalila CS, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153:910-918.

45. Li W, Teng F, Li T, et al. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol*. 2013;31:684-686.

46. Niu Y, Shen B, Cui Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*. 2014;156:836-843.

47. Auer TO, Del Bene F. CRISPR/Cas9 and TALEN-mediated knock-in approaches in zebrafish. *Methods*. 2014;69:142-150.

48. Auer TO, Duroure K, De Cian A, Concordet JP, et al. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res*. 2014;24:142-153.

49. Wu M, Wei C, Lian Z, et al. Rosa26-targeted sheep gene knock-in via CRISPR-Cas9 system. *Sci Rep*. 2016;6:24360.

50. Han H, Ma Y, Wang T, et al. One-step generation of myostatin gene knockout sheep via the CRISPR/Cas9 system. *Agr Sci Eng*. 2014;1:2-5.

51. Zhang L, Chai ML, Tian XZ, et al. Effects of melatonin on superovulation and transgenic embryo transplantation in small-tailed han sheep (*Ovis aries*). *Neuroendocrinol Lett*. 2013;34:294-301.

52. Zhao Y, Tan DX, Lei Q, et al. Melatonin and its potential biological functions in the fruits of sweet cherry. *J Pineal Res*. 2013;55:79-88.

53. Young MW, Okita WB, Brown EM, Curling JM. Production of biopharmaceutical proteins in the milk of transgenic dairy animals. *Bio Pharm*. 1997;10:34-38.

54. Ziomek CA. Commercialization of proteins produced in the mammary gland. *Theriogenology*. 1998;49:139-144.

55. Wang B, Baldassarre H, Tao T, et al. Transgenic goats produced by DNA pronuclear microinjection of in vitro derived zygotes. *Mol Reprod Dev*. 2002;63:437-443.

56. Uchida M, Shimatsu Y, Onoe K, et al. Production of transgenic miniature pigs by pronuclear microinjection. *Transgenic Res*. 2001;10:577-582.

57. Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res*. 2014;24:372-375.

58. Nagy AGM, Vintersten K, Behringer R. *Manipulating the Mouse Embryo: A Laboratory Manual*, 3rd edn. Cold Spring Harbor: Harbor Laboratory Press; 2003.

59. Li G, Shi W, Chen G, et al. Construction and in vivo evaluation of a mammary gland-specific expression vector for human lysozyme. *Plasmid*. 2014;76:47-53.

60. Valtonen M, Niskanen L, Kangas AP, Koskinen T. Effect of melatonin-rich night-time milk on sleep and activity in elderly institutionalized subjects. *Nord J Psychiatry*. 2005;59:217-221.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.