CRISPR/Cas9-mediated Tryptophan Hydroxylase 1 Knockout Decreases Calcium Transportation in Goat Mammary Epithelial Cells

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Research

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Abstract

Background: Calcium is one of the major mineral nutrients in goat milk. Tryptophan hydroxylase1 (TPH1) is a rate-limiting enzyme catalyzing hydroxylation of L-tryptophan into 5-hydroxytryptamine (5-HT) essential for maintaining calcium homeostasis. The function of TPH1 and 5-HT in goat mammary calcium homeostasis is not well known.

Methods: The CRISPR/Cas9-mediated TPH1 knockout goat mammary epithelial cells (GMEC) were constructed firstly. Then the content of 5-HT, intracellular calcium level and abundance of key genes related to calcium transportation were evaluated and compared in wild-type GMEC, TPH1 knockout GMEC, to explore the impact of TPH1 on calcium transportation, respectively. Wild-type GMEC and TPH1 knockout GMEC were further treated with exogenous 5-HTP to confirm the role of TPH1 in regulating calcium homeostasis in GMEC. 5-HT concentration was measured by enzyme-linked immunosorbent assay and fluo-3 staining was used to determine intracellular calcium content.

Results: The TPH1 knockout GMEC heterozygous clone with no off-target effects was obtained after transfection of the Cas9/sgRNA expression vector. The 5-HT synthesis and intracellular calcium level decreased in TPH1 gene knockout GMEC. The mRNA abundance of secretory-pathway Ca\(^{2+}\)-ATPase1 (SPCA1) and plasma membrane Ca\(^{2+}\)-ATPase1 (PMCA1) were up-regulated while the mRNA abundance of secretory-pathway Ca\(^{2+}\)-ATPase2 (SPCA2) was down-regulated in TPH1 knockout GMEC. Up-regulation of parathyroid hormone-related peptide (PTHrP), a key regulator of mammary calcium metabolism, induced by 5-HTP were blocked by TPH1 gene knockout. The TPH1 knockout GMEC showed a lower sensitivity to 5-HTP induced elevation of calcium content.

Conclusion: Results suggested that TPH1 plays an important role in regulating calcium homeostasis via PTHrP and calcium transportation related factors in GMEC.

Introduction

Calcium is one of the major mineral nutrients in dairy products. Calcium can regulate bone forming, maintain excitability of muscle system and nerve system, participating in the process of immune regulation and mediate normal life activities of cells[1–3]. During lactation, a large amount of calcium was transported from plasma to mammary epithelial cells[4]. The mammary gland generates a large transepithelial calcium gradient in favour of milk[5]. The plasma membrane calcium efflux pumps PMCAs (plasma membrane Ca\(^{2+}\)-ATPases), located on the apical membrane, was responsible for the transportation of Calcium from the epithelial cell to milk[6]. SPCAs (secretory-pathway Ca\(^{2+}\)-ATPases), another calcium pump given its role at Golgi, is also involved in the secretion of calcium into milk[7, 8]. Despite this, pathways and mechanisms which underlie the movement of calcium across the mammary gland are still worth studying.
Tryptophan hydroxylase1 (TPH1) is a rate-limiting enzyme catalyzing hydroxylation of L-tryptophan into 5-hydroxytryptamine (5-HT)[9]. As is reported widely, 5-HT, also named serotonin, plays an important role in regulating several aspects of lactation among species such as mammary tissue development, milk protein synthesis and lactation maintaining[10–12]. More importantly, 5-HT in the mammary gland could affect the synthesis of Parathyroid hormone-related peptide (PTHrP) which has been recognized as a regulator of calcium metabolism, and thus maintain calcium homeostasis during lactation [13] [14]. The previous studies showed that there was a positive correlation between the contents of 5-HT and PTHrP in goats and cows during pregnancy and lactation[15, 16].

Global TPH1 knockout through Cre-loxP system has been carried out to discover novel genes, pathways and functions which serotonin modulates during lactation in mice[17]. Although TPH1 has a crucial effect on lactation, there is still no evidence of such endeavors coming out in goats or goat mammary epithelial cells. The novel clustered regularly interspaced short palindromic repeats (CRISPR)-mediated gene editing technique has been used successfully to build a permanent loss-of-function model[18–21]. Because of its higher efficiency and flexibility, CRISPR/Cas9 gene knockout system was chosen in this study to investigate the function of TPH1 on calcium metabolism in goat mammary gland.

The objective of this study is to evaluate the effect of TPH1 knockout through CRISPR/Cas9 technology on 5-HT synthesis, intracellular calcium content, expression levels of local calcium regulator and relative abundance of genes-related to calcium transportation. This study provides evidence about function of TPH1 and 5-HT in regulating calcium metabolism in dairy goats.

**Materials and Methods**

**Animals and Cells**

All experimental procedures with live goats used in this study were approved by the Animal Care and Use Committee of the Northwest A&F University, Yang Ling, China (permit number: 15–516, date: 2015-9-13).

GMEC were isolated from Xinong Saanen dairy goats at peak lactation (60 d after parturition) as described previously[22]. In brief, a piece of tissue was dissected from mammary gland under sterile conditions and blood on the tissue was washed away with D-Hank’s solution supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin. Then fatty and connective tissues were removed and the granular acinar tissue was cut into pieces and then cultured with complete medium. The composition of the medium is as follows: Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12) medium (11320 – 033, Invitrogen Corporation, Waltham, MA) supplemented with insulin (5 µg/ml; Sigma-Aldrich, St. Louis, MO), penicillin and streptomycin (100 U/ml; 080092569, Harbin Pharmaceutical Group, Harbin, China), epidermal growth factor (10 ng/ml; PHG0311, Invitrogen), hydrocortisone (1 µg/ml; H0888, Sigma-Aldrich), and 10% fetal bovine serum (10099 – 141, Invitrogen). The GMEC were cultured to confluence in complete medium at 37 °C in a humidified atmosphere with 5% CO₂. Culture medium was
changed every 24 h. To promote lactogenesis, the cells were cultured in the basal medium with prolactin (L6520, 2 µg/mL, Sigma) for 48 h before performing the following experiments.

**Construction Of Cas9-sgrna Expression Vector And Plasmid Transfection**

The sgRNAs which targeted to exon 1 of *Capra hircus* TPH1 (GenBank ID: 102184739) were designed using the online CRISPR design tool (http://crispr.mit.edu/)[23]. Two sgRNAs (Table 1) were selected based on their predicted scores and lower off-target effects for Cas9/sgRNA expression vector construction. pSpCas9 (BB)-2A-Puro (plasmid 62988, PX459 V2.0, Addgene, Cambridge, MA) was chosen as the backbone plasmid for the NHEJ-mediated pathway. The sgRNAs were synthesized as single-strand DNA oligonucleotides by Invitrogen (Shanghai, China), and annealed oligonucleotides were inserted into PX459 vector containing two *Bbs*I (R3539S, NEB, USA) enzyme sites according to the previous protocol[24], resulting in PX459 - sg1 and PX459 - sg2 vector. The sgRNA with higher efficiency was used for the single clone selection.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence(5’ to 3’)</th>
<th>Primer function</th>
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<td>caccGGTTGCTTTTCCCTTTTCCGA</td>
<td>Knockout exon 1 gene of bovine <em>TPH1</em> gene</td>
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<td>TPH1-sgRNA1-R</td>
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<td>TPH1-Test-F</td>
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<td>T7 EI enzyme digestion of exon 1 gene sequence</td>
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<td>qPCR for <em>PMCA1</em> gene</td>
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<td>cacc and aaac: complementary bases</td>
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When GMEC were cultured to 70%-80% confluence in 6-well plates, the cells were transfected with 2 µg of pX459-sg1/sg2 using Lipofectamine™ 2000 (11668019, Invitrogen, Waltham, MA). The PX459 vector was used as a negative control. Forty-eight hours later, cells transfected with pX459 plasmid were cultured in basal medium containing puromycin (1 µg/mL, P8833, Sigma) for 72–96 hours. Surviving cells were expanded for the following experiments.

**Cell Selection, DNA Extraction and T7en1 Cleavage Assay**

Individual cell clones were isolated from the remaining cell pool by the limiting dilution method as described previously[25]. Briefly, cells were diluted to one cell per 100 µL of medium, inoculated into 96-well cell culture plates, and cultured for 10–14 days to obtain single clone colonies. When cells reached 90% confluence, half of the cells were collected and genomic DNA was extracted using the Universal Genomic DNA Kit (CW Biotech). The DNA fragments spanning the target site were PCR amplified using the test primers (Table 1; product size: 607 bp). The PCR products were purified by PCR Clean-Up Kit (AP-PCR-50, Axygen, CA, USA) according to the manufacturers’ instructions. Purified DNA was annealed for T7EN1 cleavage assay[26] (M0302L, NEB, USA) and the enzyme digestion product was analyzed by agarose gel electrophoresis. Cleaved bands intensity were measured by ImageJ software (ImageLab, http://imagej.net). The genome editing efficiency was calculated by the formula: \(100 \times (1 - (1 - (b + c)/(a + b + c))^{1/2})\), where a is the intensity of the undigested PCR product and b and c are the intensities of each cleavage band. The PCR products of each single clone cell were cloned into pMD19-T vector. Each plasmid was randomly picked out and sent for sanger sequencing to assess sequence modifications by Invitrogen (Shanghai, China).

**Off-target Effects Analysis**

Off-target (OT) sites were predicted using the online website tool Cas-OFFinder (http://www.rgenome.net/cas-offinder/)[27]. Mismatches ≤ 3 bp was used as criteria[28, 29]. Genomic DNA extracted from single clones were used as templates for off-target sites PCR. The primers for 10 off-target site detections are shown in Suppl. Table 1. T7EN1 cleavage assay was used and the PCR products were inserted into pMD19-T vector for sequencing.

**Cell Treatment With 5-htp**
5-Hydroxytryptophan (5-HTP, Sigma-Aldrich) was dissolved in Dimethyl sulfoxide (DMSO) according to the manufacturers’ instructions, and further dilutions were made in complete medium to reach a final concentration. When wild type GMEC and TPH1 knockout GMEC reached approximately 70–80% confluence in lactogenic medium, they were treated with 0 µg/mL, 50 µg/mL or 100 µg/mL 5-HTP respectively. Cells were used for intracellular calcium analysis or collected for RNA isolation after 24 h incubation. After 48 h incubation cells were collected for protein extraction.

**Measurement Of Intracellular Calcium**

After 24 h incubation, the GMEC were, loaded with the fluorescent Calcium indicator fluo-3, Calcium imaging was done on a laser scanning confocal microscopy (Becton Dickinson, Inc.). Dissolve fluo-3, AM (Solarbio, Beijing) to prepare 2 mM storage solution with anhydrous DMSO, then an equal volume of 20% pluronic F127 solution (Solarbio, Beijing) was added to fluo-3, AM/DMSO solution. 4 µM fluo-3, AM working solution was prepared by diluting with Hanks balanced salt solution (HBSS, Solarbio, Beijing). Fluo-3, AM working solution was then added to the cells. After cultured at 37 °C for 20 minutes, 5 times the volume of HBSS containing 1% fetal bovine serum was added to GMEC for 40 minutes. The cells were washed 3 times and then resuscitated with HEPES buffer saline (Solarbio, Beijing) to make $1 \times 10^5$ cells/ml solution. Cells were cultured for 10 minutes and intracellular calcium trafficking was detected using Fluo-3, AM (4 µM, Solarbio) by a laser scanning confocal microscopy. The intensity of fluorescence was measured by ImageJ software.

**Measurement Of 5-hT Synthesis**

5-HT synthesis was assessed by the 5-HT concentration of medium which had or had not been used to culture GEMC for 24 h. Medium concentration of 5-HT was analyzed by enzyme-linked immunosorbent assay (SHKXSM Co.Ltd., China) according to the manufacturer’s instructions. Intra-assay CV (%) is less than 10% and Inter-assay CV(%) is less than 15%. Briefly, 50 µl of collected samples were added to the appropriate wells. Then, 100 µl of enzyme conjugate was added to standard wells and sample wells except the blank well, incubate for 60 min at 37 °C. After the microtiter plate was washed five times, 50 µl of substrate A and B were added to each well and incubated for 15 min at 37 °C. Then, each well was added with a 50 µl stop solution. The absorbance of each sample at 450 nm wavelength was detected using a microplate reader within 15 min.

**Quantitative Real-time Pcr**

Total RNA was isolated using Trizol reagent (Takara Bio Inc., Japan) according to the manufacturer’s instructions. The first-strand complementary DNA was synthesized using the PrimeScript RT kit (Takara Bio Inc., Japan). Quantitative real time PCR (qPCR) primer sequences are shown in Table 1. Ubiquitously expressed transcript (*UXT*), ribosomal protein S9 (*RPS9*) and mitochondrial ribosomal protein L39
(MRPL39) were used as internal control genes[30]. The qPCR was run in triplicate in a Bio-Rad master cycler using the SYBR Green PCR Master Mix (Takara, Japan) according to the manufacturer’s protocol. The qPCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

**Western Blot**

Western Blot

Western blot was performed as described previously [15]. Cells were collected from different treatment groups, pelleted by centrifugation and lysed in RIPA buffer. Total protein was prepared and protein concentration was determined using the Bradford method. Proteins were then separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes and blocked with milk powder solution for 1.5 h at room temperature and overnight incubation with the primary antibody. The membranes were incubated with the primary antibody overnight. Anti-PTHrP, anti-TPH1, and anti-β-actin were purchased from (Abcam, Cambridge, MA). Then the membranes were washed with PBS-tween and incubated for 1.5 h with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA). β-actin was used as a housekeeping protein. Protein bands were detected after treatment of SuperSignal West Femto agent of Thermo (Thermo Scientific, Karlsruhe, Germany).

**Statistical Analysis**

All the data were presented as mean ± SEM of three independent experiments. T-test or one-way ANOVA was applied to analyze the difference between groups. $P < 0.05$ represented a significant difference. SPSS 19.0 statistics software (SPSS, Inc., Chicago, IL) and GraphPad Prism software 6.0 were used for data statistics and statistical mapping.

**Results**

**Cleavage Efficiency analysis of sgRNAs and Single Cell Clone selection**

Of the two sgRNAs, sgRNA1 targeted the anti-sense strand of the TPH1 exon1 while sgRNA2 targeted the sense strand of the TPH1 exon1 (Fig. 1A). Genomic DNA was extracted and performed T7EN1 assay after puromycin selection. As shown in Fig. 1B, sgRNA2 had 25.2% cleavage efficiency. Following selecting single clone cells from survival GMEC of sgRNA2 transfection, we obtained one single cell clone with 32.3% cleavage efficiency through T7EN1 assay (Fig. 1B). As the Sanger sequencing assay showed, there were two genotypes at the target site of single clone 1 (Fig. 1C). One allele showed 18 nucleotides deletion and 31 nucleotides insertion, the other allele showed 10 nucleotides deletion (Fig. 1C). Therefore, single clone 1 was chosen as the homozygous TPH1 knock clone in further experiments.
Off-target Effect Of Crispr/cas9 In Single Clone

On the basis of the above single clone cell selection results, T7EN1 cleavage and sanger sequencing assay were performed to analysis off-target effect of single clone cell. Ten off-target sites (Fig. 2A) were chosen for examination and no off-targets were detected (Fig. 2B).

**Knockout of TPH1 reduce serotonin synthesis and intracellular calcium content**

TPH1 is recognized as the main rate-limiting enzyme of 5-HT synthesis. Thus, western blot and enzyme-linked immunosorbent assay were performed to evaluate content of TPH1 in cells and 5-HT in medium. Compared to wild-type GMEC, the protein level of TPH1 decreased about 90% (P < 0.05) (Fig. 3A). Medium 5-HT content increased after incubated with wild type GMEC, while medium 5-HT content of TPH1 knockout GMEC was lower than the medium 5-HT content of wild type GMEC (P < 0.05) (Fig. 3B). Compared to wild type GMEC, intracellular calcium content decreased approximately 50% (P < 0.05) (Fig. 3C, 3D). Besides, the expression changes of key mammary calcium transportation-related genes were assessed in TPH1 knockout GMEC. Compared to wild type GMEC, the mRNA abundance of secretory-pathway Ca$^{2+}$-ATPase1 (SPCA1) and plasma membrane Ca$^{2+}$-ATPase1 (PMCA1) were up-regulated (P < 0.05) (Fig. 3E, 3H) while the mRNA abundance of secretory-pathway Ca$^{2+}$-ATPase2 (SPCA2) was down-regulated (P < 0.05) (Fig. 3F) in TPH1 knockout GMEC. Knockout of TPH1 had no significant effect (P < 0.05) on sarco(endo)plasmic reticulum Ca$^{2+}$ ATPase 2 (SERCA2) (Fig. 3G).

**Knockout of TPH1 blocks 5-HTP induced PTHrP expression and calcium transportation**

Previous study had reported that Parathyroid hormone-related protein (PTHrP) played an essential role in calcium mobilization and had a correlation with 5-HTP. To determine whether knockout of TPH1 has influences on the abundance of PTHrP and 5-HTP induced calcium accumulation, wild type GMEC and TPH1 knockout GMEC were treated with 5-HTP respectively. As is shown in Fig. 4A and Fig. 4B, knockout TPH1 decreased both the mRNA and protein abundance of PTHrP (P < 0.05). Treatment of 100 µM 5-HTP increased protein and mRNA level in wild type GMEC (P < 0.05) while showed no significant influence on the expression of PTHrP in TPH1 knockout GMEC (P > 0.05) (Fig. 4C, 4D). In wild type GMEC, treatment of 100 µM 5-HTP decreased mRNA expression of PMCA1, SPCA1, SERCA2 (P < 0.05) and increased the mRNA expression of SPCA2 (P < 0.05) (Fig. 4E – 4H). In TPH1 knockout GMEC, treatment of 100 µM 5-HTP also decreased the mRNA expression of PMCA1 (P < 0.05) while had no significant influence on the mRNA expression of SPCA1, SPCA2 and SERCA2 (P > 0.05) (Fig. 4E – 4H). Fluo-3 staining results showed that 5-HTP increased intracellular calcium contents in a dose dependent manner (Fig. 4I). Treatment of 100 µM 5-HTP increased intracellular calcium levels by about 50% in TPH1 knockout GMEC (P < 0.05) and by about three times in wild type GMEC (P < 0.05).

**Discussion**
5-HT participates in numerous processes in the mammary gland, including tight junction permeability, PTHrP synthesis, and Calcium transport regulation[14, 31–33]. TPH1 is responsible for hydroxylation to the 5’ position to form the amino acid 5-hydroxytryptophan (5-HTP), which is the initial and rate-limiting step in the synthesis of the nonneuronal serotonin[34]. Cognition of 5-HT system has been further deepened with the enrichment of TPH1 gene related researches[35, 36]. 5-HT’s neuronal and peripheral functions were separated through TPH1 knockout mice model[37]. Besides, the functions of 5-HT in bone regulation and energy metabolism were also explained in detail with a TPH1 knockout models[38, 39]. In the present study, we analyzed the function of TPH1 on calcium metabolism in GMEC through CRISPR/Cas9 system. Knockout of TPH1 gene decreased both the transcriptional and translation level of TPH1. In the medium of TPH1 knockout cells, the concentration of 5-HT was lower compared with wild type cells, which indicating that TPH1 was necessary for the synthesis of 5-HT[40]. Through 2D and 3D intracellular calcium imaging analysis, calcium content in GMEC decreased approximately 50% by TPH1 knockout,and this result was similar to the result of TPH1 knockout induced less bone resorption of mice [41]. These results suggested that both 5-HT synthesis and calcium metabolism in GMEC were negatively affected in the TPH1 knockout GMEC model.

To further substantiate the specific molecular components responsible for TPH1 affecting calcium transportation in GMEC, the expressions of calcium transportation related genes were detected. The plasma membrane calcium efflux pump PMCA1, responsible for the efflux of calcium from the epithelial cell[8], was increased in TPH1 knockout GMEC. Secretory pathway calcium pumps SPCA1 and SPCA2, involved in the secretion of calcium into milk, were up-regulated and down-regulated by TPH1 knockout, respectively. There was no influence on the expression of endoplasmic reticulum calcium sequestration pump SERCA2[42]. However, in our previous study, only the upregulation of PMCA1 was detected when TPH1 was knocked down by small interfering RNA[15]. The present study indicated a more realistic result that knockout TPH1 affected the expressions of those calcium transportation related genes, which led to the decrease in intracellular calcium content.

During lactation, PTHrP regulates calcium homeostasis via a feedback mechanism[43, 44]. 5-HT, synthesized from 5-HTP, is also involved in processes including milk protein biogensis and calcium homeostasis[14, 45]. Supplementation of 5-HTP increased contents of circulating 5-HT, PTHrP and milk calcium around parturition in mice and cows[14, 46, 47]. 5-HT induced PTHrP expression in bovine and goat mammary epithelial cells, while knockout of TPH1 in mice reduces the expression of mammary PTHrP and the expression can be rescued by restoring 5-HT synthesis[14, 15]. Similarly, knockout of TPH1 in GEMC decreased mRNA expression and protein abundance of PTHrP in the present study. Besides, the administration of 5-HTP increased intracellular calcium content in a dose dependent manner and rescued the expression of PTHrP. To interest us, then, TPH1 knockout appears to reduce the cell’s sensitivity to 5-HTP. Intracellular calcium levels increased about threefold when wild-type GMEC were treated with 100 µM 5-HTP, but calcium levels in TPH1 knockout GMEC increased by only about 50% under the same treatment. Further detection of calcium transportation related genes’ expression also showed that the response of TPH1 knockout GEMC to 5-HTP treatment was limited, except for PMCA1. Combined with our simultaneous study, we have confirmed that 5-HTP can inhibit the expression of
PMCA1 through miRNA-99a-3p axis in GMEC[48] and increase intracellular calcium content rather than through TPH1 axis. It is assumed that expression level of PTHrP, where there was no significant difference between wild type GEMC and 100 µM 5-HTP treated TPH1 gene knockout GEMC, mediates the phenomenon mentioned above. Despite the novel findings, the mechanism still needs further study.

CRISPR/Cas9 technique has been used for generating gene modified animal model[49] and to perform in vivo study of genes, miRNAs and long non-conding RNAs[50–52]. The CRISPR-associated protein 9, an endogenous endonuclease, directed by a single-guide RNA (sgRNA) causes a double strand break at the designed site firstly. Then, the cells tend to repair their DNA in two pathways[53]: non-homologous end-joining (NHEJ), which can cause random nucleotide insertion[54]; or homology-directed repair (HDR), which integrates a homologous DNA sequence[55]. In the present study, we obtained a heterozygous TPH1 knockout clone using the NHEJ pathway. We noticed that although the double strand was broken at the same site, the difference occurred during the repair process: One allele showed 18 nucleotides deletion and 31 nucleotides insertion, the other allele showed 10 nucleotides deletion. Off-target effect is the major and common limitation in various gene editing techniques[56]. In the present study, T7EN1 cleavage and sanger sequencing results showed that there was no off-target effect in the 10 predicted sites. Furthermore, TPH1 protein abundance was blocked in the THP1 knockout GMEC. These results indicate that the CRISPR/Cas9 system successfully knocked out the TPH1 gene.

Conclusions

In conclusion, we successfully obtained TPH1 gene heterozygous knockout GMEC by CRISPR/Cas9-mediated gene editing system. The heterozygous knockout of TPH1 blocked the cellular 5-HT synthesis, decreased the content of intracellular calcium and affected the expression of genes responsible for the efflux of calcium from the epithelial cell. The expression of PTHrP, the key regulator of mammary calcium homeostasis, was decreased in TPH1 knockout GMEC and its expression can be rescued by 5-HTP treatment. The processes of 5-HTP induced expression of PTHrP and calcium content was limited by the expression of TPH1. These results suggest that TPH1 plays an essential role in regulating calcium homeostasis in GMEC.

Abbreviations

TPH1: Tryptophan hydroxylase1; 5-HT: 5-hydroxytryptamine; GMEC: goat mammary epithelial cells; CRISPR/Cas9: CRISPR and CRISPR-associated 9; 5-HTP: 5-Hydroxytryptophan; SPCA1: secretory-pathway Ca2+ -ATPase1; PMCA1: plasma membrane Ca2+-ATPase1; SPCA2: secretory-pathway Ca2+-ATPase2; PTHrP: parathyroid hormone-related peptide; SERCA2: sarco(endo)plasmic reticulum Ca2+ ATPase2;

Declarations

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Authors’ contributions

ZZF, HLZ, JL and HBT designed the research; ZZF conducted the research and wrote the manuscript; ZZF, XYC, HYZ, HJG and WD analyzed the data; HLZ has primary responsibility for the final content. All authors read and approved the final manuscript.

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Availability of data and materials

All data measured or analyzed during this work are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All experimental procedures with live goats used in this study were approved by the Animal Care and Use Committee of the Northwest A&F University, Yang Ling, China (permit number: 15-516, date: 2015-9-13).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


Figures
Figure 1

Selection and identification of a TPH1 knockout clone. A Design of two sgRNAs (sgRNA1 and sgRNA2) targeting exon1 of the TPH1 gene. sgRNA1 and sgRNA2 are indicated in blue and underlined, and the corresponding PAM motifs are shown in red and underlined. B T7EI assays and were performed to evaluate the genome editing effect before and after puromycin selection of transfected cells, and bands densitometry was estimated by ImageJ. C Sequences of modified TPH1 alleles in single clone 1 were shown by TA clone sequencing. The regions of the mutation are marked by red and blue dotted lines in wild type sequences (WT), and the corresponding PAM motifs of sg1 are highlighted in red; deletion (−) and insertion (+) are shown to the right of each sequence.
Figure 2

Analysis of off-target effects of sgRNA1 in GEMC. A 10 potential off-target sites predicted by the online site and on-target site. The off-target nucleotide bases matching with the sgRNA and the PAM sequence are highlighted in blue and green respectively. B Detection of the off-target site genomic sequence by T7EN1 assay. OT, off-target; WT, wild type of each off-target site.
**Figure 3**

Effects of knockout of TPH1 on serotonin synthesis, intracellular calcium content and relative mRNA expression of genes related to calcium transportation in goat mammary epithelial cell (GEMC). All experiments were conducted in triplicate. Data are reported as Means ± SE, *P < 0.05. A Western blot for TPH1 protein in wild type (WT) and TPH1 knockout (TPH1-KO) cells, bands densitometry was estimated by ImageJ. B Enzyme-linked immunosorbent assay were performed to evaluate content of 5-HT in
medium after 24h of cell culture. C Intracellular calcium ion was stained by Fluo-3 AM, then was imaged by Laser scanning Confocal Microscope in both two-dimensional (2D) and three-dimensional (3D) modes, the intensity of fluorescence was measured by ImageJ software (D). E-H Total RNA was isolated using TRIzol reagent and the relative expression of genes related to calcium transportation was measured using real-time PCR.

Figure 4
Effect of knockout of TPH1 on 5-HTP induced PTHrP expression and calcium transportation. All experiments were conducted in triplicate. Data are reported as Means ± SE, *P < 0.05. A Western blot for PTHrP protein in wild type (WT) and TPH1 knockout (TPH1-KO) cells, bands densitometry was estimated by ImageJ. B Real-timePCR for relative mRNA expression in wild type and TPH1 knockout cells. C Protein abundance of PTHrP of cells treated with 100μM 5-HTP. WT, wild type GEMC treated with DMSO; WT + 5-HTP, wild type GMEC treated with 5-HTP (100μM); KO+5-HTP, TPH1 knockout GMEC treated with 5-HTP (100μM). D-H Relative mRNA expression of genes related to calcium transportation of cells treated with 100μM 5-HTP. WT, wild type GEMC; TPH1-KO, TPH1 knockout GMEC; NC, negative control, treatment of DMSO; 5-HTP, treatment of 5-HTP (100μM). I Intracellular calcium ion was stained by Fluo-3 AM in wild type (WT) and TPH1 knockout (TPH1-KO) cells treated with 0μM, 50μM and 100μM 5-HTP, and the intensity of fluorescence was measured by ImageJ software (J).

**Supplementary Files**

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