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Original Research Article

Hexokinase 1 and 2 mediates glucose utilization to regulate the synthesis of kappa casein via ribosome protein subunit 6 kinase 1 in bovine mammary epithelial cells

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ABSTRACT

Glucose plays a vital part in milk protein synthesis through the mTOR signaling pathway in bovine mammary epithelial cells (BMEC). The objectives of this study were to determine how glucose affects hexokinase (HK) activity in BMEC and investigate the regulatory effect of HK in kappa casein (CSN3) synthesis via the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway in BMEC. For this, *HK1* and *HK2* were knocked out in BMEC using the CRISPR/Cas9 system. The gene and protein expression, glucose uptake, and cell proliferation were measured. We found that glucose uptake, cell proliferation, *CSN3* gene expression levels, and expression of HK1 and HK2 increased with increasing glucose concentrations. Notably, glucose uptake was significantly reduced in *HK2* knockout (HK2KO) BMEC treated with 17.5 mM glucose. Moreover, under the same glucose treatment conditions, the proliferative ability and abundance of CSN3 were significantly diminished in both *HK1* knockout (HK1KO) and HK2KO BMEC compared with that in wild-type BEMC. We further observed that the phosphorylation levels of ribosome protein subunit 6 kinase 1 (S6K1) were reduced in HK1KO and HK2KO BMEC following treatment with 17.5 mM glucose. As expected, the levels of glucose-6-phosphate and the mRNA expression levels of glycolysis-related genes were decreased in both HK1KO and HK2KO BMEC following glucose treatment. These results indicated that the knockout of *HK1* and *HK2* inhibited cell proliferation and CSN3 expression in BMEC under glucose treatment, which may be associated with the inactivation of the S6K1 and inhibition of glycolysis.

1. Introduction

A continuous supply of glucose is required for the maintenance of lactation. Indeed, glucose requirements in lactating dairy cows are nearly 4-fold those for non-lactating ones (Bell and Bauman, 1997). The lactating mammary gland consumes up to 85% of the circulating glucose for milk production (Bickerstaffe and Annison, 1974). Consequently, glucose deficiency leads to a reduction in milk protein synthesis. One study showed that dairy cows with high milk protein concentration had higher glucose than dairy cows with low milk protein concentration (Wang et al., 2022). In addition, shortening dry period decreased milk protein in dairy cows under heat stress, accompanied by lower serum glucose concentration (Boustan et al., 2021), suggesting that glucose plays a pivotal role in milk protein synthesis. Toerien et al. (2010) reported that jugular infusion of glucose enhanced milk protein yield in feed-deprived dairy cows, with similar results in lactating dairy goats (Safayi and Nielsen, 2013). Glucose is a potent activator of milk protein synthesis (Toerien et al., 2004). Conversely, deficiency glucose suppressed casein gene and protein expression of bovine mammary epithelial cells (BMEC) (Zhang et al., 2018). Therefore, glucose uptake plays a crucial role in the mammary gland's ability to synthesize milk protein.

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Milk protein synthesis positively correlates with glucose supply (Rius et al., 2010). However, evidence suggests that, in ruminants, mammary glucose supply and glucose uptake may not always be strongly linked, and other factors may play a determinant role in regulating glucose uptake. Compared with extracellular glucose concentrations, the metabolic activity of the mammary glands was more strongly related to glucose uptake (Nielsen et al., 2001; Xiao and Cant, 2005). Hexokinases (HK) play an essential role in glucose metabolism. In the cytoplasm, HK phosphorylate the glucose taken up into cells via glucose transporters (GLUT), yielding glucose-6-phosphate (G6P), the first rate-limiting step in glucose metabolism. HK exist as four isoforms—HK1 to HK4—that differ in their catalytic and regulatory characteristics and exhibit tissue-specific expression patterns (Wilson, 2003). HK1 and HK2 are expressed in both rats' and sows' lactating mammary glands, the latter at exceptionally high levels (Kaselonis et al., 1999; Chen et al., 2017), suggesting that both enzymes are involved in milk synthesis. Protein synthesis is a highly adenosine-triphosphate (ATP)-dependent activity strongly correlated with cellular energy metabolism. Glycolysis is one of the major metabolic pathways and a key source of cellular energy. The inhibition of HK activity can result in reduced levels of G6P, which limits ATP production and protein synthesis. However, little is known regarding the role of HK in milk protein biosynthesis or the putative underlying mechanisms.

In milk-producing mammary glands, glucose is a precursor and a signaling regulator (Kimball and Jefferson, 2006; Burgos et al., 2013). However, the mechanism by which the milk-producing mammary gland detects and adjusts to glucose availability is incompletely understood. The mechanistic target of rapamycin complex1 (mTORC1) signaling pathway, which controls glucose-dependent cell proliferation, growth, and metabolism (Saxton and Sabatini, 2017), is a key pathway for elucidating this mechanism. The mTORC1 pathway activation has been documented in bovine mammary cells, as evidenced by the presence of phosphorylated (p)-mTOR (Ser^{2448}) (Appuhamy et al., 2011). The ribosomal protein S6 kinase 1 (S6K1), a rate-limiting regulator of cell growth and casein expression (Appuhamy et al., 2011; Nan et al., 2014; Yang et al., 2015), is a direct substrate for phosphorylation by mTOR. Understanding the molecular processes underlying the relationship between HK and mTORC1 pathway activation in BMEC could lead to developing strategies for increasing the efficiency with which BMEC utilizes glucose via specific adjustments in nutrient delivery.

Glucose was reported to regulate cell proliferation and casein expression through the mTORC1 signaling pathway in BMEC (Nan et al., 2014; Yang et al., 2015). Although it has previously been demonstrated that glucose promotes protein translation by activating the mTORC1 pathway in BMEC, little is known about how HK regulate casein expression. Kappa casein (CSN3) is an important component of milk protein (Wang et al., 2016). For example, the degree of glycosylation CSN3 can affect the synthesis of casein and milk protein (Robitaille et al., 1991). *CSN3* knockout mice cannot lactate and the casein micelles in the secretions are unstable (Shekar et al., 2006). We hypothesized that HK1- and HK2-mediated glucose utilization regulates CSN3 synthesis via the mTORC1 signaling pathway. Accordingly, in this study, we explored the role of HK in the mTORC1 signaling pathway associated with CSN3 synthesis in BMEC.

2. Materials and methods

2.1. Animal ethics statement

The experiments were approved by the Institutional Animal Care and Use Committee of Yangzhou University (SYXK (Su) IACUC 2012-0029), Yangzhou, China.

2.2. Cell culture

The immortalized BMEC by SV40 large T used in this study were provided by the Institute of Animal Culture Collection and Application (IACCA), Yangzhou University (Zhan et al., 2016). The BMEC were grown in DMEM/F12 (C11330500BT, Gibco, China) containing lactational hormones (5 g/mL insulin, 1 g/mL prolactin, and 1 g/mL hydrocortisone; Sigma-Aldrich, St Louis, MO, USA), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin.

2.3. Generation of HK1 and HK2 knockout BMEC

Exon 4 of the bovine *HK1* gene (GenBank ID: 280817) and exon 3 of the bovine *HK2* gene (GenBank ID: 788926) were selected as the target loci for editing. The sgRNAs were constructed using CHOPCHOP (http://chopchop.cbu.uib.no/) (Moyer and Holland, 2015). All prospective 25-bp primer sequences followed by 5′-CACCG-3′ were graded and evaluated based on predicted efficiency scores and the number of potential off-target sites (up to three mismatches in the protospacer), 40% to 60% guanine-cytosine content, and self-complementarity score ($\leq$1) (Edick et al., 2021). The sequences of the sgRNAs (synthesized by Genewiz Bioscience Co., Ltd, Suzhou, China) are shown in Table 1. The sgRNAs were subcloned into the lentiCRISPR v2 vector (52961, Addgene, USA), yielding HK1-sgRNA1, HK1-sgRNA2, HK1-sgRNA3, HK2-sgRNA1, HK2-sgRNA2, and HK2-sgRNA3.

HEK293T cells were seeded in six-well plates and cultured to 70% to 80% confluence. The cells were then co-transfected with 2.5 μg of lentiCRISPR v2-sgRNA1–3 plasmids, 1.5 μg of pMDLg/pRRE plasmid, 0.7 μg of pRSV-Rev plasmid, and 1 μg of pMD 2.G plasmid using PEI MAX (24765-1, Polysciences, USA) following the manufacturer's instructions. After culturing at 37 °C under 5% CO_2 for 8 h, the medium was replaced with a 10% FBS-supplemented growth medium, and the cells were cultured for a further 24 h, after which the medium was changed. Lentiviruses were collected between 48 and 72 h later and the media containing the viruses were collected and strained through a 0.45-μm sieve to remove cells and other debris from the supernatant. The BMEC (60% to 70% confluence) were infected with lentivirus supernatants for 72 h and were then extracted using 0.05% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) solution; half the BMEC were harvested to extract genome DNA. The remaining BMEC continued to grow in 6-well plates. Genomic DNA was extracted from a single-cell colony using a genomic DNA extraction kit (DP214-02, Tiangen, China). The genomic area encompassing the CRISPR/Cas9-gRNA target site was amplified using PCR SuperMix (Thermo Fisher Scientific). The HK1- and HK2-specific primers (5′ to 3′) were as follows: Forward: GTCTTAACACTGAACCTCCA, Reverse: CAGACCATCCAGTAAGAGAA;

Table 1
Bovine signal guide RNA (sgRNA) used in this study.

sgRNA	Sequence (5′ to 3′)	Exon
HK1-sgRNA1	F: CACCGCTATTCTGGATTGCCGACAA R: AAACTTGTCGGCAATCCAGAATAGC	4
HK1-sgRNA2	F: CACCGTCGGCAATCCAGAATAGACG R: AAACCGTCTATTCTGGATTGCCGAC	4
HK1-sgRNA3	F: CACCGAAGGACAAGAAATTACCTGT R: AAACACAGGTAATTTCTTGTCCTTC	4
HK2-sgRNA1	F: CACCGATCCCCGAGGACATCATGCG R: AAACCGCATGATGTCCTCGGGGATC	3
HK2-sgRNA2	F: CACCGTCGCATGATGTCCTCGGGGA R: AAACTCCCCGAGGACATCATGCGAC	3
HK2-sgRNA3	F: CACCGTACCCAAAGCACACGGAAGT R: AAACACTTCCGTGTGCTTTGGGTAC	3

F = forward; R = reverse; *HK1* = hexokinase 1; *HK2* = hexokinase 2.

and Forward: TCACCTAGAGATGACCAGTC, Reverse: AAATCCCTCCCACTAAAGCA, respectively (Figs. 5A and 6A). The sequencing assay confirmed whether the target *HK1* and *HK2* genes were mutated. Scramble peaks at the gRNA target site indicate that the target was active. Then mutated BMEC were extracted using 0.05% trypsin–0.02% EDTA solution, and then it was diluted and aliquoted into 96-well plates, and the medium was replaced every 5 days. After 1 month, positive clones were subjected to sequencing analysis. Finally, the amplicon sequences were cloned into the T vector (6013, Takara, Beijing, China) and sequenced to prove the absence of base number.

2.4. Experimental design

To examine the effect of glucose in BMEC, cells were plated into six-well plates at a density of 1×10^5 cells per well. At 80% confluence, the BMEC were starved in a medium without glucose (D6540, Solarbio, China) for 2 h and subsequently incubated in a medium containing different concentrations of glucose (0, 2.5, 10, or 17.5 mM) as previously reported (Zhang et al., 2018). To determine the effect of HK in response to glucose treatment, *HK1* knockout (HK1KO), *HK2* knockout (HK2KO), and wild-type BMEC were plated into six-well plates at a density of 1×10^5 cells per well. At 80% confluence, the cells were starved in a medium without glucose (D6540, Solarbio, China) for 2 h and subsequently incubated in the presence (17.5 mM) or absence of glucose. All the media (D6540, Solarbio, China) used for treatments were serum-free and adjusted to pH 7.4. The cells were harvested after 24 h of incubation to assess their proliferative ability (performed in sextuplicate) and after 6 h incubation for all other experiments (performed in triplicate).

2.5. Analysis of glucose uptake and cell proliferation

A colorimetric glucose oxidase/peroxidase assay kit (E1010-1, Applygen, China) was used to determine the amount of glucose present in the culture medium. The amount of glucose absorbed was estimated as the difference between the amounts before and after 6-h incubation and presented as miligrams of glucose per miligram of protein per hour (Silva et al., 2022). A BCA Protein Assay Kit (P0012, Beyotime, Beijing, China) was used to assess the total protein content of the cells, and a Cell Counting Kit-8 (CCK-8) assay kit (A311, Vazyme Biotech Co., Ltd, China) was used for the estimation of cell proliferation, as previously described (Yang et al., 2022). The HK1KO, HK2KO, and wild-type BMEC were seeded into 96-well plates at 1250, 2500, 5000, 10,000, and 20,000 cells per well to generate a standard curve. After treatment, the cells were washed five times with 200 μL of sterile water and then cultured in 100 μL of DMEM supplemented with 10 μL of CCK-8 reagent at 37 °C with 5% CO_2 for 3 h. The absorbance of each well at 450 nm was determined using an automated microplate reader (Multiskan GO, Thermo Scientific, Shanghai, China).

2.6. Determination of HK activity and G6P levels

HK activity was assessed using a total HK assay kit (BC0740, Solarbio). Briefly, the cells were lysed and cleared by centrifugation, 5 μL of the lysate was transferred into 195 μL of reaction buffer on ice, and the absorbance at 340 nm was quickly measured using an automated microplate reader (Thermo Scientific, Shanghai, China). Total HK activity was calculated as the optical density (OD) determined after 5 min minus the value measured at 0 min, which was reported as nanomole NADPH generated per milligram of protein per minute. The G6P levels were determined using a Glucose-6-phosphate Colorimetric Assay Kit (K011, Elabscience, China) according to the manufacturer's guidelines (www.elabscience.cn).

2.7. Quantitative RT-PCR

The cells were seeded at a density of 2×10^5 cells/well in six-well plates for mRNA expression analysis. Total RNA was extracted using a FastPure Cell/Tissue Total RNA Isolation Kit (RC101, Vazyme Biotech Co., Ltd, Nanjing, China). RNA quality was assessed using 2% agarose gel electrophoresis, while RNA purity and concentration were assessed using an OD-1000+ microspectrophotometer (One drop-1000+, Thermo Scientific, Shanghai, China). The total RNA $OD_{260/280}$ ratio between 1.9 and 2.0 and the 28S ribosomal RNA band intensity in total RNA samples was approximately 2-fold that of the 18S ribosomal RNA band, indicating the total RNA was of excellent quality (Ma et al., 2021a,b). The RNA was reverse transcribed using an RT kit from Takara (RR047, Beijing, China) in a reaction mixture containing 1 μg of total RNA and 1× PrimeScript RT Master Mix in a final volume of 20 μL (37 °C for 15 min). Reverse transcriptase was inactivated at 85 °C for 5 s. Quantitative real-time PCR (qPCR) was performed using SYBR Premix Ex Taq II (RR091Q, Takara, Beijing, China). The 20-μL reaction mixture contained SYBR Premix Ex Taq II, 0.4 μM of each forward and reverse primer, and 100 ng of cDNA. qPCR comprised an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Where possible, the primers were designed to span exon–exon junctions. Primer dimer formation was assessed by melting curve analysis after amplification to validate the occurrence of a single product. Table 2 contains a list of the primers used. A no-template negative control reaction was included. RefFinder (http://www.leonxie.com/referencegene.php), which uses Normfinder, geNorm, and the comparative ΔCT approach, was used to rank the candidate genes, from which the first-rank reference genes (actin beta [*ACTB*], ribosomal protein S9 [*RPS9*], and glyceraldehyde-3-phosphate

Table 2
The sequences of the primers used in this study.

Gene	Primer sequence (5′ to 3′)	Source	Size, bp
GAPDH	F: TTGTCTCCTGCGACTTCAACA R: TCGTACCAGAAATGAGCTTGAC	NM_001034034.2	103
RPS9	F: CCTCGACCAAGAGCTGAAG R: CCTCCAGACCTCACGTTTGTTC	NM_001101152.2	64
ACTB	F: GACCCAGATCATGTTCGAGA R: CTCATAGATGGGCACCGTGT	NM_173979.3	145
CSN3	F: CCAGGAGCAAAACCAAGAAC R: TGCAACTGGTTTCTGTTGGT	NM_174294.2	148
HK1	F: GTGTGCTGTTGATAATCTCC R: AATAACTGTTGGACGAATGC	NM_001012668.2	149
HK2	F: AAGATGCTGCCCACCTACG R: TCGCTTCCCGTTCCGCACA	XM_015473383.2	123
PFKL	F: TGACTCGTATGGGCATTTAC R: AACTGGATGATGTTGGAGAC	NM_001080244.2	127
PFKM	F: ACGAGATTGACTTGGATACC R: GAGAATACACGGACTTGGAT	NM_001075268.1	155
GPI	F: AACTTCACTGAGGATCGGGC R: CTTGCCCGAGTACCCTTTCC	NM_001040471	171
ALDOA	F: GTCCACTCCGCCAGCC R: CTTGGCAATGCTCCCGGT	NM_001101915	200
ALDOB	F: ACCATGGCCCACCAGTTTC R: GGTTCCCCATCGTGCCTAC	NM_001034485	130
ALDOC	F: AGAAACCACCACTCAAGGGC R: CACAATCCCATTCTGCTGGC	NM_001097984.2	190

F = forward; R = reverse; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *RPS9* = ribosomal protein S9; *ACTB* = actin beta; *CSN3* = kappa casein; *HK1* = hexokinase 1; *HK2* = hexokinase 2; *PFKL* = phosphofructokinase liver type; *PFKM* = phosphofructokinase muscle; *GPI* = glucose-6-phosphatase isomerase; *ALDOA* = fructose-bisphosphate A; *ALDOB* = fructose-bisphosphate B; *ALDOC* = fructose-bisphosphate C.

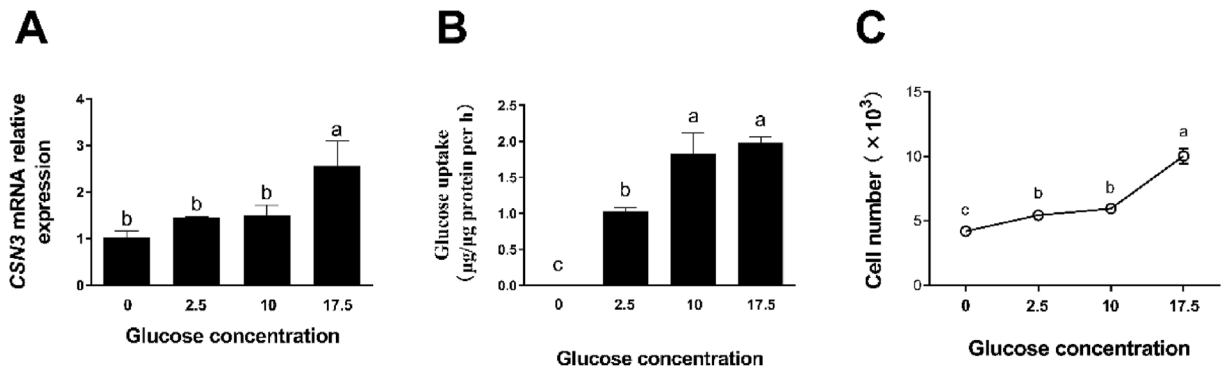

Fig. 1. The effect of different glucose concentrations on bovine mammary epithelial cells (BMEC). The BMEC were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h and then incubated with different concentrations of glucose (0, 2.5, 10, or 17.5 mM) for 6 or 24 h. (A) The relative gene expression levels of kappa casein (*CSN3*) were assessed by quantitative real-time PCR (qPCR) and normalized to those of actin beta (*ACTB*). (B) Glucose uptake was measured using a commercially available kit. (C) The effect of glucose on BMEC proliferation. Values are shown as means ± SEM of three independent experiments for glucose uptake and gene expression analysis and six independent experiments for the assessment of cell proliferation. Different lowercase letters denote significant differences among treatment groups at $P < 0.05$. Error bars represent the SEM.

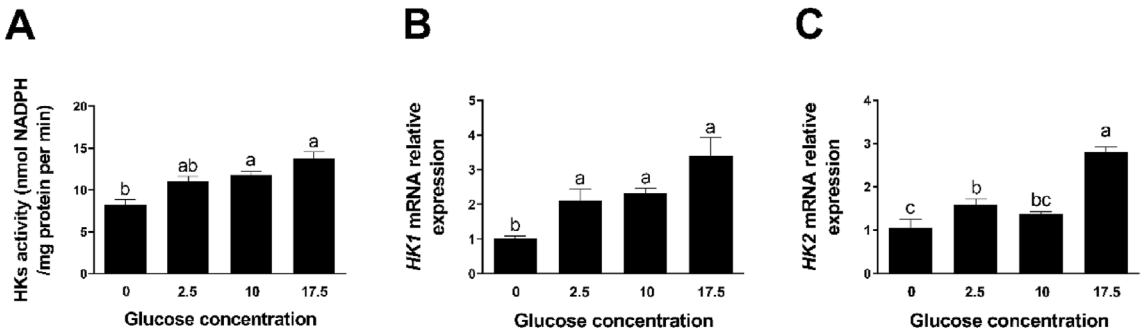

Fig. 2. Hexokinase (HK) activity and the mRNA abundance of hexokinase 1 (*HK1*) and hexokinase 2 (*HK2*) in bovine mammary epithelial cells (BMEC) under different glucose concentrations. The BMEC were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h and then incubated with different concentrations of glucose (0, 2.5, 10, or 17.5 mM) for 6 h. (A) HK activity was measured using a commercially available kit. (B, C) The gene expression levels of *HK1* and *HK2* were assessed by quantitative real-time PCR (qPCR) and were normalized to those of actin beta (*ACTB*). All values are shown as means ± SEM of three independent experiments. Different lowercase letters denote significant differences among treatment groups at $P < 0.05$. Error bars indicate the SEM.

dehydrogenase [*GAPDH*]) were selected. The final ranking was determined by assigning a suitable weight to each gene and then calculating the geometric mean of their weights. A lower geometric mean of ranking denoted greater expression stability. Finally, *ACTB* was screened for subsequent study. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each trial was conducted in triplicate.

2.8. Western blotting analysis

Cells were seeded in a 10-cm dish (2×10^6 cells/well) for protein expression analysis and cultured at 37 °C with 5% CO_2. Cells were lysed in RIPA lysis buffer (89900, Thermo Scientific, Shanghai, China) containing 1× protease inhibitor cocktail (1862209, Thermo Scientific, Shanghai, China) and 1× phosphatase inhibitor cocktail (1862209, Roche, Shanghai, China), yielding total protein. Protein concentrations were measured using a BCA kit (P0010, Beyotime, China) according to the protocols specified by the manufacturer. Equal amounts (40 μg) of protein were separated by SDS–PAGE and transferred to nitrocellulose membranes (PALL, Shanghai, China). The membranes were then blocked in blocking buffer (5% horse serum in Tris-buffered saline with Tween 20 [TBS-T: 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20]) and incubated overnight at 4 °C with primary antibodies (diluted in blocking buffer) targeting GAPDH (2118, 1:1,000; CST, Shanghai, China), mTOR (2972, 1:750; CST, Shanghai, China), p-mTOR (2971, 1:750; CST, Shanghai, China), S6K1 (9202, 1:750; CST, Shanghai, China), p-S6K1 (9234, 1:750; CST, Shanghai, China), HK1 (AF1726, 1:750; Beyotime, China), HK2 (ab209847, 1:750; Abcam, USA), and CSN3 (orb482191, 1:750; Biorbyt). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG [7074, 1:5000; CST, Shanghai, China]). Pierce ECL Plus Western Blotting Substrate or Super Signal West Femto Maximum Sensitivity Substrate (46640, Thermo Scientific, Shanghai, China) were employed for the detection of target bands.

2.9. Immunofluorescence analysis

Immunofluorescence analysis was undertaken as previously described (Zhan et al., 2020). Briefly, cells cultured on chamber slides were fixed in methanol and acetone for 20 min at 20 °C, blocked for 1 h at room temperature in PBS containing 3% horse serum, and then incubated with anti-HK1 and anti-HK2 primary antibodies overnight at 4 °C. After gentle rinsing in PBS, the cells were incubated for 45 min with FITC-conjugated anti-rabbit IgG (1:400; sc-3753, Santa Cruz, Shanghai, China), rinsed in PBS, and counterstained with DAPI for 5 min. Finally, the samples were mounted in PermaFluor mounting medium (TA006FM, Thermo

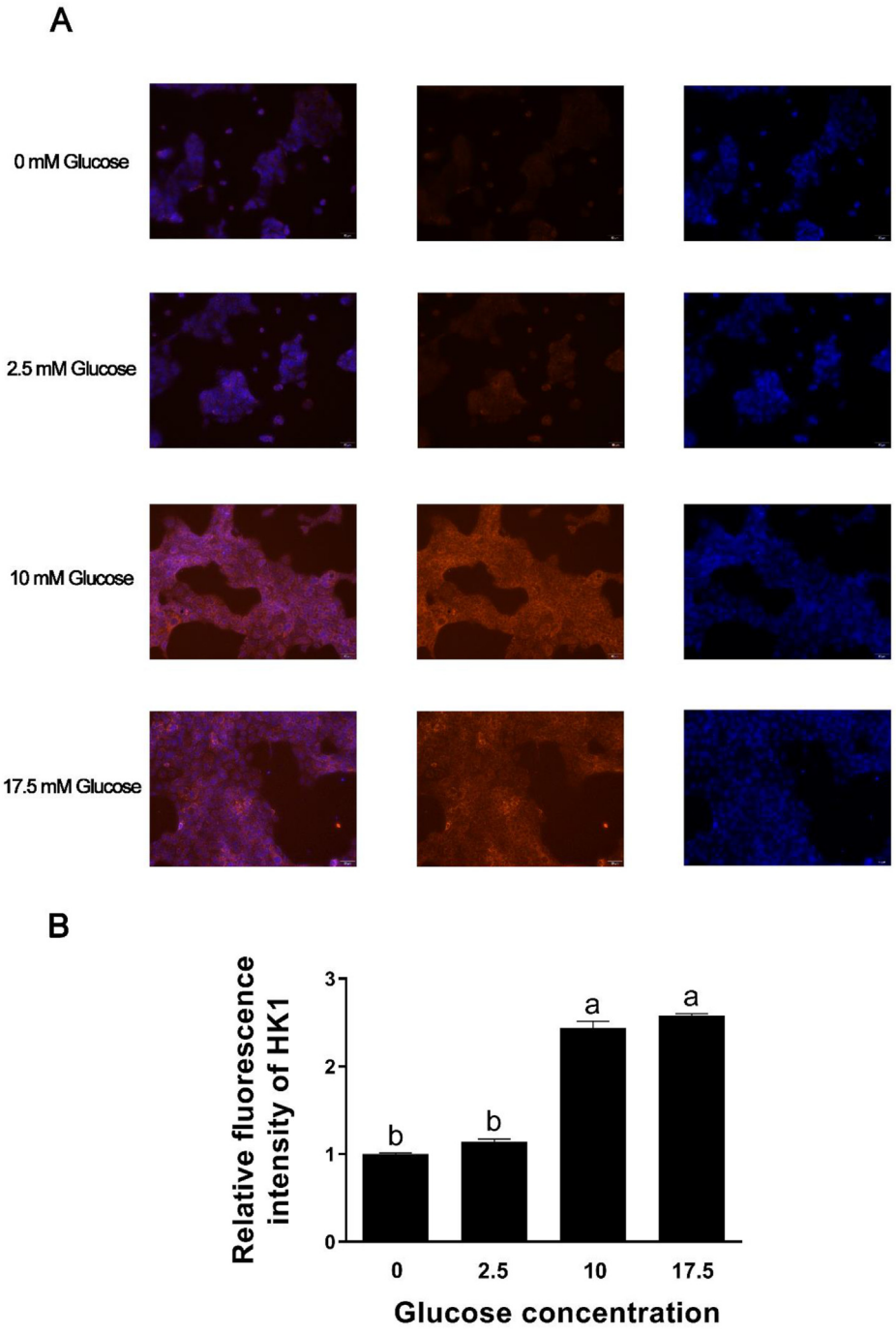

Fig. 3. The effect of different glucose concentrations on the protein expression of hexokinase 1 (HK1) as determined by immunofluorescence staining. Bovine mammary epithelial cells (BMEC) were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h, incubated with different concentrations of glucose (0, 2.5, 10, or 17.5 mM) for 6 h, and then subjected to immunofluorescence staining. HK1 is shown in red and nuclei (DAPI) are shown in blue. The data are representative of three independent experiments. Different lowercase letters denote significant differences among treatment groups at $P < 0.05$.

Scientific, Shanghai, China), coverslipped, and observed and imaged using a confocal laser scanning microscope (BX53, Olympus, Tokyo, Japan).

2.10. Statistical analysis

All data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The data are shown as the mean and pooled standard error of the mean (SEM). The effects of glucose were calculated using one-way ANOVA with Bonferroni's multiple-range test to evaluate the difference between different treatment groups. Other studies were subjected to two-way ANOVA analysis, and the statistical model included the effects of the cell type and glucose levels and their interactions. A post hoc test was conducted using Bonferroni's multiple comparison test for significant interactions. *P*-values < 0.05 were considered significant.

3. Results

3.1. The effect of glucose on CSN3 expression and HK activity in BMEC

A profile of the mRNA expression of *CSN3* in BMEC treated with various glucose concentrations is presented in Fig. 1A. The gene expression of *CSN3* was 2-fold greater in BMEC treated with 17.5 mM than in untreated BMEC ($P = 0.037$); however, compared with the negative controls, no differences in *CSN3* gene expression were observed with glucose treatments at the 2.5 and 10 mM concentrations ($P > 0.05$). Higher *CSN3* expression was accompanied by increased glucose uptake. Compared with the 2.5 mM group, glucose uptake by BMEC was greater in both the 10 ($P = 0.035$) and 17.5 ($P = 0.012$) mM glucose treatment groups (Fig. 1B). BMEC viability was increased with increasing glucose availability, with the greatest effect seen in the 17.5 mM group relative to that in the negative control (glucose deficiency) group ($P < 0.001$) (Fig. 1C).

Next, the expression levels of HK1 and HK2 were examined to further investigate the mechanisms underlying how glucose addition influences *CSN3* gene expression in BMEC. We found that the activities of overall HK in BMEC were diminished with decreasing glucose concentrations (Fig. 2A), whereas the opposite was observed with increasing glucose availability (2.5 mM, $P = 0.015$; 10 mM, $P = 0.026$; 17.5 mM, $P = 0.002$), compared with the negative control group. Similarly, the mRNA abundance of *HK1* in BMEC was significantly enhanced under all glucose concentrations tested (all $P < 0.05$), and the gene expression of *HK2* was remarkably increased under 2.5 and 17.5 mM concentration treated ($P < 0.05$), relative to

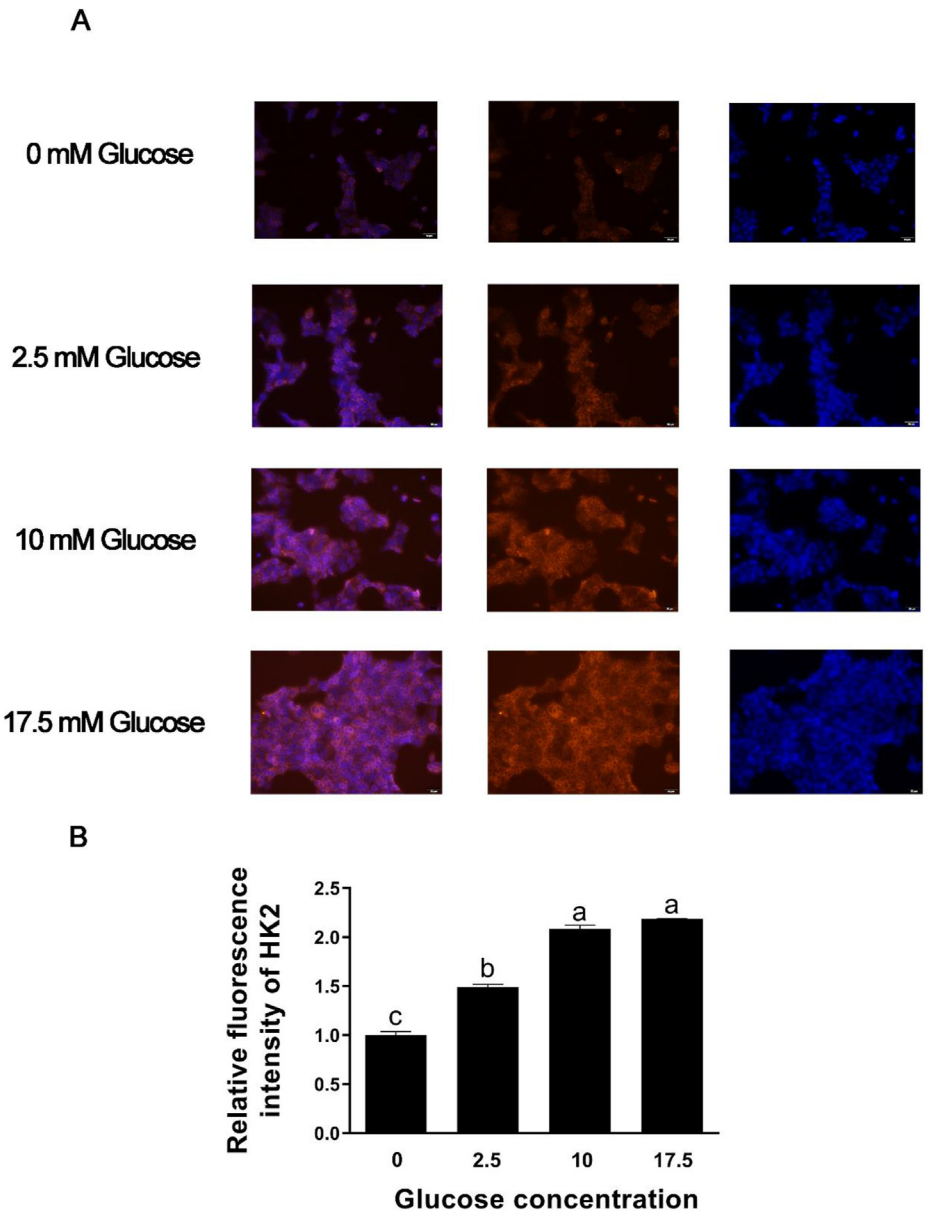

Fig. 4. The effect of different glucose concentrations on the protein expression of hexokinase 2 (HK2) as determined by immunofluorescence staining. Bovine mammary epithelial cells (BMEC) were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h, incubated with different concentrations of glucose (0, 2.5, 10, or 17.5 mM) for 6 h, and then subjected to immunofluorescence staining. HK2 is shown in red and nuclei (DAPI) are shown in blue. The data are representative of three independent experiments. Different lowercase letters denote significant differences among treatment groups at $P < 0.05$.

that in the negative controls (Fig. 2B and C). Immunofluorescence results showed that the protein expression of HK1 increased in 10 ($P < 0.001$) and 17.5 mM ($P < 0.001$) glucose, and HK2 was upregulated in all glucose treatments (all $P < 0.05$), relative to the negative control group (Figs. 3 and 4).

3.2. The establishment of HK1 and HK2 knockout BMEC lines

To assess the functions of the *HK1* and *HK2* genes, we generated HK1KO and HK2KO BMEC lines using CRISPR/Cas9. First, three gRNAs targeting exon 4 of HK1 and three targeting exon 3 of HK2 were designed. The PCR products of the targeted HK1 and HK2 genomic regions were subjected first to a cruiser enzyme digestion assay for Cas9-sgRNA activity assessment and then to sequencing analysis. Sequence analysis showed that the HK1 clone and the 8th clone of HK2 sgRNA1 (HK2 1 to 8 clone) were associated with scrambled peaks at the gRNA target sites (Figs. 5A and 6A). In the HK1 and HK2 1 to 8 clone, one allele had a deletion flanking the gRNA3 and gRNA1 regions, respectively, whereas the other allele was unedited. To clarify the precise target site of the deletion in the HK1 clone, the relevant PCR products were cloned into the T vector and subjected to sequence analysis. The results showed that the HK1 clone had a 2-bp deletion beside the gRNA3 target site (Fig. 5B and C). In addition, the 6th clone of HK2 sgRNA2 (HK2 2 to 6 clone) had a 5-bp deletion flanking the gRNA2 target sequence (Fig. 6B and C). The expression of HK1 in HK1KO BMEC and HK2 in HK2KO

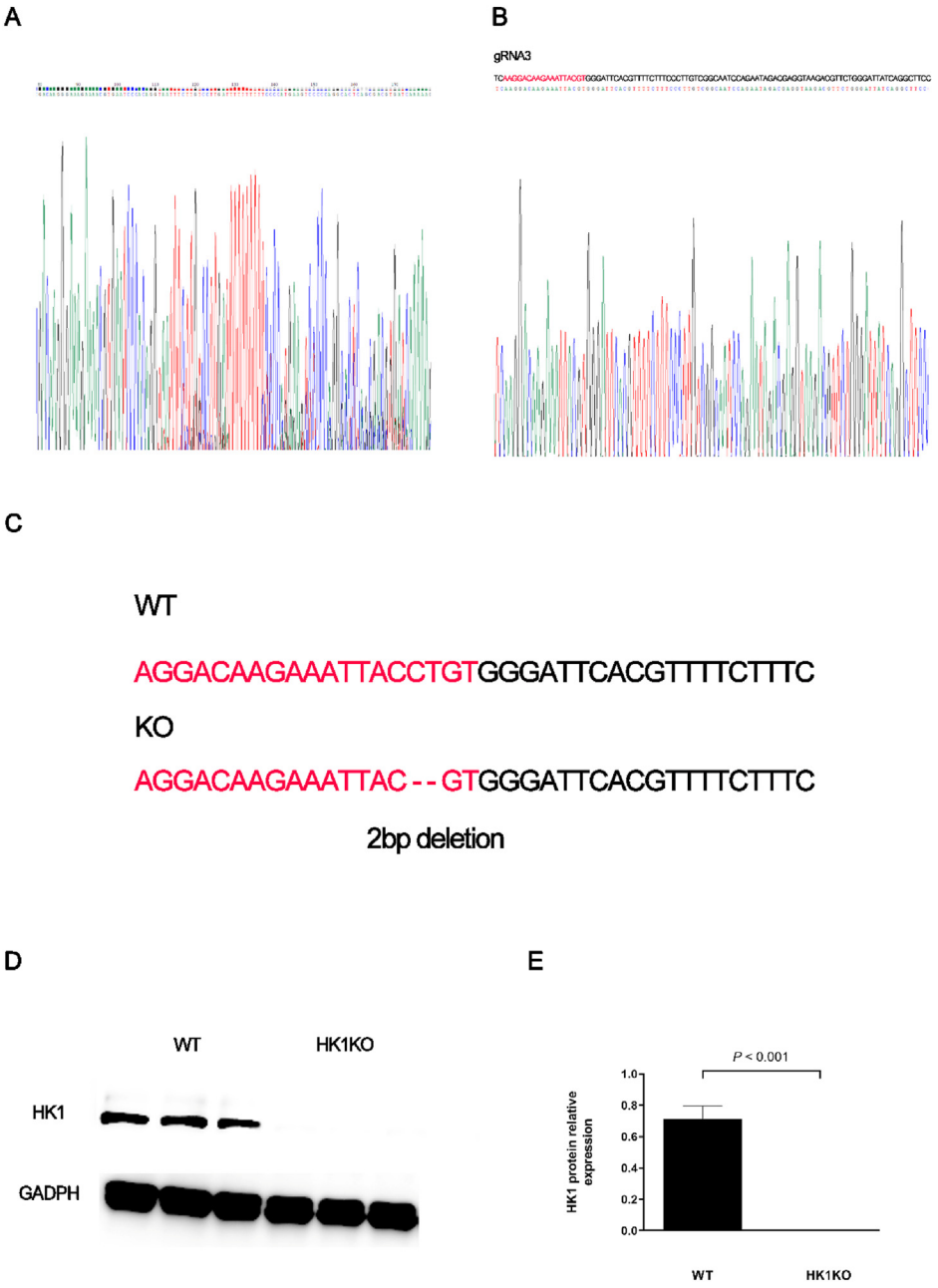

Fig. 5. The generation of hexokinase 1-knockout (HK1KO) bovine mammary epithelial cell (BMEC) lines using the CRISPR/Cas9 system. (A) Sequence analysis of a gRNA3 target-positive clone using the HK1 R primer. (B, C) Sequence analysis of the T's and A's (TA) clone. The deletions in HK1KO BMEC are presented. (D, E) Western blotting analysis of the HK1 protein level in HK1KO BMEC. WT = wild type.

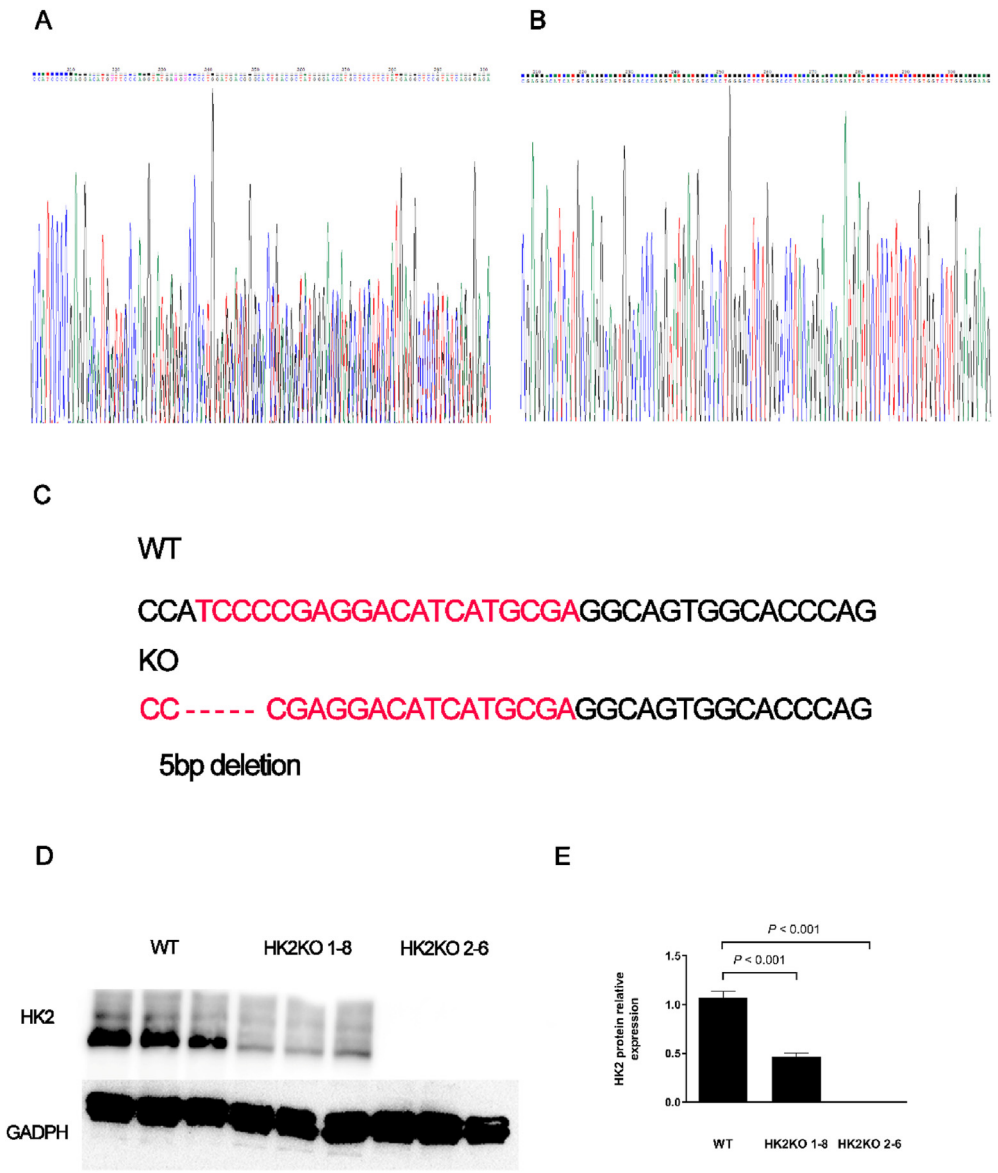

Fig. 6. The generation of hexokinase 2-knockout (HK2KO) bovine mammary epithelial cell (BMEC) lines using the CRISPR/Cas9 system. (A) Sequence analysis of a gRNA1 target-positive 1 to 8 clone using the HK2 F primer. (B, C) Sequence analysis of a gRNA2 target-positive 2 to 6 clone using the HK2 F primer. The deletions in HK2KO BMEC are shown. (D, E) Western blotting analysis of HK2 protein levels in HK2KO BMEC. WT = wild type.

BMEC was assessed by Western blotting (Figs. 5D and E and 6D, E). Our dates showed that compared with WT BMEC, HK1 KO BMEC and HK2 2 to 6 clone BMEC abolished ($P < 0.001$) the protein expression of HK1 and HK2, respectively. Accordingly, HK1KO and HK2KO BMEC lines originating from the HK1 and HK2 2 to 6 clone were used in subsequent experiments.

3.3. The effect of HK1KO and HK2KO on protein synthesis in BMEC treated with glucose

After generating the HK1KO and HK2KO model BMEC lines, we next evaluated the effect of HK deletion on the ability of BMEC to respond to glucose. We found that glucose uptake was decreased in HK2KO BMEC compared with that in wild-type cells ($P < 0.001$), whereas no difference in glucose uptake was observed between wild-type and HK1KO BMEC ($P = 0.394$) (Fig. 7A). The proliferative ability of both HK1KO ($P = 0.003$) and HK2KO ($P < 0.001$) BMEC was decreased in the presence of glucose, compared with wild-type BMEC treated with 17.5 mM glucose; in addition, the proliferation of HK2KO BMEC, but not HK1KO BMEC, was also decreased ($P = 0.037$) under conditions of glucose deprivation, related to wild-type BMEC (Fig. 7B). Meanwhile, compared with wild-type BMEC treated with 17.5 mM glucose, the mRNA abundance of *CSN3* was decreased in both HK1KO ($P < 0.01$) and HK2KO ($P < 0.01$) BMEC treated with 17.5 mM glucose (Fig. 7C). The protein expression of CSN3 showed a similar trend (Fig. 7D and E). These results showed that HK1 and HK2 mediated the regulatory effects of glucose on cell proliferation and CSN3 gene and protein expression in BMEC.

Glycolysis is a key source of the cellular energy required for protein synthesis and growth. As expected, we found that G6P levels were higher ($P = 0.050$) in BMEC treated with glucose than in those subjected to glucose deprivation. However, in the presence of glucose, the G6P levels were lower in both HK1KO BMEC ($P = 0.045$) and HK2KO BMEC ($P = 0.003$) than in wild-type BMEC (Fig. 8B). Next, we evaluated the effect of HK1 and HK2 on the expression of glycolysis-related genes by qPCR (Fig. 8C and D). The

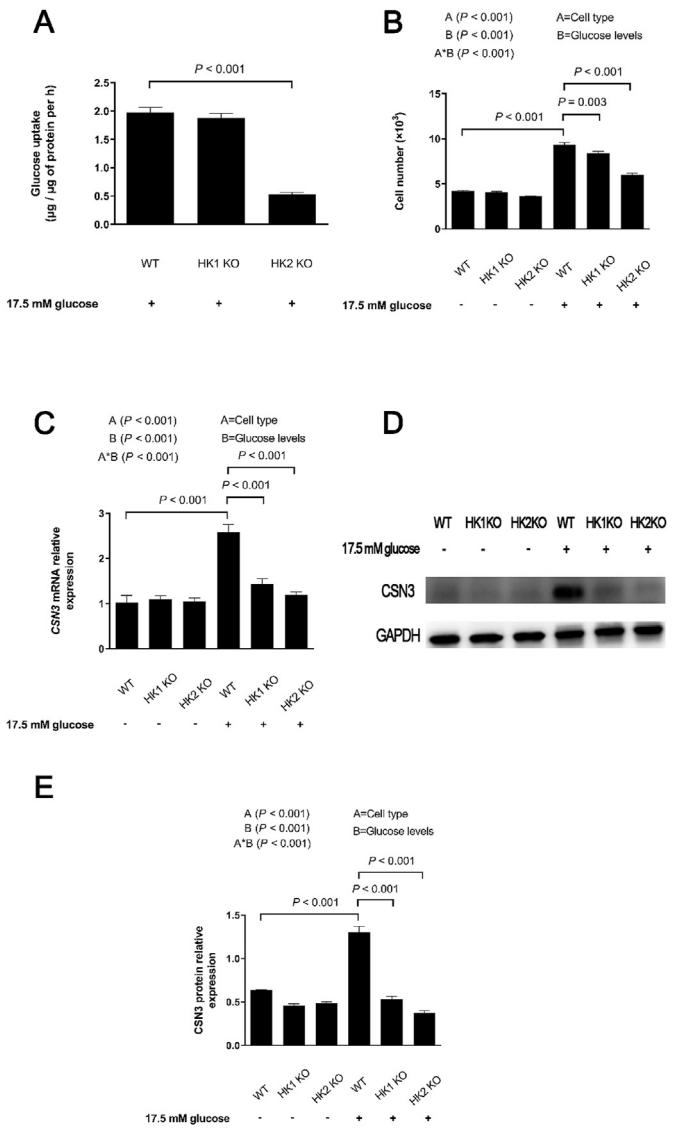

Fig. 7. The effect of hexokinase 1 (*HK1*) and hexokinases 2 (*HK2*) on glucose uptake, cell proliferation, and kappa casein (*CSN3*) gene expression in bovine mammary epithelial cells (BMEC). *HK1* knockout (HK1KO) BMEC and *HK2* knockout (HK2KO) BMEC, and BMEC were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h and then incubated with different concentrations of glucose (0 or 17.5 mM) for 6 or 24 h. (A) Glucose uptake was measured using a commercially available kit. (B) The effect of glucose on cell proliferation in BMEC. (C) *CSN3* mRNA expression levels were assessed by quantitative real-time PCR (qPCR); actin beta (*ACTB*) was used for normalization. (D, E) CSN3 protein expression levels were assessed by western blotting; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Values are shown as means ± SEM of three independent experiments for glucose uptake and gene expression analysis and six independent experiments for the assessment of cell proliferation. Error bars indicate the SEM.

results showed that the mRNA expression levels of glucose-6-phosphatase isomerase (*GPI*), fructose-bisphosphate A (*ALDOA*), and fructose-bisphosphate C (*ALDOC*) were unaffected ($P > 0.005$) in response to either glucose addition or HK knockdown. The relative mRNA expression levels of phosphofructokinase muscle (*PFKM*) ($P = 0.046$) and *ALDOB* ($P = 0.050$) were higher in BMEC supplied with glucose than in those subjected to glucose deprivation. In the case of glucose deprivation, compared with wild-type BMEC, the relative mRNA expression levels of *ALDOB* decreased in HK1KO BMEC ($P = 0.026$). With glucose supplementation, meanwhile, the relative mRNA abundance of phosphofructokinase liver type (*PFKL*) and *ALDOB* was lower in both HK1KO BMEC ($P = 0.050$ and $P < 0.001$, respectively) and HK2KO BMEC ($P = 0.018$, and $P < 0.001$, respectively) than in wild-type BMEC. In addition, HK2KO BMEC treated with glucose decreased the mRNA gene of *PFKM* ($P = 0.001$) compared to wild-type BMEC treated with glucose.

The mTORC1 signaling pathway was related to both cell proliferation and casein expression. Compared with wild-type BMEC, following glucose treatment, the p-S6K1 level was reduced ($P < 0.001$) in both HK1KO and HK2KO BMEC (Fig. 9A and B).

4. Discussion

Glucose supply can influence the mTOR signal transduction pathway and milk protein synthesis in immortalized mammary cells (Zhang et al., 2018). Zhao et al. (2012) also proposed that HK2 might perform a vital function in glucose uptake in BMEC. However, relatively little is known regarding the role of HK in milk protein biosynthesis in BMEC in the presence of glucose or the putative underlying mechanism.

Glucose plays a central role in metabolism in almost all organisms and can serve as a precursor for the synthesis of nucleotides. In this study, cells were starved overnight and treated with three concentrations of glucose, with the highest concentration being more than 3-fold higher than that typically seen in the blood of dairy cows. We found that increased glucose availability was accompanied by a concomitant increase in glucose uptake by BMEC, which is consistent with that reported by Rius et al. (2010). Cell growth requires a high rate of protein synthesis and a large amount of cellular energy. In the same pattern, the highest glucose concentration was sufficient for cell proliferation. Lunt and Vander Heiden (2011) argued that enhanced glucose metabolism is selected for in proliferating cells throughout nature because increased glucose uptake can supply sufficient amounts of the chemical building blocks, such as nucleotides, amino acids, and lipids, that are required for the synthesis of macromolecules necessary for cell growth. In addition, although glucose has a key role in milk protein synthesis, milk protein production is also an energy-dependent process (Lobley, 1990). Our data showed that glucose deficiency leads to lower *CSN3* gene expression, consistent with previously reported (Zhang et al., 2018). These findings support that glucose can increase the *CSN3* expression in BMEC.

HK are essential enzymes for glucose metabolism and have been suggested to play a vital role in milk synthesis during lactation. To test this hypothesis, we investigated the activity of these enzymes in BMEC in this study. We found that increasing glucose availability was associated with greater HK activities, which was consistent with the results of a previous study suggesting that HK activities were positively correlated with glucose uptake (Yamada et al., 2005). HK1 and HK2 are expressed in the mammary glands of lactating rats (Kaselonis et al., 1999), and it is interesting to identify the vital function exerted by each HK during milk protein synthesis. The qPCR and immunofluorescence results indicated that the expression of both HK1 and HK2 was significantly increased in BMEC following glucose treatment, suggesting that the two enzymes may perform an important function in milk protein synthesis.

To explore the mechanisms underlying the effects of HK on milk protein synthesis, we generated HK1KO and HK2KO BMEC lines. We observed that glucose uptake was unaffected in HK1KO BMEC; however, glucose uptake was markedly decreased in HK2KO BMEC, as also previously shown in BMEC exposed to 3-bromopyruvate, an inhibitor of HK2 (Zhao et al., 2012). This supports the previous assumption that HK2, rather than HK1, may perform a vital function in glucose uptake in BEMC. However, in our study, the gene expression of *CSN3* was decreased in both HK1KO and HK2KO

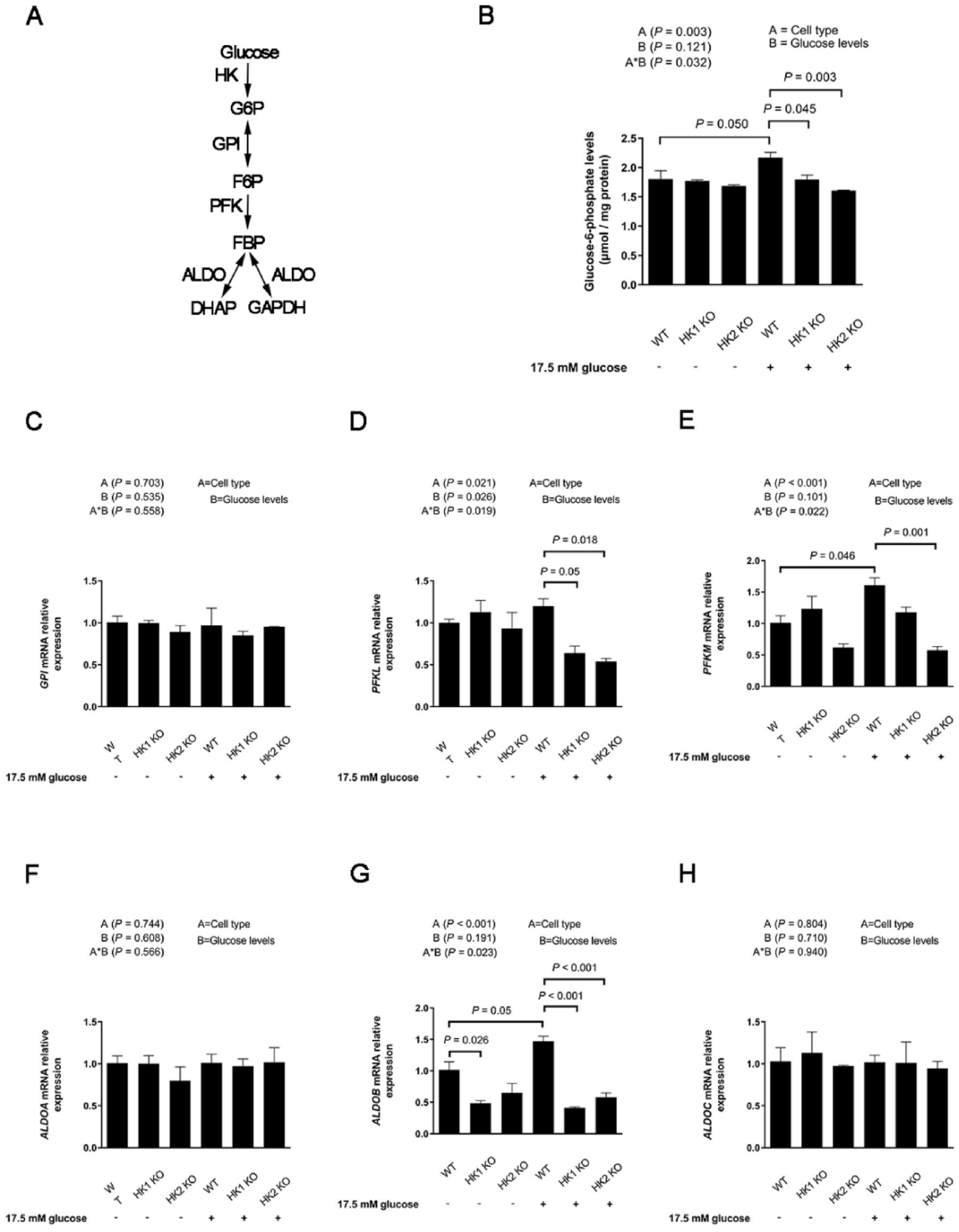

Fig. 8. The effect of hexokinase 1 (*HK1*) and hexokinases 2 (*HK2*) on the expression of glycolysis-related genes in bovine mammary epithelial cells (BMEC) in response to glucose supplementation. BMEC, *HK1* knockout (HK1KO) BMEC, and *HK2* knockout (HK2KO) BMEC were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h and then incubated with (17.5 mM) or without glucose for 6 h. (A) A schematic diagram of glucose metabolism. (B) The glucose-6-phosphate levels were measured using a commercially available kit. (C to H) The relative gene expression levels of glucose-6-phosphatase isomerase (*GPI*), phosphofructokinase liver type *(PFKL*), phosphofructokinase muscle (*PFKM*), fructose-bisphosphate A (*ALDOA*), fructose-bisphosphate B (*ALDOB*), and fructose-bisphosphate C (*ALDOC*) were assessed by quantitative real-time PCR (qPCR) and were normalized to those of actin beta (*ACTB*). Different lowercase letters denote significant differences between treatment groups at $P < 0.05$. Error bars denote the SEM. HK = hexokinase 1; G6P = glucose-6-phosphate; GPI = glucose-6-phosphatase isomerase; F6P = fructose-6-phosphate; PFK = phosphofructokinase; FBP = fructose bisphosphate; ALDO = fructose-bisphosphate; DHAP = dihydroxyacetone phosphate; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; WT = wild-type.

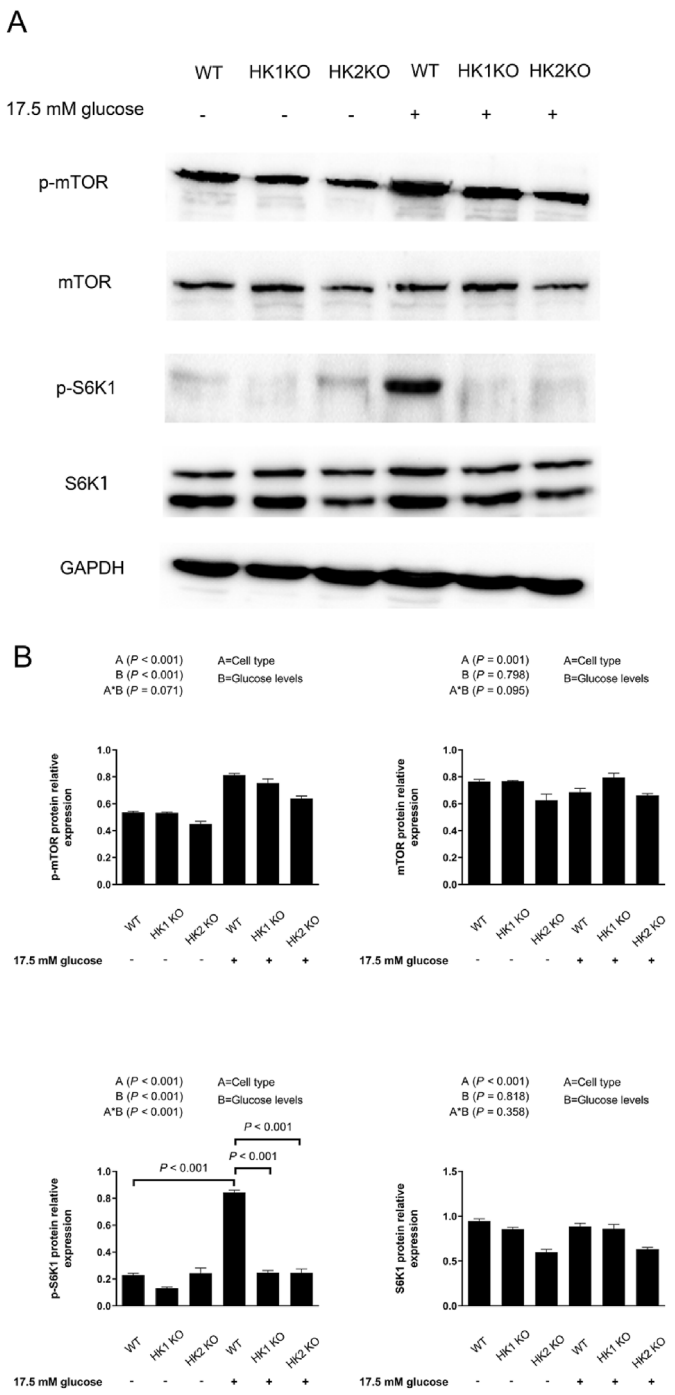

Fig. 9. The effect of hexokinase 1 (*HK1*) and hexokinase 2 (*HK2*) on the expression of key proteins involved in the mammalian target of rapamycin complex 1 (mTORC1) pathway in bovine mammary epithelial cells (BMEC). BMEC, *HK1* knockout (HK1KO) BMEC, and *HK2* knockout (HK2KO) BMEC were treated with Dulbecco's modified Eagle's medium (DMEM) without glucose for 2 h, incubated with (17.5 mM) or without glucose for 6 h, and then subjected to Western blotting analysis. (A) Representative Western blots for mammalian target of rapamycin (mTOR), phosphorylated (p)-mTOR, ribosome protein subunit 6 kinase 1 (S6K1), and p-S6K1, normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with different treatments. (B) Immunoblotting and measurement of the intensity. Protein expression was normalized by the respective abundance of GAPDH. The data are based on triplicate experiments. WT = wild-type.

BMEC following glucose treatment. Moreover, although we found that the proliferative capacity of HK1KO/HK2KO BMEC was reduced compared with that of wild-type BMEC, these differences were only noticeable under conditions of glucose supplementation. It has been reported that the inhibition of HK1 and HK2 can induce apoptotic cell death (Pastorino and Hoek, 2003). These decreases in cell proliferation rates may have partly resulted from the activation of mitochondrial apoptotic signaling cascades. These results may indicate that glucose uptake is not the main factor affecting protein synthesis and cell proliferation in BMEC.

During glycolysis, which is a key mediator of cellular energy metabolism (Brooks, 2009), glucose is transformed into pyruvate, NADH, and ATP (Tran and Wang, 2019). Protein turnover contributes to approximately half of the ATP consumption in the mammary epithelium, owing to the high demand for protein in milk production (Hanigan et al., 2009). Accordingly, glycolysis plays a vital role in protein synthesis. Our data showed that the relative mRNA expression levels of the glycolysis-related genes *PFKL*, *PFKM*, and *ALDOB* were decreased in HK1KO and HK2KO BMEC in response to glucose treatment, whereas those of *GPI*, *ALDOA*, and *ALDOC* were unaffected. HK phosphorylate glucose to generate G6P, which is subsequently converted to fructose-6-phosphate (F6P) by the activity of GPI. We found that glucose supplementation to HK1KO and HK2KO BMEC decreased G6P levels, which may have resulted in reduced ATP production. However, *GPI* mRNA expression was not decreased in HK1KO and HK2KO BMEC in response to glucose treatment, which may have been due to the G6P-to-F6P conversion being a reversible reaction. Phosphofructokinases convert F6P into fructose-1,6-bisphosphate (FBP) while aldolase (ALDO), of which there are three subunits (ALDOA, ALDOB, and ALDOC), splits FBP into dihydroxyacetone phosphate (DHAP) and GAPDH. The results of the present study show that, compared with that in wild-type BMEC, the mRNA expression levels of *PFKL* and *ALDOB* were decreased in HK1KO and HK2KO BMEC; meanwhile, HK2KO also decreased the mRNA expression levels of *PFKM* under glucose treatment; suggesting that the knockout of *HK1* and *HK2* can lead to a decrease in FBP, DHAP, and GAPDH production for glycolysis. The inhibition of glycolysis can interfere with ATP production and activate the AMPK pathway, thereby inhibiting protein synthesis (Jiang et al., 2008; Inoki et al., 2003). The effects of *HK1* and *HK2* loss on *PFKL*, *PFKM*, and *ALDOB* expression in BMEC under glucose treatment may prevent glucose conversion into ATP, resulting in less available energy for milk protein synthesis.

The role of mTORC1 in milk protein synthesis has been well characterized. Studies have demonstrated that glucose infusion in feed-deprived dairy cows can increase the levels of p-mTOR and p-S6K1, thereby enhancing protein synthesis in mammary tissue (Toerien et al., 2010). Zhang et al. (2018) reported that glucose enhances protein synthesis in BMEC by promoting mTORC1 signaling. The levels of S6K1 phosphorylation in HK1KO and HK2KO BMEC were only decreased in the presence of glucose. Sharma et al. (2007) found that glucose phosphorylation is required for mTORC1 signaling activation and that G6P can induce the phosphorylation of S6K1. Our data were consistent with these observations, which suggests that the decrease in p-S6K1 levels may have been due to the downregulation of G6P expression. Accordingly, the suppression of phosphorylation of S6K1 kinase may have occurred owing to lower levels of G6P. Additionally, the effect of HK1 and HK2 deficiency on casein synthesis suggests that increasing HK1 and HK2 expression in BMEC may represent a vital strategy for stimulating mammary protein production. However, this possibility requires further investigation in dairy cows.

5. Conclusions

In this study, we found that glucose treatment increased the mRNA expression of *CSN3*, promoted cell proliferation, and enhanced HK activity in BMEC. The knockout of *HK1* and *HK2*

inhibited cell proliferation and CSN3 gene and protein expression in BMEC under glucose treatment, which may have been associated with the inactivation of the S6K1 kinase and inhibition of glycolysis. These observations highlight that HK1 and HK2 exert vital functions in CSN3 protein expression by regulating S6K1 kinase.

Author contributions

Guoqi Zhao and **Tianyu Yang**: designed the experiments. **Jia Guo** and **Han Song**: assisted in collection samples under the direction of **Tianyu Yang**. **Osmond Datsomor** and **Yuhang Chen**: assisted in preparing the manuscript. **Maocheng Jiang** and **Kang Zhan**: assisted in data curation. **Guoqi Zhao**: contributed to providing financial support. All authors have read and approved the final manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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