MicroRNA-216b inhibits heat stress-induced cell apoptosis by targeting Fas in bovine mammary epithelial cells

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Abstract

Heat stress affects milk yield and quality in lactating dairy cows in summer. Bovine mammary epithelial cells (bMECs) play a key role in milk secretion, and microRNAs (miRNAs) regulate numerous functions of bMEC. Previous reports have verified that miR-216b regulated cell apoptosis through repressing target genes in several cancer cells. So, our purpose was to explore the potential involvement of miR-216b in heat stress-induced cell apoptosis in bMECs. Firstly, the heat stress model was constructed and we found that apoptotic rates of bMECs significantly increased under heat stress. The expression of miR-216b, Bax mRNA, and caspase-3 mRNA was upregulated. However, Bcl-2 mRNA level was detected to differentially downregulated. Overexpression of miR-216b remarkably downregulated the expression of caspase-3 and Bax mRNA and protein, and the mRNA and protein level of Bcl-2 was increased. Inhibition of miR-216b increased the activity of caspase-3 and Bax, and the level of Bcl-2 was inhibited. Moreover, Fas was identified as a target gene of miR-216b through bioinformatic analysis and dual-luciferase reporter assay. Fas activity was significantly inhibited and enhanced respectively after transfecting miRNA mimics and inhibitor. Finally, inhibition of Fas via the small interfering RNA (siRNA) also inhibited cell apoptosis induced by heat stress. Taken together, our results indicated that miR-216b exerted as an anti-apoptotic effect under heat stress in bMECs by targeting Fas.

Keywords miR-216b · Fas · Heat stress · Apoptosis

Introduction

Heat stress is sum of the animals for non-specific response of thermal environment, it causes the imbalance of internal metabolism and damage to the tissue and organ. For dairy cattle, the long-term breeding for increased milk production has led to higher metabolic heat generation and therefore increases susceptibility to undergo heat stress (Kadzere et al. 2002). So, milk

yield and milk quality are decreased under heat stress (Khate and Yadav 2010), which indicates the dysfunction of the mammary gland. Mammary epithelial cells (MECs) are important secretory cells of the mammary gland, and they are affected by various stimuli, especially by heat stress. Although heat-induced bMECs injury has been studied, its mechanism remains poorly understood. Thus, this study was aimed to investigate the regulatory mechanism of bMECs under heat stress.

Apoptosis or programmed cell death is a naturally occurring and very useful process that organisms use to remove unwanted cells during development and for tissue homeostasis. There are two main pathways of apoptosis. The extrinsic signaling pathway leading to apoptosis involves transmembrane death receptors, which bind to extrinsic ligands and transduce intracellular signals. The intrinsic signaling pathway involves no-receptor mediated intracellular signals, inducing activities in the mitochondria that initiate apoptosis (Elmore 2007). Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues; nevertheless, there are a wide variety of stimuli and conditions can trigger apoptosis, such as

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physiological and pathological. Heat stress is one of the important apoptosis-inducing stresses and is well known to synthesize a set of proteins called heat shock proteins, which can influence Fas-mediated apoptosis in Jurkat cells (Liossis et al. 1997). Fas (also called Apol or CD95) was first discovered to induce the apoptosis of various human cells in 1989 (Trauth et al. 1989), which is a transmembrane protein belonging to the death receptor subfamily and can be stimulated by its ligand, FasL, to recruit different adaptor proteins which activate the initiator caspase-8 and caspase-3 to initiate apoptosis (Ji et al. 2014). Recently, in germ cells, apoptosis induced by heat exposure was mainly mediated through the Fas/FasL system (Miura et al. 2002). In addition, Fas/FasL signaling pathway has been reported to play an important role in the protection of heat stress-induced apoptosis of bovine sertoli cells (Guo et al. 2015). However, the detailed functional role of Fas in bMECs apoptosis induced by heat stress is not clear.

MicroRNAs (miRNAs) are a group of small, endogenous and non-coding RNA molecules with 18–24 nucleotides, which contribute to the post-transcriptional regulation of target genes by base pairing with specific binding sites located in the 3' untranslated region (UTR) of the target mRNAs, causing the degradation or translational repression of the mRNAs (Mendell and Olson 2012; Sonkoly et al. 2008). Hundreds of miRNAs have been identified to regulate up to 30% of all protein-encoding genes in humans, which act as key regulators in a wide variety of biological processes (Bartel 2004). Accumulating evidence has shown that the apoptotic machinery is regulated by miRNAs. For example, upregulated miR-34a potentiated Fas-mediated endplate chondrocyte apoptosis by repression of Bcl-2 (Chen et al. 2015). Also, miR-25 was reported to downregulate Fas/FasL to inhibit cerebral ischemia/reperfusion injury-induced apoptosis (Zhang et al. 2016). In addition, it was confirmed that miR-196b modulated apoptosis in colorectal cancer cells by partially repressing Fas expression and could be a potential candidate in colorectal cancer therapy (Mo et al. 2015). These finding highlight the ability of miRNAs to regulate cell apoptosis by targeting critical genes and signaling pathways.

Materials and methods

Cell culture

Bovine mammary epithelial cells (bMECs) were purchased from CELLBIO (Shanghai, China), and HEK-293 cell line was purchased from the Cell Resource Centre of Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/

mL streptomycin and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

Heat stress model

For heat stress model construction, cells were incubated under two experimental conditions: 42 °C (HS) and 37 °C (control). Culturing cells at 42 °C was done to represent a severe heat stress, because environmental conditions could reach 42 °C and extreme condition could exceed this temperature in summer. Then, bMECs were cultured at 42 °C for 0, 1, 6, 12, and 24 h for the corresponding heat stress treatment. And the cells were harvested and analyzed with flow cytometry assay (FACS) and qRT-PCR to investigate cell apoptosis (Fig. S1) and expression of genes (Fig. S2). Finally, the cells cultured at 42 °C for 6 h were constructed as heat stress model.

Analysis of bMECs apoptosis by flow cytometry assay

The bMECs were harvested by enzymatic digestion and washed with cold PBS for three times. Then, cells were stained with FITC Annexin V/PI apoptosis detection kit I (BD bioscience) according to the manufacturer's protocol. Briefly, cells were resuspended with binding buffer at a concentration of 1×10^6 cells/mL, then 100 µL was transferred to a 5-mL culture tube. Gently vortex the cells and incubate for 15 min at room temperature in the dark after adding FITC Annexin V and PI. The labeled cells were analyzed by using a flow cytometer.

Transfection of miR-216b mimics, inhibitor, and siRNA

For transfection of miR-216b, miR-216b mimics, or siRNA, the medium was transfected by Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, bMECs were seeded in 24-well or 6-well plates and miR-216b mimics and inhibitor, chemically synthesized in Sangon (Shanghai, China), were transfected when the cell density reached at 70–80% confluence at a concentration of 200 nM respectively for 6 h. The same protocol was used for transfection of siRNA. Subsequently, the transfected cells were subjected to heat stress treatment, then harvested for further analysis.

MTT assay

The cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and cells were treated as described above for indicated time. Then, 20 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Carlsbad, CA, USA) was added into each well for an incubation of 4 h. Then, the medium was carefully discarded, and 150 µL of dimethyl sulfoxide (DMSO, Invitrogen) was added

into each well for 10 min to solubilize the formazan produced from MTT by the viable cells. Finally, the absorbance was measured at 490 nm using an automatic microplate reader (Thermo, USA).

Dual-luciferase reporter assay

The bioinformatic analysis was conducted via Targetscan. The 3'-UTR of Fas containing the target sites of miR-216b was synthesized and amplified, then inserted into the Sac I-Xba I site of the pmirGLO Vector (Promega, Madison, WI). The mutated 3'-UTR was also constructed by replacing some complementary sites in 3'-UTR with other bases and inserted into the vector. For luciferase reporter assays, HEK-293 cells were seeded into 24-well plates in triplicate. Then, the wild type (WT) and mutated type (Mut) 3'-UTR vectors, miR-mimics, and NC miRNA were co-transfected into HEK-293 cells, alternately. Forty-two hours after transfection, firefly luciferase (luc2) activity was measured and normalized to the Renilla luciferase (hRluc-neo) activity according to the TransDetect® Double-Luciferase Reporter Assay Kit (Transgen, China).

RNA extraction and real-time qPCR

Total RNA was extracted using RNAiso Plus (Takara, Japan) following the manufacturer's protocols and then 1 µg of total RNA was subjected to a reverse transcription reaction. For miR-216b quantification, a real-time quantitative PCR was performed by using mir-X™ miRNA First-Strand Synthesis Kit (Takara, Japan) and the SYBR Premix Ex Taq II reagent (Takara, Japan) on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, and 60 °C 30 s and 72 °C for 1 min. The expression of miR-216b was normalized to that of U6 as a reference. For the mRNA quantification, RT-PCR was performed by using SYBR Premix Ex Taq II reagent (Takara, Japan) under the following conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, and 60 °C 30 s and 72 °C for 1 min. The expression of mRNAs was normalized to that of the house-keeping gene β -actin as an endogenous control. All reactions were performed in triplicate, and the relative fold expression was calculated using $2^{-\Delta\Delta C_t}$. And the primers are listed in Table 1.

Western blot analysis

For the total protein, cells were incubated with cold RIPA (Beyotime, Shanghai, China) reagent. After 10 min of treatment, the supernatant was collected, and the protein amount was quantified with a BCA kit (Beyotime, Shanghai, China). Approximately 20 µg of total protein were loaded per well onto a 12% acrylamide gel and subsequently transferred to a

Table 1 Primer sequences used for quantitative real-time PCR

DNA oligonucleotide	Primer sequence (5' → 3')
Fas	Sense ATGGCTGGTATCAACTCTG Anti-sense GATGGCTCTGTCTGTGTA
Bax	Sense GGAGATGAATTGGACAGTAAC Anti-sense GTTGAAGTTGCCGTCAGA
Bcl-2	Sense CCTGTGGATGACCGAGTA Anti-sense GCTGCTTCTTGAATCTTCTG
Caspase-3	Sense GAGGACAATAAGGTGCTGAA Anti-sense CCAACTGACTGACTGACTG
Caspase-9	Sense TTATTGAAGACCAGCAGACA Anti-sense AGTGAATCCTCCAGAACCA
β -actin	Sense GCCCATCTATGAGGGGTACG Anti-sense TCCTTGATGTCACGGACGATT
miR-216b	Sense AAATCTCTGCAGGCAAATGTGA Anti-sense TGGTGTCTGGAGTCG

polyvinylidene difluoride membrane (PVDF) (Millipore, Billerica, MA, USA). Next, PVDF membranes were incubated overnight at 4 °C with the primary antibodies (anti-Fas at 1: 2000 dilution (Abcam, USA), anti-caspase-3 at 1: 1000 dilution (Santa Cruz, CA, USA), anti-Bax at 1: 1000 dilution (Santa Cruz, CA, USA), anti-Bcl-2 at 1: 1000 dilution (Santa Cruz, CA, USA), and anti- β -actin at 1: 2000 dilution (Servicebio, Wuhan, China)). Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 5000; Servicebio, Wuhan, China) at room temperature for 2 h. The protein bands were visualized by enhanced chemiluminescence (ECL) detection system (Beyotime, China).

Statistical analysis

The data were assessed using the GraphPad Prism software 5.0 (GraphPad, USA) and presented as mean $\pm$ standard deviation (SD). Student's *t* test and one-way analysis of variance (ANOVA) were used to analyze the significance of the different levels. The $p < 0.05$ was considered statistically significant difference and $p < 0.01$ extremely significant difference.

Results

Effects of heat stress-induced cell apoptosis and expression of genes

Firstly, the apoptosis of bMECs under heat stress was investigated. As shown in Fig. 1a, b, compared with the control, the percentage of early apoptotic (EA) (14.69%) and late apoptotic (LA) (13.66%) cells was significantly increased under heat stress (Fig. 1c). In addition, the expression of miR-216b (Fold change = 6.66) showed

Fig. 1 Heat stress-induced cell apoptosis and changed expression of miR-216b and mRNAs. **a** and **b** Flow cytometry analyses of apoptosis in bMECs in the condition of control and heat stress. **b** Percentages of early apoptosis (OA) and late apoptosis (LA). **c** The expression of miR-216b

post heat stress. **d–h** The levels of Bax, Bcl-2, caspase-3, and caspase-9 were confirmed by quantitative RT-PCR post heat stress. * $p < 0.05$, ** $p < 0.01$. Each experiment was performed in triplicate

significantly upregulated under heat stress (Fig. 1d). Based on the above results, we subsequently focused on the mRNA expression of apoptotic genes. The expression of Bax mRNA shown markedly increased under heat stress (Fig. 1e). And the expression of Bcl-2 mRNA shown downregulated (Fig. 1f). For the other three pro-apoptotic genes, caspase-3 (Fig. 1g), and caspase-9 (Fig. 1h), they were identified to up-regulate post heat stress. Taken together, the treatment of heat stress in bMECs could induce the differential expression of apoptotic genes and miRNAs, and led to cell apoptosis finally.

miRNA-216b overexpression enhances cell viability, whereas inhibits cellular apoptosis

To investigate whether miR-216b was directly involved in cell apoptosis under heat stress, the expression of miR-

216b was upregulated by transfection of miR-216b mimics into bMECs. The results showed a 54-fold ($p < 0.01$) increase of miR-216b expression after miR-216b mimics transfection compared with the NC treatment (Fig. 2a). The result of MTT assay showed that heat stress significantly decreased cell viability, and transfection of miR-216b mimics showed markedly enhanced cell viability (Fig. 2b). Then, we further investigated the role of overexpressed miR-216b in cell apoptosis. The results indicated that heat treatment significantly improved the mRNA level of caspase-3 ($p < 0.01$) (Fig. 2c) and Bax ($p < 0.01$) (Fig. 2g), whereas inhibited Bcl-2 mRNA expression ($p > 0.05$) (Fig. 2e). After transfection of miR-216b mimics, caspase-3 and Bax mRNA expression were diminished and Bcl-2 mRNA expression was upregulated. The expression of proteins exhibited similar as the above

Fig. 2 miR-216b overexpression enhanced cell viability and inhibited apoptosis. **a** Cells were transfected with miR-216b mimics, and the expression of miR-216b was confirmed by qRT-PCR ($n = 3$). **b** Heat stress markedly suppressed and miR-216b notably promoted cell viability ($n = 5$). **c** and **d** Caspase-3 mRNA and protein expression were inhibited by

miR-216b ($n = 3$). **e** and **f** Bcl-2 mRNA and protein expression were promoted by miR-216b ($n = 3$). **g** and **h** Bax mRNA and protein expression were repressed by miR-216b. **i** The expression of mRNA and protein ratio of Bax to Bcl-2 ($n = 3$). * $p < 0.05$, ** $p < 0.01$

descriptions (Fig. 2d, f, h). In addition, we investigated the ratio of Bax to Bcl-2, which showed an increase post heat stress, but decreased after miR-216b mimics were transfected (Fig. 2i). These results revealed that overexpression of miR-216b could inhibit cell apoptosis and increase cell viability in heat stress conditions.

miRNA-216b knockdown promotes cell apoptosis

In order to further verify the role of miRNA-216b in heat stress, the expression of miR-216b was downregulated by transfection of miR-216b inhibitor. The result presented a high level of endogenous miR-216b expression (Fig. 3a). After

tolerating heat stress, the qRT-PCR and Western blot analyses indicated that the inhibition of miR-216b resulted in a significant increase in caspase-3 expression at both the mRNA and protein levels (Fig. 3b, c). Inversely, the mRNA and protein levels of Bcl-2 showed a remarkable decrease compared with transfection of INC (Fig. 3d, e). Furthermore, the expression of Bax mRNA and protein showed a significantly increase post heat stress (Fig. 3f, g). The ratio of Bax/Bcl-2 was upregulated by transfecting miR-216b inhibitor. In total, these results indicated the knockdown of miR-216b might promote

cell apoptosis. Combined with the results of miR-216b over-expression, the data showed that miR-216b was involved in cell apoptosis in bMECs in the condition of heat stress.

Fas is a direct target of miR-216b

To unravel the mechanism underlying miR-216b regulated cell apoptosis, we searched for positive regulators of cell apoptosis using the Targetscan database (www.targetscan.org/vert_71/). Of these predicted candidate genes, Fas stood out

Fig. 3 miR-216b knockdown promotes apoptosis. **a** Cells were transfected with miR-216b inhibitor, and the levels of miR-216b were confirmed by qRT-PCR. **b** and **c** The expression of caspase-3 mRNA and

protein. **d** and **e** The expression of Bcl-2 mRNA and protein. **f** and **g** The expression of Bax mRNA and protein. **h** The ratio of Bax/Bcl-2. Each experiment was performed in triplicate. * $p < 0.05$, ** $p < 0.01$

as an attractive target because it was an important death receptor. Sequence alignment demonstrated that Fas contains a conserved domain in the 3'-untranslated region (3'-UTR) that has complementarity with miR-216b (Fig. 4a, b). Next, we cloned the 3'-UTR of Fas harboring the complementary sequence to miR-216b seed sequence in a reporter vector. The result showed that fusion of miR-216b to the Fas 3'-UTR significantly reduced the activity of the luciferase reporter gene (Fig. 4d). In parallel, we mutated the 3'-UTR of Fas (Fig. 4c) and constructed a reporter vector. The miR-216b co-transfection had no effect on the luciferase activity of the reporter containing the mutant Fas 3'-UTR (Fig. 4d). These results suggested that miR-216b interacted directly with the Fas 3'-UTR.

miR-216b is inversely correlated with the expression of Fas

To further investigate whether miR-216b could affect endogenous Fas activity, miR-216b mimics and inhibitors were transfected into bMEC cells. The results indicated that Fas mRNA and protein expression were significantly reduced after transfecting miR-216b mimics (Fig. 5a, b). Furthermore, the expression of Fas mRNA and protein showed a remarkable increase after transfecting miR-216b inhibition (Fig. 5c, d). These results further supported the notion that Fas was a target gene of miR-216b.

Inhibition of Fas is correlated with cell viability and cell apoptosis

In order to further confirm the role of Fas in cell apoptosis in bMECs, MTT assay was performed to investigate the effects of Fas silencing on cell apoptosis in the condition of heat stress. As shown in Fig. 6a, expression of Fas mRNA was

significantly decreased, and cell viability was enhanced by the transfection of Fas silencing (Fig. 6b). In addition, the interactions between Fas expression and cell apoptosis were investigated by evaluating the expression of caspase-3, Bax, and Bcl-2. The expression of caspase-3 mRNA was significantly increased in the condition of heat stress, but decreased dramatically after siRNA transfection (Fig. 6c). And the Western results exhibited the similar results (Fig. 6d). Conversely, the anti-apoptotic gene, Bcl-2, showed decreased expression under heat stress, but it was remarkably increased post Fas silencing (Fig. 6e, f). Another pro-apoptotic gene, Bax, showed significantly downregulated mRNA and protein levels (Fig. 6g, h), and the Bax/Bcl-2 decreased in the presence of siRNA (Fig. 6i). In other words, these results demonstrated that the inhibition of Fas enhanced cell survival and consequently suppressed heat stress-induced cell apoptosis. Collectively, all these results revealed the regulatory mechanism that miR-216b repressed heat stress-induced cell apoptosis, which most likely involved the inhibition of Fas.

Discussion

In this study, we constructed the heat stress model and focused on the study of subsequent cell apoptosis in bovine mammary epithelial cells. As a result, under heat stress conditions, miR-216b was shown to inhibit heat stress-induced cell apoptosis and Fas expression. Repression of Fas expression also showed decreased apoptosis. Here, we proposed a mechanism for this effect by which miR-216b negatively regulated cell apoptosis of bMECs via targeting the 3'-UTR of Fas mRNA, and might help to develop a candidate miRNA that could lead to relieve the effects of heat stress for dairy cattle.

Severe heat stress has been reported to trigger a series of stress responses of the body, such as imbalance of internal

Fig. 4 Fas is a direct target of miR-216b. **a** Predicted the binding site of miR-216b in the 3'-UTR of Fas that is complementary of miR-216b. **b** The homology of complementary sites in different species. **c** The sequences of the wild type and the mutant Fas 3'-UTR (red marked) used

for the luciferase reporter constructs. **d** miR-216b inhibited the wild type Fas 3'-UTR reporter but not the mutant Fas 3'-UTR ($n = 3$). * $p < 0.05$, ** $p < 0.01$

Fig. 5 miR-216b negatively regulates Fas expression. Quantitative RT-PCRs of Fas mRNA expression in bMECs after the transfection of miR-216b mimics (a) and inhibitor (c) in the condition of HS. Western blots of the Fas protein were confirmed by transfection of mimics (b) and inhibitor (d). Each experiment was performed in triplicate. * $p < 0.05$, ** $p < 0.01$

metabolism (Maglara et al. 2003) and cytotoxic effect (Moulin and Arrigo 2006). Furthermore, heat stress can induce cell apoptosis in several cells, such as cardiomyocytes (Wang et al. 2015), sertoli cells (Guo et al. 2015), and human umbilical vein endothelial cells (Gu et al. 2015). Cell apoptosis could be triggered via three different pathways, including death receptors, mitochondria, and endoplasmic reticulum, respectively. And a series of specific proteins regulate the process in each of these pathways. Bax is a pro-apoptotic member of the Bcl-2 family proteins, and it plays an important role in the mitochondrial apoptosis pathway through alteration of permeability of the mitochondrial outer membrane (MOM) (Vaux and Korsmeyer 1999; Li et al. 2013). Bcl-2 inhibits cell apoptosis by obstructing the pro-apoptotic effects of Bax and blocking the release of cytochrome c from the mitochondria. The ratio of Bax to Bcl-2 is confirmed to lower in various cancers, and higher ratio could induce the release of caspase-3 and leads to cell apoptosis subsequently (Salakou et al. 2007). Moreover, previous studies have demonstrated that Bax/Bcl-2 regulated cell apoptosis by activating the cleaving of caspase-9 and caspase-3 (Degenhardt et al. 2002). In this study, the results revealed that the expression of Bax and Bcl-2 were significantly increased and downregulated in heat stress exposed cells. And we observed increased caspase-9 and caspase-3 activity in bMECs subjected to heat stress. Taken

together, our results suggested that heat stress could induce cell apoptosis by triggering the mitochondrial pathways. These results are consistent with the report from YL Hsu which showed that heat shock triggered the mitochondrial and death receptor apoptotic pathway indicated by Bax/Bcl-2 and Fas activity respectively (Hsu et al. 2011).

In this study, miR-216b overexpression inhibited the expression of Bax and caspase-3, and promoted the expression of Bcl-2. On the contrary, miR-216b inhibition induced the upregulation of caspase-3 and Bax activity and the decrease of Bcl-2 activity. In the previous studies, miR-216b has been identified to play important roles in cancer as a tumor suppressor. Deng M et al. showed that miR-216b could exert its function by inhibition of the KRAS-related AKT and ERK pathways, which suppressed tumor growth and invasion (Deng et al. 2011). Furthermore, other studies have proposed several targets of miR-216b, such as insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) (Liu et al. 2015) and syndecan binding protein (SDCBP) (Jana et al. 2017) which exerted the tumor-suppressor function of miR-216b in different cancers. In addition, Xu et al. showed that miR-216b could directly target the 3'-UTR of Beclin 1 and significantly decreased HCPT-triggered autophagy and apoptosis (Xu et al. 2014). These results demonstrated that miR-216b might take part in the regulation of cell apoptosis, which were consistent

Fig. 6 Fas silencing promoted cell survival. **a** The expression of Fas mRNA was shown to be downregulated post the transfection of siRNA. **b** Fas silencing significantly enhanced cell viability in the presence of HS. **c** and **d** Fas silencing downregulated the expression of caspase-3 mRNA and protein. **e** and **f** Fas silencing upregulated the expression of Bcl-2

mRNA and protein. **g** and **h** Fas silencing markedly inhibited Bax mRNA and protein levels. **i** Effects of Fas silencing on the ratio of Bax/Bcl-2 via qRT-PCR and Western blot. Each experiment was performed in triplicate. * $p < 0.05$, ** $p < 0.01$

with our observations. On the other hand, the present study demonstrated that miR-216b served important roles with caspase-3, Bax, and Bcl-2 in the occurrence of cell apoptosis under heat stress. Caspase-3 is a crucial effective molecule in the early stage of apoptosis. To be specific, caspase-3 is a kind of cleavage enzyme, which leads to cleave poly (ADP-ribose) polymerase and active endonuclease, and finally, cell apoptosis. Bcl-2 is the first member, one of a

growing family that regulates cell death, which was identified to promote cell survival by inhibiting apoptosis (Boise et al. 1993). Thus, miR-216b is an important regulator in cell apoptosis induced by heat stress.

Regulation of Fas plays an important role in the apoptosis processes (Zhang et al. 2014), which has been identified that Fas demethylation could induce the upregulation of Fas apoptotic pathway (Liu et al. 2017). Also, the expression of Fas

could be regulated by histone acetyltransferase and resulted in lung cancer cell apoptosis (Han et al. 2017). However, detail regulation of Fas suffered from heat stress remained unclear. FLIP, also known as Casper, iFLICE, FLAME-1, CASH, CLARP, MRIT, or usurpin, was identified to be downregulated post heat stress and sensitized Fas-induced cell apoptosis in Jurkat and HeLa cells (Tran et al. 2003). In addition, another protein, Hsp72, was proved to act upstream of the mitochondria to inhibit Fas-mediated apoptosis (Clemons et al. 2005). In the present research, we first identified that Fas was a target of miR-216b, and speculated that miR-216b might act as a regulator in cell apoptosis via Fas. Subsequently, Fas was silenced to verify our hypothesis. So, it is predictable that some regulatory factors play crucial roles in heat stress-induced cell apoptosis. And this proposes a hypothesis that there exists some connection between the regulators. Here, our study focused on the regulation of miR-216b in heat stress-induced cell apoptosis, and identified that miR-216b targeted Fas to inhibit cell apoptosis. And it is important and worth to investigate the relationship between miR-216b and FLIP/Hsp72 in our future studies.

In total, the present study demonstrates for the first evidence that miR-216b could play a crucial role in the regulation of heat stress-induced apoptosis by targeting Fas in bMECs. This study highlights the potentially important role of miRNAs in heat stress; however, some drawbacks still exist.

Author contributions SJL and MCC contributed to concept design. YSH and THZ cultured the cells and constructed the heat stress models; MCC, THZ, and HBH performed RNA and protein isolation and Western blot; WDX, YS, and BWL performed data analysis; MCC, JW, and SYC performed manuscript preparing. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interest.

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