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MicroRNA-124 reduces caveolar density by targeting caveolin-1 in porcine kidney epithelial PK15 cells

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Abstract Caveolin-1 is the principal component of caveolae, and it is implicated in endocytosis, cholesterol homeostasis, signal transduction and tumorigenesis. MicroRNAs play key regulatory roles in many cellular processes. However, the molecular mechanism by which porcine caveolin-1 is regulated by microRNAs remains unclear. In the present study, we found that miR-124 could directly target caveolin-1 in porcine kidney epithelial cells (PK15). A luciferase reporter assay revealed that miR-124 directly bound to *Cav1* mRNA. Ectopic expression of miR-124 decreased porcine *Cav1* expression at both the mRNA and protein levels. Furthermore, we used transmission electron microscopy to count caveolae in the cytosolic space next to the membrane and we found that the overexpression of miR-124 in PK15 cells reduced the density of the caveolae. Our results suggested that miR-124 reduced caveolar density by targeting caveolin-1 in PK15 cells; therefore, miR-124 could play an important role in the caveolae-mediated endocytosis of pathogens and signal transduction.

Keywords wmiR-124·Caveolin-1·Caveolardensity·PK15cells

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Introduction

Small, noncoding RNAs, approximately 22 nucleotides in length, which are known as microRNAs (miRNAs), play a crucial role in the post-transcriptional regulation of genes involved in fundamental biological processes, including cell differentiation, proliferation, apoptosis and cell signaling [1–4]. In mammals, miRNAs bind to the 3′UTR of target mRNAs, leading to translational repression or mRNA degradation [5, 6]. These miRNAs are predicted to regulate the activity of approximately 30 % of all protein-coding genes [7]. The number of miRNAs that have been identified in humans and pigs is growing, and a total of 2,042 human and 306 porcine mature miRNAs are currently registered in miRBase (release 19, http://www.mirbase.org).

Caveolae, flask-shaped invaginations of the plasma membrane, were first identified in the 1950s as endocytic structures that play important roles in the regulation of many cellular functions, including endocytosis, lipid metabolism and cell signaling [8–10]. Caveolin-1 is a principal component of caveolae membranes; it belongs to a family of three genes including Cav2 and Cav3. Caveolin-1 and caveolin-2 are co-expressed in most cells and co-localized within the plasma membrane and other internal cellular membranes, while caveolin-3 expression is essentially restricted to muscle cells [11, 12]. Previous studies

have shown that the loss of caveolae through the knockout of caveolin-1 contributes to cancer, diabetes and dyslipidemia [13–15]. Porcine caveolin-1 was first identified in a *Haemophilus parasuis* porcine infection model [16]. However, the molecular mechanism by which porcine *Cav1* is regulated by microRNAs remains unclear.

Recently, several miRNAs that target human and mouse caveolin-1 were identified, including miR-103, miR-107, miR-802, miR-203, and miR-133a, and these miRNAs regulate insulin sensitivity, caloric restriction, ROMK channels, tumor cell migration and invasion by targeting caveolin-1 [17–20]. However, no miRNAs have been reported to target porcine caveolin-1. We show herein that miRNA-124 decreased the number of caveolae by directly targeting caveolin-1 in PK15 cells. Therefore, forced expression of miR-124 may prevent the invasion of pathogenic microbes into host cells by caveolae-mediated endocytosis.

Materials and methods

Cell culture and transfection

PK15 and baby hamster kidney (BHK-21) cell lines (obtained from the American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM, High glucose, Thermo Scientific Hyclone, Beijing, China) supplemented with 10 % fetal bovine serum and maintained in a humidified incubator at 37 °C and 5 % CO_2. The miRNA mimics were synthesized by GenePharma (Shanghai, China) and were transfected into cells at the indicated concentrations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA and protein were prepared at 48 h after transfection for qPCR or Western blot analysis, respectively.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Primer pairs used for qRT-PCR are presented in Table 1. Total RNA was extracted from cultured cells using the TRIzol reagent (Invitrogen) following the manufacturer's instructions. The synthesis of cDNA was conducted with 1 μg of total RNA using an RT-PCR reagent kit (TOYOBO), and cDNA was subjected to quantitative PCR using the SYBR Green Master Mix (Roche). The relative expression level of caveolin-1 was normalized to Ribosomal protein L32 gene (RPL32) expression using the $2^{-\Delta\Delta CT}$ method [21]. The PCR program was as follows: 95° for 5 min followed by 40 cycles of 95° for 20 s, 60° for 30 s, and 72° for 20 s.

Protein extraction and western blot

Cells were harvested at 48 h after transfection and lysates were prepared using RIPA buffer (Beyotime, China). The protein concentration was determined with the BCA Protein Assay kit (Solarbio, China). Equal amounts of protein lysate were separated on 12 % SDS–polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween-20 and then incubated with a primary antibody against porcine caveolin-1 (#3238, 1:1,000, Cell Signaling) or β-Actin (#4967, 1:1,000, Cell Signaling) overnight at 4 °C. Membranes were then washed and incubated with an HRP-conjugated secondary antibody, and finally visualized by the addition of the SuperSignal West Pico chemiluminescent substrate (Pierce).

Luciferase reporter assay

For the luciferase reporter assays, miR-124 target sequences were inserted between the *Xho*I and *Not*I restriction sites in the 3′UTR of the hRluc gene in the psiCHECK-2 vector

Table 1 Primers used for microRNA reverse transcription and real-time quantification PCR

Name	Sequence (5′–3′)	T_m (°C)	Size (bp)
CAV1- S	CAGCCTCCCTAAAGACCAAA	60	185
CAV1-A	GCTTGACCCCATTTATCCACC		
MiR-124-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCA GTTGAGTTGGCATT		
MiR-124-S	TCGGCAGGTAAGGCACGCGGTG	62	64
MiR-124-A	TCAACTGGTGTCGTGGAGTCGGC		
RPL32-S	CGGAAGTTTCTGGTACACAATGTAA	60	96
RPL32-A	TGGAAGAGACGTTGTGAGCAA		
U6_S	GTGCTCGCTTCGGCAGCACATAT	62	106
U6_A	AAAATATGGAACGCTTCACGAA		

S sense primer, *A* anti-sense primer, *RT* loop primer for microRNA reverse transcript

(Promega, Madison, WI, USA). The fragments of porcine *Cav1* 3′UTR were amplified from the genomic DNA using primers as indicated in Table 2. BHK-21 cells were co-transfected with 200 ng psiCHECK2 constructs and 30 nM miR-124 mimics or miR-negative control in 24-well plates. Twenty-four hours after transfection, firefly and Renilla luciferase activities were measured using the Dual-Glo Luciferase assay system (Promega) according to the manufacturer's instructions. Normalized data were calculated as the quotient of Renilla/firefly luciferase activities. Three independent experiments were performed in triplicate.

Electron microscopy

The cells were transfected with miR-124 mimics or miR-negative controls in 6-cm plates, and 48 h post-transfection, cells were washed twice with PBS and then digested with 5 mmol/L EGTA-5 mmol/L EDTA PBS for 15 min. They were scraped off and centrifuged at 1,500×*g* for 5 min and then the pellets were fixed in glutaraldehyde (2.5 %) in PBS (0.1 M at pH 7.2) at room temperature for 1 h before being stored at 4°. The cells were washed, stained with 1 % osmium tetroxide, washed, dehydrated in 50, 70, 90, 95, and 100 % ethanol, placed in propylene oxide, and embedded in the epoxy resin Quetol-812 according to the manufacturer's instructions (Nissin EM). Ultrathin (80 nm) sections were cut on a LEICA EM UC6 microtome then stained with uranyl acetate and lead citrate [22]. Images were acquired using a FEI TECNAI spirit microscope with an operating voltage of 80 kV and a CCD camera.

For quantitation of caveolae, distinctly flask-shaped (50–80 nm in diameter) structures found on the plasma

Table 2 Primers used for luciferase reporter gene vector construct

Name	Sequence (5′–3′)	T_m (°C)	Size (bp)
CAV1-3UTR-S	AA**CTCGAG**ACCCACTCTTTGAGGCTATT	64	1,813
CAV1-3UTR-A	AA**GCGGCCGC**GCACAAGGGACTAAGCATCT		
CAV1-3UTR-B1-S	AA**CTCGAG**GTTGATCATTTTATGGTAAGGG	60	257
CAV1-3UTR-B1-A	AA**GCGGCCGC**GATATAACAAATACTTGGGC		
CAV1-3UTR-B2-S	AA**CTCGAG**CTGTGATTACTTTCTATGCC	58	307
CAV1-3UTR-B2-A	AA**GCGGCCGC**TCCACCTTAATTCATTTC		

The underline indicates additional bases and the restriction enzyme recognition sites are bold

S sense primer, *A* anti-sense primer

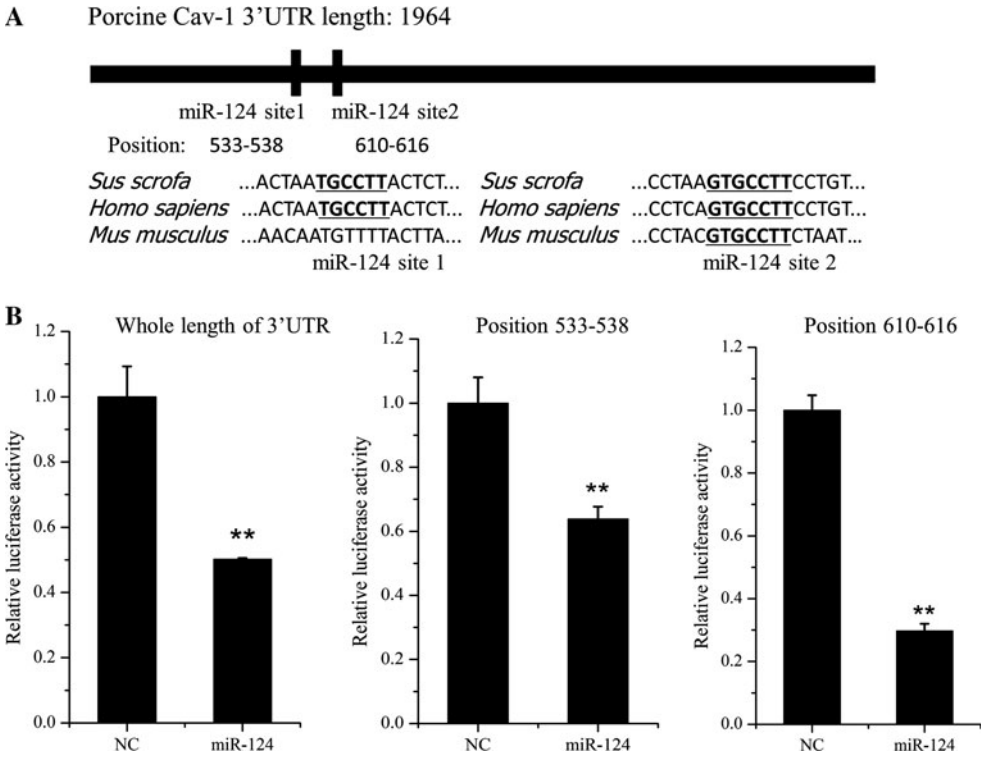

Fig. 1 Putative target sites in the *Cav1* 3′UTR were identified. **a** The two binding sites are at positions 533–538 and 610–616; the first nucleotide after the stop codon of porcine caveolin-1 is defined as position "1." **b** BHK-21 cells were co-transfected with luciferase reporter vectors containing the 3′UTR of porcine *Cav1* gene and miR-124 mimics or miR-negative control. The luciferase assay was performed at 24 h after the transfection. Renilla luciferase values were normalized against firefly luciferase values, $^{**}P < 0.01$

membranes were scored as caveolae. The quantification of the abundance of caveolae was performed as described previously [23]. At least 30 cells were selected randomly at the same magnification, and the cells were overlaid with appropriate double-lattice grids (using Adobe Photoshop software) to measure intersections inside the cell. The number of caveolae in the cell was counted. This was repeated three times from different fields in two independent experiments.

Statistical analysis

Data are presented as the mean ± SD, and Student's *t* test was used for comparisons. *P* values < 0.05 were considered significantly different between groups.

Results

MicroRNA-124 was predicted to target porcine caveolin-1

To identify which miRNAs could target porcine caveolin-1, we performed computational analyses using the miRNA target prediction database targetscan (http://www.targetscan.org/) [24]. The miR-124 was predicted to have two potential target sites in the 3′UTR of the *Cav1* gene, and one of the binding sites (position 610–616) is highly conserved in mammals (Fig. 1a). A previous study had shown that *Cav1* gene expression is downregulated in miR-124-transfected cells [25], leading us to ask whether miR-124 could directly bind and post-transcriptionally regulate caveolin-1, so we chose to further characterize its activity.

Caveolin-1 is a direct target of miR-124

To investigate whether miR-124 targets the 3′UTR of *Cav1*, a luciferase reporter vector was created by inserting the whole length and a partial 3′UTR into the psiCHECK2 vector. The luciferase reporter gene and either miR-124 mimics or negative control oligonucleotides were co-transfected into BHK-21 cells. The luciferase activity was significantly decreased in constructs containing the whole length of *Cav1* 3′UTR and two miR-124 target sites (positions 533–538 and 610–616 in the 3′UTR of *Cav1*) compared to the control (Fig. 1b). The trend expressions of porcine *Cav1* gene and miR-124 in tissues and organs were opposite: *Cav1* gene had high expression in heart, lung, fat, and leg muscle, while miR-124 revealed high-level expression in brain; however, *Cav1* gene had low

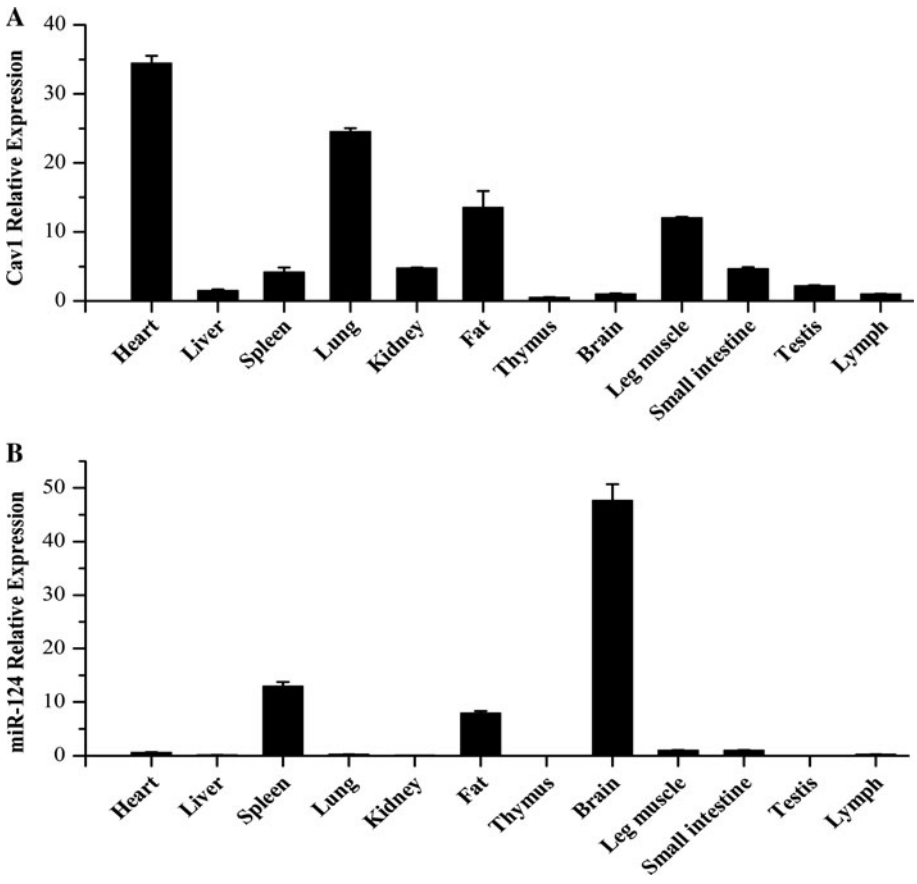

Fig. 2 Analysis of the tissue expression of the porcine *Cav1* gene and miR-124 in the Large White pig by real-time RT-PCR. RPL32 and U6 snRNA were used as reference genes

expression in brain (Fig. 2). Therefore, we assessed whether endogenous caveolin-1 would be downregulated by overexpression of miR-124. Western blot analysis and quantitative RT-PCR were performed to evaluate caveolin-1 expression after miR-124 or negative control oligonucleotide transfection. PK15 cells were transfected with the miR-124 mimics at the concentration of 50 nM, the expression of miR-124 was significantly increased in the miR-124 transfection group compared to oligonucleotide transfection (Fig. 3a), and the transcriptional expression of the *Cav1* gene was significantly reduced in the miR-124 transfection group (Fig. 3b). Subsequently, miR-124 also repressed the *Cav1* protein level (Fig. 3c). Taken together, these results suggest that miRNA-124 could negatively regulate the caveolin-1 protein though the degradation of *Cav1* mRNA.

MicroRNA-124 reduces caveolar density in PK15 cells

Caveolin-1 plays a critical role in the formation of caveolae; therefore, the function of miR-124 in caveolar biogenesis was investigated. PK15 cells were transfected with miR-124 mimics or control oligonucleotides, then we counted the number of morphologically defined caveolae using transmission electron microscopy. Cells (>30) were randomly selected from control and miR-124-transfected samples, and the caveolar density was calculated as described in the materials and methods. The data showed that miR-124-transfected cells displayed a significant reduction in caveolar membrane invaginations compared to the control (Fig. 4).

Discussion

In order to fully understand the functional roles of miRNAs, numerous studies have been performed in human and mouse systems; however, the functions of microRNAs in pigs are largely unknown. In the present work, we demonstrate for the first time that miR-124 overexpression reduces the density of caveolae by directly targeting

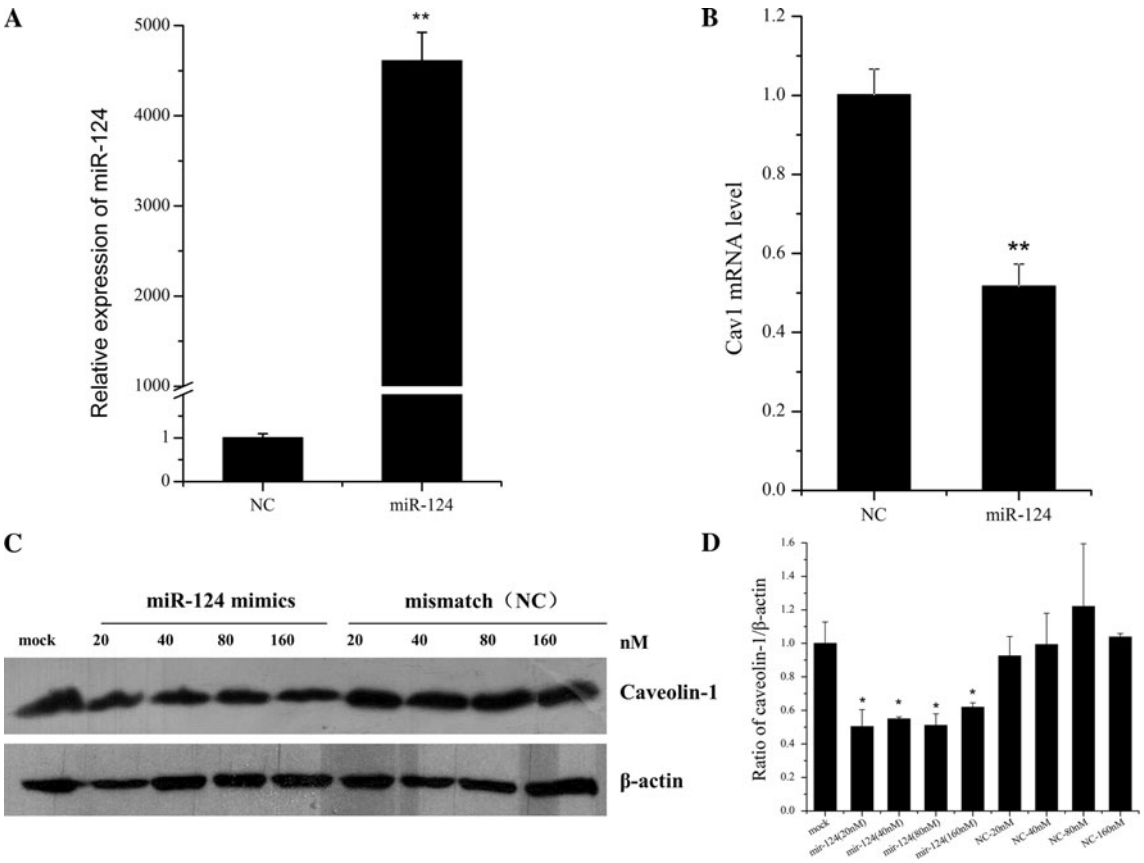

Fig. 3 Addition of miR-124 suppresses porcine caveolin-1 expression. **a** PK15 cells were transfected with miR-negative control or miR-124 mimics. The expression level of miR-124 was assessed by real-time RT-PCR at 48 h after transfection. Expression levels were normalized to the small nuclear U6 snRNA. **b** The endogenous porcine *Cav1* mRNA was repressed in PK15 cells transfected with miR-124 mimics when compared to a miR-negative control group. **c** Western blot analysis of caveolin-1 expression in PK15 cells transfected with a negative control and miR-124 mimics at 48 h after transfection, increasing amounts of miR-124 mimic oligonucleotides (20, 40, 80, 160 nM) as indicated. β-Actin was used as a loading control. **d** The quantitative protein level of (**c**). $*P < 0.05$; $**P < 0.01$

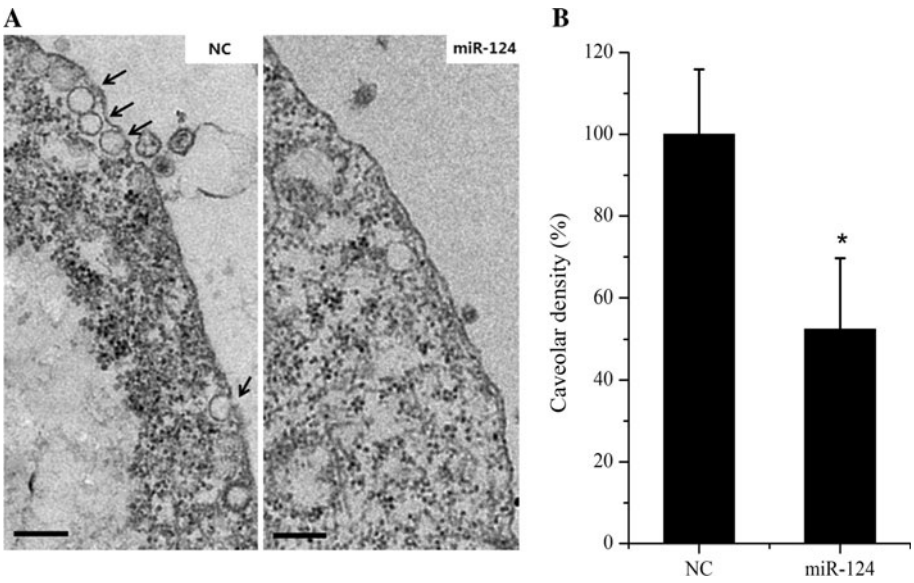

Fig. 4 Addition of miR-124 reduces caveolar density in PK15 cells. PK15 cells were transfected with miRNA-124 mimics (miR-124) or negative control oligonucleotides (NC). **a** Electron micrograph of part of a PK15 cell; arrows indicate caveolae. *Scale bar* 200 nm. **b** Quantification of the abundance of caveolae in cells reveals a significant reduction in cells transfected with miR-124. *Error bars* indicate SD. The *P* value was calculated using Student's *t* test, $*P < 0.05$

caveolin-1 in PK15 cells. MicroRNA-124, highly expressed in neurons, was first cloned from the mouse brain and mediates neuronal differentiation [26, 27]. Recent studies have shown miR-124 to be attenuated in several tumors, and the overexpression of miR-124 inhibits the metastasis of breast cancer [28]. MicroRNA-124 was downregulated in hepatocellular carcinoma cells, and the ectopic expression of miR-124 in HCC cells inhibits cell invasion and metastasis [29, 30]. In addition, the overexpression of caveolin-1 in HCC cells enhanced oncogenic properties, including invasion and migration [31]; these results therefore suggest that miR-124 may regulate HCC cells' tumorigenesis by targeting caveolin-1.

Caveolin-1 is a basic component of caveolae in most mammalian cells. Caveolae play a crucial role in the invasion of pathogens, as simian virus 40 and the murine leukemia virus enter cells via a caveola-dependent endocytic pathway [32, 33]. Depletion or sequestration of cholesterol from the membrane using chemicals and siRNA or dominant-negative constructs targeting caveolin-1 has been shown to impair caveolae-mediated endocytosis, thereby reducing viral infection [34, 35]. Importantly, miR-124 decreases the density of caveolae on the membrane; thus, miR-124 may help the cell to resist the viral invasion and act as an antiviral factor. In addition, viral infection causes severe economic losses to the swine industry worldwide, such as porcine reproductive and respiratory syndrome virus (PPRSV), Pseudorabies virus (PRV), and classical swine fever virus (CSFV). The mechanisms of cell entry by these porcine viruses are poorly understood; thus, our findings could be helpful for antiviral research. On the other hand, many signaling molecules, such as epidermal growth factor receptor (EGFR), G-proteins, endothelial nitric oxide synthase (eNOS) and insulin receptors, gather in the caveolae [36–39] and the overexpression of miR-124 may diminish the number of these signaling molecules in caveolae-enriched plasma membrane microdomains and affect downstream signaling pathways. Because caveolin-1 has multiple functions, miR-124 may mediate other effects that contribute to its phenotype.

In summary, our findings demonstrate that miR-124 decreases the number of caveolae in PK15 cells by directly regulating the expression of caveolin-1. MicroRNA-124 is also the first identified miRNA to regulate the expression of porcine caveolin-1. These findings suggest that miR-124 may be involved in the regulation of caveolae-associated signaling molecules and act to inhibit infections by pathogens.

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