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ORIGINAL ARTICLE

Identification of miR-192 target genes in porcine endometrial epithelial cells based on miRNA pull-down

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Abstract

Introduction MicroRNAs (miRNAs)—a class of small endogenous non-coding RNAs—are widely involved in post-transcriptional gene regulation of numerous physiological processes. High-throughput sequencing revealed that the miR-192 expression level appeared to be significantly higher in the blood exosomes of sows at early gestation than that in non-pregnant sows. Furthermore, miR-192 was hypothesized to have a regulatory role in embryo implantation; however, the target genes involved in exerting the regulatory function of miR-192 required further elucidation.

Methods In the present study, potential target genes of miR-192 in porcine endometrial epithelial cells (PEECs) were identified through biotin-labeled miRNA pull-down; functional and pathway enrichment analysis was performed via gene ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment. Bioinformatic analyses were concurrently used to predict the potential target genes associated with sow embryo implantation. In addition, double luciferase reporter vectors, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), and Western blot were performed to verify the targeting and regulatory roles of the abovementioned target genes.

Results A total of 1688 differentially expressed mRNAs were identified via miRNA pull-down. Through RT-qPCR, the accuracy of the sequencing data was verified. In the bioinformatics analysis, potential target genes of miR-192 appeared to form a dense inter-regulatory network and regulated multiple signaling pathways, such as metabolic pathways and the PI3K-Akt, MAPKs, and mTOR signaling pathways, that are relevant to the mammalian embryo implantation process. In addition, CSK (C-terminal Src kinase) and YY1 (Yin-Yang-1) were predicted to be potential candidates, and we validated that miR-192 directly targets and suppresses the expression of the CSK and YY1 genes.

Conclusion We screened 1688 potential target genes of miR-192 were screened, and CSK and YY1 were identified as miR-192 target genes. The outcomes of the present study provide novel insights into the regulatory mechanism of porcine embryo implantation and the identification of miRNA target genes.

Keywords miRNA pull-down · miR-192 · Target gene · Porcine endometrial epithelial cells · CSK · YY1

Introduction

MicroRNAs (miRNAs) are a class of short-stranded non-coding RNAs that are responsible for indirectly regulating several physiological processes in the body through the inhibition or degradation of mRNA expressions [1]. More than 1/3 of the genes in the human are reported to be directly regulated by the corresponding miRNAs additionally, a single miRNA can regulate hundreds or thousands of target genes [2]. The identification of miRNA target genes helps us to understand the regulatory role of miRNAs.

Identification of miRNA target genes has been performed primarily through computer software to establish predictions

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about them. Bioinformatics software, including TargetScan and miRBase, and prediction algorithms for miRNA target genes are fundamentally designed based on a perfect pairing of the 2–8 sites at the 5′ end of the miRNA mature strand, i.e., the seed region (NT2-7), with the 3′ UTR region of the mRNA [1]. However, the identification of the target gene is challenging owing to the short length of miRNA sequences and the presence of only partial complementarity with the target gene recognition element (microRNA response elements, MRE). First, although MRE pairing in the seed region of miRNAs significantly facilitates the identification of target genes and is a fundamental algorithm for target gene prediction [3], even a perfect seed region match could not guarantee their target relation. Second, miRNA–mRNA interactions are susceptible to the effects of the secondary structure of mRNAs. miRNA reportedly binds to both the CDS and 5′UTR regions of mRNA in addition to the 3′ UTR region of mRNA [4, 5]. Therefore, bioinformatic predictions typically have a high rate of false positive outcomes. A high rate of false negatives is concurrently observed, owing to which it is possible to miss key target genes during investigations. Although bioinformatic analyses are critical in the identification of the miRNA target genes, the high false-positive and -negative rates of bioinformatics predictions can often be cost- and time-consuming.

However, biochemical experimental methods including CLIP, AGO2-IP, HITS-CLIP, RIP, immunopurification, and biotin-labeled miRNA pull-down are considered feasible analyses modalities to identify miRNA target genes in practice by experimentally mimicking the mechanism underlying miRNA action in cells [6–8]. However, among these approaches, researchers have reported a progressive increase in the application of biotin-labeled miRNA pull-down owing to its high specificity for the identification of individual miRNA target genes [3, 8–10]. Orom et al. [10] first used this approach to validate the Drosophila head degeneration incomplete gene (hid gene) as a target gene for bantam miRNAs. Subsequently, Lal et al. [3] used this approach to report that transcripts of 982 genes were enriched in the pull-down sequences of miR-34a, and subsequent studies demonstrated that 90% of these genes may directly be regulated by miR-34a. In this regard, we discovered that identifying miRNA target genes using a biotin-labeled miRNA pull-down technique is a vital step to understating the molecular mechanism underlying miRNA function.

A literature review revealed that current national and international research on miR-192 has focused on the regulation of human diseases [11]. However, to the best of our knowledge, no study on its use in pig pregnancy has been identified thus far. Our group demonstrated that the expression of miR-192 was significantly higher in the blood exosomes of pregnant (15 days) sows than in the non-pregnant sows. This consequently indicated that miR-192 may influence early embryo attachment in sows as a novel regulatory factor (unpublished). Therefore, we used a biotin-labeled miRNA pull-down to identify and analyze the target genes that are directly regulated by miR-192 in porcine endometrial epithelial cells. This subsequently assisted us in providing reference data for the biological study of miR-192 on the process of embryo attachment in sows and the identification of miR-192 target genes.

Materials and methods

Test materials

Porcine endometrial epithelial cells (PEECs), item no. (iCell-0048a), were purchased from Shanghai iCell Bioscience Inc; The PK15 cells and psiCHECK-2 vector are typically stored and maintained in this laboratory. Furthermore, miR-192 mimics (Sense, 5′-CUGACCUAUGAAUUGACAGCC-3′ and antisense, 5′-GGCUGUCAAUUCAUAGGUCAG-3′) and miR-192 inhibitor (5′-GGCUGUCAAUUCAUAGGUCAG-3′) were synthesized by the RiboBio Company (Guangzhou, China).The miRNA negative control (miR1N0000001-1-5) sequence, which is not provided by RiboBio Com, has no homology to the human, mouse and pig genomes and can be used in human, mouse and pig-related experiments [12, 13].

Culture of cells and their transfection

PEEC cells were cultured using an epithelial cell-specific culture system (iCell-0048a-001b) (iCell Bioscience Inc, Shanghai). In addition, the PK15 cells were cultured using DMEM culture medium containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin mixture double antibody (Gibco). Both the abovementioned cell lines were cultured at 37 °C with 5% CO_2. When the cells reached 60–80% confluence, the cells were transfected according to the Guangzhou Ribo Biological miRNA instructions and Lipofectamine™ 3000 (Thermo Fisher) instructions for subsequent experiments.

miRNA pull-down assay

The biotin-labeled miR-192 pull-down assay was performed according to the method reported in the literature [14]. Namely, biotin-labeled miR-192 mimics (bi-miR-192) and biotin-labeled miR-192 mimics negative control (bi-CTL) (RiboBio, Guangzhou) were first transfected with porcine endometrial epithelial cells. After 24 h of culture, these cells were lysed using Trizol lysis solution (Thermo Scientific) (1 mL of lysis solution was added to each well of a 6-well plate). Furthermore, 50 μL of total cellular RNA—i.e., the

input group, which included miR-192 and miRCTL inputs—was extracted after complete lysis, and the remainder was extracted using Dynabeads™ M-280 streptavidin (Thermo Scientific). Following biotin adsorption, the miRNA pull-down technique was used to capture the miR-192 RISC complex, and a series of RNAs were obtained via incubation and washing—i.e., the pull-down group, which included miR-192 and miRCTL pull-downs—and then transferred to Shanghai cloud-seq for sequencing.

Bioinformatics analysis

Raw data were obtained by sequencing on an Illumina NovaSeq 6000 sequencer. Raw data were first quality-controlled using Q30 values. The cutadapt software (v1.9.3) was used to disconnect and remove low-quality reads to obtain high-quality clean reads. These clean reads were subsequently compared with the pig reference genome (Sscrofa.11.1) using the hisat2 software. FPKM values for the mRNAs were then obtained using the cuffdiff software guided through the Ensembl gtf gene annotation file. The target gene determination method was slightly modified from that reported in the literature [3, 14]. We determined a fold change of > 2 of (Bi-miR-192 pull-down/Bi-CTL pull-down)/(Bi-miR-192 input/Bi-CTL input) as a potential target gene for miR-192; Additionally, enrichment analysis was performed and potential target genes were mapped using the online software DAVID (https://david.ncifcrf.gov/) and Microbioscape (http://www.bioinformatics.com.cn). The regulatory network of miRNA-mRNA interactions was mapped using Cytoscape 3.6.1.

Identification of embryonic attachment-related target genes

To perform multidimensional screening for potential target genes associated with early embryo attachment in sows, we used the miR-192 pull-down data from this study; proteomic data for each tissue of the sow's 15-days gestation attachment site, non-attachment site, and non-pregnant endometrium (referred to as porcine endometrial tissue differential proteomic data) (unpublished); miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/); TargetScan 7 (http://www.targetscan.org/vert_72/); STRING (https://string-db.org/) with parameters set to (minimum required interaction score > 0.9), and a review of the literature.

Dual luciferase validation

MREs of potential target genes with miR-192 were analyzed using the RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) software. Based on the MREs, we designed wild-type and mutant primers (Table S1) and constructed their plasmids using miR-192 mimics and mimics negative control (Ribobio, Guangzhou, China) to cotransfect PK15 cells. Firefly luciferase and renilla luciferase activities were measured 24 h after transfection using a dual luciferase reporter assay kit (Promega, USA).

RT-qPCR

When PK15 cells reached 70–80% confluence, miR-192 mimics, mimics NC, miR-192 inhibitor, and inhibitor NCs were transfected into PK15 cells. The cells were subsequently harvested after 48 h, and total cellular RNA was extracted. The primers for the reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) reaction were synthesized by Shanghai Biotech (Table S2). The total volume of the RT-qPCR reaction system was 20 μL, which included 1 μL of cDNA template, 10 μL of the SYBR® Premix Ex Taq™ (2×) mixture, 7 μL of ddH_2O, and 1 μL of upstream and downstream primers (10 mol/L). RT-qPCR reaction procedure was as follows: 94 °C pre-denaturation for 5 min, (denaturation at 94 °C for 15 s, annealing for 20 s, cyclic extension at 72 °C for 20 s) 35 cycles. This procedure was performed in triplicates for each sample, and RT-qPCR values were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot

A total of 48 h after the transfection, total cell protein was extracted from PK15 cells, and protein concentration was determined using the BCA kit (Shanghai Biotech). Following this, we performed SDS–polyacrylamide gel electrophoresis, constant pressure electrophoresis at 80 V, and constant pressure at 100 V for membrane transfer. After blocking with 5% skimmed milk for 2 h, the primary antibody was incubated 12 h at 4 °C. The samples were incubated with primary antibodies (CSK, 1:1000, CST; YY1, 1:1000, ABcam; β-actin, 1:10000, ABcam) at 25 °C for 2 h. We then incubated the samples with secondary antibodies (DyLight™ 680 Conjugate labeled goat anti-mouse and goat anti-rabbit IgG, CST) to acquire sample images using the Odyssey clx infrared fluorescence imaging system. The target and reference proteins were analyzed for greyscale values using Image J software.

Data analysis

SSPS 26.0 was used for data analyses, and all results were expressed as "mean ± SD". Intergroup differences were assessed using an independent samples t-test; furthermore, $p < 0.05$ indicated statistical significance.

Results and analysis

Quality control of the sequencing data of the samples

High-throughput sequencing was used to obtain > 99% clean reads from bi-CTL input, bi-miRNA-192 input, bi-CTL pull-down, and bi-miRNA-192 pull-down samples and a Q30 of > 85% (Table 1). Therefore, sequencing data from this study appears to be of good quality.

Enrichment analysis of differentially expressed mRNAs by combining libraries from both the input and pull-down groups

In the miRNA pull-down assay, input is used as a positive control, which contains all changes in gene expression directly or indirectly induced by miR-192 following cell transfection with bi-miR-192 and bi-CTL.

We used FPKM numbers for differential analysis of input and pull-down sequencing data, by combining the input and pull-down results. That is, when the fold change of > 2 of (Bi-miR-192pull-down/Bi-CTL pull-down)/(Bi-miR-192 input/Bi-CTL input) as a potential target gene for miR-192. In this study, we screened 1688 differential mRNAs. To further obtain a clustering model for differentially expressed target mRNAs, we performed a clustering analysis based on the FPKM values of these 1688 differentially expressed genes (DEGs). The clustering heat map revealed that, in the input group, the miR-192 input group showed lower expression levels than the miR-CTL input group; additionally, in the pull-down group, miR-192 pull-down showed higher expression levels than the miR-CTL pull-down group (Fig. 1, Table S3).

Validation of differentially expressed mRNAs

Ten differentially expressed genes (ATG16L1, EIF1, PGK1, ITM2B, CSK, PCBP1, YY1, PLXNA1, PLS3, and ARL4C) were randomly selected for RT-qPCR validation. The outcomes of the RT-qPCR analyses (Fig. 2) demonstrated that the expression of the abovementioned 10 differentially expressed genes in the input and pull-down groups was consistent with the sequencing results. Therefore, this result verified the accuracy of the sequencing outcomes recorded in this study.

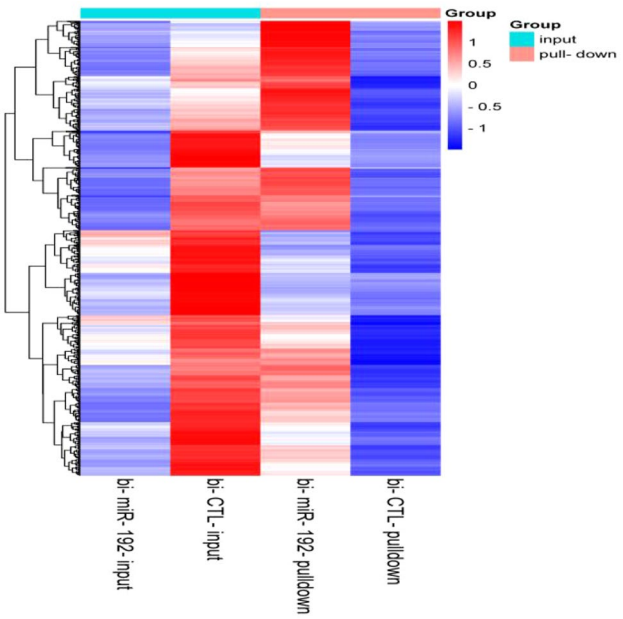

Fig. 1 Clustering of potential miR-192 target genes on a heat map. (Color figure online)

Enrichment analysis of miR-192 differentially expressed mRNA

We performed gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on target genes captured via biotin-labeled miR-192 pull-down—an important bioinformatics statistic designed to represent gene and gene product properties uniformly across all species. Furthermore, GO covered the following three domains: cellular component (CC), molecular function (MF), and biological process (BP) [15]. GO enrichment results revealed that we significantly enriched a total of 263 significant GO entries ($p < 0.05$), including 92 BPs, 98 CCs, and 73 MFs (Table S4). They were primarily associated with the composition of organelles, the metabolic activity of cells, the biosynthesis of compounds required by the organism, cytoskeleton synthesis, intracellular protease complexes, and enzyme activity (Fig. 3A).

Table 1 Shows the total number of sample sequences and annotation matches

Category	bi-CTL-input	bi-miRNA-192-input	bi-CTL-pulldown	bi-miRNA-192-pulldown
Raw reads	40,744,546 (100%)	44,309,696 (100%)	40,744,546 (100%)	47,183,932 (100%)
Clean reads	40,742,562 (> 99%)	44,309,41 (> 99%%)	45,995,766 (> 99%)	47,183,528 (> 99%)
Q30	88.22%	85.39%	85.55%	89.72%
Size (bp)	325	355	327	337

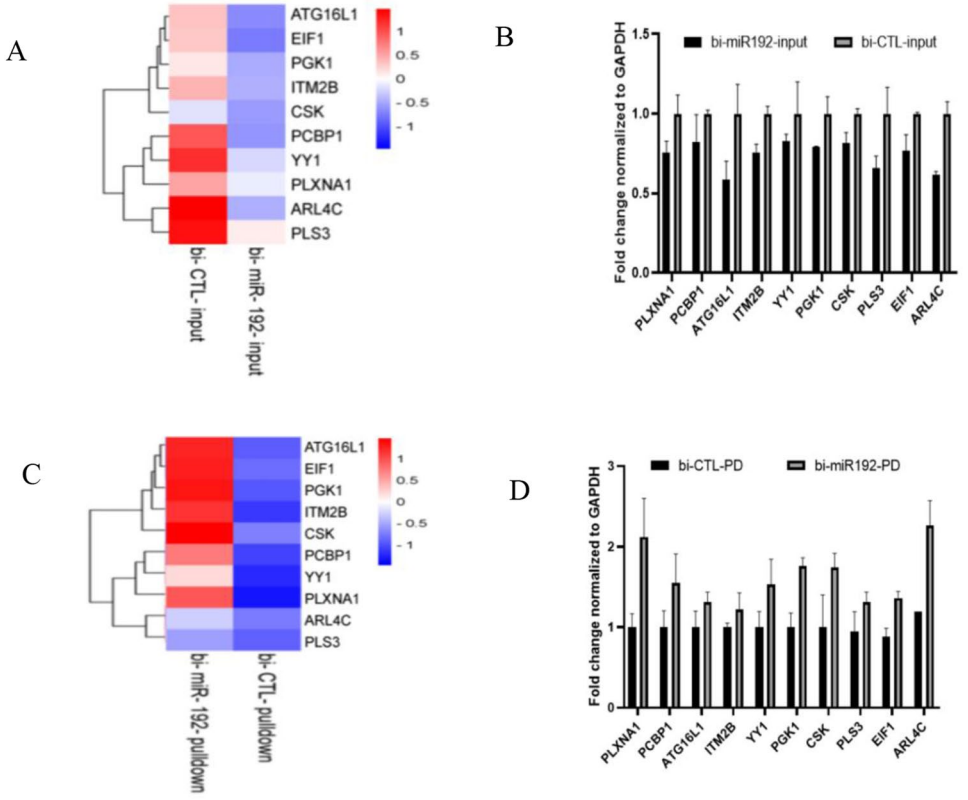

Fig. 2 RT-qPCR validation of differentially expressed target genes. **A, C** Cluster maps of some of the differential target mRNAs in the input and pull-down groups, respectively; **B, D** RT-qPCR verification of partial differential target mRNAs in the input and pull-down groups, respectively. (Color figure online)

The annotation of KEGG signaling pathways showed that we significantly enriched 104 signaling pathways in total ($p < 0.05$) (Table S5). These pathways include metabolic pathways and PI3K-Akt, MAPK, and mTOR signaling pathways related to mammalian embryonic development and attachment (Fig. 3B). In addition, we observed that metabolic pathways were the most enriched signaling pathways, with 183 differentially expressed genes (10.8% of the total number of genes in this study). MAPK1, MAPK3, MTOR, WNT7A, FEGR2, CDC42, LIPG, and GLUL, the main genes involved in the abovementioned four signaling pathways, were associated with the regulation of pregnancy in sows (Fig. 3C). Additionally, the role of other pathways such as protein serine/threonine ligases, the cytoskeleton, and adherent spots in influencing the regulation of embryonic pregnancy cannot be overlooked in this study [16, 17].

Enrichment analysis of metabolism-related genes

Metabolic changes are influenced by important physiological and biochemical processes in organisms, including the regulation of pregnancy in mammals [18]. Analyses of the 182 metabolism-related differential genes enriched in this study revealed that these genes were mainly involved in the synthesis and oxidative catabolic pathways of 44 substances, including sugars, lipids, proteins, coenzymes, and various amino acids ($p < 0.05$) (Fig. 4, Table S6). During mammalian pregnancy, these processes, which involve oxidative catabolism and the synthesis of sugars, lipids, and proteomes, interact to maintain the physiological changes of term pregnancy. The energy they produce sustains the energy needs of the mother and embryo for development and attachment during gestation, in addition to the various intermediates produced by these pathways that are necessary for the growth and development of the embryo during pregnancy.

Screening of embryo attachment-related target genes

The potential target genes captured by miR-192 pull-down were intersected with two sets of data from the subject's proteins that were differentially expressed between the attachment site and non-attachment site of endometrial tissue of pregnant sows and endometrial tissue of nonpregnant sows, and four intersections were found (CSK, SERPINB9, PLXNA1, and PLS3) (Fig. 5A). However, intersections with the common bioinformatics prediction software miRWalk 3.0 and TargetScan 7 revealed a total of 14 intersections (EIF1, XPO4, ARL4C, KPNA6, SRSF6, ABHD2, CDC6, ALG6, MAPK1, MYLK, ANKRD44, YY1, SLC11A2, and B3GALNT2) (Fig. 5B). A relevant literature review and collation of these 18 genes demonstrated that the CSK [19, 20]. Gene was associated with early pregnancy regulation

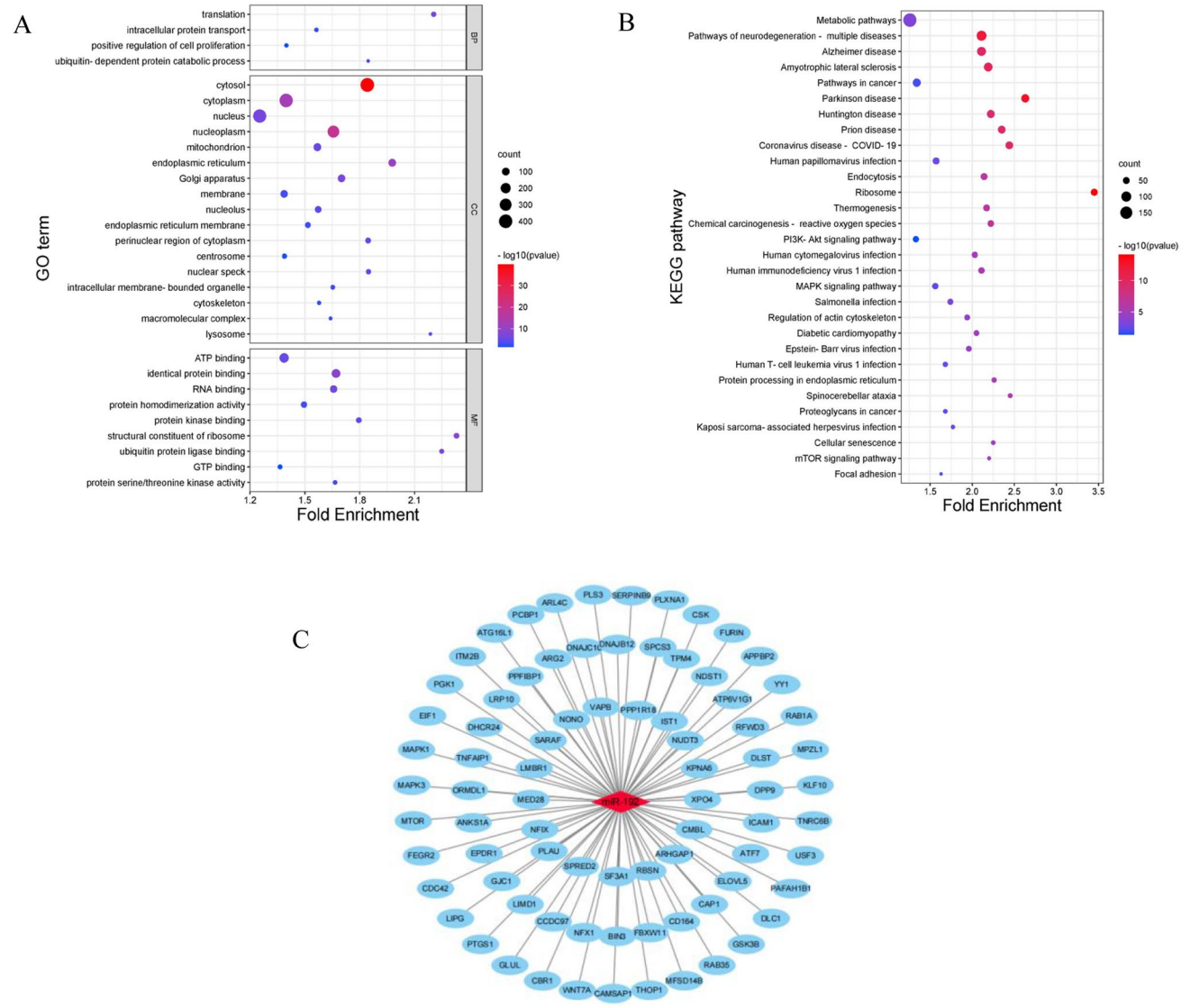

Fig. 3 Enrichment analysis and annotation of miR-192. **A** The top 30 gene ontology classes of miR-192 regulated target genes. **B** The top 30 miR-192 regulated target gene pathways; **C** miRNA–mRNA regulatory network. (Color figure online)

in humans or mice. As a multifunctional transcription factor, YY1 is involved in regulating embryonic development, cytoskeleton formation, and trophoblast egg invasion and attachment processes by regulating CBS, MMP2, ITGB3/7, PVT1, and RTCB [21–23]. Additionally, protein phase-work network analysis revealed that CSK proteins showed direct interactions with several proteins (Fig. 5C), which include the epidermal growth factor receptor, receptor double-regulated protein, pregnancy-associated glycoprotein 1, epidermal regulator and protein complexine phosphatase non-receptor type 22, among other proteins directly related to uterine tolerance, embryo development and attachment during mammalian pregnancy [24–26]. Therefore, we hypothesized that CSK and YY1 genes act as potential target genes for miR-192 in the regulation of embryo attachment in sows.

Validation of miR-192 targeting relationships with CSK and YY1 genes

RNAHybrid predicted the presence of MREs on the 5′ UTR of the CSK gene and the 3′UTR of the YY1 gene for miR-192, and based on the MREs, we successfully constructed wild-type and mutant vectors (Fig. S1); The dual luciferase reporter showed that the luciferase activity of the wild-type CSK gene was very significantly downregulated compared to the mutant ($p < 0.01$), and the luciferase activity of the wild-type YY1 gene was significantly downregulated ($p < 0.05$) compare to mutant (Fig. 6A, B). RT-qPCR results showed that miR-192 significantly downregulated mRNA expression amount of CSK ($p < 0.05$) and YY1 ($p < 0.01$) (Fig. 6C). The results of western blot analyses showed that miR-192

Fig. 4 Metabolism-related gene enrichment analysis. (Color figure online)

significantly inhibited YY1 protein expression ($p < 0.05$), and tended to inhibit CSK protein expression; however, the difference was non-significant ($p > 0.05$) (Fig. 6D, E). According to these findings, miR-192 directly targets and regulates the expression of the CSK and YY1 genes.

Discussion

Embryo attachment is a key aspect of mammalian reproductive physiology, and this step involves a large and complex regulatory network of multiple regulators [27]. miRNAs are indirectly involved in embryo attachment as transcriptional regulators of gene expression. Studies on humans, mice, cattle, and pigs have reported that miRNAs are specifically expressed in the maternal uterus or embryonic tissues in early gestation and can be used as biomarkers of embryo attachment in mammals [28–30]. Previous studies by our group suggest that miR-192 may be a novel regulator of embryo attachment in sows; however, the target genes through which miR-192 exerts its regulatory function require further elucidation.

Identification of the miRNA target genes is necessary to understand the role of miRNAs. Biotin-labeled miRNA pull-down facilitates the specific identification of specified miRNA target genes and is one of the most important tools for miRNA target gene identification and functional

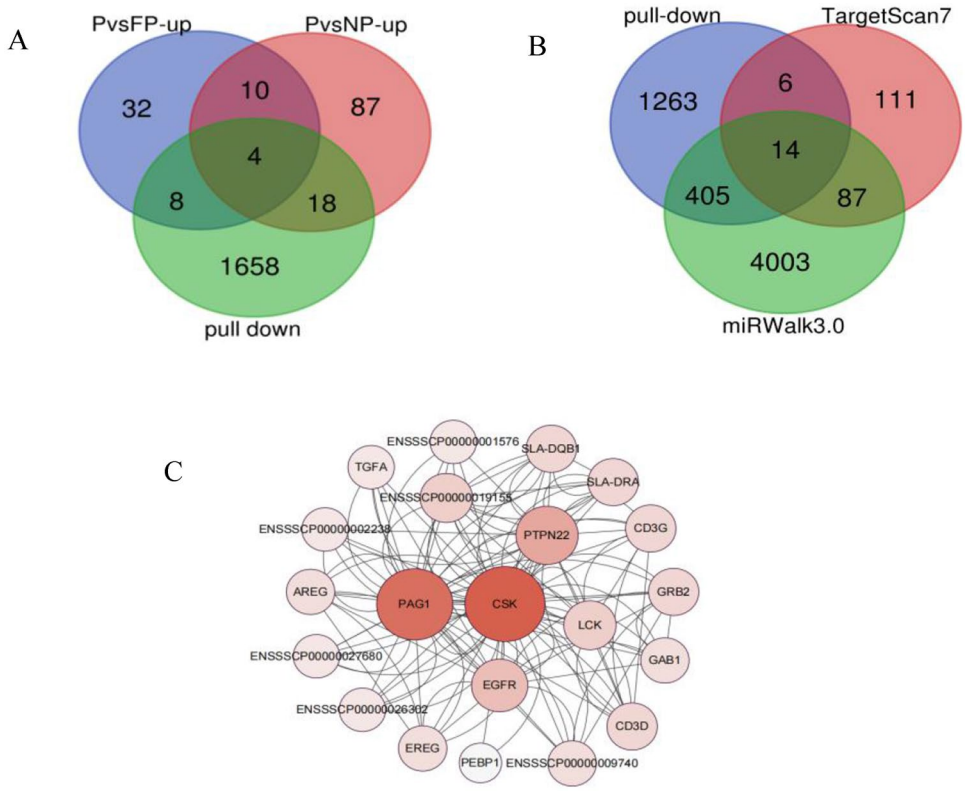

Fig. 5 Embryonic attachment-associated gene screen. **A** Venn diagram of miR-192 pull-down and porcine endometrial differential protein, (P) endometrial attachment site (FP) endometrial non-attachment site (NP) non-pregnant sow endometrium (pull-down) potential target genes identified by miR-192 pull-down; **B** Venn diagram of miR-192 pull-down with miRWalk 3.0 and TargetScan 7; **C** CSK-interacting protein Network diagram

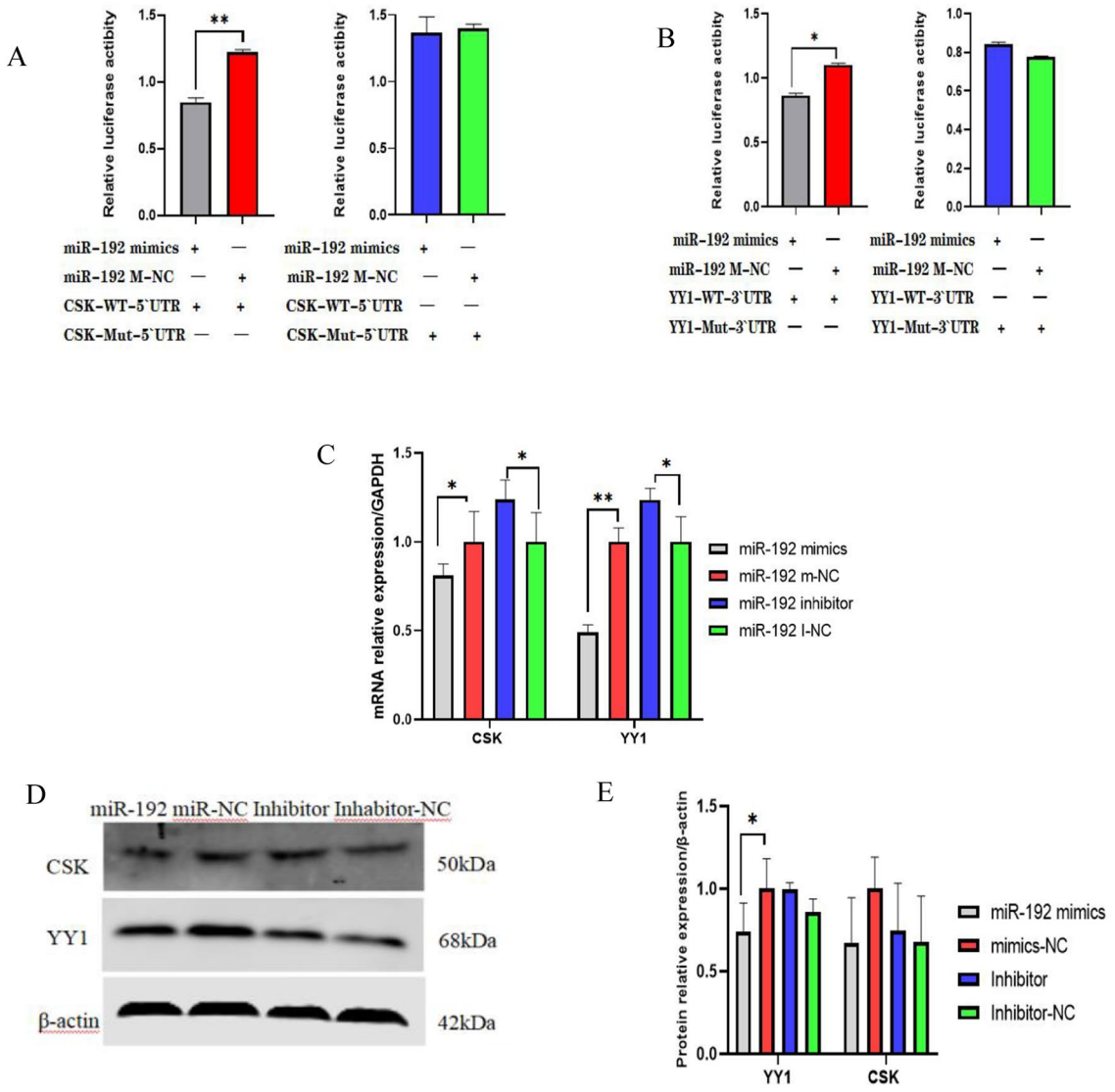

Fig. 6 Validation of miR-192 for CSK and YY1 gene targeting relationships. **A, B** double luciferase reporter for CSK and YY1 genes; **C** effect of miR-192 on CSK and YY1 gene mRNA expression; **D, E** effect of miR-192 on CSK and YY1 gene protein expression. (Color figure online)

analysis [3, 9]. In the present study, we transfected porcine endometrial epithelial cells with biotin-labeled miR-192 and performed high-throughput sequencing after RNA capture via the pull-down assay. This was done to identify potential miR-192-regulated target genes in porcine endometrial epithelial cells. Genes showing a minimum 20% decrease in mRNA levels after miRNA transfection were considered to be directly or indirectly regulated target genes, and biotin-labeled miRNA transfected cells captured using magnetic beads were enriched for miRNA-bound target genes [3]. Therefore, after transfection of the specified miRNA, we performed association analysis—considering the intersection—of the downregulated and captured target genes. We then identified these genes as potential target genes that are directly regulated by the miRNA. We screened miR-192 for directly regulated target genes in porcine endometrial epithelial cells by identifying 1688 potential target genes after integration analysis of the input and pull-down groups. The accuracy of the sequencing results was verified by randomly selecting 10 differentially expressed target genes for RT-qPCR validation, and the results demonstrated that the RT-qPCR results of the input and pull-down groups of the 10 differential mRNAs were consistent with the sequencing trends. Furthermore, our dual luciferase reporter assay verified that miR-192 could directly target and regulate the YY1 and CSK genes at the pull-down screening threshold in the present study. In their study, Ji et al. [31] demonstrated that miR-192 can target the YY1 gene and the results of this study are consistent with this. Therefore, these observations indicated that the pull-down results of this study are reliable.

Gene enrichment analysis facilitates the identification of the major signaling pathways associated with the candidate genes and predicts the possible mechanisms of the genes in an attempt to establish a foundation for future studies [15]. In the present study, we performed KEGG enrichment analysis on the differentially expressed genes screened, and the enrichment results revealed that the miR-192-regulated potential target genes were enriched in metabolic, PI3K-Akt, mTOR, and MAPK signaling pathways related to embryonic attachment, the metabolic pathway in particular, which had the highest number of enriched genes in the present study.

During mammalian pregnancy, complex metabolic changes were observed between the implantation-competent blastocyst and the recipient uterus, which included changes in maternal lipoproteins, cholesterol, proteins, and amino acids to meet the nutritional requirements of the fetus [18]. However, metabolic responses trigger the immune system to respond to pregnancy by increasing levels of pro-inflammatory cytokines to further enhance energy stores [18, 32]. Our study data suggest that the metabolic pathway is the signal transduction pathway showing the highest number of genes enriched in the present study and may be involved in the regulation of porcine pregnancy. In pigs, the embryo rapidly lengthens and attaches to the uterine wall through cell proliferation or migration during embryo attachment. A large amount of energy is consumed during this proves which increases the metabolic demands; in addition, glucose, fructose, and numerous amino acids increase in the uterine cavity and enter the trophoblast through specific pathways [33]. The coordination of biosynthetic pathways is a major factor in embryonic development, whereas oxidative metabolism promotes aerobic glycolysis through pathways such as the tricarboxylic acid (TCA) cycle, whose intermediate products can be shunted into the pentose phosphate pathway and one-carbon metabolism for the ab initio synthesis of nucleotides. This consequently provides energy for the embryo during attachment and in addition to the production of various substances required for embryonic development [34]. In the present study, potential target genes for miR-192 were enriched in porcine endometrial epithelial cells for 44 metabolic pathways associated with nucleotide, amino acid, fatty acid, sugar synthesis, and oxidative catabolism. These include carbon metabolism, oxidative phosphorylation, fatty acid metabolism, TCA cycle, arginine and proline metabolism, pyruvate metabolism, glycolysis/glycogenesis, and amino acid biosynthesis pathways. The energy stored and released during the breakdown and synthesis of these metabolic pathways in vivo can be used for the purposes of elongation, migration, hormone synthesis, implantation, early embryonic development, and the link between mother and embryo in the pig embryo.

The regulation of endometrial tolerance and embryonic development is a crucial step in mediating embryo attachment. In clinical practice, activation of the PI3K/Akt/eNOS signaling pathway reportedly improved endometrial tolerance and embryonic invasion processes [35]. In mice, the VEGF/PI3K/Akt pathway in endometrial cells is involved in regulating Ca2+-mediated angiogenesis, which facilitates embryo implantation into the endometrium [36]. The endometrium is closely linked to embryo attachment, and the conditional absence of the uterus is molecularly linked to mTOR activation [37], which activates the PI3K/PKB/mTOR/NO signaling pathway to promote embryo implantation and attachment [38]. MAPK signaling pathways have different and interrelated biological effects in the preimplantation embryo, which include regulating differentiation and apoptosis, transducing stimulatory signals, and participating in embryonic development.Inhibition of MAPK signaling delays mouse blastocyst development, reduces embryonic trophoblast growth, and inhibits the maturation of porcine oocytes [39, 40]. In the present study, certain genes associated with the pertinent metabolic pathways and the PI3K-Akt, mTOR, and MAPK signaling pathways, including MAPK1 and MAPK3 are involved in the regulation of embryomaternal recognition during sow gestation [41], mTOR is associated with the regulation of sow embryo growth and development [42], and WNT7A is involved in endometrial morphology and embryo implantation in sows during gestation [43]. Changes in CDC42 may limit stromal cell invasion by porcine embryos [44], and LIPG and GLUL are linked to nutrient and energy supply for maternal and embryonic development during pregnancy [33, 45]. In this regard, we speculate that miR-192 may be indirectly involved in metabolic, PI3K-Akt, mTOR, MAPK, and other signaling pathways through the regulation of target genes.

YY1 (Yin-Yang-1) is a ubiquitously expressed transcription factor that activates or represses specific genes to influence cellular regulatory mechanisms, including cell growth control, apoptosis, and pregnancy regulation [46]. The YY1 gene is reportedly implicated in the regulation of human uterine artery endothelial cell development, cytoskeleton formation, and trophoblast cell invasion and adhesion [21–23, 47]. In addition, YY1 is involved in early uterine metaphase and embryo implantation in mice through the regulation of Rtcb promoter activity [48], and YY1 mutations are associated with embryonic lethality in mice [49]. CSK (C-terminal Src kinase) is a non-receptor tyrosine kinase that is widely expressed in cells and plays an important role in cell growth and differentiation, metabolism, motility, and localization [50]. Decidualization is the progesterone and estrogen-induced differentiation of endometrial fibroblast-like stromal cells into ecdysteroid cells, which is essential for the establishment and maintenance of pregnancy. Activation of c-Src is associated with in vitro metaphase of human embryonic stem cells (hSCs) and the decidualization process of human stromal cells [25, 26]. These studies confirm

that YY1 and CSK can regulate the pregnancy process in humans or mice; however, to the best of our knowledge, no reports of regulation in porcine pregnancy exist. In this study, we combined miR-192 pull-down data, the subject's early pregnancy attachment endometrial proteomic data, bioinformatics, and literature data to predict that CSK and YY1 genes may also be candidate target genes affecting the porcine embryo attachment process from multiple perspectives. The direct targeting of miR-192 to CSK and YY1 genes to suppress their expression was verified using dual luciferase, RT-qPCR, and western blot analyses. However, the exact mechanism of miR-192 targeting and regulation of CSK and YY1 genes during embryo attachment needs to be verified by further experiments.

Conclusion

In this study, 1688 potential target genes of miR-192 were identified in porcine endometrial epithelial cells, and their regulated biological roles were predicted. This study provides a new reference for the identification of miR-192 in the sow embryo attachment process and target genes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-023-08349-w.

Author contributions TH guided this work and reviewed the article. QL finished the article. RG, FH, YS, SL, XC, MQ and ML, help to finish the experiment. YC, SX, and XS collected relevant information. QL modified the figures. The final draft was read and approved by all of the writers.

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Declarations

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

Ethical approval This research does not contain any studies with human or animal participants by any of the authors.

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