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ORIGINAL ARTICLE

Maternal obesity downregulates microRNA *let-7g* expression, a possible mechanism for enhanced adipogenesis during ovine fetal skeletal muscle development

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BACKGROUND: Obesity in women of childbearing age is increasing at an alarming rate. Growing evidence shows that maternal obesity induces detrimental effects on offspring health, including pre-disposition to obesity. We have shown that maternal obesity increases fetal intramuscular adipogenesis at mid-gestation. However, the mechanisms are poorly understood. MicroRNAs (miRNAs) regulate mRNA stability. We hypothesized that maternal obesity alters fetal muscle miRNA expression, thereby influencing intramuscular adipogenesis.

METHODS: Non-pregnant ewes received a control diet (Con, fed 100% of National Research Council (NRC) recommendations, $n = 6$) or obesogenic diet (OB; 150% NRC recommendations, $n = 6$) from 60 days before to 75 days after conception when the fetal *longissimus dorsi* (LD) muscle was sampled and miRNA expression analyzed by miRNA microarray. One of miRNAs with differential expression between Con and OB fetal muscle, *let-7g*, was further tested for its role in adipogenesis and cell proliferation in C3H10T1/2 cells.

RESULTS: A total of 155 miRNAs were found with a signal above 500, among which, three miRNAs, *hsa-miR-381*, *hsa-let-7g* and *bta-miR-376d*, were differentially expressed between Con and OB fetuses, and confirmed by quantitative real-time PCR (QRT-PCR) analyses. Reduced expression of miRNA *let-7g*, an abundantly expressed miRNA, in OB fetal muscle was correlated with higher expression of its target genes. Overexpression of *let-7g* in C3H10T1/2 cells reduced their proliferation rate. Expression of adipogenic markers decreased in cells overexpressing *let-7g*, and the formation of adipocytes was also reduced. Overexpression of *let-7g* decreased expression of inflammatory cytokines.

CONCLUSION: Fetal muscle miRNA expression was altered due to maternal obesity, and *let-7g* downregulation may enhance intramuscular adipogenesis during fetal muscle development in the setting of maternal obesity.

INTRODUCTION

Obesity is now an epidemic in the United States, with more than one-third of the population aged $\geqslant 20$ are obese (2007–2008), including women of child bearing age.[1] Associated with the prevalence of obesity in adults, nearly 17% of children and teenagers aged 2–19 years old are obese.[2] Maternal obesity caused by high-energy diets increases the risk of obesity in offspring.[3,4] Skeletal muscle is one of the principal sites for glucose and fatty acid utilization and comprises 40–45% of body mass in adults.[5] In precocial species, the embryo-fetal period is crucial for skeletal muscle development, because no net increase of muscle fiber numbers occurs after birth.[6] Muscle cells and adipocytes are both derived from mesenchymal stem cells,[7] and myogenesis and adipogenesis are considered to be competitive processes as inhibition of adipogenesis promotes myogenesis.[8,9] Fetal adipogenesis begins around mid-gestation, a stage of development which partially overlaps with secondary myogenesis.[10] We have conducted a series of studies using the pregnant sheep as a model and shown that maternal obesity enhances intramuscular adipogenesis during fetal skeletal muscle development,[11–14]

leading to altered muscle properties in offspring sheep.[15] However, the detailed mechanisms remain poorly understood.

MicroRNAs (miRNAs) are single-stranded RNA molecules 21–23 nucleotides in length, which play a crucial role in developmental processes by regulating expression of target genes, many of which govern cell proliferation and differentiation.[16,17] It has been estimated that about 30% of all protein-coding genes are regulated by miRNAs.[18]

Based on our previous observations that myogenesis was impaired while adipogenesis was elevated in fetal muscle of obese sheep by mid-gestation,[12–14] we hypothesized that maternal obesity altered miRNA expression in fetal muscle, which may have an important role in adipogenic differentiation during fetal muscle development.

Following miRNA screening we identified three miRNAs that were differentially expressed. Of particular interest in relation to adipogenesis was the observation of lower *let-7g* in obesogenic diet (OB) compared with Con fetal muscle. MiRNA *let-7* was first discovered in *C. elegans* where it was shown to regulate development, cellular proliferation and differentiation.[19] Shortly after, *let-7*

was found in other species and conserved from mollusks to vertebrates.[20] Numerous studies have been conducted on *let-7* and 13 different members of the human *let-7* family have been identified on nine different chromosomes.[21] *Let-7g* has been reported to be related to colon cancer,[22] and subsequently shown to repress the growth of lung cancer cells[17] and lung tumor development.[16] Because of the similarity between cancer cells and stem cells, it has been suggested that there may be similar regulatory signaling by *let-7g* in stem cells.[23]

We found that reduced expression of miRNA *let-7g* in OB fetal muscle was correlated with higher expression of its target genes and that overexpression of *let-7g* in C3H10T1/2 cells reduced their proliferation rate, expression of adipogenic markers, adipocyte formation and expression of inflammatory cytokines. For the first time, our data indicate that *let-7g* inhibits adipogenesis.

MATERIALS AND METHODS

Care and use of animals

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. From 60 days before conception to day 75 of gestation (day of mating = day 0; term 150 days), multiparous Rambouillet/Columbia ewes were fed either a highly palatable diet of 100% NRC recommendations (control, Con; $n = 6$) or an OB of 150% NRC recommendations (OB; $n = 6$) on a metabolic body weight (BW) basis ($BW^{0.75}$).[12] All ewes were weighed at weekly intervals and rations adjusted for weight gain, and body condition scored at monthly intervals to evaluate changes in fatness. A body condition score of 1 (emaciated) to 9 (obese) was assigned by two trained observers after palpation of the transverse and vertical processes of the lumbar vertebrae (L2 through L5) and the region around the tail head.[24] There was no difference in body weight and body condition score before dietary treatments; after 60 days of treatments, both body weight and body condition score were much higher in OB group than in Con group.[12]

Following a 12-h overnight fast, 12 pregnant sheep (6 Con and 6 OB) were weighed on day 75 of gestation (term 150 days). Sheep were sedated with intravenous ketamine ($10 \, mg \, Kg^{-1}$) and general anesthesia was induced and maintained by isoflurane inhalation. The abdomen and uterus were opened and fetuses were quickly removed to obtain weight and length, and the *longissimus dorsi* (LD) muscle on both sides was immediately collected and snap frozen in liquid nitrogen for analysis. Fetuses from five twin pregnant ewes in each group were selected for further analyses. Though no difference in weight was observed among fetuses of different sexes, the sex of fetuses in each group was balanced. The fetal weight was 268 ± 12 versus $374 \pm 10 \, g$ (Con versus OB), while the weight of LD muscle was 2.40 ± 0.16 versus $3.46 \pm 0.20 \, g$ (Con versus OB).[12]

MiRNA microarray analysis

miRNA was extracted from the LD muscle of three fetuses of an average body weight in each treatment using QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA) and purified by a miRNeasy Mini Kit (Qiagen). Purified miRNA was then sent for miRNA microarray analysis (LC Sciences, Houston, TX, USA) based on all known miRNAs of human, rat, mouse, cattle and sheep. Briefly, Con and OB miRNA were labeled with green and red fluorescent dyes, respectively. Then, labeled miRNA was applied to an uParaFlo microfluidics chip (Custom Microarray Platform, LC Sciences, Houston, TX, USA) for hybridization, microarray scan and data extraction. A locally-weighted regression method was used for data normalization, which was performed by LC Sciences.

Quantitative real-time PCR

Total mRNA was extracted from the fetal LD muscle or C3H10T1/2 cells using TRI reagent (Sigma, St. Louis, MO, USA) and reverse transcribed into complementary DNA using a kit (Qiagen). Complementary DNAs were used for real-time PCR (RT-PCR) analyses using a SYBR Green RT-PCR kit from Bio-Rad (Hercules, CA, USA). Primers used for sheep samples were: follistatin (FST) forward, 5′-GGCTCCGCCAAGCAAAGA-3′, and reverse, 5′-CG TTGAAAATCATCCACTT-3′; tumor necrosis factor receptor super family, member 4 (TNFRSF4) forward, 5′-GCCCACCAGGACATTTCTC-3′, and reverse, 5′-GGCGTCCGAGCTGCTATT-3′; interleukin (IL)-6 forward, 5′-TCATCCTGAG AAGCCTTGAGA-3′, and reverse, 5′- TTTCTGACCAGAGGAGGGAAT-3′; and tumor necrosis factor (TNF) α forward, 5′-ACACCATGAGCACCAAAAGC-3′,

and reverse, 5′-AGGCACAAGCAACTTCTGGA-3′. Mouse primer sets used were: FST forward, 5′-CCGCCAAGCAAAGAACGGCC-3′, and reverse, 5′-GG GCGCCCCGTTGAAAATC-3′; TNFRSF4 forward, 5′-GCAGGACAGCGGCTACA AGC-3′, and reverse, 5′-GTGGCGGGTCTGCTTCCAG-3′; Cyclin D1 forward, 5′-GCGTACCCTGACACCAATCT-3′, and reverse, 5′-ATCTCCTTCTGCACGCACT T-3′; Cyclin dependent kinase 4 (CDK4) forward, 5′-CTAGCTCGCGG CCTGTGTCTA-3′, and reverse, 5′-AGCAGGGGATCTTACGCTCGG-3′; proliferating cell nuclear antigen forward, 5′-TAGTCGCCACAACTCCGCCAC-3′, and reverse, 5′-CGTCCCAGCAGGCCTCATTGA-3′; peroxisome proliferator-activated receptor (PPAR) γ forward, 5′-GCCTGCGGAAGCCCTTTGGT-3′, and reverse, 5′-CAGCAAGCCTGGGCGGTCTC-3′; CCAAT-enhancer binding protein α (C/EBPα) forward, 5′-GGTACGGCGGGAACGCAACA-3′, and reverse, 5′-GAAGATGCCCCGCAGCGTGT-3′; TNFα forward, 5′-TGGGACAGTGACCTG GACTGT-3′, and reverse, 5′-TTCGGAAAGCCCATTTGAGT-3′;[25] IL6 forward, 5′-GCTGGTGACAACCACGGCCT-3′, and reverse, 5′-AGCCTCCGACTTGTGAAG TGGT-3′. The 18S RNA was used as a control, forward, 5′-TTGTACACACCGC CCGTCGC-3′, and reverse, 5′-CTTCTCAGCGCTCCGCCAGG-3′. PCR conditions were as following: 20 s at 95 °C, 20 s at 58 °C and 20 sec at 72 °C for 35 cycles. After amplification, a melting curve (0.01 C per s) was used to confirm product purity. Data were expressed relative to 18S ribosomal RNA as previously described.[11]

Cell culture

C3H10T1/2 cells were purchased from American type culture collection (Manassas, VA, USA) and cultured in maintaining medium containing Dulbecco's modified Eagle's medium (Sigma), 10% fetal bovine serum and 1% antibiotics at 37 °C.[26] Medium was changed every 72 h. HEK 293T cells were purchased from GenHunter Corporation (Nashville, TN, USA) and maintained in the same medium as C3H10T1/2 cells.

Plasmids

All plasmids were obtained from Addgene (Cambridge, MA, USA), which include pCMV-VSV-G (Cat. No. 8454), pCMV-dR8.2 dvpr (Cat. No. 8455), Puro.let-7g.Cre (Cat. No. 17409), Puro.let-7gsm.Cre (Cat. No. 17410) and green fluorescent protein Cre empty vector (Cat. No. 20781). Plasmids were recovered in LB medium for 16 h at 30 °C, and DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) and dissolved in sterile TE buffer. DNA concentration was measured by Nanodrop 2000 (Thermo scientific, Wilmington, DE, USA) and plasmid DNA was adjusted to a concentration of $200 \, ng \, \mu l^{-1}$.

Lentivirus production and infection

One day before transfection, 1.5×10^5 HEK293T cells were seeded in a 60-mm plate. Transfection was conducted using FuGENE transfection reagent following the instructions of the manufacturer (Promega, Madison, WI, USA). Briefly, plasmid DNA was added at a ratio of 10:5:1 (2 µg of target plasmid, 1 µg of pCMV-dR8.2 dvprm and 200 ng of pCMV-VSV-G). The ratio of total DNA to FuGENE was 4:1. DNA and transfection reagent were mixed for 10 min and the mixture added dropwise to cells. The transfection lasted for 24 h and, the expression of green fluorescent protein was examined under a fluorescence microscope. Viruses were collected at 48, 72, and 96 h after transfection. The collected viral supernatant was filtered through a syringe with 0.45 µm filter and then concentrated with a Lenti-XTM concentrator (Cat. No. 631231, Clontech, Mountain View, CA, USA). The titer of lentivirus was estimated by using a Lenti-XTM GoStixTM kit (Cat. No. 631243, Clontech). A titer of approximately 10^6 infection units (IFU) ml^{-1} was reached after concentration. The viral infection of C3H10T1/2 cells was performed with $8–10 \, \mu g \, ml^{-1}$ polybrene and approximately 10^5 IFU of lentivirus for 24 h. C3H10T1/2 cells were left to recover for at least 48 h before puromycin was used to select drug-resistant cells. The selection was continued for 2 weeks.

Cell proliferation analysis

Cell proliferation rate was measured by using a CyQUANT NF Cell Proliferation Assay Kit (Cat. No. C35007, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Adipogenic differentiation

Adipogenic differentiation was induced as described previously with minor changes.[27] Briefly, C3H10T1/2 cells were seeded to 20% confluence and treated with 10 µM 5′-azacytidine for 3 days to demethylate genomic DNA and induce multipotency. After reaching confluence, cells were

grown in adipogenic medium (5 µg ml^{-1} insulin, 1 µg ml^{-1} dexamethazone, 27.8 µg ml^{-1} isobutylmethylaxanthine and 10 µM troglitazone) for 3 days, and then the above step was repeated once. Following which the medium was changed to Dulbecco's modified Eagle's medium supplemented with insulin only (5 µg ml^{-1}) for 2 days, and this step was repeated once.[26,28–30]

Oil-Red O staining

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, rinsed with 60% ethanol and stained with 0.2% (w/v) Oil-Red O in 60% ethanol (Sigma Chemical Co.) for 10 min as described previously.[26] Then, cells were destained with 60% ethanol for 5 min and used for microscopic observation.

Immunoblotting

Immunoblotting was conducted as previously described, and data were normalized to tubulin.[11] Antibodies against PPARγ (Cat. No. 2443) and CEBPα (Cat. No. 2295) were purchased from Cell Signaling (Danvers, MA, USA), and tubulin antibody from Sigma (Cat. No. T4026).

Vector construction and luciferase assay

Expression vector was constructed as described previously.[31] Briefly, a 1086 bp 3′ UTR fragment of FST gene containing the predicted hsa-let-7g binding site was amplified and subcloned into the XbaI site of the pGL3-control luciferase vector (Cat. No. E1741, Promega). The primer sequences used for amplification were (XbaI sites are in bold): forward 5′-GC**TCTAGA**ACTCTCCACCAATGTTCAGTG-3′ and reverse 5′-GC**TCTAGA**TGCACATTTACA TGGTAG-3′. The construct was verified by XbaI digestion and sequencing.

The luciferase assay was performed as previously reported.[31,32] Briefly, C3H10T1/2 cells infected with Puro.let-7g.Cre or Puro.let-7gsm.Cre were seeded at 50 000 cells per well in a 12-well plate the day before transfection. These cells were cotransfected with 400 ng of pGL3-control luciferase vector and 50 ng of Renilla luciferase vector. Luciferase activity was measured 48 h after transfection using the Dual-Glo Luciferase Assay System (Promega). The Renilla luciferase activity served as an internal control.

Table 1. MiRNAs differentially expressed between Control (con) and obesogenic diet (OB) fetal skeletal muscle

MicroRNA	Con	OB	Log2 (OB/Con)	P-value
Hsa-let-7g	23 060 ± 106	21 379 ± 433	− 0.12	0.066
Hsa-miR-381	2367 ± 46	3306 ± 93	0.50	0.002
Bta-miR-376d	6991 ± 178	4835 ± 583	− 0.52	0.091
Hsa-let-7b	63 ± 2	83 ± 5	0.38	0.04
Hsa-miR-1306	36 ± 1	30 ± 2	− 0.32	0.10

Three muscle samples of fetuses at an average body weight within each group were used for microRNA microarray analyses ($n = 3$) based on all known miRNAs of human, rat, mouse, cattle and sheep.

Statistical analysis

Statistical analyses were conducted according to our previous studies in sheep[6,11,33] and cultured cells.[32] Briefly, each animal was considered as an experimental unit. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000, Cary, NC, USA). The differences in the mean values were compared by the Tukey's multiple comparison, and mean ± s.e.m. were reported. Statistical significance was considered as $P < 0.05$ for cell culture studies. For miRNA analyses in fetal muscle, however, due to the limited sample size (three samples in each treatment), $P < 0.10$ was considered as significant.

RESULTS

MiRNA expression in fetal muscle

We first analyzed miRNA expression in muscle from fetuses of both control and obese mothers using the miRNA microarray (Supplementary Table 1). To our knowledge, this is the first time to thoroughly analyze the expression of miRNAs in fetal sheep. Of 1030 miRNAs tested, 155 were expressed in sheep fetal muscle (signals > 500). Among these 155 miRNAs, three were differentially expressed in fetal muscle of control and obese mothers, which are *hsa-miR-381*, *hsa-let-7g* and *bta-miR-376d* ($P < 0.10$, Table 1, Figure 1a). Besides, three other miRNAs with signals less than 500 were also differentially expressed (Table 1 and Supplementary Figure 1). We then confirmed the expression level of those miRNAs using quantitative RT-PCR. For those three miRNAs expressed at relatively high levels, the results were pretty consistent. The expression of *miR-381* was 67.0 ± 17.6% higher in OB compared with control fetal muscle ($P < 0.05$, Figure 1a), while the expression of *let-7g* and *miR-376d* was lower in the OB group (24.6 ± 3.3% and 26.0 ± 8.8% respectively, $P < 0.05$, Figure 1a). There were no significant differences between the two groups of fetuses for those three miRNAs expressed at low levels ($P > 0.10$, Supplementary Figure 2). We focused on miRNA *let-7g*, as its expression level was much higher than other two miRNAs with differential expression (Table 1) and its reported role in carcinogenesis, a process related to stem cell proliferation and differentiation, which likely has a role in adipogenic differentiation.

We then analyzed the mRNA expression of two potential target genes of *let-7g*, TNFRSF4 and FST, which were both higher in OB fetal muscle (increased by 63.0 ± 21.6% and 45.2 ± 13.6%, respectively, $P < 0.05$, Figure 1b).

In order to identify whether there was a change in the expression of inflammatory cytokines in Con and OB tissues, we further analyzed tumor necrosis factor α and IL-6 mRNA expression, both of which were higher in OB compared with Con tissue ($P < 0.05$, Figure 1c), indicating inflammatory response in OB fetal muscle.

Effect of *let-7g* on expression of potential target genes

We overexpressed *let-7g* in mesenchymal stem cell line C3H10T1/2 cells using a lentiviral system. To test the efficiency
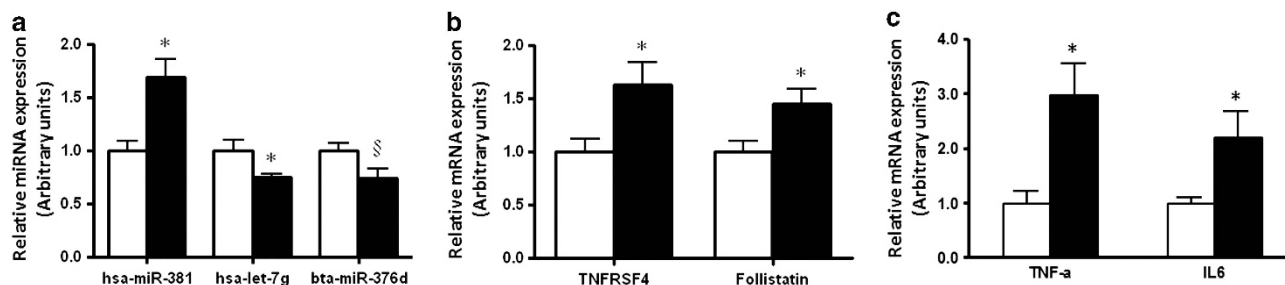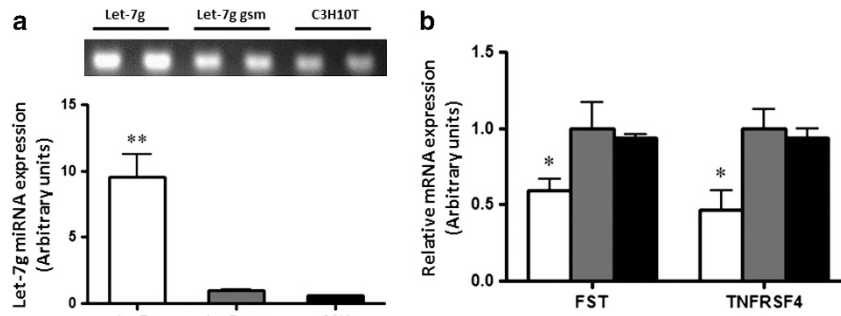

Figure 1. Expression of microRNAs (miRNAs) and potential targets of miRNA *let-7g* in fetal LD muscle of obese (OB ■) versus control sheep (Con □). (**a**) Quantitative RT-PCR showed higher expression of *miR-381*, but less *let-7g* and *miR-376d* in OB fetal muscle than in Con muscle. (**b**) mRNA expression of TNFRSF4 and FST were increased in OB compared with Con fetal muscle, both are potential targets of *hsa-let-7g*. (**c**) mRNA expression of tumor necrosis factor α and IL-6 were higher in OB compared with Con fetal muscle (*$P < 0.05$, §$P < 0.10$ when compared with Con; Mean ± s.e.m.; $n = 10$ in Con and $n = 10$ in OB group).

Figure 2. *Let-7g*, FST and TNFRSF4 expression in stably selected C3H10T1/2 cells. (**a**) *Let-7g* expression in C3H10T1/2 cells infected with lentiviral plasmids Puro.let-7g.Cre (□), Puro.let-7gsm.Cre (■), and C3H10T1/2 cells without infection (■); cells infected with Puro.let-7gsm.Cre used as control. (**b**) *Let-7g* overexpression reduced the mRNA expression of FST and TNFRSF4 (**$P<0.01$, *$P<0.05$ when compared with *let-7gsm*; Mean ± s.e.m.; $n = 4$).
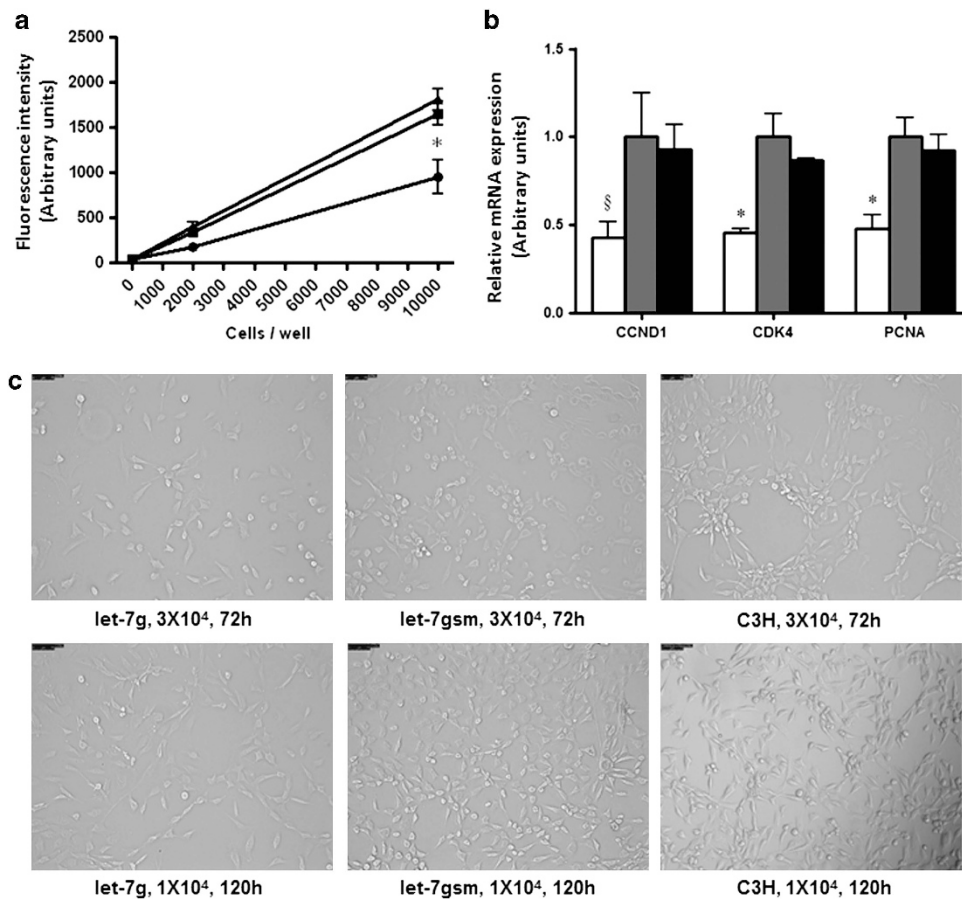

Figure 3. *Let-7g* suppresses the proliferation of mesenchymal stem cells. (**a**) Cell proliferation rate was lower in C3H10T1/2 cells infected with lentiviral plasmids Puro.let-7g.Cre compared with C3H10T1/2 cells or C3H10T1/2 cells infected with Puro.let-7gsm.Cre. Either 2×10^3 or 10^4 cells were plated in 96-well plates for analysis. (**b**) Cell proliferation related genes, cyclin D1, CDK4 and proliferation cell nuclear antigen were decreased after *let-7g* overexpression. (**c**) C3H10T1/2 cells infected with Puro.let-7g.Cre grew slower than C3H10T1/2 cells and C3H10T1/2 cells infected with Puro.let-7gsm.Cre. Either 10^4 or 3×10^4 cells were plated in 6-well plates for analysis (*$P<0.05$, $^\S P<0.10$ when compared with *let-7gsm*; Mean ± s.e.m.; $n = 4$ in each group).

of transfection, we included a green fluorescent protein empty vector in the lentiviral system, which showed an above 90% transfection and transduction efficiency (Supplementary Figure 3). In cells transfected with lentiviral plasmid Puro.let-7g.Cre, the expression level of *let-7g* was much higher in overexpressed cells compared with non-treated cells, as well as cells infected with a plasmid carrying scrambled sequence ($P<0.01$, Figure 2a). We next evaluated the effects of *let-7g* overexpression on target gene expression; both FST and TNFRSF4 mRNA levels were decreased

after *let-7g* overexpression ($40.3 \pm 7.0\%$ and $53.5 \pm 13.2\%$ lower, respectively, $P<0.05$, Figure 2b), consistent with the data obtained in fetal sheep muscle *in vivo*.

Effect of *let-7g* on the proliferation of mesenchymal stem cells
C3H10T1/2 cells infected with *let-7g* grew much slower than control cells, with the cell proliferation rate only $57.3 \pm 11.1\%$ of control cells ($P<0.05$, Figure 3a). In addition, several genes related
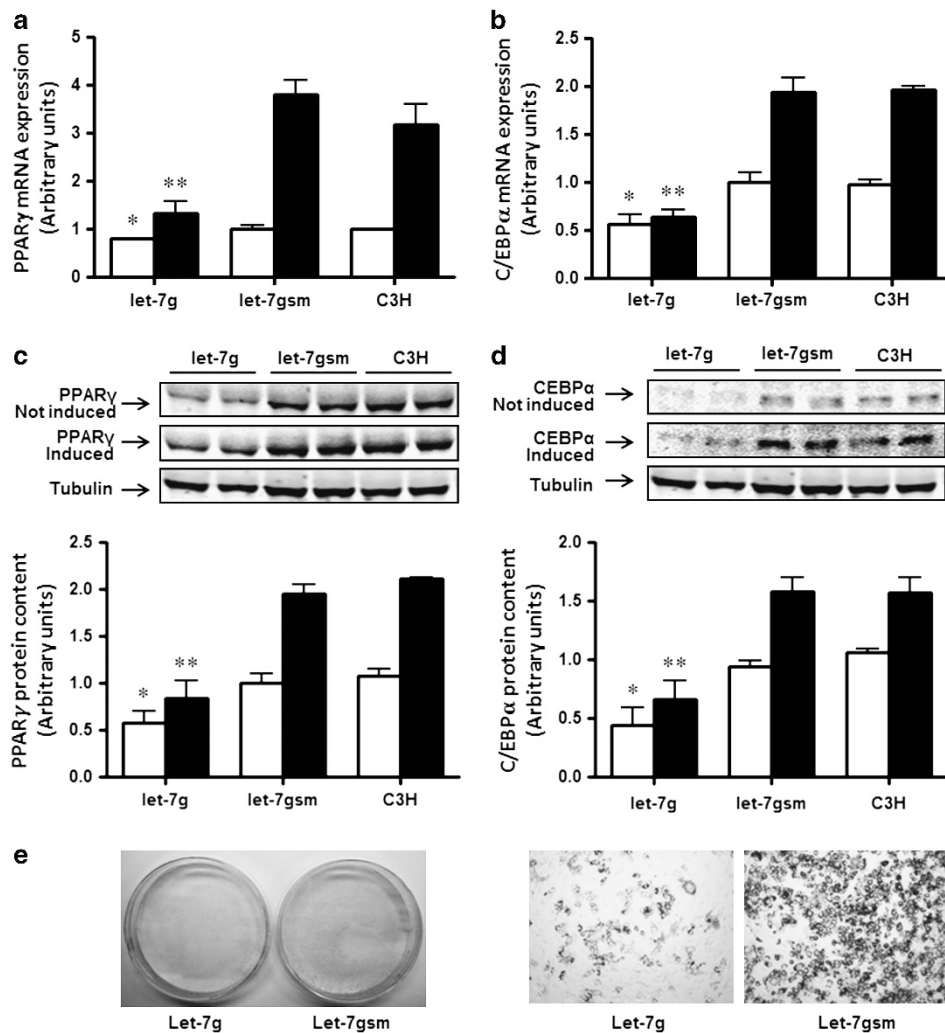
Figure 4. *Let-7g* inhibits expression of adipogenic differentiation markers and adipogenesis in C3H10T1/2 cells treated with 5′-azacytidine. (**a**) mRNA expression of PPARγ without or with adipogenic induction, which were both lower in cells infected with Puro.let-7g.Cre. (**b**) mRNA expression of CCAAT/enhancer-binding protein alpha (C/EBPα) without or with adipogenic induction, which were both lower in cells infected with Puro.let-7g.Cre. (**c**) Protein content of PPARγ without or with adipogenic induction, which were both lower in cells infected with Puro.let-7g.Cre. (**d**) Protein expression of C/EBPα without or with adipogenic induction, which were both lower in cells infected with Puro.let-7g.Cre. (**e**) After cultured in adipogenic differentiation medium for 10 days, there were less adipocytes formed in C3H10T1/2 cells infected with lentiviral plasmid Puro.let-7g.Cre compared with cells infected with Puro.let-7gsm.Cre plasmid (left: x1magnification; right: x100 magnification). (*$P < 0.05$, **$P < 0.01$ when compared with *let-7gsm*; mean ± s.e.m.; $n = 4$; □, cells without adipogenic induction; ■, cells after adipogenic differentiation).

to cell proliferation, including cyclin D1, CDK4 and proliferating cell nuclear antigen, were all lower in *let-7g* overexpressed cells (57.3 ± 9.1%, 54.6 ± 2.4%, and 52.3 ± 8.3% lower for *let-7g*, scrambled, and control cells respectively, $P < 0.05$, Figure 3b), further supporting the role of *let-7g* in cell proliferation. We then plated the same amount of cells in 6-well plates, and after 72 and 96 h, the density of cells was different in cells infected *let-7g* compared with non-infected and scrambled infected cells (Figure 3c).

Let-7g and adipogenic differentiation of mesenchymal stem cells

After 6 days of culture without adipogenic induction, *let-7g* inhibited the mRNA expression of PPARγ (20.6 ± 1.4% lower, $P < 0.05$, Figure 4a) and CCAAT-C/EBPα (44.0 ± 9.9% lower, $P < 0.05$, Figure 4b), both of which are markers of adipogenesis, and also the protein content of PPARγ (42.9 ± 12.7% lower, $P < 0.05$, Figure 4c) and C/EBPα (56.1 ± 15.2% lower, $P < 0.05$, Figure 4d). After 6 days of adipogenic differentiation, the mRNA expression

of PPARγ and C/EBPα was still much lower in cells overexpressing *let-7g* (65.0 ± 6.2% and 67.0 ± 3.7% lower respectively, $P < 0.05$, Figures 5a and b), and the protein content of PPARγ and C/EBPα was also less in *let-7g* overexpressed cells (56.9 ± 9.2% and 58.1 ± 10.3% lower, respectively, $P < 0.05$, Figures 5c and d). After 10 days of adipogenic induction, less adipocyte were formed in cells infected with plasmid Puro.let-7g.Cre, overexpressing *let-7g* (Figure 4e). These reductions in the expression of adipogenic markers and adipocyte formation likely were not due to the decrease in cell proliferation, because these data were normalized for the amount of RNA and proteins, or the total number of cells used for analyses.

To further analyze the role of *let-7g* in adipogenesis, we measured the time course change of *let-7g* expression during adipogenesis. The level of *let-7g* did not change during the initial stage of adipogenesis, but slightly increased during the late stage of differentiation (Supplementary Figure 4). These data indicate that *let-7g* expression is not closely correlated with adipogenic
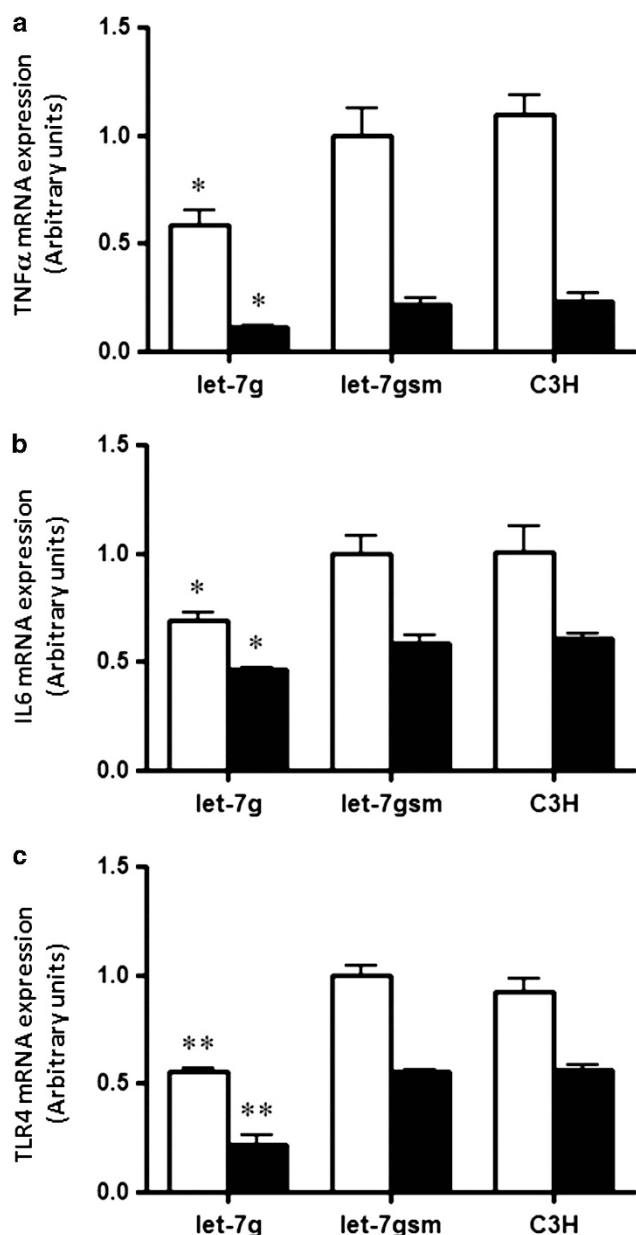
Figure 5. *Let-7g* inhibits the expression of inflammatory cytokines in C3H10T1/2 cells treated with 5′-azacytidine. (**a**) Quantitative RT-PCR showed a decreased expression of TNFα mRNA in C3H10T1/2 cells infected with lentiviral plasmid Puro.let-7g.Cre; after adipogenic differentiation, mRNA level of TNFα was reduced in all 3 types of cells, but there was difference between cells infected with lentiviral plasmid Puro.let-7g.Cre and C3H10T1/2 cells. (**b**) mRNA expression of IL6 was lower in C3H10T1/2 cells infected with lentiviral plasmid Puro.let-7g.Cre than C3H10T1/2 cells before and after adipogenic differentiation. (**c**) Lentiviral plasmid Puro.let-7g.Cre infection decreased the mRNA level of TLR4 in C3H10T1/2 cells both before and after adipogenic differentiation (**$P < 0.01$, *$P < 0.05$ when compared with *let-7gsm*; mean ± s.e.m.; $n = 4$; □, cells without adipogenic induction; ■, cells after adipogenic differentiation).

differentiation. The mechanisms regulating Let-7g expression need to be further explored.

Let-7g and the expression of inflammatory cytokines

Let-7g overexpression inhibited the expression of inflammatory cytokines TNFα, IL6 and TLR4 (by 41.2 ± 6.3%, 30.5 ± 3.6% and

44.4 ± 1.6%, respectively, $P < 0.05$, Figures 5a, b and c), demonstrating the anti-inflammatory effect of *let-7g*. Interestingly, the mRNA expression of those inflammatory cytokines was all decreased following adipogenic differentiation (49.2 ± 4.8%, 21.0 ± 1.5% and 60.8 ± 8.3% lower for *let-7g*, scrambled, and control cells, respectively, $P < 0.05$, Figures 5a–c).

FST is as a potential target of *let-7g*

We next investigated whether *let-7g* directly binds to the mRNA of its potential target gene FST. We cloned the 1086 bp 3′ UTR fragment of FST gene containing the predicted *hsa-let-7g* binding site and subcloned it into the 3′ UTR of luciferase gene. If the 3′ UTR fragment of FST gene is a target of *let-7g*, the stability of luciferase mRNA will be impaired and less luciferase will be translated. Using luciferase assay, we found that *let-7g* overexpression led to a modest decrease in luciferase activity (by 26.6 ± 5.7%, $P < 0.10$, Supplementary Figure 5), suggesting that FST is a potential direct target of *let-7g*, which were consistent with the changes in FST expression in both fetal muscle *in vivo* and cultured cells *in vitro*.

DISCUSSION

Obesity is an increasingly serious problem in the United States, as well as many countries around the world. Childhood obesity is also increasing. The high rate of maternal obesity predisposes offspring to obesity.[34,35] Our previous studies have shown that myogenesis is impaired and adipogenesis increased in fetal muscle of obese sheep,[11,12,14] and we have demonstrated that chronic inflammation may have a role.[11,14] However, the causative mechanisms remain poorly understood. In the current study, we analyzed miRNA expression of fetal sheep muscle at mid gestation. Our results showed that there are several miRNAs with differential expression in fetal muscle of control and obese mothers. Because of the high abundance of *let-7g*, we next analyzed expression of its potential target genes, including FST and TNFRSF4, which were both upregulated. The association between *let-7g* and the expression of FST and TNFRSF4 was further tested *in vitro*. In C3H10T1/2 cells, with *let-7g* overexpression, both FST and TNFRSF4 were decreased, indicating that FST and TNFRSF4 are targets of *let-7g*.

We then tested the role of *let-7g* in cell proliferation. In *let-7g* overexpressed cells, we observed a lower proliferation rate, consistent with previous reports that *let-7g* suppresses growth of cancer cells and lung tumors.[16,17] We also observed downregulation of the cell proliferation markers cyclin D1, CDK4 and proliferating cell nuclear antigen. Cyclin D1 has been used as a marker of cell proliferation and its lower expression represses cell proliferation.[31] Moreover, knocking down cyclin D1 suppresses cancer cell growth.[36] CDK4 forms a complex with cyclin D and has an important role in cell cycle control.[37] Proliferating cell nuclear antigen is another marker of cell proliferation.[38] In combination, these data show that cell proliferation was decreased by *let-7g* overexpression.

The detailed mechanisms reducing cell proliferation remain to be investigated. A recent study using hepatocellular carcinoma cells showed that *let-7g* suppresses expression of c-Myc and increases expression of p16 (INK4A).[39] Our data both *in vivo* and *in vitro* also showed that FST is one of the targets of *let-7g*. FST is a myostatin-binding protein, which is known to inhibit the activity of myostatin, a negative regulator in cell proliferation.[40] In addition, FST induces satellite cell proliferation, which then leads to muscle hypertrophy.[41] Moreover, FST overexpression in human myoblasts promotes proliferation and differentiation, further supporting the role of FST in inducing cell proliferation.[42] We speculated that the inhibitory effect of *let-7g* on cell proliferation is partially mediated by targeting FST mRNA. Less *let-7g* expression

in OB fetal muscle promoted FST expression, which enhances cell proliferation. Indeed, the muscle and weights were higher in fetuses of OB compared with those of control sheep.[12] We next investigated whether *let-7g* directly binds to the 3′ untranslated region of FST mRNA. We cloned the 1086bp 3′ UTR fragment of FST and subcloned it into the 3′ UTR of luciferase gene. Using luciferase assay, we found that *let-7g* overexpression led to a modest decrease in luciferase activity. The relatively weak inhibitory effect on luciferase expression could be due to the fact that the cloned FST 3′ UTR might not include all *let-7g* targeted sequences, and another possibility is that FST is only an indirect target of *let-7g*, which requires further investigation.

Let-7g also affected adipogenesis. Expression of adipogenic markers was decreased in cells overexpressing *let-7g*. miRNAs have been reported to be involved in adipogenesis; for example, *MiR-143* was reported to regulate adipocyte differentiation, but only in preadipocytes.[43] *Let-7* family miRNAs, including *let-7b* and *let-7c*, have also been reported to regulate adipogenesis of 3T3-L1 cells, but again in preadipocytes.[44] To our knowledge, this is the first report of a potential role for *let-7g* in adipogenic differentiation of mesenchymal stem cells. However, there is a chance that the inhibitory role of *let-7g* on adipogenesis is derived from its ability to inhibit cell proliferation, which needs further studies to elucidate.

Our results further indicated that miRNA *let-7g* is involved in inflammation. We overexpressed *let-7g* in C3H10T1/2 cells, resulting in a decrease in the expression of inflammatory cytokines TNFα, IL6 and TLR4. Thus, it is highly possible that miRNA *let-7g* has a role in the development of chronic inflammation in fetal muscle of obese sheep. Indeed, we observed that the expression of both TNFα and IL6 mRNA was upregulated in OB compared with Con fetal muscle, showing inflammatory response in OB fetal muscle.

We also analyzed the expression of *let-7g* during adipogenesis. Its expression is slightly increased during the late stage of adipogenesis, which indicates that adipogenic differentiation is not a major factor regulating *let-7g* expression. Up to now, there is no report describing factors governing *let-7g* expression, which warrants further investigation.

In conclusion, our findings demonstrate that maternal obesity affects miRNA expression in fetal skeletal muscle, in which miRNA *let-7g* could have an important role because of its effects on the suppression of stem cell proliferation and adipogenic differentiation. *Let-7g* also inhibits the expression of inflammatory cytokines. Therefore, *let-7g* may be an important mediator linking obesity, inflammation and adipose tissue development. Such mechanism should also be applicable not only to intramuscular adipogenesis, but also in other adipose depots due to the uniformity in mechanisms regulating adipogenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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