Downregulation of lncRNA *USP2-AS1* in the placentas of pregnant women with non-diabetic fetal macrosomia promotes trophoblast cell proliferation

YIWEN LU[1,2*], QIUQIN TANG[3*], SHANSHAN YANG[4], YUTING CHENG[1,2], MEI LI[1,2], DAN GUO[1,2], ZIQIANG FU[1,2], HUA JIANG[3] and WEI WU[1,2]

[1]State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University;
[2]Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health,
Nanjing Medical University, Nanjing, Jiangsu 211166; [3]Department of Gynecology and Obstetrics,
Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, Nanjing,
Jiangsu 210004; [4]School of Public Health, Nanjing Medical University, Nanjing, Jiangsu 211100, P.R. China

Abstract. Macrosomia is a common perinatal complication, with a series of adverse effects on newborns and pregnant women. However, the effects of long non-coding RNAs (lncRNAs) on non-diabetic fetal macrosomia (NDFMS) remain unclear. The aim of the present study was to investigate whether aberrant lncRNA expression in the placenta is involved in the pathogenesis of NDFMS and to elucidate its biological mechanisms. The expression profile of lncRNAs in the placentas of pregnant women with NDFMS was investigated using an Agilent Human LncRNA Microarray. Differentially expressed lncRNAs were selected for validation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Additionally, the function of lncRNA ubiquitin-specific peptidase 2 antisense RNA 1 (*USP2-AS1*) was investigated using a trophoblast cell line. The results revealed that 763 lncRNAs were upregulated and 129 lncRNAs were downregulated in the placentas of women in the NDFMS group (|FC| ≥2.0). A total of 10 lncRNAs (|FC| ≥4.0, signal value ≥50) were selected for validation using two-stage RT-qPCR, indicating that the expression trends of the 10 differentially expressed lncRNAs in the NDFMS group (n=8 vs. 8 and 48 vs. 48) were consistent with the microarray data. In addition, a significant downregulation in the levels of lncRNA *USP2-AS1* was observed in both the microarray data and second-stage verification. The overexpression of lncRNA *USP2-AS1* induced G1 phase cell cycle arrest and the number of cells entering S phase was reduced. In addition, the viability of HTR-8/SVneo cells was significantly inhibited when lncRNA *USP2-AS1* was overexpressed. Therefore, these findings demonstrated that lncRNAs were significantly differentially expressed in the placentas of pregnant women with NDFMS and that the downregulation of lncRNA *USP2-AS1* may be involved in the pathogenesis of NDFMS, by promoting trophoblast cell viability.

Introduction

Macrosomia is a common perinatal complication that has been defined as a full-term infant with a birth weight of ≥4,000 g. In recent decades, the incidence of macrosomia has been increasing, affecting 15-45% of newborns of women with gestational diabetes mellitus and 12% of newborns of women without gestational diabetes mellitus (1). Compared to normal infants, macrosomia increases the risk of childhood obesity, adult obesity, hypertension, diabetes and other age-related diseases (2,3). Diabetes is a risk factor for the development of macrosomia (1,4); however, an effective possible strategy for the prevention and treatment of non-diabetic fetal macrosomia (NDFMS) has not yet been proposed, at least to the best of our knowledge. The underlying pathogenesis of NDFMS remains unclear and further studies are required.

The placenta is composed of the amniotic membrane, leaf-shaped chorion and maternal decidua. The placenta is the interface of nutrition exchange between the mother and fetus, which is essential for the maintenance of the normal functional fetal development (5,6). Therefore, abnormal

Correspondence to: Professor Wei Wu, State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, 101 Longmian Avenue, Nanjing, Jiangsu 211166, P.R. China
E-mail: wwu@njmu.edu.cn

Professor Hua Jiang, Department of Gynecology and Obstetrics, Women's Hospital of Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, 123 Tianfei Road, Nanjing, Jiangsu 210004, P.R. China
E-mail: jianghua@njmu.edu.cn

*Contributed equally

placental development and placental dysfunction adversely affect fetal growth (7-9). The proliferation and apoptosis of placental trophoblasts play a key role in the development and maturation of the placenta during pregnancy. Previous studies have demonstrated that the excessive proliferation and reduced apoptosis of placental trophoblasts result in the occurrence of diabetic fetal macrosomia (10,11). However, there is only limited information available regarding the molecular mechanisms of placental development in NDFMS (12,13).

Long non-coding RNAs (lncRNAs), which have a length of >200 nucleotides, play a crucial role in disease development, by regulating the mechanisms of DNA methylation, histone modification, post-transcriptional regulation, RNA interference, imprinted genes and microRNA regulation (14-17). Previous studies have reported that lncRNAs may potentially participate in the pathogenesis of placental development (18-22); however, the specific biological effects of lncRNAs remain largely unknown, particularly concerning the regulatory role of ubiquitin-specific peptidase 2 antisense RNA 1 (*USP2-AS1*) in NDFMS.

In the present study, the expression profiles of lncRNAs in the placentas of pregnant women with NDFMS group and healthy controls were examined using an Agilent Human LncRNA Microarray, containing 40,916 lncRNA probes. Subsequently, 10 lncRNAs (|FC| ≥4.0, signal value ≥50) from the microarray results were selected for validation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Furthermore, the function of lncRNA *USP2-AS1* was investigated using HTR-8/SVneo, a trophoblast cell line. The present study aimed to provide new insight into the potential pathogenesis of NDFMS by analyzing the role of lncRNAs in placental development.

Materials and methods

Sample collection. The placental tissues used in the present study were provided by Changzhou Maternal and Child Health Care Hospital during the period from September, 2014 to June, 2015. A total of 96 participants were enrolled in the present study, including 48 pregnant women with NDFMS (newborn weight, ≥4,000 g) and 48 women with normal pregnancies (newborn birth weight, ≥2,500 g and <4,000 g). All participants were monotocous primigravida with full-term birth (≥37 weeks and <42 weeks). The mothers in both groups were free of diabetes or other complications (placental abruption, gestational hypertension, placenta previa, and other complications) during pregnancy. Following the removal of the placental tissue fetal membranes, three sections of placental tissues were randomly collected. Subsequently, the placental tissues were immediately stored at -80°C. The present study was conducted in compliance with the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of Nanjing Medical University (approval no. FWA00001501). Written informed consent was obtained from all pregnant women prior to their participation in the present study.

RNA extraction and RT-qPCR. Total RNA was extracted from the placental tissues and cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Placental tissues of appropriate size were placed in TRIzol® for RNA extraction. All procedures were performed on ice, to prevent RNA degradation. The concentration and purity of the RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.), and electrophoresis with 1.5% denaturing agarose gels was used to assess RNA integrity. The PrimeScript RT reagent kit (Takara Bio, Inc.) was used to reverse transcribe the RNA samples into cDNA (RR036A; Takara Bio, Inc.), and qPCR was performed using SYBR® Premix Ex Taq™ on a LightCycler 480 II real-time fluorescent quantitative PCR system (Roche Diagnostics). For reverse transcription, the thermal cycling conditions were: 37°C for 15 min, 85°C for 5 sec and 4°C to end the reaction. For qPCR, the thermal cycling conditions consisted of a step of denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The $2^{-\Delta\Delta Cq}$ method was used for the calculation of lncRNA relative expression levels. *GAPDH* was used as an internal control for lncRNA quantification (23). All the reactions were run in triplicate. The primer sequences for RT-qPCR are listed in Table I.

lncRNA microarray analysis. A total of eight samples from the NDFMS group and eight samples from the control group were prepared into two merged RNA samples, one per each group, for microarray screening. RNA labeling and microarray hybridization were performed according to the manufacturer's protocol using the Agilent Human LncRNA Microarray V4.0 (Agilent Technologies, Inc.), which contains ~77,000 probes that can detect 40,916 lncRNAs. All sequence information was selected from public curated transcriptome databases [including RefSeq (https://www.ncbi.nlm.nih.gov/refseq/), UCSC known genes (http://genome.ucsc.edu) and GENCODE (https://www.gencodegenes.org)]. The datasets are available in the NCBI GEO repository database (GSE199148).

The reverse transcribed cDNA products were used as templates, and random sequence primers were used. The products were quantitatively labeled for microarray hybridization. Each dot array was hybridized with a mixed sample, using 2 dot matrices in total. The hybridized arrays were washed, fixed and scanned using the Agilent DNA Microarray Scanner (G2565CA; Agilent Technologies, Inc.). Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies, Inc.) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies, Inc.). The differential expression of lncRNAs between the two groups was screened by fold change (FC) filtering (|FC| ≥2.0). Differentially expressed lncRNAs (|FC| ≥4.0, signal value ≥50) identified in the microarray were selected using RT-qPCR.

Cells and cell culture. HTR-8/SVneo cells were generously provided by Professor Yanling Wang (Institute of Zoology, Chinese Academy of Sciences, Beijing, China). The HTR-8/SVneo cell line (https://web.expasy.org/cellosaurus/CVCL_7162) was initially developed by Graham *et al* (24). The HTR-8/SVneo cell line was generated using freshly isolated evCTB from a first-trimester placenta, which was transfected with a plasmid containing the simian virus 40 large T antigen (SV40). A recent study demonstrated that this cell line contains two distinct populations, one of

Table I. Sequences of primers for lncRNAs used in RT-qPCR.

lncRNA ID	Gene symbol	Primers	Sequences (5'-3')
ENST00000580048.1	*ENSG00000264247.1*	Forward	TCACATCCCATGGCCAGAAG
		Reverse	GCCACAGGTAGAGCTGACAC
ENST00000453774.1	*ENSG00000228262.2*	Forward	GGCCATGGCTTCAACTAGACT
		Reverse	AGAAAAGGAAGTGAGCACGGG
ENST00000604250.1	*ENSG00000228262.4*	Forward	TGCAAGAGCATGTGGGTCAA
		Reverse	ACTCCCAGCCACTATGCATTC
NR_002791.2	*EMX2OS*	Forward	ACGATCCACTCCCTGGTACA
		Reverse	CGGAAAAGGGTTGGTGCAAG
uc011fns.2	*HLA-DQA1*	Forward	AAGCCACCCAGCTACCTAATTC
		Reverse	ACATTTCTGAGCCAAAGGCAGAG
NR_034160.1	*USP2-AS1*	Forward	GGAACTCACAACACACGGGA
		Reverse	TTGCACAAGATGACAGGGCT
HIT000332651	*HIX0040474*	Forward	AGAGTGTGAGACCTGTGGAG
		Reverse	CAACAAGTTCGTGACCGTGC
LIT3502	*LIT3502*	Forward	ATGAAGGTGGCCTGGGTAGA
		Reverse	TCCCATGTACTCTATAAGCAGCTC
TCONS_00001644	*XLOC_000983*	Forward	GAAACACGACGGGGGACTTA
		Reverse	AAGGTCCATCGGATTCCACA
ENST00000587085.1	*ENSG00000228262.2*	Forward	TCTAAGCCCTGGTGAATGCTG
		Reverse	AGTGTGTCCTGAACCCCATT
	GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
		Reverse	GGATCTCGCTCCTGGAAGATG

lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR.

epithelial origin and one of mesenchymal origin (25). The trophoblasts were cultured in RPMI-1640 medium (Shanghai Basal Media Technologies Co., Ltd.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin (Biosharp Life Sciences) in humidified air at 37˚C with 5% CO_2. Fresh medium was replaced every 2 days, depending on the cell status.

Vector construction and cell transfection. The lncRNA *USP2-AS1* overexpression lentiviral vector plasmid (Shanghai OBiO Technology Corp., Ltd.) was constructed to overexpress full-length lncRNA *USP2-AS1*. The pLenti-EF1a-EGFP-F2 A-Puro-CMV-USP2-AS1 overexpression plasmid (4 μg) was transfected into the HTR-8/SVneo cells using Lipofectamine 2000® (Invitrogen, Thermo Fisher Scientific, Inc.). The control was an empty vector. Cells were collected to determine infection efficiency following 48 h incubation at 37˚C. The infection efficiency were verified by analyzing the relative expression of lncRNA *USP2-AS1*.

Cell viability assay. The Cell Counting Kit-8 (CCK-8; Vazyme Biotech Co., Ltd.) assay was used to determine cell viability. After the transfected cells were cultured at 37˚C for 24 h, they were counted and transferred to a 96-well orifice plate to ensure that the number of cells in each sample was the same. After 12 h, the serum-free RPMI-1640 medium (PM150110; Procell Life Science & Technology Co., Ltd.) without penicillin and

streptomycin was replaced, and the cells were cultured at 37˚C for an additional 4 h. CCK-8 (10 μl) was then added to each well, and the ultraviolet absorbance value was measured at a wavelength of 450 nm using an enzyme standard instrument (Infinite M200 Pro; Tecan Group, Ltd.) after 10 and 30 min.

Cell apoptosis assay. The cells were seeded in 6-well plates ($1x10^3$ cells/well). Following a 24-h transfection, the treated cells were washed twice with cold PBS. Cell suspensions ($5x10^6$ cells in 400 μl of combination solution) were stained with FITC-labeled Annexin V (C1062S; Annexin V-FITC apoptosis detection kit; Beyotime Institute of Biotechnology) and PI (P-CA-201; Procell Life Science & Technology Co., Ltd.) for 15 min at room temperature in the dark. Binding buffer (400 μl; C1062S; Beyotime Institute of Biotechnology) was then added, and the cells were analyzed using flow cytometry (BD FACSCalibur and CellQuest Pro, v6.0; BD Biosciences). Annexin V-positive and PI-positive cells were considered apoptotic cells.

Cell cycle assay. Following a 24-h transfection, the cells were washed twice with PBS. The supernatant was discarded, and 1 ml pre-cooled 75% ethanol was added to the cell pellet. The cells were mixed and incubated at 4˚C for >12 h for fixation. The cells were then washed twice with PBS (Gibco; Thermo Fisher Scientific, Inc.) and centrifuged at 111 x g at 4˚C for 5 min. Cells were resuspended in 100 μl PBS (Gibco; Thermo Fisher Scientific, Inc.) and 50-100 μl of PI (P-CA-201; Procell

Table II. Clinical characteristics of the study population.

Characteristic	Control (n=48)	NDFMS (n=48)	P-values
Maternal age (years)	26.33±2.68	26.88±3.27	0.69
Gestational age (weeks)	39.66±1.01	39.74±0.95	0.63
Pre-pregnant BMI (kg/m^2)	20.06±2.64	21.21±3.90	0.08
Gravidity	1.25±0.60	1.34±0.73	0.51
Pregnant weight gain (kg)	16.84±4.11	19.83±4.89	0.002
Placental weight (g)	593.33±102.31	760.73±124.14	<0.001
Birth weight (g)	3322.29±309.60	4238.04±235.86	<0.001
Infant sex, n (%)			0.008
Male	21 (43.75)	34 (70.83)	
Female	27 (56.25)	14 (29.17)	

Values are the mean ± SD. NDFMS, non-diabetic fetal macrosomia; BMI, body mass index.

Life Science & Technology Co., Ltd.) were then added, followed by incubation at room temperature in the dark for 30 min. The cell cycle phase was determined using flow cytometry (BD CellQuest Pro, Version 6.0; BD Biosciences).

Statistical analysis. Data are presented as the mean ± SD. If data were in normal distribution, the Student's t-test was used to compare two groups. Otherwise, the Mann-Whitney U-test was used. The maternal age, gestational age, pre-pregnant BMI, gravidity, pregnant weight gain, placental weight, birth weight between two groups were analyzed using Student's t-test. The infant sex was analyzed using Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software Inc.). In all cases, P<0.05 was considered to indicate a statistically significant difference.

Results

Patient clinical characteristics. The maternal and infant clinical characteristics of the study cohorts are summarized in Table II. The median maternal age of NDFMS and control group were 26. The age range (minimum-maximum) of NDFMS and control group were 21-26 and 21-34, respectively. Birth weight, placental weight and weight gain during pregnancy were significantly higher in the NDFMS group than in the control group (P<0.05). A significant difference was also found between the NDFMS group and the control group regarding the sex of the infants (P<0.05). However, maternal age, gestational age, pre-pregnancy body mass index and gravidity did not differ significantly between the two groups (P>0.05).

lncRNA microarray analysis. To investigate the potential role of lncRNAs in NDFMS, an Agilent Human LncRNA Microarray (potential to detect 40,916 lncRNAs) was used to analyze the lncRNA expression profiles in the NDFMS group and control group. In total, 892 (2.18%, 892/40,916) differentially expressed lncRNAs were identified with 763 (85.54%, 763/892) significantly upregulated lncRNAs (FC≥2) and 129 (14.46%, 129/892) significantly downregulated lncRNAs

(FC ≤-2) in the placentas of women in the NDFMS group in comparison with the control placentas. A scatter plot was drawn to demonstrate the changes in lncRNA expression between the two groups (Fig. 1A), and cluster analysis revealed the clustering association of differentially expressed lncRNAs between the two groups (Fig. 1B). A total of eight samples from the NDFMS group and eight samples from the control group were prepared into two merged RNA samples. Therefore, it was not possible to calculate a P-value for the statistics. The lncRNA with the largest FC, among the upregulated lncRNAs, was HIT000075832 (FC=9.19), and the lncRNA with the lowest FC among the downregulated lncRNAs was ENST00000513672.1 (FC=-11.48). The top 10 upregulated and downregulated lncRNAs in the placentas of women in the NDFMS group are presented in Table III. Subsequently, the lncRNA microarray data were further screened, and the genomic locations of differentially expressed lncRNAs were classified and analyzed (Fig. 1C). The distribution of differentially expressed lncRNAs in gene sites may imply the potential role of lncRNAs. According to their positions in the genome, lncRNAs can be divided into five categories, as follows: bidirectional, antisense, sense, intergenic and intronic. Among the 892 differentially expressed lncRNAs between the two groups, a total of 364 out of 892 (40.81%) lncRNAs were classified into these five categories. Intergenic lncRNAs accounted for the largest proportion in the classification of differentially expressed lncRNAs. Intergenic lncRNAs have a higher evolutionary conservatism and tissue specificity, and they demonstrate active transcriptional activities. lncRNAs in other genomic locations may also play a variety of important potential biological roles, including gene regulation, cell differentiation and chromatin remodeling (26). Additionally, the chromosome distribution of upregulated and downregulated lncRNAs is demonstrated in Fig. 1D.

lncRNA RT-qPCR verification. In general, when the signal value of the microarray was >50, the detection result was reliable. A total of 12 differentially expressed lncRNAs (|FC| ≥4.0, signal value ≥50) are depicted in Table IV. The two lncRNA (RNA147334|p0438_imsncRNA843 and LIT3501) could
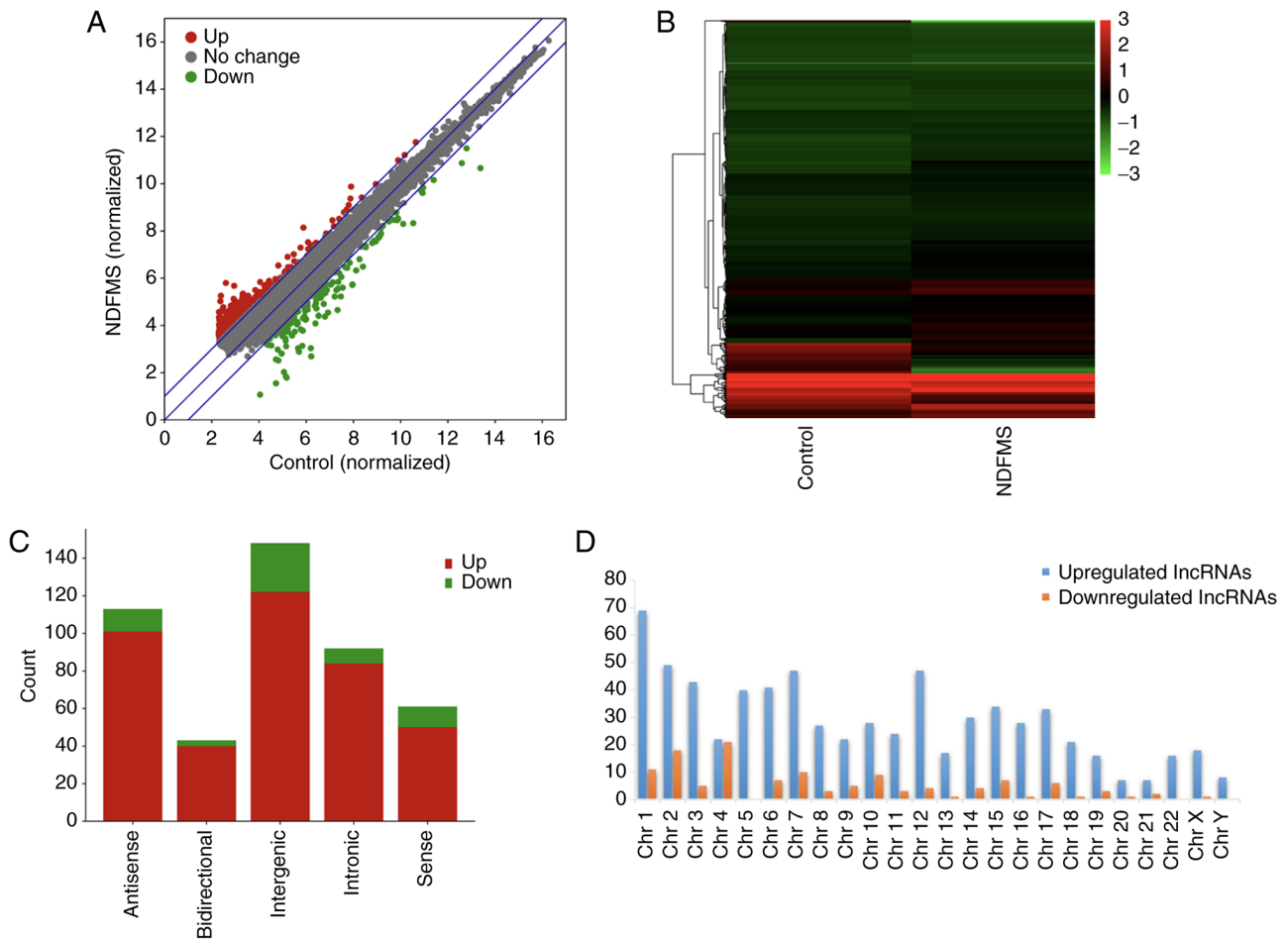
Figure 1. Differentially expressed lncRNAs in placental tissues of women in the NDFMS group compared with the control group examined using lncRNA microarray detection. (A) Scatter plot of changes in placental lncRNA expression in the NDFMS and control group. The values displayed on the x- and y-axis of the scatter plot are the normalized signal values of each sample (log2 scale). The blue line represents the fold change (given the default fold change value is 2.0). The red markers above the top blue line represent upregulated lncRNAs, and the green markers below the bottom blue line represent downregulated lncRNAs. (B) Clustering heatmap of differentially expressed lncRNAs. Colors represent lncRNA relative expression levels (red, higher; green, lower). (C) Classification of genomic locations of differentially expressed lncRNAs. (D) The chromosome distribution of upregulated and downregulated lncRNAs in the NDFMS group. lncRNA, long non-coding RNA; NDFMS, non-diabetic fetal macrosomia.

not be amplified with RT-qPCR using the designed primers. The remaining 10 differentially expressed lncRNAs were selected for verification. RT-qPCR verification was divided into two stages. Firstly, samples analyzed using an lncRNA microarray (n=8 vs. 8) were used for phase I RT-qPCR verification (Table V). Subsequently, more placental tissue samples from the NDFMS and control group (n=48 vs. 48) were used for phase II lncRNA expression verification (Fig. 2). The two-stage RT-qPCR verification results shared a consistent similar trend as compared with the microarray expression data results (the expression trend of 10 lncRNAs was consistent with the microarray data). Among these, ubiquitin-specific peptidase 2 antisense RNA 1 (*USP2-AS1*) demonstrated a significantly decreased expression in both microarray and two-stage RT-qPCR verification results. Therefore, *USP2-AS1* was selected as the candidate lncRNA for the subsequent experiments.

Effects of lncRNA USP2-AS1 on human chorionic trophoblasts. The proliferation and apoptosis of trophoblasts are fundamental for the development of the placenta and the

pathogenesis of NDFMS. In the present study, HTR-8/SVneo cells were used to elucidate the role of lncRNA *USP2-AS1* in placental development. The transfection efficiency of lncRNA *USP2-AS1* was first examined. Compared with the control group, the *USP2-AS1* expression levels in HTR-8/SVneo cells in the *USP2-AS1* overexpression group were significantly increased following transfection (Fig. 3A). The overexpression of lncRNA *USP2-AS1* arrested the cells in the G1 phase and reduced the number of cells entering the S phase (Fig. 3B). The overexpression of lncRNA *USP2-AS1* also significantly decreased cell viability (Fig. 3C). However, the overexpression of lncRNA *USP2-AS1* significantly decreased cell apoptosis compared with the control group (Fig. 3D).

Discussion

The occurrence of macrosomia is dependent on various factors. For environmental factors, including the occurrence of diabetes in pregnant women, the probability of producing macrosomia is ~26%, and the probability of producing macrosomia of pregnant women without diabetes is limited

Table III. The top 10 upregulated and downregulated lncRNAs in NDFMS, compared with the control.

lncRNA ID	Gene symbol	FC[a]	Regulation	Chromosome
HIT000075832	*HIX0114733*	9.19	Up	7
ENST00000567862.1	*ENSG00000261310.1*	7.31	Up	16
TCONS_00007242	*XLOC_003223*	6.64	Up	3
HIT000067251	*HIX0030109*	6.32	Up	2
ENST00000430463.1	*ENSG00000215498.4*	4.87	Up	22
uc011fns.2	*HLA-DQA1*	4.80	Up	6
TCONS_00006281	*XLOC_002882*	4.80	Up	3
ENST00000503357.1	*ENSG00000249290.1*	4.78	Up	3
ENST00000433249.1	*ENSG00000236556.1*	4.60	Up	10
uc021vkt.1	*abParts*	4.54	Up	2
ENST00000513672.1	*ENSG00000248322.1*	-11.48	Down	1
ENST00000607437.1	*ENSG00000228262.4*	-10.41	Down	2
ENST00000415714.1	*ENSG00000235142.2*	-9.81	Down	6
ENST00000610239.1	*ENSG00000272727.1*	-9.00	Down	4
ENST00000594455.1	*ENSG00000230768.2*	-8.94	Down	1
ENST00000564381.1	*ENSG00000261045.1*	-8.17	Down	16
ENST00000598737.1	*ENSG00000228486.3*	-7.87	Down	2
TCONS_00001693	*XLOC_001052*	-7.02	Down	1
ENST00000433965.1	*ENSG00000235142.2*	-6.87	Down	6
ENST00000580048.1	*ENSG00000264247.1*	-6.58	Down	18

[a]FC, fold change; positive numbers represent upregulation and negative numbers represent downregulation. lncRNAs, long non-coding RNAs; NDFMS, non-diabetic fetal macrosomia.

Table IV. List of differentially expressed lncRNAs in the Agilent Human LncRNA Microarray results.

lncRNA ID[a]	Gene symbol	FC[b]	Regulation
ENST00000580048.1	*ENSG00000264247.1*	-6.58	Down
RNA147334lp0438_imsncRNA843	Null	-6.41	Down
ENST00000453774.1	*ENSG00000228262.2*	-5.36	Down
ENST00000604250.1	*ENSG00000228262.4*	-5.21	Down
NR_002791.2	*EMX2OS*	-4.96	Down
uc011fns.2	*HLA-DQA1*	4.80	Up
NR_034160.1	*USP2-AS1*	-4.73	Down
LIT3501	*LIT3501*	-4.71	Down
HIT000332651	*HIX0040474*	-4.68	Down
LIT3502	*LIT3502*	-4.60	Down
TCONS_00001644	*XLOC_000983*	-4.34	Down
ENST00000587085.1	*ENSG00000228262.2*	-4.22	Down

[a]Signal value is >50. [b]FC, Fold change; positive numbers represent upregulation and negative numbers represent downregulation; their absolute values are >4. lncRNAs, long non-coding RNAs; NDFMS, non-diabetic fetal macrosomia.

to 5-8% (1). Pregnant women with excessive nutrition, obese pregnant women and overweight pregnant women have been reported to also be susceptible to macrosomia (27-29). Normal placental function exists in only a few overdue pregnancies, and the fetal weight increases with the period of pregnancy. The incidence of a large amount of amniotic fluid in pregnant women is high. Genetic factors also have a certain effect on the weight of the fetus (30). Usually, the incidence of fetal macrosomia is high in tall parents (31). Among these confounding factors, the policy of encouraging one child in family planning implemented by the Chinese government and the corresponding inclusion and exclusion criteria of the present study were effectively controlled. However, after controlling the aforementioned factors, there is still a certain risk for

Table V. Expression of lncRNAs in 8 placental tissues of women in the NDFMS and control group.

Gene symbol	Control (n=8)	NDFMS (n=8)	P-value
ENSG00000264247.1	0.104±0.074	0.031±0.008	0.172
ENSG00000228262.2	0.115±0.054	0.141±0.026	0.142
ENSG00000228262.4	0.155±0.070	0.110±0.029	0.208
EMX2OS	0.115±0.045	0.087±0.057	0.600
HLA-DQA1	0.002±0.002	0.034±0.011	0.0008
USP2-AS1	0.344±0.416	0.087±0.018	0.002
HIX0040474	0.00006±0.00002	0.00009±0.00004	0.075
LIT3502	0.0007±0.001	0.00007±0.00003	0.916
XLOC_000983	0.109±0.57	0.064±0.016	0.093
ENSG00000228262.2	0.122±0.023	0.105±0.024	0.093

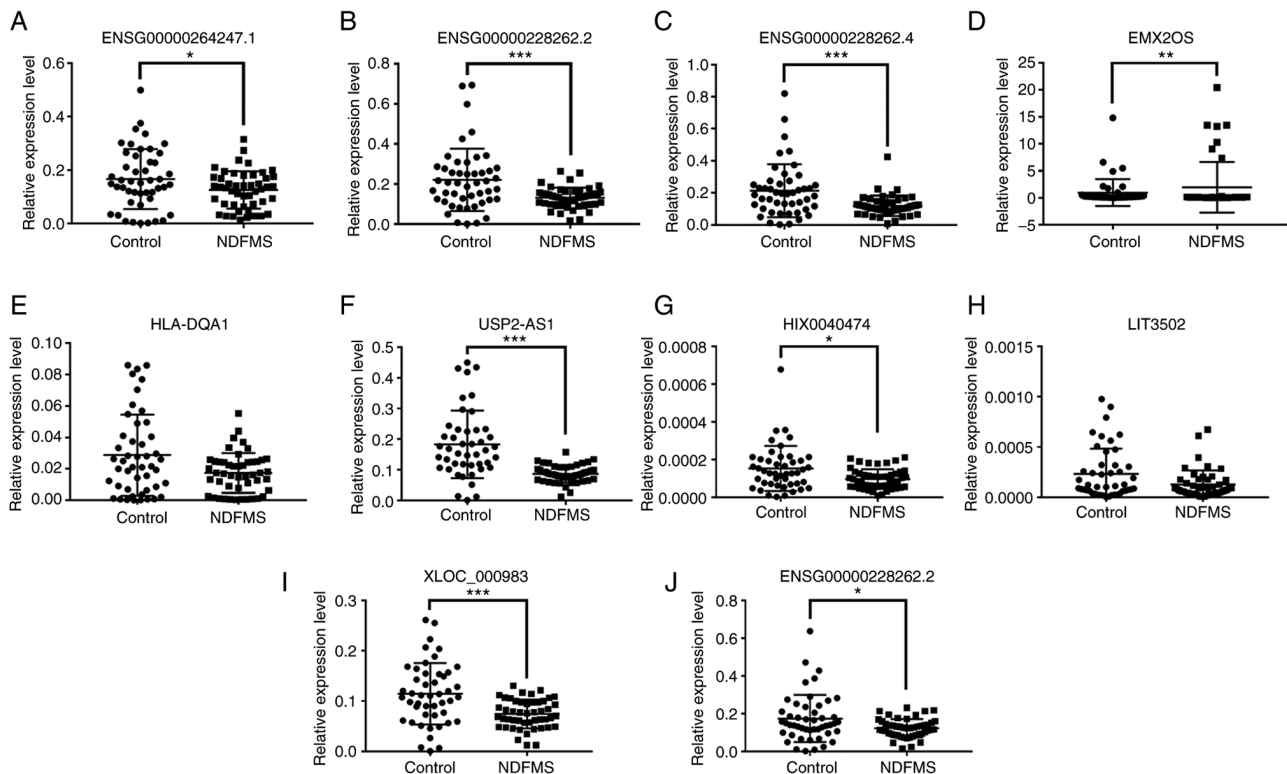Values are the mean ± SD. lncRNAs, long non-coding RNAs; NDFMS, non-diabetic fetal macrosomia.

Figure 2. Verification results of expression levels of lncRNAs in the placental tissues of women in the NDFMS group and control group. Expression levels of 10 lncRNAs in placental tissues of women in the NDFMS group and control group: (A) *ENSG00000264247.1*, (B) *ENSG00000228262.2* (lncRNA ID: ENST00000453774.1), (C) *ENSG00000228262.4*, (D) *EMX2OS*, (E) *HLA-DQA1*, (F) *USP2-AS1*, (G) *HIX0040474*, (H) *LIT3502*, (I) *XLOC_000983*, (J) *ENSG00000228262.2* (lncRNA ID: ENST00000587085.1). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, vs. the control group. lncRNA, long non-coding RNA; NDFMS, non-diabetic fetal macrosomia.

macrosomia: the weight of the placenta is a variable exerting marked influence on the occurrence of macrosomia (32). A comparison of the data suggested that the weight of the placenta was associated with fetal birth weight. The size of the placenta has been demonstrated to affect the birth weight of the fetus (33).

All the nutrients required for the growth of the fetus are supplied by the mother through the placenta. However, the purpose of the placenta is not merely for material exchange; it also has a number of other functions, as follows: i) defense function: it functions as a barrier against a number of bacteria, pathogens and drugs (34); ii) cooperative function: chorionic gonadotropin, placental lactogen, estrogen, progesterone, oxytocin enzyme, thermostable alkaline phosphatase, cytokines and growth factors are secreted (34); iii) storage function: a large number of nutrients (protein, glycogen, calcium and iron) are stored in placental cells for fetal growth requirements (35); and iv) metabolic regulation function: the
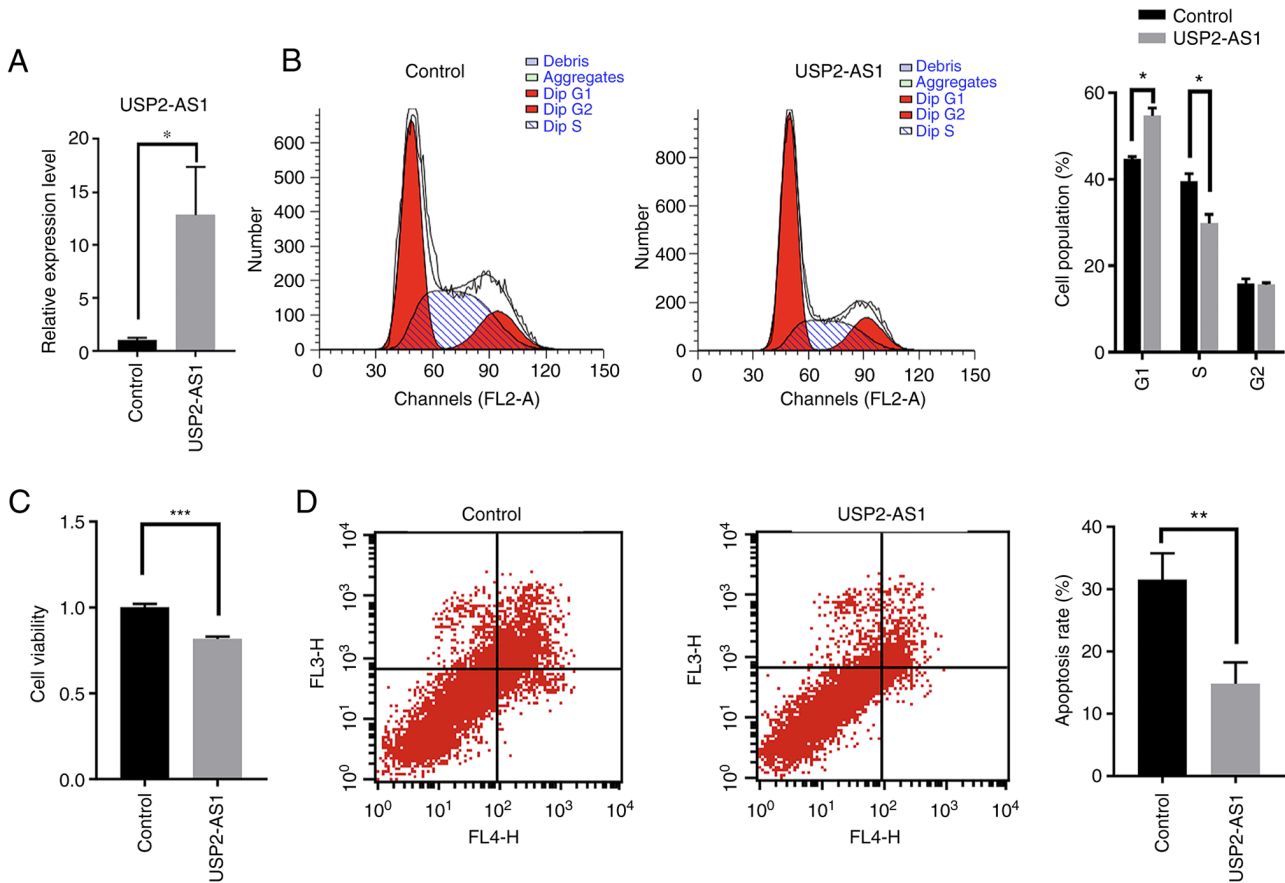
Figure 3. Effects of lncRNA *USP2-AS1* overexpression on the cell cycle, viability and apoptosis of HTR-8/SVneo cells. (A) Verification of lncRNA *USP2-AS1* transfection efficiency in HTR-8/SVneo cells. (B) Effect of lncRNA *USP2-AS1* overexpression on cell cycle of HTR-8/SVneo cells. (C) Effect of lncRNA *USP2-AS1* overexpression on the viability of HTR-8/SVneo cells. (D) Effect of lncRNA *USP2-AS1* overexpression on the apoptosis of HTR-8/SVneo cells. The control in Fig. 3A is an empty vector. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

placenta may regulate the metabolism of the body similar to that of the liver (36). Previous research results by the authors revealed that the placental weights of the macrosomia group were significantly higher than those of the normal group (12). Thus, it was considered worthy of investigation to define which factors lead to the overdevelopment of placentas and the occurrence of macrosomia.

lncRNAs are non-coding RNAs with a length of >200 nucleotides, and are related to numerous pregnancy complications, including gestational diabetes (37). In the present study, the expression profiles of lncRNAs in the placentas of pregnant women with NDFMS were investigated using an Agilent Human LncRNA Microarray V4.0. In the placentas of the women in the NDFMS group, 763 lncRNAs were upregulated and 129 lncRNAs were downregulated. Subsequently, 10 differentially expressed lncRNAs were selected to validate the preliminary results, and the two-stage RT-qPCR verifications were consistent with the microarray results. In addition, lncRNA *USP2-AS1* exhibited a significant downregulation in both the microarray data and second-stage RT-qPCR verification. Therefore, lncRNA *USP2-AS1* was the most prominent candidate lncRNA, and was used for subsequent analysis.

lncRNA *USP2-AS1*, located on the human chromosome 11q23.3, is a lncRNA with a length of 2,486 nucleotides. A previous study revealed that *USP2-AS1* promotes the growth

and metastasis of colon adenocarcinoma cells and may play a carcinogenic effect in colon adenocarcinoma (38). In addition, lncRNA *USP2-AS1* has been demonstrated to be upregulated in ovarian cancer. Mechanistic analysis have revealed that *USP2-AS1* promotes ovarian cancer progression via the miR-520d-3p/KIAA1522 axis (39). lncRNAs have also been revealed to play vital biological regulatory effects in the development of the placenta (18-22); however, the role and mechanisms of action of *USP2-AS1* in NDFMS remain unclear.

The growth patterns of placental cells bear similarities to those of tumor cells, which are often referred to as 'pseudotumors'. Therefore, the present study focused on the key molecules that regulate the biological function of placental cells. Firstly, a cell model in which the target lncRNA was overexpressed in a trophoblast cell line was generated, and the viability and apoptosis of the cells was evaluated. Following *USP2-AS1* overexpression, the cells were blocked in the G1 phase, and the cell viability and apoptotic rates were decreased. It was hypothesized that the decrease in the apoptosis of HTR-8/SVneo cells overexpressing USP2-AS1 may have been a compensation effect. These results suggested that *USP2-AS1* mainly promotes placental development by affecting the proliferative activity of placental cells, which may lead to NDFMS. However, further studies are required for the elucidation of the precise

molecular mechanisms of *USP2-AS1* in the placentas of pregnant women with macrosomia. The combination of basic and clinical research will provide a breakthrough point for the research of non-diabetic macrosomia and a theoretical basis for the prevention and treatment of clinical non-diabetic macrosomia.

The present study had several limitations. Firstly, the subjects were women who resided in the vicinity of Jiangsu Province, resulting in regional limitations. Secondly, the present study did not predict the lncRNA target genes or explore their functions in NDFMS. Finally, the function of aberrantly expressed lncRNA *USP-AS1* was not verified further with the use of an animal model. In a follow-up project by the authors, the biological functions of lncRNA *USP-AS1* in macrosomia will be further explored *in vivo* and also by applying molecular mechanism research.

In conclusion, the present study identified the expression profile of lncRNAs in the placentas of women with NDFMS and revealed for the first time, to the best of our knowledge, that lncRNA *USP2-AS1* participates in the pathogenesis of NDFMS by regulating cell function. The present study provides new insight into exploring the post-transcriptional regulatory mechanisms of NDFMS, suggesting potential biological targets for future clinical treatment of NDFMS.

Acknowledgements

Funding

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI GEO repository database (GSE199148), [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199148].

Authors' contributions

WW and HJ were involved in the conceptualization and design of the study. DG and QT were involved in the acquisition of data. YL and YC were involved in data analysis. YL, ML, YC, WW and HJ were involved in the interpretation of the data. YL, SY, DG and ZF performed the experiments. WW, QT and HJ contributed materials/analysis tools. YL and YC were involved in the preparation of the original draft. WW and HJ were involved in the reviewing and editing of the manuscript. YL, ZF and QT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was supervised and approved by the Institutional Review Board of Nanjing Medical University (FWA00001501). Written informed consent was obtained from all pregnant women prior to their participation in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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