WNT5A, β-catenin and SUFU expression patterns, and the significance of microRNA deregulation in placentas with intrauterine growth restriction

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Abstract. Placental insufficiency is a common cause of intrauterine growth restriction (IUGR). It affects ~10% of pregnancies and increases fetal and neonatal morbidity and mortality. Although Wnt and Hh pathways are crucial for embryonic development and placentation, their role in the pathology of IUGR is still not sufficiently explored. The present study analyzed the expression of positive regulators of the Wnt pathway, WNT5A and β-catenin, and the expression of the Hh pathway negative regulator suppressor of fused (SUFU). Immunohistochemical and reverse transcription-quantitative PCR (RT-qPCR) assays were performed on 34 IUGR and 18 placental tissue samples from physiologic singleton-term pregnancies. Epigenetic mechanisms of *SUFU* gene regulation were also investigated by methylation-specific PCR analysis of its promoter and RT-qPCR analysis of miR-214-3p and miR-378a-5p expression. WNT5A protein expression was higher in endothelial cells of placental villi from IUGR compared with control tissues. That was also the case for β-catenin protein expression in trophoblasts and endothelial cells and SUFU protein expression in trophoblasts from IUGR placentas. The *SUFU* gene promoter remained unmethylated in all tissue samples, while miR-214-3p and miR-378a-5p were downregulated in IUGR. The present results suggested altered Wnt and Hh signaling in IUGR. DNA methylation did not appear to be a mechanism of *SUFU* regulation in the pathogenesis of IUGR, but its expression could be regulated by miRNA targeting.

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Introduction

Intrauterine growth restriction (IUGR) (or fetal growth restriction-FGR) implies fetal incapability to achieve its genetically determined growth potential. The American College of Obstetricians and Gynecologists (ACOG) and Society for Maternal-Fetal Medicine (SMFM) define IUGR based on a sonographic finding of estimated fetal weight (EFW) or abdominal circumference (AC) below the 10th centile for gestation age (1) and is diagnosed in about 10% of pregnancies (2). IUGR is a heterogeneous entity since the growth restriction could be caused by fetal, maternal, or placental pathology with possible common overlapping. Abnormal placentation and placental vascular disease lead to chronic uteroplacental hypoxia and IUGR (2). Our interest is IUGR due to uteroplacental insufficiency and dysfunction since the placental origin is the most common cause of late-onset IUGR (3). Adequate diagnosis and appropriate pregnancy monitoring and delivery timing of growth-restricted fetuses are essential because of the increased risk of fetal and neonatal morbidity and mortality. That said, perinatal mortality with IUGR is 6- to 10- fold increased (4). Also, numerous studies found that infants born with IUGR had an increased risk for neurodevelopmental abnormalities and lower cognitive performance (5-7).

The specific pattern of balanced differentiation of various trophoblastic cell types is crucial for normal placentation (8,9), and numerous studies reported a decisive role of appropriate Wingless (Wnt) signaling in normal placental development (10-12).

Wnt canonical pathway is an evolutionary conserved cell-signaling system essential for the regulation of cell differentiation, migration, invasion, and apoptosis (13), thus contributing to multiple organ system development (14-17). Consequently, abnormal Wnt signaling has been reported in various diseases and abnormalities such as pregnancy-related diseases-preeclampsia or IUGR (18), birth defects, different malignancies (19-22) and the pathophysiology of various neuropsychiatric disorders (23). β-catenin is a cytoplasmic protein with an essential role in Wnt canonical signaling pathway. Wnt ligands activate Wnt signaling. WNT5A is one of these ligands that plays a critical role in convergent extension (CE), epithelial-mesenchymal transition (EMT), and planar cell polarity (PCP) regulation during the embryonic period (24). Once Wnt ligand binds to the transmembrane Frizzled (Fz) receptor and LRP5 and LRP6 co-receptors (lipoprotein receptor-related protein 5 and 6), cytoplasmic Dishevelled (DVL) protein activates and degrades the APC (adenomatous polyposis coli)/Axin/CK1a (casein kinase 1a)/GSK3ß (glycogen synthase kinase 3b) destruction complex causing the stabilization and accumulation of β-catenin in the cytoplasm, in an active, non-phosphorylated form. Accumulated β-catenin then migrates to the nucleus. It associates to TCF/LEF (T-cell factor/lymphoid-enhanced binding factor) family of transcription factors that subsequently activate the target genes' transcription. In the absence of Wnt ligands, when Wnt canonical pathway is not activated, the APC/Axin/CK1a/GSK3ß destruction complex binds to β-catenin, causing the phosphorylation (i.e., inactivation) of β-catenin and its proteasomal degradation.

The Hh signaling pathway also has a decisive role during embryonic development due to its function in trophoblast EMT (25), cell growth and patterning (26), angiogenesis and vasculogenesis, as well as various tissue and organ systems development (27,28).

Signaling begins when one of three Hedgehog homologs, Indian (IHH), Desert (DHH) or Sonic (SHH), binds to the membrane Patched receptor (PTCH), and then seven transmembrane spanning protein Smoothened (SMO) initiates a signaling cascade and activates GLI (glioma-associated oncogene) transcription factors and target genes. Suppressor of fused (SUFU) exerts its function as an essential negative regulator of the Hh signaling pathway by binding to GLI, thus causing phosphorylation and inactivation of GLI and further signaling. Min *et al* (29) demonstrated that SUFU could be a negative regulator of the Wnt and Hh signaling pathways, showing a potentially strong linkage between these pathways. This could be very significant since SUFU might be an essential link between the two pathways and their crosstalk.

DNA methylation is one of the most important and well-studied DNA epigenetic modifications that have a decisive role in placental development. Altered placental methylation patterns have been associated with the disruption of placental morphology and linked to pregnancy pathology (30). It has been shown that treating pregnant rats with DNA methyltransferase inhibitor (5azaC) at different stages of pregnancy results in altered trophoblast proliferative activity, disruption of placental structure, and reduced placental weight (31). The altered DNA methylation patterns have also been reported in placentas from pregnancies with underlying placental pathologies such as preeclampsia or IUGR (32). Also, another study found growth-related *WNT2* gene promoter methylation to be associated with low birth weight (33). Based on the knowledge of the importance of DNA methylation in regulating gene expression in the placenta and the fact that it is the best known and studied epigenetic modification, we wanted to elucidate if this mechanism also regulates the *SUFU* gene expression.

MicroRNAs (miRNAs), a class of small (19-25 nucleotides) evolutionary conserved endogenous single-stranded non-coding RNAs, also play an important role in epigenetic regulation of gene expression. They prevent protein production in a sequence-specific manner by participating in either cleavage and degradation or translation inhibition of target mRNAs (34-36). Up to now, aberrant expression of regulatory miRNAs has been associated with the initiation and progression of various pathological processes (37-39). Recent evidence also highlights their regulatory roles in human fetoplacental growth (40-43). Some of them, such as miR-214 and miR-378, have also regulated the Hh signaling pathway (44-46).

Based on these assumptions and since the role of the Wnt and Hh signaling pathways is still insufficiently explored in the placenta and IUGR, we wanted to investigate the expression of WNT5A, β-catenin, and SUFU in placentas from IUGR and gather more information regarding the role of these pathways in placental pathology related to IUGR. Also, our study aimed to explore if DNA methylation and miR-214-3p and miR-378-5p targeting could be epigenetic mechanisms involved in regulating *SUFU* gene expression in placentas.

To our knowledge, up to date, *SUFU* gene methylation status and the status of miR-214-3p and miR-378-5p in the term IUGR placentas have not been reported.

Materials and methods

Tissue samples. The samples used in the study were a part of a collection of placental tissue samples belonging to the University of Zagreb School of Medicine. They were collected in collaboration with the University Hospital 'Merkur' Zagreb. Both institutions are parts of the Scientific Center of Excellence for Reproductive and Regenerative Medicine (CERRM).

In the examination of placentation, a control group consisted of eighteen formalin-fixed paraffin-embedded (FFPE) placentas, obtained from physiological singleton complication-free pregnancies, delivered at term (between 38 and 42 weeks of gestation) of a newborn with normal body weight (between 10th and 90th percentile for gestational age, newborn sex, and mother's parity). The experimental group consisted of 34 term placentas from pathological pregnancies with IUGR based on serial ultrasound measurements of fetal biparietal diameter (BPD), head and abdominal circumference (HC and AC, respectively), and femur length (FL), with the assessment of the bodyweight below 10th percentile for the duration of pregnancy, fetal sex, mother's parity, and confirmed at birth by measuring newborn body weight. The only pregnancy pathology that was included in the study was IUGR.

Table I. Primer sequences for MSP and RT-qPCR.

Targeted gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Product length, bp
MSP primers				
SUFU methylated	NC_000010.11	GTTTCGGGGAGTTTTATTTATC	GAAAACCGAAAAAACAATCG	180
SUFU unmethylated	NC_000010.11	GTTTTGGGGAGTTTTATTTATTGA	AAACAAAAACCAAAAAAACAATCA	183
RT-qPCR primers				
WNT5A	NM_003392.7	GCACCAGAGCAGACAACC	TCACAACACGGAGGAATCAG	89
SUFU	NM_016169.4	GCTGCTGACAGAGGACCCACA	GTGCAGACACCAACGATCTGGA	84
CTNNB1	NM_001904.4	TGCGTACTGTCCTTCGGGCT	ATGGCAGGCTCAGTGATGTCT	52
GAPDH	NM_002046.7	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87

CTNNB1, catenin β1; MSP, methylation-specific PCR; RT-qPCR, reverse transcription-quantitative PCR; *SUFU*, suppressor of fused.

Exclusion criteria for pathological pregnancies and controls were as follows: Multiple pregnancies, tobacco and drug use, intrauterine viral infections (TORCH and Parvovirus B19), chorioamnionitis, hypertension, preeclampsia, fetal malformations, and genetic abnormalities as well as autoimmune diseases or eating disorders of the mother. The board-certified pathologist (A.S.) examined each placenta and rendered the diagnosis. A disc-shaped tissue sample comprising an entire thickness of the placenta from the fetal to maternal side, about 5 cm from the umbilical cord, was taken from each placenta.

Immunohistochemistry (IHC). IHC was performed on 34 IUGR placentas and 18 control placentas. FFPE tissue sections (4 μm thickness) were placed on silanized glass slides (DakoCytomation) and analyzed by immunohistochemistry as previously described (47). Antigen retrieval was performed by heating the sections in Dako Target Retrieval Solution (Dako Corporation) in a steamer for 20 min. Sections were incubated with primary antibody overnight at 4˚C. Dako REAL Envision detection system (cat. no. K0679; Dako; Agilent Technologies, Inc.) was utilized for visualization as suggested by the manufacturer, and the sections were counterstained with hematoxylin at room temperature (RT) for 1 min. The following primary antibodies were used: anti-WNT5A (mouse monoclonal anti-human; Cat. No. ab86720, Abcam, dilution 1:1,000), anti-β-catenin (rabbit polyclonal anti-human; Cat. No. ab16051, Abcam, dilution 1:500) and anti-SUFU (rabbit polyclonal anti-human; Cat. No. 26759-1-AP, Proteintech, dilution 1:500). Tonsils (WNT5A), colon tissue (β-catenin), and kidney (SUFU) were used as positive controls. Negative control was treated the same way with the omission of incubation with primary antibodies.

The expression pattern of WNT5A, β-catenin, and SUFU in placentas was interpreted independently by two pathologists (S.V., A.S.) as follows: 0 if no staining was observed; 1 if <10% cells were stained; 2 if 10-50% cells were stained; and 3 if >50% cells were stained (48). Protein expression was observed in trophoblasts, stromal cells, and endothelial cells. In discordant interpretations, the pathologists reviewed cases together to obtain an agreement.

Methylation-specific PCR (MSP). DNA was isolated from two 10 μm sections of FFPE control placental tissue (n=14) and IUGR placental tissue (n=10) as previously described (49) and treated with bisulfite using the MethylEdge Bisulfite Conversion System (Promega, Madison, Wisconsin, USA) according to the instructions from the manufacturer. Bisulfite-treated DNA was used for methylation-specific PCR reaction (MSP). Primers for *SUFU* promoter region (Table I) were synthesized according to Paluszczak *et al* (50). All PCRs were performed using TaKaRa EpiTaq HS (for bisulfite-treated DNA) (TaKaRa Bio): 1XEpiTaq PCR Buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 10 pmol of each primer (Sigma-Aldrich), 25 ng of DNA, and 0.75 Units of TaKaRa EpiTaq HS DNA Polymerase in a 25 μl final reaction volume. PCR cycling conditions were as follows: initial denaturation at 95˚C for 30 sec, followed by 40 cycles consisting of three steps: 95˚C for 30 sec, the respective annealing temperature for 30 sec, 72˚C for 30 sec, followed by a final extension at 72˚C for 7 min. For the amplification of the methylated *SUFU* promoter region, the annealing temperature was 58˚C, while for the unmethylated *SUFU* promoter region was 55˚C. PCR products were separated on 2% agarose gel stained with GelStar nucleic acid stain (Lonza Rockland, Inc.) and visualized on a UV transilluminator. Methylated Human Control (Promega) was used as a positive control for methylated reaction, and unmethylated human EpiTect Control DNA (Qiagen) was used as a positive control for unmethylated reaction, and nuclease-free water was used as a negative control.

RNA extraction, reverse transcription, and RT-qPCR. For mRNA analysis, total RNA was isolated from five consecutive 5-μm thick sections of FFPE control placental tissue (n=14) and IUGR placental tissue (n=14). Shortly, all tissue sections were deparaffinized by incubation in 1.0 ml xylene (Invitrogen; Thermo Fisher Scientific, Inc.) for 3 min at 50˚C, followed by centrifugation (three times for 5 min at RT at 12,000 x g each). The supernatant was then discarded, and the obtained tissue pellet was washed three times with 1.0 ml absolute ethanol and subsequently incubated overnight at 55˚C in 350 μl of protease K digestion buffer (20 mM Tris-HCl pH 8.0; 1 mM $CaCl_2$; 0.5% sodium dodecyl sulfate and 500 μg/ml protease K; all reagents were obtained from Sigma-Aldrich; Merck KGaA). Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's recommended procedure. The purity and quantity of total RNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, one μg of total RNA from each sample was reverse transcribed using the high-capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The mRNA expression levels of targeted genes (*CTNNB1, WNT5A, SUFU*) were determined using a CFX-96 real-time qPCR detection system (Bio-Rad Laboratories, Inc.). All qPCR reactions were performed in triplicate using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus PCR master mix; Takara Biotechnology Co., Ltd.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The CFX96 manager software version 3.1 (Bio-Rad Laboratories, Inc.) was used to generate the cycle threshold values, and the data were analyzed using the $2^{-\Delta\Delta Cq}$ method (51). The relative mRNA expression levels of *CTNNB1*, *WNT5A*, and *SUFU* were normalized against the mRNA expression levels of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an endogenous control. The specificity of qPCR amplification was confirmed using melting curve analysis. The primer sequences used for mRNA analysis are presented in Table I.

For microRNA analysis, total RNA was isolated from five consecutive 5-μm thick sections of FFPE control placental tissue (n=14) and IUGR placental tissue (n=14) as described above and purified with the 'NucleoSpin miRNA Plasma' kit (Macherey-Nagel, Germany). Reverse transcription was performed using the 'TaqMan MicroRNA Reverse Transcription Kit' (Applied Biosystems) and specific loop primers for Hsa-miR-214-3p (assay ID 002306, Applied Biosystems, USA) and Has-miR-378a-5p (assay ID 000567, Applied Biosystems, USA) following the manufacturer's procedure. The expression levels of targeted miRNAs were determined using the 'TaqMan microRNA Assay' and 'TaqMan™ Universal Master Mix II, no UNG' (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Small nuclear RNA U6 (U6 snRNA assay ID 001973, Applied Biosystems, USA) was used as an endogenous control. The following thermocycling conditions (CFX-96 real-time qPCR detection system; Bio-Rad Laboratories, Inc.) were used: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. All qPCR reactions were performed in triplicate, and data were analyzed using the $2^{-\Delta\Delta Cq}$ method as described above (51).

Statistical analysis. The normality of data distribution was tested using the Shapiro-Wilk test. Continuous variables are shown as mean ± standard deviation (SD) or median with interquartile range (Q1, Q3). Categorical variables were presented as frequencies (n) and percentages (%). Based on the normality of data distribution, the group differences for quantitative variables were analyzed using the unpaired Student's t-test (normally distributed data) or the Mann-Whitney U test (non-normally distributed data), correspondingly. Categorical variables were compared using the Chi-square or Fisher's exact test, as appropriate. After Chi-square or Fisher's exact test, and to correct for multiple comparisons, where necessary, Bonferroni correction was used and corrective t-values stated, as appropriate. The two-tailed $P<0.05$ was considered statistically significant. All analyses were performed using the SPSS Statistics software version 27.0 (IBM Corp.).

Results

Clinical data - physiological and IUGR pregnancies. The following clinical variables were analyzed: maternal age, maternal body weight and height, maternal weight gain and body mass index (BMI) before pregnancy and at time of delivery, fetal body weight and height, placental weight and fetal/ placental weight ratio. Moreover, the gender of the newborns and mode of delivery was also analyzed. As expected, a statistically smaller fetal weight, height and placental weight were found in newborns from IUGR pregnancies (P<0.001). IUGR was more frequent in female newborns (P=0.033), and pregnancies complicated with IUGR were significantly more often completed with cesarean section delivery than physiological pregnancies (P=0.001), which was also expected. The mean maternal age in the IUGR group was 31.6 and was significantly higher compared with healthy controls with a mean age of 28.5 years (P=0.035). Median gestational age at delivery was significantly lower in the IUGR group compared with the control group, 38+3/7 (38+2/7, 39+4/7) and 39+3/7 weeks (39+3/7, 40+5/7), respectively (P=0.001) (Table II).

Expression levels of WNT5A, SUFU, and CTNNB1 mRNA. The RT-qPCR analysis showed that all targeted genes (*WNT5A, SUFU,* and *CTNNB1*) were transcriptionally active in both IUGR and control placenta tissue samples. The relative mRNA expression levels of *WNT5A, SUFU,* and *CTNNB1* genes were higher in the IUGR than in the control tissue (Fig. 1). However, none of the observed differences in mRNA expression levels was statistically significant. Regarding the mRNA expression levels of targeted genes in IUGR tissue samples analyzed as a separate group, the *CTNNB1* gene showed the highest transcriptional activity, followed by the *SUFU* and *WNT5A* genes. In control tissue samples, the *CTNNB1* gene also showed the highest expression levels among the targeted genes analyzed. In contrast, the expression levels of the *SUFU* gene were lower than those observed for the *WNT5A* gene (data not shown). No statistically significant gene expression was detected in the last analysis as well.

WNT5A, β-catenin, and SUFU protein expression in IUGR placentas. In IUGR placentas, WNT5A and β-catenin were expressed in >10% of endothelial cells in 70.5 and 79.4% samples, respectively. In contrast, these proteins were expressed in <10% of endothelial cells in physiological placentas in 94.4 and 55.6% of samples. In IUGR placentas, β-catenin was expressed in >10% of trophoblast cells in 94.1% of the samples, compared with 66.7% of the samples from placentas with uncomplicated pregnancies. On the other hand, in both

Table II. Clinical parameters of mothers and newborns from IUGR and physiological pregnancies.

Variable	Control (n=18)	IUGR (n=34)	P-value
Maternal age, years	28.5±3.6	31.6±5.5	0.035[a]
Gestational age at delivery, weeks+days	39+3/7 (39+2/7, 40+5/7)	38+3/7 (38+2/7, 39+4/7)	0.001[a]
Pre-pregnancy BMI, kg/m^2	21.9 (19.3, 22.9)	20.2 (19.0, 21.7)	0.532
BMI at delivery, kg/m^2	26.6±2.7	26.0±3.0	0.532
Maternal body height, cm	168.4±5.5	166.2±7.0	0.253
Maternal body weight at delivery, kg	75.4±8.0	72.0±9.6	0.210
Total weight gain during pregnancy, kg	16.0 (13.0, 18.5)	14.0 (11.0, 20.0)	0.159
Fetal birth weight, g	3,516.7±336.9	2435.9±195.9	<0.001[a]
Fetal height, cm	51.0 (50.0, 51.2)	46.0 (45.7, 48.0)	<0.001[a]
Placental weight, g	554.6±76.6	369.7±80.2	<0.001[a]
Fetal/placental weight ratio	6.4±0.9	6.9±1.5	0.253
Sex, n (%)			
Male	13 (72.2)	14 (41.2)	0.033[a]
Female	5 (27.8)	20 (58.8)	
Mode of delivery, n (%)			
Cesarean section	0 (0.0)	14 (41.2)	0.001[a]
Vaginal delivery	18 (100.0)	20 (58.8)	

[a]Statistically significant difference. Values are presented as the mean ± standard deviation, median (Q1, Q3) or n (%). IUGR, intrauterine growth restriction.

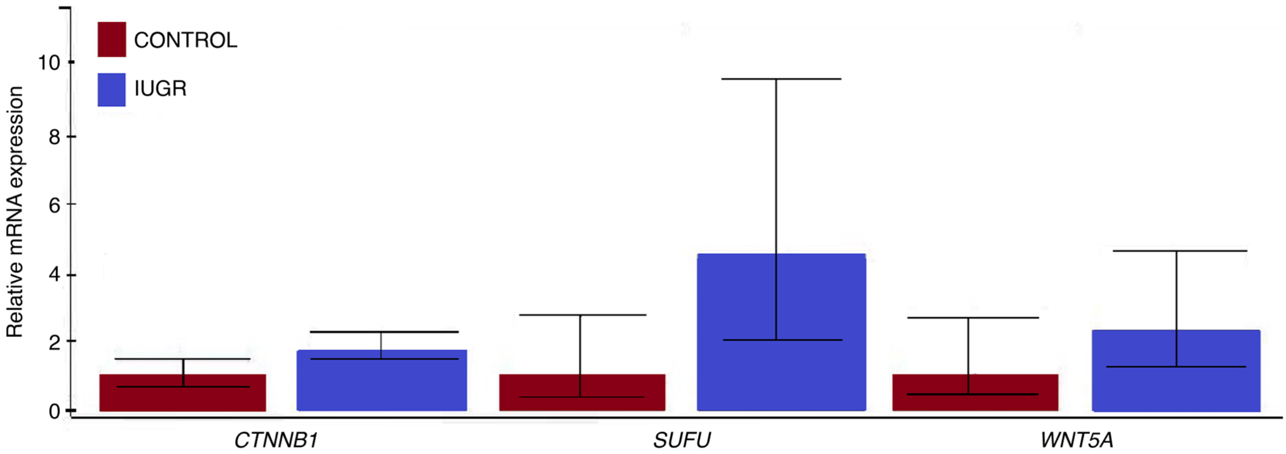

Figure 1. *WNT5A*, *SUFU* and *CTNNB1* mRNA expression in IUGR vs. control placental tissue normalized to *GAPDH* and relative to control tissue. *CTNNB1*, catenin β1; IUGR, intrauterine growth restriction; *SUFU*, suppressor of fused.

placental groups, WNT5A and β-catenin expression in >10% of stromal cells were observed in 100% of the samples (Table III).

The expression of WNT5A, the positive regulator of the Wnt signaling pathway, was significantly higher in endothelial cells of placental villi (P<0.001) in placentas with IUGR compared with term placentas from physiologic pregnancies. β-catenin also exhibited a significantly higher expression in trophoblasts (P=0.026) and endothelial cells of placental villi (P<0.001) in IUGR placentas compared with the physiologic placentas (Figs. 2 and 3).

SUFU protein expression was observed in >10% of trophoblast cells in 100% samples of IUGR placentas and 77.6% samples of physiological placentas. The protein expression in >10% of stromal cells of IUGR placentas was observed in 100% of samples in the IUGR group and 83.3% in the control group. SUFU protein expression was significantly higher in trophoblasts in IUGR placentas (P=0.029) than in physiologic ones (Table III; Figs. 2 and 3).

Expression levels of miR-214-3p and miR-378a-5p. The RT-qPCR analysis results showed that targeted miRNAs (miR-214-3p and miR-378a-5p) were expressed in all tissue samples. Furthermore, both miR-214-3p (P=0.040) and miR-378a-5p (P<0.001) showed a significantly lower expression in the IUGR compared to control placental tissue (Fig. 4). Also, in control tissue samples analyzed as a separate group, the miR-378a-5p showed higher

Table III. WNT5A, β-catenin and SUFU protein expression in IUGR and normal (control) placentas.

	Throphoblasts			Stromal cells			Endothelial cells		
Protein	Normal placentas, n (%) (n=18)	IUGR placentas, n (%) (n=34)	P-value	Normal placentas, n (%) (n=18)	IUGR placentas, n (%) (n=34)	P-value	Normal placentas, n (%) (n=18)	IUGR placentas, n (%) (n=34)	P-value
WNT5A, %			0.718			0.873			<0.001[a]
>50	16 (88.9)	29 (85.3)		11 (61.1)	20 (58.8)		0 (0.0)	6 (17.6)	
10-50	2 (11.1)	5 (14.7)		7 (38.9)	14 (41.2)		1 (5.6)	18 (52.9)	
<10	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		9 (50.0)	3 (8.8)	
0	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)		8 (44.4)	7 (20.7)	
β-catenin, %			0.059			0.602			0.002[a]
>50	3 (16.7)	11 (32.4)		16 (88.8)	32 (94.1)		0 (0.0)	16 (47.1)	
10-50	9 (50.0)	21 (61.7)		2 (11.2)	2 (5.9)		8 (44.4)	11 (32.3)	
<10	5 (27.8)	2 (5.9)		0 (0.0)	0 (0.0)		2 (11.2)	3 (8.8)	
0	1 (5.5)	0 (0.0)		0 (0.0)	0 (0.0)		8 (44.4)	4 (11.8)	
SUFU, %			0.030[b]			0.105			0.440
>50	5 (27.8)	17 (50.0)		8 (44.4)	20 (58.8)		0 (0.0)	2 (5.9)	
10-50	9 (50.0)	17 (50.0)		7 (38.9)	14 (41.2)		10 (55.5)	18 (52.9)	
<10	2 (11.1)	0 (0.0)		2 (11.2)	0 (0.0)		5 (27.8)	12 (35.3)	
0	2 (11.1)	0 (0.0)		1 (5.5)	0 (0.0)		3 (16.7)	2 (5.9)	

[a]Statistically significant with Bonferroni correction of 0.006. [b]Statistically not significant with Bonferroni correction of 0.006. IUGR, intrauterine growth restriction; SUFU, suppressor of fused.

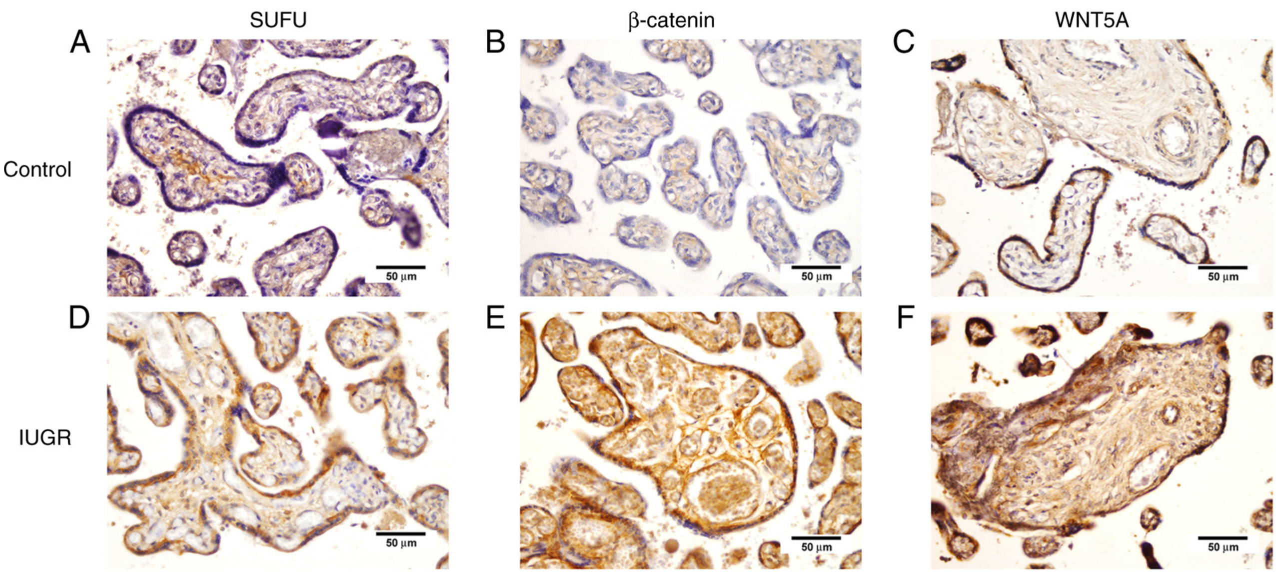

Figure 2. SUFU protein expression analysis in placental villi from (A) normal (control) and (D) IUGR placenta revealed higher expression in trophoblasts of IUGR placenta. β-catenin protein expression analysis in placental villi from (B) control and (E) IUGR placentas revealed higher β-catenin expression in trophoblasts and endothelial cells in IUGR placentas. WNT5A protein expression analysis in placental villi from (C) control and (F) IUGR placentas indicated higher expression in endothelial cells in IUGR placentas. Scale bar, 50 μm. IUGR, intrauterine growth restriction; SUFU, suppressor of fused.

transcriptional activity. However, the observed difference was insignificant (data not shown). Contrary to the IUGR tissue group, both targeted miRNAs showed almost the same expression levels that were lower than their expression values in the control tissue group (data not shown).

DNA promoter methylation status of SUFU gene in IUGR placentas. DNA promoter methylation of the *SUFU* gene was analyzed by the methylation-specific PCR assay in 10 IUGR and 14 physiologic placentas. *SUFU* gene promoter was unmethylated in all physiologic placentas, while in the IUGR

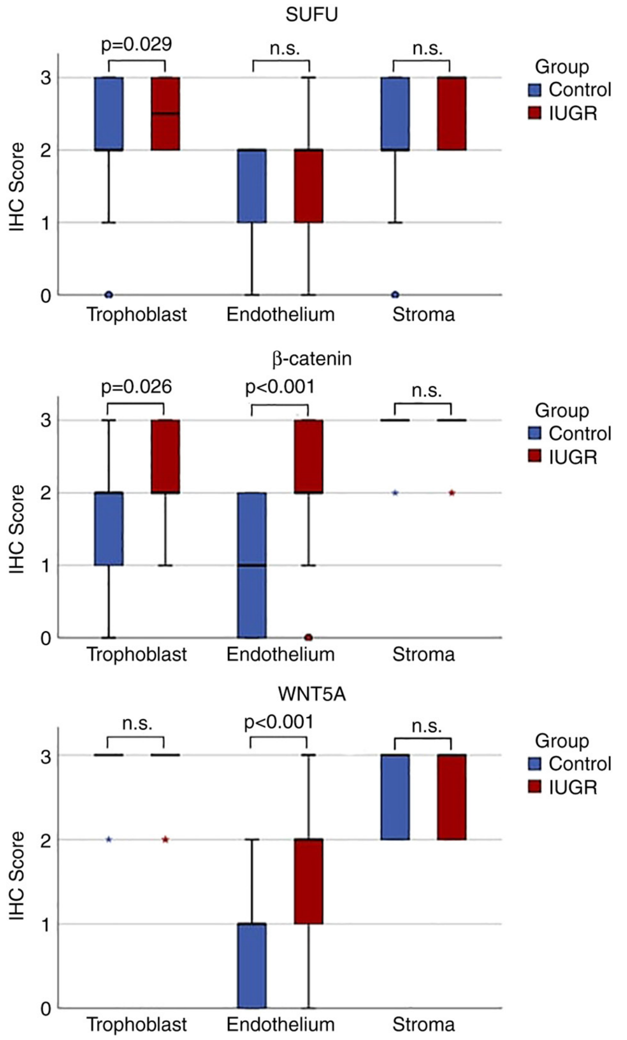

Figure 3. Boxplots of WNT5A, β-catenin and SUFU protein expression in IUGR and normal (control) placentas in trophoblasts, endothelium and stroma. 0, no staining observed; 1, <10% cells were stained; 2, 10-50% cells were stained; and 3, >50% cells were stained. Asterisks denote extreme outliers, while small circles denote outliers. IHC, immunohistochemistry; IUGR, intrauterine growth restriction; n.s., not statistically significant; SUFU, suppressor of fused.

group, one placenta showed weak methylation of the *SUFU* gene promoter. In other IUGR placentas, the *SUFU* gene promoter was unmethylated (Fig. 5).

Discussion

Since WNT5A and β-catenin are positive regulators of the Wnt pathway, their significantly lower expression in placentas from IUGR compared to the placentas from physiologic, uncomplicated pregnancies could have been expected. Our results showed significantly higher protein expression of WNT5A and β-catenin in IUGR placentas compared to placentas from uncomplicated pregnancies. These results align with our previous study that revealed significantly higher expression of all three Dishevelled proteins (DVL1-3) in IUGR placentas, indicating their potential effects on placental hypoxia and angiogenesis in IUGR (52).

Uteroplacental insufficiency causes placental hypoperfusion and chronic hypoxia and is one of the leading causes of IUGR. Numerous studies report the association between oxidative stress and IUGR (53-55) and the contribution of oxidative stress to the IUGR metabolic sequelae (56). Zhang *et al* (57) reported that oxidative stress upregulates Wnt signaling in a concentration-dependent manner and induces angiogenic activity, thus contributing to neovascularization. Funato *et al* (58) also found that reactive oxygen species (ROS) promoted β-catenin stabilization. Vikram *et al* (59) reported that suppressing oxidative stress by antioxidants prevents β-catenin dephosphorylation in endothelial cells. At the same time, the β-catenin expression also increased ROS in endothelial cells and whole blood vessels, suggesting that ROS could be both upstream mediators and downstream effectors of Wnt signaling (59). Moreover, it has been reported that active WNT3A and β-catenin could upregulate tumor necrosis factor-alpha (TNF-α) in endothelial cells, thus promoting endothelial dysfunction (60). Increased expression of β-catenin also diminished vascular nitrogen oxide (NO) bioavailability and impaired endothelium-dependent vasorelaxation (59). Moreover, endothelial cells from patients suffering from type 2 diabetes mellitus had a 1.3-fold higher WNT5A expression. Furthermore, inhibition of WNT5A restored endothelial NO synthase activity, improved nitric oxide production and abrogated endothelial dysfunction (61).

This data suggests that placental dysfunction was triggered by hypoxia and oxidative stress. This may be partially explained by the higher β-catenin expression in endothelial and trophoblast cells and higher WNT5A expression in endothelial cells in IUGR placentas obtained in our research.

Various studies emphasized the importance of Wnt signaling in regulating apoptosis and suggested its antiapoptotic activity (62,63). That said, Wnt signaling activation, as showed in our IUGR placentas, could be a protective mechanism that, besides inducing angiogenesis and supporting vasorelaxation, could improve uteroplacental blood flow and fetal oxygenation and be protective by reducing apoptotic activity and negative consequences of oxidative stress.

On the other hand, other studies are not in accordance with our results. Fan *et al* (64) reported active, dephosphorylated β-catenin and Matrix Metallopeptidase 9 (MMP-9) levels to be significantly lower in preeclampsia, especially a severe form, compared with placentas from normal pregnancies, thus suggesting the shallow invasion that is associated with preeclampsia (65) to be regulated by β-catenin via Snail and MMP-9 (64). Other studies also confirmed lower β-catenin expression in trophoblast cells affected by hypoxia and inhibited proliferation, weakened migration, invasion, and excessive apoptosis (66). Trophoblast invasion is vital for normal embryonic development. The extravillous trophoblast is formed in epithelium-mesenchymal transition (EMT) (67). Its capacity to invade the spiral arteries, mediate the destruction of the arterial wall and replace the endothelium is essential for pregnancy progress (68). There is evidence that Hh signaling plays an essential role in EMT and invasion. Tang *et al* (69) found higher expression of Hh ligands in the villous core as Wnt-producing tissues and higher expression of Hh receptors PTCH1 and SMO in trophoblast layers as Wnt-responding tissues. They also found that the Hh ligand stimulates the EMT of human cytotrophoblast cells.

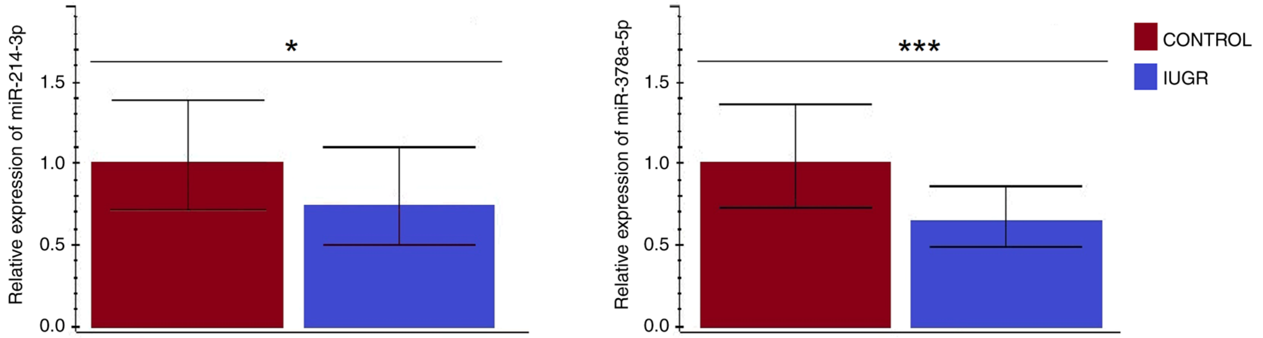

Figure 4. miR-214-3p and miR-378a-5p expression in IUGR vs. control placental tissues normalized to U6 small nuclear RNA and relative to control tissue. *P<0.05; ***P<0.001. IUGR, intrauterine growth restriction; miR, microRNA.

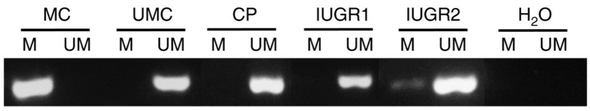

Figure 5. A representative example of methylation-specific PCR analysis for the suppressor of fused gene promoter in term placentas from physiologic pregnancies (CP) and term placentas from pregnancies complicated with IUGR. IUGR1 and IUGR2 are samples from different patients. IUGR1 is a representative example of the unmethylated promoter of the *SUFU* gene in IUGR placentas. IUGR2 is one sample of IUGR placenta with the presence of methylated promoter of the *SUFU* gene. CP, control placenta; H_2O, water, negative control; IUGR, intrauterine growth restriction; M, methylated reaction; MC, methylated human control, positive control for methylated reaction; UM, unmethylated reaction; UMC, unmethylated human control, positive control for unmethylated reaction.

In contrast, knockdown of Gli1 and Gli2 attenuated SHH-induced EMT (indicated by lower vimentin and higher E-cadherin expressions) and colony formation (69). Zhang and Zhang found that Forkhead Box C2 (FOXC2) facilitates trophoblast invasion through the activation of the Hh pathway-trophoblast cells with overexpression of FOXC2 also experienced higher expression levels of SHH, Gli and Snail and were more invasive (70). The effect was reverted when the samples were treated with siRNA targeting FOXC2 (70). All these data emphasize the importance of Hh signaling in human trophoblast physiology. To the best of our knowledge, the role of SUFU in placentation and IUGR has not been addressed by any previous study. Our results showed that its expression is significantly higher in the trophoblast cells of the IUGR placentas, whereas there were no differences in endothelium and stromal cells. As a negative regulator of the Hh pathway, higher expression of SUFU in IUGR trophoblast cells may contribute to lower activity of Hh signaling and subsequently to impaired trophoblast function in IUGR placentas.

Since SUFU is a negative regulator of the Hh pathway and possibly the Wnt pathway, its greater protein expression in IUGR placentas could be expected. Our results align with that, but higher protein expression of positive Wnt pathway regulators, WNT5A and β-catenin, was also found in IUGR placentas than normal ones. Higher expression of SUFU protein in that context could reveal another role of SUFU. Liu *et al* (71) demonstrated that SUFU could also be a positive regulator of the Hh pathway, thus maximizing Hh pathway activation. Moreover, it has been reported that RIO kinase 3 (RIOK3) acts as a SUFU-dependent positive regulator of Hh signaling (72), suggesting that SUFU could exert its double function as a positive regulator through other compounds.

In contrast to their protein expressions, the RT-qPCR analysis revealed no significant difference in *WNT5A, SUFU,* and *CTNNB1* mRNA expressions between IUGR placentas and controls. This could be explained by the fact that IHC analysis enabled protein expression analysis and quantification in different placental compartments (trophoblasts, stromal cells and endothelial cells). In contrast, RT-qPCR analysis was performed in whole placental tissue sections.

In our study, other than the *SUFU* gene and protein expression, we also wanted to perceive the *SUFU* gene promoter methylation status. Our results show that the *SUFU* gene promoter is unmethylated in all but one IUGR placenta. It is also unmethylated in all the placentas from uncomplicated pregnancies. We conclude that other epigenetic mechanisms might regulate *SUFU* gene expression based on these results. Although it has been demonstrated that DNA methylation is an essential epigenetic mechanism in the human placenta and that IUGR is significantly associated with altered DNA methylation patterns in the placenta (30,73), growing evidence, including our results, suggests other epigenetic mechanisms could also be fundamental in placental gene expression regulation. Kimura *et al* (74) demonstrated that histone post-translational modifications could be an essential mechanism of placental gene expression regulation. Moreover, Chuang *et al* (75) reported that histone modification is linked with the expression of genes that are decisive for mediating trophoblastic fusion and, therefore, proper placental structure and function.

MiRNAs appear to be actively involved in placental gene regulation and development (76). It has been reported that the expression of several placenta-specific miRNAs has been reduced in placentas from pregnancies with IUGR than in placentas from uncomplicated pregnancies (77). Pineles *et al* (78) reported specific miRNA expression patterns associated with preeclampsia, which was also confirmed by Zhu *et al* (79). Another study also confirmed functional miRNAs in the trophoblast. The specific miRNAs in the placenta can be up or down-regulated by the varying oxygen levels, primarily in a hypoxic environment (80). This is an interesting finding that could be important in IUGR since

IUGR is associated with chronic hypoxia, as we already stressed earlier. Peng *et al* (81) reported that *SUFU* was regulated by miRNA-20b and induced cell proliferation, migration and EMT by negatively regulating both Wnt and Hh signalling pathways.

Several studies reported *SUFU* gene expression to be epigenetically downregulated by miRNAs and thus silenced in various tumors such as breast, gastric, basal cell, or non-small cell lung carcinomas (82-85). Alimirah *et al* (82) reported that miR-214 targets the *SUFU* gene, which then inhibits its expression in breast cancer. This finding was also confirmed in another study that found *SUFU* gene expression negatively correlated with miR-214 expression, indicating that miR-214 directly targeted *SUFU* expression and Hh signaling in promoting liver fibrosis (86). Moreover, He *et al* (44) reported precisely the miR-214-3p-SUFU-GLI1 axis as the critical signaling pathway responsible for smooth muscle cell (SMC) differentiation and generation from adventitial stem/progenitor cells (AdSPCs) important for controlling neointimal hyperplasia. MiR-214-3p controls vascular SMC proliferation and migration, while *SUFU* is identified as its true target gene that operates as a transcriptional repressor of SMC contractile genes, which is essential in the context of vascular remodeling after injury (44).

MiR-378a-5p was reported to negatively regulate the expression of *SUFU* as a target gene in melanomas. That is important since miR-378a-5p was found to increase cell migration and invasion and to have proangiogenic activity by significantly inducing angiogenic growth factor VEGF secretion, which then increases *in vivo* and *in vitro* angiogenesis (87). Earlier studies also reported miR-378a-5p as a promoter of angiogenesis by upregulating VEGF, thus inducing neovascularization in hypoxia by targeting the *SUFU* gene (45,88).

Based on these studies on other tissues showing miR-214-3p and miR-378a-5p to modulate *SUFU* expression, we wanted to identify their potential involvement in the regulation of *SUFU* expression in placentas and, especially, in IUGR placentas. This is particularly interesting due to the role of these miRNAs in vascular remodeling and angiogenesis. Our results showed that miR-214-3p and miR-378a-5p expressions were increased in control placentas compared with IUGR placentas. This aligns with protein expression results where SUFU protein expression was increased in IUGR term placentas compared with physiological ones. Our results also suggest that miR-214-3p and miR-378a-5p targeting could be involved in epigenetic regulation of *SUFU* gene expression in normal and IUGR placental tissue.

We found mean maternal age to be significantly higher in the IUGR group compared with the control group. This is interesting since there are conflicting reports regarding the association between maternal age and risk for IUGR. Some studies found increased maternal age to be an independent risk factor for IUGR, which is in line with our findings (89,90). Other studies did not find any association between maternal age and IUGR risk (91-93), while Yu *et al* (94) reported younger maternal age as a risk factor for IUGR.

There are several limitations of the present study. First, the sample size was moderate. Second, the study did not explore the functional impact of the targeted mRNA and miRNA expression on trophoblast cells. It would also be interesting to focus on other epigenetic mechanisms besides the reported miR-214-3p, miR378a-5p and DNA methylation in placentas with IUGR. Our future studies will focus on the shortcomings of the current study.

Our study provides new insights on the involvement of the Wnt and Hh signaling pathways and epigenetic regulation of *SUFU* gene expression in the placenta and IUGR. However, our results should be further explored in a larger cohort to specify more closely their exact functional roles in placental insufficiency, detection or surveillance of IUGR, and optimal delivery planning. The precise functional impact of miR-214-3p and miR-378a-5p on *SUFU* gene expression in these tissue and pathological settings should be scrutinized as well.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IMS contributed to conceptualization, data interpretation, data acquisition and analysis, performed experimental work, wrote and edited the manuscript, and revised the manuscript for important intellectual content. VKK contributed to conceptualization, interpretation and design of experiments, edited the manuscript, and revised the manuscript for important intellectual content. FP contributed to data analysis and interpretation, performed experimental work, and revised the manuscript for important intellectual content. LL contributed to data analysis and interpretation, and revised the manuscript for important intellectual content. MG contributed to data interpretation, performed experimental work and revised the manuscript for important intellectual content. NS contributed to data interpretation, and revised the manuscript for important intellectual content. TD contributed to data interpretation and performed experimental work. AS contributed to data analysis and interpretation, and revised the manuscript for important intellectual content. KK contributed to data interpretation, and revised the manuscript for important intellectual content. SV contributed to data analysis and interpretation, and revised the manuscript for important intellectual content. LS conceived the idea, contributed to conceptualization, data collection and analysis and interpretation of the results, and revised the manuscript for important intellectual content. LS and IMS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the School of Medicine, University of Zagreb, Zagreb, Croatia (641-01/22-02/01; 30th October 2018) and the Ethics Committee of the University Hospital Merkur Zagreb, Zagreb, Croatia (03/1-1341; 14th February 2018). Written informed consent was obtained from all participants involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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