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From Cell Biology to Tissue Engineering

Altered expression of human endogenous retroviruses syncytin-1, syncytin-2 and their receptors in human normal and gestational diabetic placenta

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Summary. Introduction: Syncytins belong to the Human Endogenous Retrovirus family. The syncytin-1 receptor, SLC1A5, and syncytin-2 receptor, MFSD2, interact with their respective syncytin proteins to induce syncytiotrophoblast formation. However, there is no information about syncytins in gestational diabetic placenta. Therefore, we studied the expression and localization of syncytins and their receptors during normal placental development and in gestational diabetic placenta.

Methods: Immunohistochemistry and Western-blot methods were performed with antibodies against syncytin-1, syncytin-2, SLC1A5 and MFSD2 in human first trimester placental tissues, normal term and gestational diabetic placentas. Syncytin-1, syncytin-2 and MFSD2 mRNA transcripts were determined by qRT-PCR in normal and diabetic term placentas.

Results: Cytoplasmic syncytin-1, syncytin-2, SLC1A5 and MFSD2 immunoreactions were observed in the trophoblastic layers in all placental samples. Some of the stromal cells showed strong cytoplasmic punctate staining. There were significantly weak syncytin-2 and MFSD2 immunoreaction intensities in diabetic placentas by ImageJ analysis, in parallel with decreased syncytin-2 and MFSD2 proteins in diabetic placentas by Western-blot. Protein expression of SLC1A5 increased dramatically in early pregnancy compared to term placenta. Syncytin-1, syncytin-2 and MFSD2 mRNA transcripts showed similar relative expression pattern by qRT-PCR.

Discussion: Syncytins were localized not only in cytotrophoblast cells and the basement membrane of the syncytiotrophoblast but also in the apical microvillous membrane, cytoplasm of syncytiotrophoblast, some of the stromal cells and endothelium. Decreased syncytin-2 and MFSD2 proteins in gestational diabetic placentas might cause abnormal syncytiotrophoblast formation and possibly be involved in the pathology. Therefore, our study highlights an important potential relationship between syncytins and gestational diabetic placenta.

Key words: Syncytin, SLC1A5, MFSD2, Human placenta, Gestational diabetes

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Introduction

The placenta is unique among all other organs in that it conducts nutrient and gas exchange between mother and fetus; it participates in metabolic, catabolic, and secretory functions and protects the fetus from the maternal immune system during pregnancy (Demir, 1979; 1980; Benirschke et al., 2006; Rawn and Cross, 2008). Many of these placental functions are carried out by the villous syncytiotrophoblast which is the principle boundary layer between maternal and fetal tissues (Chen et al., 2006). A healthy pregnancy is closely related to normal placental development which is dependent on the appropriate formation and expansion of the syncytiotrophoblast. This layer expands through intercellular fusion with the underlying mononuclear villous cytotrophoblast, although the precise molecular mechanisms involved in trophoblast turnover are poorly

understood (Esnault et al., 2008).

Human endogenous retroviruses (HERVs) are one of the major groups of retrotransposons (Smit, 1999). HERVs infected the germ line during earlier stages of evolution and were inherited as endogenous retroviral elements comprising approximately 8% of the human genome (Harris, 1998; Griffiths, 2001). Because of subsequent genetic degradation and mutations in the genome, most retroviral sequences contain in-frame stop codons or deletions which make these sequences transcriptionally inactive. However, some retroviral elements have large open reading frames (ORF) and retain their potential transcriptional capacity with important functions for the host's physiology (Rote et al., 2004). A systematic search in human genome has showed that 18 *ENV* genes, which serve to form viral envelope in virus, encode a full length ORF whose products might have a function during human development (Villesen et al., 2004; Esnault et al., 2008). To date, three biologically relevant placental HERV proteins, HERV-W (syncytin-1), HERV-FRD (syncytin-2) and ERV-3 have been studied in placenta (Rote et al., 2004). Two members of HERV, syncytin-1 (HERV-W) and syncytin-2 (HERV-FRD), are highly and specifically expressed in the trophoblastic layer of human placenta which is considered as a possible contributor to normal placental architecture, especially in trophoblast turnover (Mi et al., 2000; Malassine et al., 2007). Syncytin-1 (HERV-W) is a glycoprotein with cell fusogenic activity and binds to its receptor, SLC1A5/ASCT2/RDR (a neutral amino acid transporter and type D mammalian retrovirus receptor) to promote formation of the multinucleated syncytiotrophoblast layer (Rote et al., 2004; Malassine et al., 2005). Another retroviral protein, syncytin-2, entered the primate genome earlier than syncytin-1 (de Parseval and Heidmann, 2005). Syncytin-2 has fusogenic activity and sequence similarity to syncytin-1, but differs in several points. Firstly, syncytin-2 binds to a member of the carbohydrate transporter superfamily MFSD2 (Major Facilitator Superfamily Domain Containing 2). Secondly, syncytin-2 has an immunosuppressive domain that might further play a role in protecting the fetus from the maternal immune system (Blaise et al., 2003; Esnault et al., 2008). Although an additional member of HERV, ERV-3, is also expressed in human placenta, a fusogenic activity is not indicated (Rote et al., 2004).

Since syncytin-1 and syncytin-2 are important for syncytiotrophoblast formation, there are studies investigating whether these proteins are crucial for healthy placental formation (Mi et al., 2000; Blaise et al., 2003). Various studies have shown the altered expression of syncytin proteins in placental abnormalities, such as pre-eclampsia (PE) and intrauterine growth restriction (IUGR) (Lee et al., 2001; Chen et al., 2006; Ruebner et al., 2010; Holder et al., 2012; Toufaily et al., 2013). However, there is no information about syncytins in gestational diabetic placenta pathology. Gestational Diabetes Mellitus (GDM) is indicated by abnormal glucose tolerance with onset or first recognition during pregnancy (O'Sullivan and Mills, 1985). In gestational diabetic pregnancies, the detrimental effect of increased glucose levels may cause an increased rate of early pregnancy loss and maternal hypertensive disorders. The fetus is affected by congenital birth defects, various metabolic abnormalities and is exposed to increased hypoxic stress with associated morbidity and mortality (Mondestin et al., 2002; Cheng et al., 2013; El Hajj et al., 2013; Ruchat et al., 2013; West et al., 2013). Chronic fetal hypoxia causes villous immaturity and increased fibrinoid necrosis, potentially altering placental structure, and affecting oxygen and nutrient exchange in gestational diabetic placenta (Evers et al., 2003; Daskalakis et al., 2008). Thus, we hypothesized that the expression of syncytin proteins, syncytin-1 and syncytin-2, and their receptors, SLC1A5 and MFSD2, alter during normal placental development and such changes might be involved in gestational diabetic placental pathology as indicated in PE and IUGR. Therefore, we aimed to determine the expression levels and cellular localization of syncytin-1, syncytin-2, and their receptors SLC1A5 and MFSD2 in the early human placenta, healthy term placenta and gestational diabetic placenta.

Materials and methods

Tissue collection

First trimester placentas (n=12) were taken from patients undergoing discretionary termination of pregnancy (4-9 weeks of gestation) by curettage for psychosocial reasons, which was unlikely to affect placental structure. Normal term placentas from uncomplicated pregnancies (n=6) and gestational diabetic placentas (n=6) were obtained by cesarean section at term (Table 1). The diagnosis of gestational diabetes mellitus (GDM) was based on oral glucose tolerance test (OGTT). If fasting (a zero time) blood glucose level >92 mg/dl, 1 hour (after loading 75 g glucose) blood glucose level >180 mg/dl, and 2 hours blood glucose level >153 mg/dl, patients were diagnosed as GDM (Metzger et al., 2010). All patients diagnosed with GDM were on oral insulin medication. None of the

Table 1. Demographics of study subjects at cesarean section.

	Normal (Term) (n=6)	Gestational diabetes (n=6)
Gestational age (weeks)	38±0.4	38±0.3
Maternal age (weeks)	32±2	37.6±0.8
BMI (kg/m^2)	30.4±1.5	33.6±2.1
Birth weight (grams)	3702.5±222	3911.6±607
Fetal sex	Female (n=3) Male (=3)	Female (n=2) Male (n=4)

All women had singleton pregnancies. There were no statistical differences between normal and gestational diabetes ($p>0.05$).

patients had a previous history of diabetes mellitus or were receiving hormone treatment. Tissues were obtained from the Department of Obstetrics and Gynecology, School of Medicine, Akdeniz University, and Clinic of Obstetrics and Gynecology, Training and Research Hospital, Antalya. Informed consent forms to use the tissues were approved by Ethical Committee of School of Medicine in Akdeniz University.

Vacuumed aspiration conceptus was washed in normal saline, dissected under the stereo microscope (Zeiss Stemi SV 11) to separate decidual and villous tissue. Term placentas were washed in normal saline and each placental specimen was obtained near the cord. For further analysis, samples were embedded in paraffin for immunohistochemical analysis and also snap frozen in liquid nitrogen directly for Western-blot and qRT-PCR analysis.

Immunohistochemistry

First trimester placenta samples were fixed in 10% neutral formalin for 18 h while term placenta samples were fixed for 24 h. Placenta samples were dehydrated in serials of ethanol, cleared in xylene and embedded in paraffin. Serial sections at 5 μm thickness were placed on positive charged slides (Thermo Fisher Scientific, Waltham, MA, USA) and incubated overnight at 56°C. Sections were de-paraffined in xylene, rehydrated in ethanol/water and finally rinsed in distilled water. An antigen retrieval procedure was performed by heating the samples with Tris-EDTA Buffer, pH 9 in a microwave oven at 750W for 7 min twice. After cooling for 20 min at room temperature, the sections were washed three times in phosphate buffered saline (PBS) and endogenous peroxidase activity was blocked (3% H_2O_2 for 30 min). The sections were washed three times in PBS prior to incubation with Ultra V blocking reagent (Lab Vision, Fremont, CA) for 7 min to block non-specific binding. The primary antibodies were incubated overnight at +4°C in a humidified chamber (Table 2). The sections were washed three times in PBS and incubated with related secondary antibodies for 45 min at room temperature (Table 2). After washing in PBS three times, the antigen-antibody complexes were amplified using a streptavidin-peroxidase complex (TS-125-HR; LabVision, Fremont, CA, USA) for 30 min. The sections were washed three times and diaminobenzidine (DAB) tablets (D-4168; Sigma Aldrich, MO, USA) were used for chromogenic detection. The sections were counterstained with Mayer's Hematoxylin (1.09249.1000; Merck, Darmstadt, Germany) and mounted on glass slides. For negative controls, instead of primary antibody, slides were incubated with normal rabbit IgG or mouse IgG depending on primary antibody used. The sections were visualized with an Axioplan microscope.

Threshold analysis of immunostaining intensities

Immunohistochemistry images were captured using Spot Imaging software version 4.6 (Diagnostic Instruments, Inc., MI, USA) at x 400 magnification. Ten photomicrographs were randomly selected for each group and analyzed by ImageJ Version 1.46 (NIH). All tissue components except background were measured by moving brightness slider until all stained areas were selected and recorded to the excel sheet. To measure only stained areas, the hue slider was decreased without changing the brightness slider, until only the IHC stained areas were selected and recorded, as well. Finally, integrated density values of IHC stained areas were normalized to the integrated density values of the total area (Jensen, 2013).The calculated ratio of the immunostained area to total area was graphed as previously published (Sati et al., 2016).

SDS-PAGE and Western-blotting

Proteins from first trimester, normal term and gestational term placentas were extracted with lysis

Table 2. Primary and secondary antibody dilutions used for immunohistochemistry and Western-blot methods.

	Company/ Catolog no	IHC Dilution	Western-blot Dilution
Primary Antibody			
Syncytin-1	Santa Cruz Biotechnology Inc/sc-50369	1:200	1:200 (in 5% non-fat dry milk)
Syncytin-2	Sigma Aldrich/ HPA011812	1:50	1:200 (in 2.5% non-fat dry milk)
SLC1A5	Abcam/ab58690	1:100	1:200 (in 5% non-fat dry milk)
MFSD2	Santa Cruz Biotechnology Inc/ sc-135305	1:200	1:200 (in 5% non-fat dry milk)
Cytokeratin-7	Dako/M7018 (for IHC) and Abcam/ab9021 (for WB)	1:100	1:500 (in 5% non-fat dry milk)
Vimentin	Abcam/ab92547	1:400	
βeta-actin	Santa Cruz Biotechnology Inc/ sc-47778		1:5000 (in 5% non-fat dry milk)
Secondary Antibody			
Biotinylated anti-rabbit	Vector Laboratories/ BA-1000	1:500	
Biotinylated anti-mouse	Vector Laboratories/ BA-2000	1:500	
HRP-conjugated anti-rabbit	Vector Laboratories/PI-1000		1:3000 (in 5% non-fat dry milk)
HRP-conjugated anti-mouse	Vector Laboratories/PI-2000		1:3000 (in 5% non-fat dry milk)

buffer (containing 0.1 M Tris, pH 7.4; 0.1 M Sodium orthovanadate and 1% Sodium dodecyl sulfate) and protease inhibitor cocktail (P8340; Sigma Aldrich, MO, USA). Samples were loaded on 10% Tris-HCl gels, electrophoretically separated and transferred to PVDF membranes (Bio-Rad Laboratories, CA, USA). To block non-specific binding, the membranes were incubated in 5% non-fat dry milk in tris buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) for 1 h. The membranes were incubated overnight at +4°C with primary antibodies (Table 2). After primary antibody incubation, the membranes were washed in TBS-T for 1 h and incubated with horseradish peroxidase conjugated secondary antibodies, listed in Table 2, for 1 h at room temperature. The membranes were washed in TBS-T for 1 h and incubated with SuperSignal Chemiluminescent Kit (Pierce Biotechnology, Rockford, IL, USA). The proteins were visualized by light emission on film (Amersham Biosciences; Buckinghamshire, England). After immunoblot bands were obtained, the proteins were stripped with Restore Plus Stripping Buffer (46430; Pierce Biotechnology, Rockford, IL, USA), the membranes were washed in TBS-T for 30 min, blocked with 5% non-fat dry milk in TBS-T for 1 hour and incubated with Beta-actin antibody (Table 2) for 2 h at room temperature. Beta-actin was used as an internal control. Immunoblot bands were quantified using ImageJ Version 1.46 (NIH).

RNA isolation and Quantitative Real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from normal term and diabetic placenta samples using RNeasy mini kit (74104; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured with Epoch™ Spectrophotometer system (BioTek, Winooski, USA). DNase treated total RNA (600ng) was reverse transcribed by QuantiTect Reverse Transcription Kit (205311; Qiagen, Valencia, CA, USA). Quantitative real-time PCR was performed in a LightCycler 1.5 Instrument (Roche) in triplicates in a final volume of 20 µl in iQ SYBER Green Supermix (170-8880; Bio-Rad Laboratories, CA, USA). Data were normalized and analyzed. The primer sequences for gene amplification were as follows: syncytin-1, (F) 5′-CCCCATCGTATAGGAGTCTT-3′ and (R) 5′-CCCCATCAGACATACCAGTT-3′; syncytin-2, (F) 5′-CGACTCAGTGTAAACAGCCA-3′ and (R) 5′-CCACAGAAGGAAGACAAAGAAAAT-3′; MFSD2, (F) 5′-CTCCTGGCCATCATGCTCTC-3′ and (R) 5′-GGCCACCAAGATGAGAAA-3′; β-actin, (F) 5′-CCAACCGCGAGAAGATGA-3′ and (R) 5′-CCAGAGGCGTACAGGGATAG-3′. Syncytin-1 PCR reaction was carried out as follows, preincubation at 95°C for 5 min and amplification at 95°C for 5 s, 60°C for 30 s and 72°C for 30 s for 45 cycles and cooling at 40°C for 10 s. Syncytin-2 PCR reaction was performed at 98°C for 5 min, followed by 45 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 15 s. MFSD2 PCR condition was 95°C for 10 s, followed by 50 cycles of 94°C for 3 s, 60°C for 15 s, 72°C for 15 s. The analysis module for absolute quantification of LightCycler software 4.1 was used to analyze the data.

Statistical analysis

Statistical calculations were performed using Sigma Stat for Windows Version 3.5 (Chicago, IL, USA). Samples were grouped by gestational age (first trimester and normal term placentas) or pathological conditions (normal versus gestational diabetic term placentas). The differences were evaluated by Mann-Whitney Rank Sum and Student t-test. Statistical significance was defined as $P<0.05$.

Results

Immunolocalization of syncytin-1 and its receptor SLC1A5 in first trimester, term and diabetic placenta

In first trimester placenta, strong cytoplasmic syncytin-1 immunoreaction was observed in both syncytiotrophoblast and cytotrophoblast cells. Some of the stromal cells and villous blood vessels were also immunopositive with syncytin-1 (Fig. 1a). SLC1A5 immunoreaction showed a similar staining pattern with its ligand, syncytin-1, in first trimester placentas (Fig. 1b).

As seen in first trimester placenta, moderate to strong cytoplasmic syncytin-1 and SLC1A5 reactions were detected in syncytiotrophoblasts of normal term placenta. Cytoplasmic punctuate staining was observed in some of the stromal cells and endothelial cells in normal term placenta. There was also some microvillous membrane immunoreactivity with syncytin-1 and SLC1A5 (Fig. 1c,d). Syncytin-1 and SLC1A5 immunoreactions were observed in a similar pattern in diabetic placenta (Fig. 1e,f). However, syncytin-1 immunostaining in the endothelial lining was weak in diabetic placenta compared to normal term placenta.

According to ImageJ analysis, there was no obvious difference in syncytin-1 staining intensity between first trimester and term placenta ($p=0.328$). However, SLC1A5 showed a statistically significant decrease in term placenta compared to first trimester ($p=0.022$) (Fig. 1g). In diabetic placenta, there was no statistically significant difference in syncytin-1 ($p=0.777$) or SLC1A5 ($p=0.432$) staining intensity compared to normal term placenta (Fig. 1h).

Immunolocalization of syncytin-2 and its receptor MFSD2 in first trimester, term and diabetic placenta

In first trimester placenta, syncytin-2 and MFSD2 showed strong immunostaining in syncytiotrophoblast with or without apical microvillus membrane immunoreaction, and also in cytotrophoblast cells. Weak to

moderate syncytin-2 and MFSD2 immunoreactions were also observed in some of the stromal cells, hematopoietic and endothelial cells in first trimester placenta (Fig. 2a,b).

In normal term placenta, strong syncytin-2 and MFSD2 reactivities were noted in syncytiotrophoblast layer (Fig. 2c,d). Weak to moderate syncytin-2 and MFSD2 immunoreactivities were seen on some of the microvillous membrane. Some of the stromal cells showed strong cytoplasmic punctuate staining with syncytin-2 and MFSD2. Immunoreactions in stromal core were less than syncytiotrophoblast. Compared to term placenta, a decreased immunostaining of syncytin-2 and MFSD2 in syncytiotrophoblast and stromal cells were observed in diabetic placental samples (Fig. 2e,f).

According to ImageJ analysis, there was no difference in syncytin-2 (p=0,673) and MFSD2 (p=0,979) staining intensities between first trimester and term placenta (Fig. 2g). Although syncytin-2 and

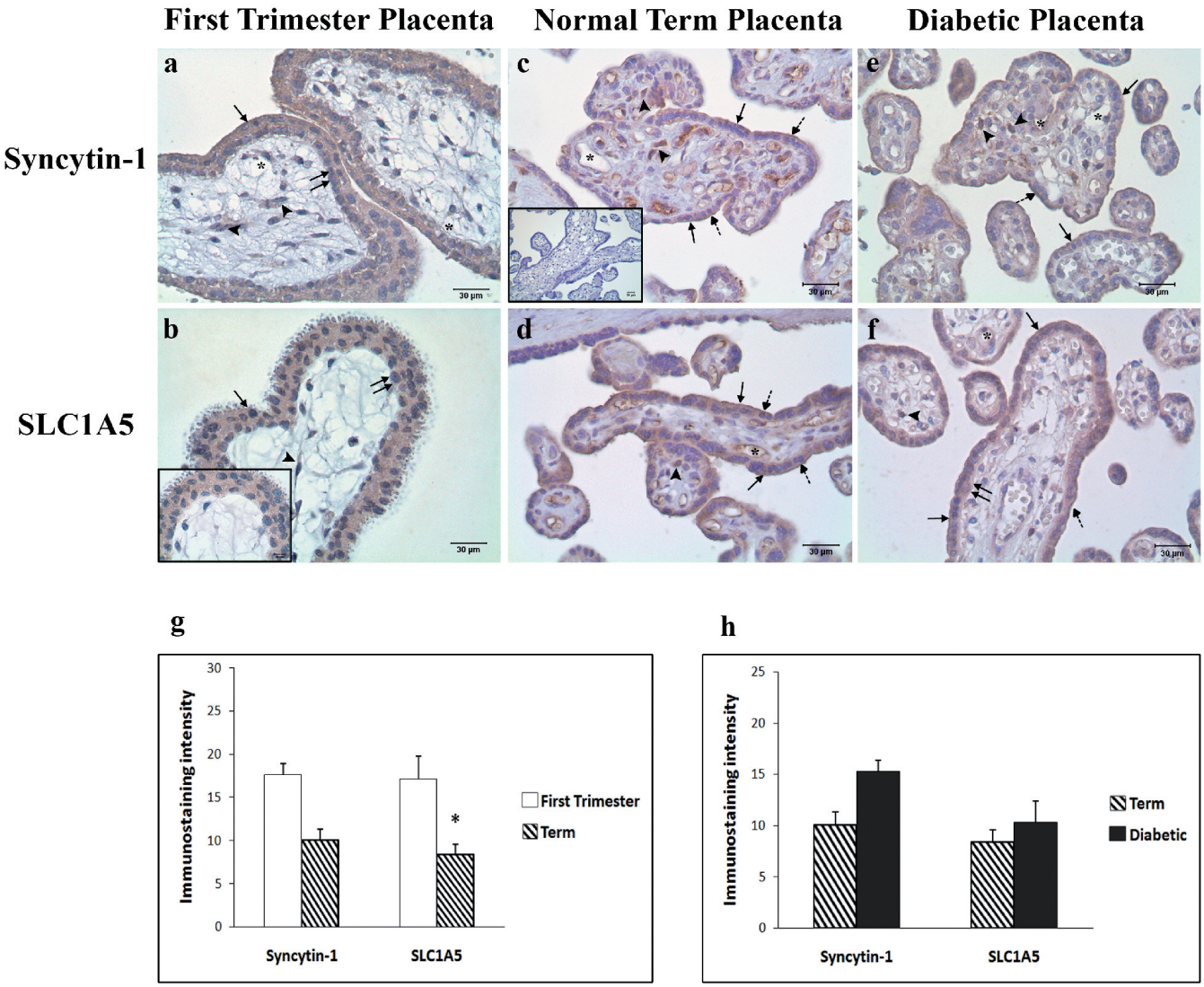

Fig. 1. The immunolocalization **(a-f)** and ImageJ analysis **(g-h)** of syncytin-1 and SLC1A5 proteins in first trimester, normal term and diabetic placenta. Syncytin-1 **(a)** and SLC1A5 **(b)** immunostaining were observed in syncytiotrophoblast (arrows), cytotrophoblast cells (double arrows), some of the stromal cells (arrow heads) and the endothelium of some vessels (asterisks) in first trimester placenta. In normal term placenta, syncytin-1 **(c)** and SCL1A5 **(d)** showed moderate to strong immunostaining in syncytiotrophoblast, some of the stromal cells, vascular endothelium and some parts of microvillous membrane (dashed arrows). Similar syncytin-1 **(e)** and SLC1A5 **(f)** expression patterns were observed in diabetic placenta. The intensities of syncytin-1 and SLC1A5 immunostaining were higher in first trimester than normal term placenta **(g)**. Immunostaining intensities of syncytin-1 and SLC1A5 in normal term and diabetic placenta were also graphed **(h)**. Inset in **(b)** and **(c)** are x 1000 magnification and negative control, respectively.

MFSD2 showed a significant decrease in diabetic placenta compared to term placenta, ($p<0.001$) and ($p=0.005$) respectively (Fig. 2h).

Characterization of trophoblast cells and mesenchymal cells

Cytokeratin 7 antibody was used to characterize the trophoblastic layers in placenta samples by immunohistochemistry (data not shown) and Western blot methods (Fig. 3). Western blot analysis was performed to see whether the difference in protein levels was not due to variation in the abundance of villous trophoblast proportion versus other cell types in the overall placenta mass. Our data indicated that the proportion of trophoblast cells in the overall placenta mass was similar between placenta lysates from different groups (Fig. 3). Moreover, the mesencyhmal cells were also characterized by using vimentin immunohistochemistry in the chorionic villous tree.

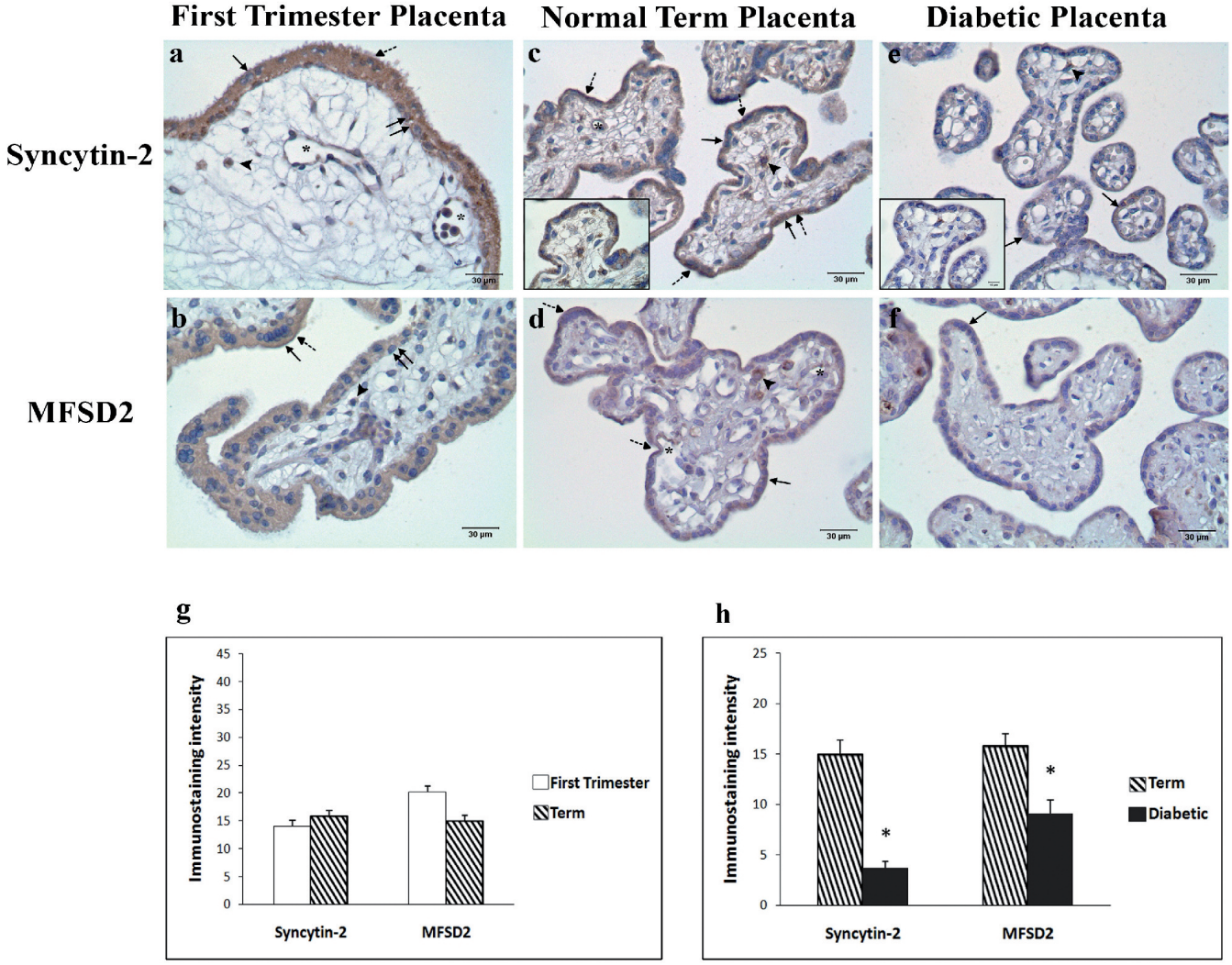

Fig. 2. The distribution **(a-f)** and ImageJ analysis **(g-h)** of syncytin-2 and MFSD2 proteins in first trimester, normal term and diabetic placenta. Syncytin-2 **(a)** and MFSD2 **(b)** were mainly expressed in syncytiotrophoblast (arrows), cytotrophoblast cells (double arrows), some parts of microvillous membrane (dashed arrows), some of the stromal cells (arrow heads) and the endothelium of some vessels (asteriks) in first trimester placenta. In normal term placenta, syncytin2 **(c)** and MFSD2 **(d)** immunostaining were observed in syncytiotrophoblast, some parts of microvillous membrane, some of the stromal cells and vascular endothelium. A decreased immunostaining of syncytin-2 **(e)** and MFSD2 **(f)** in syncytiotrophoblast and stromal cells was observed in diabetic placenta. In first trimester and normal term placenta, there was no significant difference in syncytin-2 and MFSD2 immunostaining intensities **(g)**. Unlike normal term placenta, syncytin-2 and MFSD2 immunostaining intensities were decreased in diabetic placenta **(h)**. Inset in **(c)** and **(e)** are x 1000 magnification of micrographs.

Protein expression levels of syncytin proteins and their receptors in first trimester and term placentas

The protein level of syncytin-1 (~60 kDa) had a tendency to be higher in first trimester placenta compared to term placentas. However, this increase was not statistically significant (p=0.606) (Fig. 4a). SLC1A5 protein (~54 kDa) expression was statistically higher in first trimester placentas compared with term placentas (p=0.027) (Fig. 4a). Neither syncytin-2 (~59 kDa) nor MFSD2 (~60 kDa) protein expression was statistically different between first trimester and term placentas (p=0.793) and (p=0.856), respectively (Fig. 4b).

Protein expression levels of syncytin proteins and their receptors in normal term and gestational diabetic placentas

Even if the protein expression of syncytin-1 and its receptor, SLC1A5, tended towards lower in normal term placentas than gestational diabetic placentas, no significant difference was found for syncytin-1 (p=0.166) and SLC1A5 (p=0,548) proteins between gestational diabetic and normal term placentas (Fig. 4c). In contrast, syncytin-2 and its receptor, MFSD2, expressions decreased significantly in diabetic placenta compared to normal term placenta, (p=0.029) and (p=0.006), respectively (Fig. 4d).

Syncytin-1, Syncytin-2 and MFSD2 mRNA levels in normal term and gestational diabetic placentas

According to qRT-PCR, syncytin-1 mRNA transcripts tended to be higher in diabetic placenta compared to normal term placenta (p=0.625) (Fig. 5a). On the other hand, syncytin-2 and MFSD2 mRNA levels were found to be decreased in diabetic placenta compared to normal term placenta, (p=0.525) and (p=0.032) respectively (Fig. 5b,c). Unlike syncytin-1 and syncytin-2 transcripts, only MFSD2 mRNA level was found to be statistically significant (Fig. 5c).

Discussion

Successful formation of human placenta is crucial not only for intrauterine development but also important in programming health in later life. It is known that the fusion of trophoblast cells constitutes a key process for normal placental development. The human placenta is a rich source of endogenous retroviral gene products. The role of these retroviral elements, especially syncytin proteins, in trophoblastic fusion process and placental morphogenesis, was hypothesized about 15 years ago (Harris, 1998; Mi et al., 2000). Today it is known that among other HERV proteins, especially syncytin-1 and syncytin-2 possess fusogenic activity and are abundantly expressed in human placenta. Many reports have shown the presence of syncytin proteins and their receptors during placental development and in some pathological conditions such as IUGR and PE (Blond et al., 2000; Mi et al., 2000; Lee et al., 2001; Chen et al., 2006, 2008; Muir et al., 2006; Malassine et al., 2007; Vargas et al., 2011; Holder et al., 2012). However, the results are inconsistent between several groups and there are several deviations in protocols that must be discussed, including antibodies and sensitivities of the methods

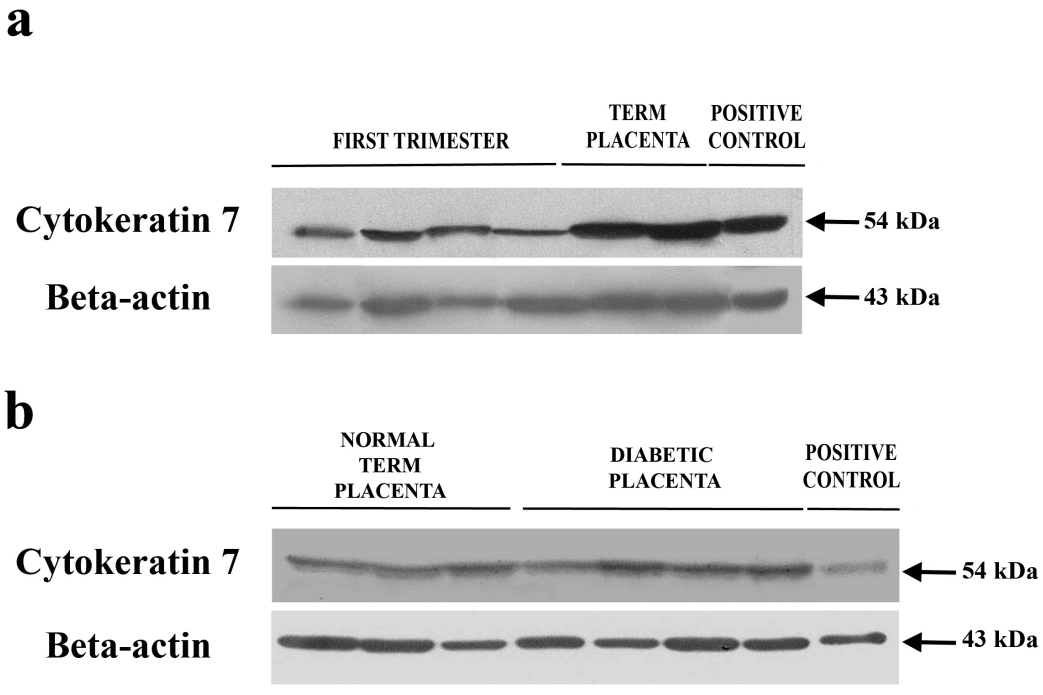

Fig. 3. Representative Western-blot bands of cytokeratin 7 in first trimester **(a)**, normal term and diabetic placenta samples **(b)**. β-actin was used as an internal control and mouse ovary lysate was used as a positive control for cytokeratin 7 antibody.

used. Besides, there is no clear evidence about the possible relationship between syncytins and gestational diabetic placenta pathology. Thus, in the light of the current literature we comprehensively analyzed the expressions of syncytin-1, syncytin-2, and their receptors in the early human placenta, healthy term and gestational diabetic placenta, to investigate whether these expression patterns would alter between normal and diabetic placenta samples.

We studied the first trimester samples from as early as 4 weeks. To our knowledge, these are the earliest tissue samples studied so far for the expression of syncytins and their receptors. Previous studies showed that syncytin-1, syncytin-2 and SLC1A5 protein localizations were present either in cytotrophoblast or syncytiotrophoblast or both cell types; and in some apical microvillous membrane (Mi et al., 2000; Lee et al., 2001; Muir et al., 2006; Hayward et al., 2007; Malassine et al., 2007, 2008; Chen et al., 2008). However, Holder et al. (2012) showed syncytin-1 immunolocalization in some of the villous stromal cells in the first trimester placenta and also in the endothelium at term (Holder et al., 2012). In line with these expression patterns indicated in the literature, we have observed syncytin-1, syncytin-2, SLC1A5 and MFSD2 immunostaining in syncytiotrophoblast with or without apical microvillous membrane staining, cytotrophoblastic cells as well as some of the stromal cells and endothelium in the first trimester and term placentas. The different expression patterns reported from previous studies, possibly with the usage of different antibodies, makes it complicated to understand their additional roles

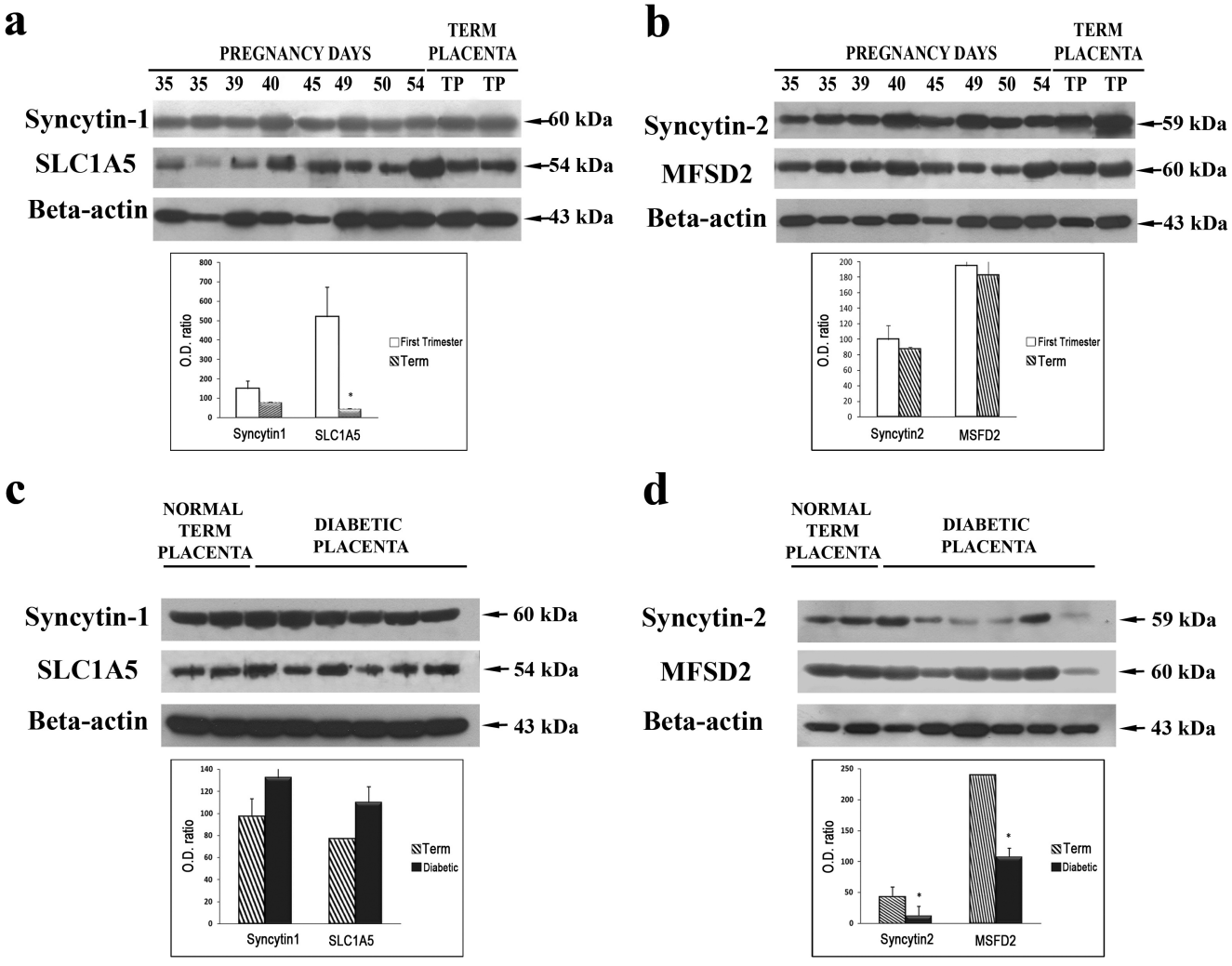

Fig. 4. Representative Western-blot bands of syncytin-1 and SLC1A5 **(a, c)**, syncytin-2 and MFSD2 **(b, d)** proteins in the first trimester, normal term and diabetic placenta. Even though only two normal term placenta samples were included in the figure, all of the first trimester, normal term and diabetic placenta samples were analyzed and then relative density for all tissue samples was normalized to β-actin and graphed.

besides syncytium formation. Thus, it would be of interest to study the possible roles of syncytin proteins and their receptor interactions particularly in stromal core of placental villi.

Syncytin-1 knockdown in BeWo cells leads to cell cycle arrest at G1 phase suggesting G1/S transition phase of the cell cycle is promoted by syncytin-1 (Huang et al., 2013). Based upon this finding, syncytin-1 expression in stromal core could indicate additional roles of syncytins (e.g. cell cycle) apart from fusogenic activity. Moreover, the apical microvillous membrane staining is also interesting since it covers the syncytiotrophoblastic surface, forming a maternofetal contact zone (Teasdale and Jean-Jacques, 1986). This region is of immunological interest (Smith et al., 1977) and the expression of syncytins and their receptors on the microvillous membrane may therefore be indicative of an immunosuppressive function of the syncytins.

We also found that syncytin-1, syncytin-2 and their receptors, SLC1A5 and MFSD2, were higher in first trimester in comparison to term placenta at protein level. However, only the SLC1A5 protein expression was found to be increased by a statistically significant amount. Chen et al. (2006) have reported higher SLC1A5 mRNA expression in first trimester compared to term placenta. However, the protein level of SLC1A5 was not analyzed since there was no suitable antibody. One can speculate that higher syncytialisation in trophoblastic layer might occur through SLC1A5 and its ligand syncytin-1 in early placental development. It is also known that regulatory mechanisms of amino acid transport may differ in early versus late pregnancy (Lager and Powell, 2012). As a neutral amino acid transporter, higher SLC1A5 expression might be related to higher neutral amino acid transport throughout the syncytiotrophoblast during the first trimester.

Besides complex physiological properties of syncytins, HERVs are also epigenetically regulated. DNA methylation, as one of the major epigenetic mechanism, generally suppresses the retrotransposons (Jaenisch and Bird, 2003; Kudaka et al., 2008). Human placenta shows lower methylation levels than other tissues (Nelissen et al., 2011). Hypomethylated pattern of placenta might explain why placenta is a rich source for actively transcribed HERVs. In human placenta, DNA methylation increases as gestational age progresses (Fuke et al., 2004). It has also been reported that the expression of syncytin-1 protein was decreased in term as compared to first trimester placenta (Smallwood et al., 2003). Our results also showed decreased syncytin-1 protein expression in term placenta. Increased DNA methylation at term might be potentially related to the decrease in syncytin-1 expression in term placenta but needs further evaluation.

Altered syncytin-1 or syncytin-2 expressions were previously reported in PE and IUGR placentas (Lee et al., 2001; Chen et al., 2006, 2008; Ruebner et al., 2010; Holder et al., 2012). However, there is no information about syncytins in gestational diabetes which is characterized by villous immaturity and the increased fibrinoid necrosis in placenta (Daskalakis et al., 2008; Gabbay-Benziv and Baschat, 2015). According to our results, there was a significant decrease in protein levels of syncytin-2 and MFSD2 in diabetic placentas compared to normal term. In addition to decreased MFSD2 protein, we also determined the decreased MFSD2 expression in diabetic placentas compared to normal term placentas at mRNA level. Although there is no statistical difference between the mRNA level of syncytin-2 in normal and diabetic placentas, it does not exclude the possibility of post-transcriptional alterations of syncytin-2 in gestational diabetic placenta. The reduced syncytin-2 and MFSD2 in diabetic placenta could potentially be involved in the interruption of the normal cellular kinetics, which is crucial for continuous syncytium formation in the healthy placenta. Abnormal syncytium formation due to decreased syncytin-2 and MFSD2 expression might be covered by fibrin structures to act as a barrier in maternal fetal transport of all metabolites in gestational diabetic placenta.

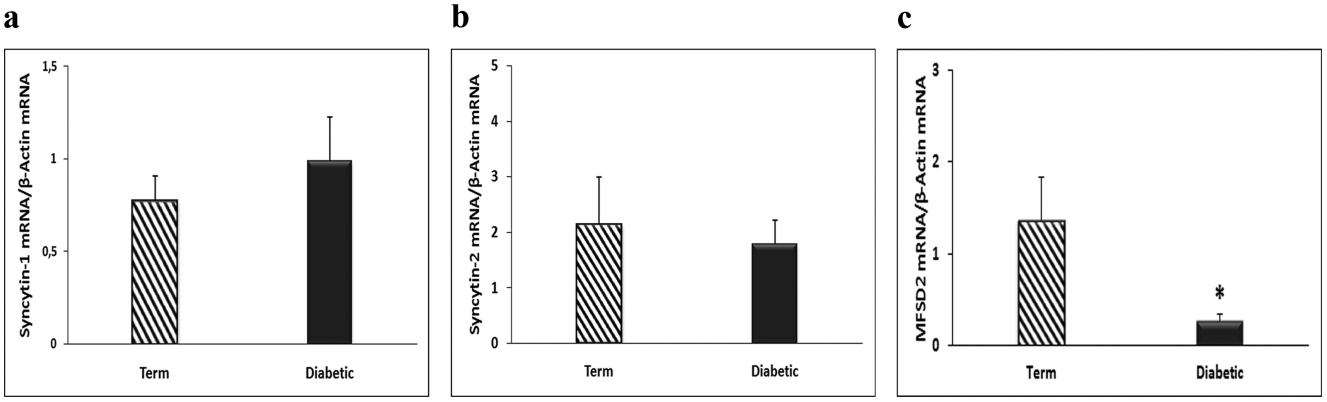

Fig. 5. The qRT-PCR analysis of syncytin-1 **(a)**, syncytin-2 **(b)** and MFSD2 **(c).** The normalized mRNA transcripts were graphed and presented.

In contrast to dramatic changes of syncytin-2 and MFSD2 expressions in diabetic placentas, syncytin-1 and SLC1A5 expression was only slightly higher in diabetic placentas. Vargas et al. (2011) reported altered expression levels of syncytin-1 and syncytin-2 in PE placentas and a more dramatic decrease in syncytin-2 expression was reported compared to syncytin-1 (Vargas et al., 2011). In our study, we did not find any significant difference for syncytin-1 between normal term and diabetic placental samples. Although syncytin-1 and syncytin-2 have fusogenic activity and sequence similarity, syncytin-2 but not syncytin-1 has an immunosuppressive domain (Blaise et al., 2003). Therefore, the altered expression of syncytin-2 and its receptor, MFSD2, might play the more dominant role in the pathogenesis of diabetic placenta.

The placenta secretes extracellular microvesicles such as exosomes (30-100/150 nm) and syncytiotrophoblast microparticles (0.2-2 μm) (Mincheva-Nilsson and Baranov, 2014). The presence of syncytin-1 and syncytin-2 were defined on the surface of placental exosomes (Vargas et al., 2014). Recently, using exosomes as biomarkers in different pathologies has had remarkable importance in diagnosis (Roberson et al., 2010; Lokossou et al., 2014). Decreased syncytin-2 protein expression in the trophoblastic layer might affect the concentration or functions of exosomes in gestational diabetic placentas. Thus, different exosome profiles might be potentially useful for diagnostic purposes in gestational diabetic pregnancies (Salomon et al., 2014).

In conclusion, our results showed altered syncytin-2 and MFSD2 expressions in gestational diabetic placental pathology. Further functional studies are needed to highlight how these retroviral proteins are differently regulated and whether altered syncytin-2 and MFSD2 expression might either be the consequence or cause of gestational diabetes.

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