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Effects of *Grp78*-Knockout on the Functionalities of Goat Trophoblast Cells

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LZ, FZ and YJ conceived and designed the experiments. LZ and FZ performed the experiments and collected the data. LZ, FZ and YJ interpreted the data. LZ and FZ wrote the manuscript.

Key words
Goat trophoblast cells, GRP78, Adhesion, Invasion, Hormone secretion

ABSTRACT

As the main components of the outermost membrane during embryonic development, trophoblast cells play an important role in embryo implantation and placenta formation. However, the molecular mechanism of trophoblast cell proliferation, invasion and hormone secretion remains elusive. In this study, we explored the role of GRP78 in the functionalities of goat trophoblast cells (GTCs). The *Grp78* gene was efficiently knockout by using the CRISPR/Cas9 system, which resulted in altered morphology and function of GTCs. The cell shape showed a subrounded configuration, and the cell size also significantly increased. Furthermore, *Grp78* knockout significantly decreased proliferation and adhesion activity, while increasing invasion activity. The secretion of estradiol and progesterone was also dramatically decreased after *Grp78* knockout. Our results strongly suggest that GRP78 plays an important role in maintaining the normal functionalities of GTCs.

INTRODUCTION

Trophoblast cells are the first cell type to differentiate during mammalian embryogenesis. Trophoblasts emerged during the late morula stage and acted as an envelope for the nonpolarized inner cell mass (ICM). ICM will give rise to all embryonic tissues and some of the extraembryonic membrane, while trophoblast cells will ultimately form the outer chorionic sac and the fetal component of the placenta (Baines and Renaud, 2017). Trophoblast cells play critical roles in sustaining pregnancy and supporting fetal growth and nutrition. Moreover, trophoblast cells can produce hormones, such as placental lactogen, to maintain pregnancy and to promote maternal angiogenesis (Turco and Moffett, 2019). However, the mechanisms involved in trophoblast cell differentiation, hormone secretion function, and fusion into syncytiotrophoblasts are still unclear.

Trophoblast specification at the morula stage reflects a unique combination of regulatory proteins in that zone of cells and the influence of various environmental cues on them (Roberts *et al*., 2004). For example, Eomes, a member of the T-box protein family, is a transcription factor determining trophoblast development in preimplantation mouse embryos (Russ *et al*., 2000). The Cdx-2 homeobox protein is highly expressed in trophectodermal cells before implantation (Chawengsaksophak *et al*., 1997). In addition, several other positive and negative regulators have been reported to be involved in the formation of trophoblast cells. These include NOSTRIN (Chakraborty and Ain, 2018), SOCS3 (a suppressor of cytokine signaling) (Takahashi *et al*., 2003), STAT5B and NR4A3 (Kusama *et al*., 2018). Arnaudeau *et al*. (2009) and Fradet *et al*. (2012) found that glucose-regulated protein 78 (GRP78) is highly expressed in trophoblastic cells. Their studies revealed that GRP78 is involved in the inactivation and stabilization of p53 and may function as a regulator of trophoblastic cell invasion. However, the direct role of GRP78 in the functionality of trophoblast cells has not been studied systematically.

GRP78 (also known as Bip) is a major endoplasmic reticulum (ER) chaperone protein that is critical for protein quality control of the ER. GRP78 is involved in the activation of ER transmembrane sensors, including inositol-requiring kinase 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like eukaryotic initiation factor 2α kinase (PERK) (Wang *et al*., 2009). Under normal physiological conditions, GRP78/BiP is bound to the luminal domain of each sensor. However, when misfolded proteins accumulate in the ER, GRP78 is released from the ER sensors to trigger the unfolded protein response (Trujillo-Alonso *et al*., 2011). GRP78 is a multifunctional protein that participates in various cellular processes, including translocating newly synthesized polypeptides,

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targeting misfolded proteins for proteasome degradation, and regulating calcium homeostasis (Luo *et al*., 2006). Recent studies have shown that GRP78-regulated ER stress is closely related to mammalian reproduction, such as placental development and fetal growth restriction (Kawakami *et al*., 2014), progesterone secretion, and steroidogenic enzyme expression (Park *et al*., 2014). Moreover, homozygous GRP78-null embryos do not hatch from the zona pellucida *in vitro*, fail to grow in culture, and exhibit proliferation defects and a massive increase in apoptosis in the ICM (Luo *et al*., 2006).

In this study, we aimed to investigate whether GRP78 is essential in maintaining the functionality of goat trophoblast cells (GTCs). The CRISPR/CAS9 system was used to knock out the *Grp78* gene in GTCs, and the knockout efficiency was confirmed by Western blot assay. The proliferation, adhesion, invasion, estradiol and progesterone hormone production activities of the knockout cells were compared with those of their wild-type counterparts.

MATERIALS AND METHODS

Plasmid construction

The lentiviral expression vector containing a *cas9* gene (lenti CRISPR) was obtained from Addgene (www.addgene.org). gRNAs targeting the goat *Grp78* gene were designed using the online-based tool Benchling (www.benchling.com) with "NGG" as PAM, and four high scored gRNAs were generated. After adding the restriction enzyme sites, four oligonucleotide pairs were synthesized (sequences are shown in Table I). The lentiCRISPR vector was digested with *BsmBI* and then gel purified using the Gel Extraction Kit (Qiagen). A pair of oligos for each targeting site were annealed and ligated into the linearized lenti CRISPR vector to generate the knockout plasmid.

Lentivirus packaging

A total of 1×10^6 HEK 293T cells/mL were plated 24 h before transfection. A total of 9 μg plasmid DNA (lentiCRISPR 4 μg, pVSVg 2 μg, psPAX2 3 μg) and 12 μL of Turbofect were diluted in 100 μL of Opti-MEM medium. The plasmids and Turbofect were incubated for 20 min at room temperature. The mixture of DNA and Turbofect was gently added to the medium and incubated overnight at 37 °C in a CO_2 incubator. After 16 h, the medium was changed to conditioned medium (advanced DMEM, 2% FBS, 0.01 mM cholesterol, 0.01 mM egg lecithin and 1 × chemically defined lipid concentrate). After an additional incubation for 48 h, cultures were centrifuged at 3000 g for 10 min, and the supernatant was filtered through a 0.45-μm PVDF filter (Millipore) and stored at −80 °C.

Table I. Sequences of the gRNA targeting *Grp78*.

gRNA	Forwar primer (5`-3`)
1	F5` CACCGGAAGGAGGACGTGGGCACGG 3`
	R AAACCCGTGCCCACGTCCTCCTTCC
2	F5` CACCGGTGCTGCTGCTGCTGCTCGG 3`
	R AAACCCGAGCAGCAGCAGCAGCACC
3	F5` CACCGGTTTGCGGCCTGTGGCTGG 3`
	R AAACCCAGCCACAGGCCGCAAACC
4	F5` CACCGGCTGCCTGCTGACCGACTGG 3`
	R AAACCCAGTCGGTCAGCAGGCAGCC

Cell culture and knockout mutant generation

The goat trophoblast cell (GTC) line was provided by Prof. Dewen Tong (Dong *et al*., 2013). GTCs were grown in complete DMEM/F12 medium (supplemented with penicillin and streptomycin and 10% fetal bovine serum) at 37°C with 5% CO_2 to achieve 60–70% confluence. Complete culture medium was replaced by a solution containing 2 mL of lentivirus with 2 μL (8 mg/mL) of polybrene (GeneChem). After 12 h, the lentivirus solution was replaced with complete culture medium and cultured for 48 h. Then, the medium was replaced with puromycin (2 mg/mL)-containing medium, and *Grp78* mutant cells were selected with puromycin. GTCs with decreased GRP78 expression (gRNA-1 group) were digested by trypsin to obtain single cells. Purified cultures from single cells were subjected to two more rounds of puromycin selection as described above, after which two cell lines designated KO-1 and KO-2 with significantly decreased GRP78 expression were obtained. To confirm the knockout efficiency, KO-1 and KO-2 cell lines were treated with tunicamycin (Tm). Tm is a pharmacological chaperone that strongly mounts ER stress-induced apoptosis and enhances GRP78 expression. When KO-1 and KO-2 cells reached approximately 70% confluence, Tm (1 μM) was added and incubated for 6 h. Protein was extracted, and the expression of GRP78 was detected by Western blot.

Cell morphology assay

Cells were digested with trypsin and reseeded at 2×10^4 cells/well in a standard 96-well plate, followed by additional culturing for 18 h. The cells were subsequently washed and imaged under bright field on a Zeiss AX10 microscope. Fifteen fields of view (5 replicates from 3 independent experiments) of control cells or KO-1 GTCs were analyzed using ImageJ software (NIH). For cell size calculation, cell boundaries were manually selected using "freehand selections". After all the cells were circled, the tool "Analyze-Measure" was used to calculate the cell area

(number of pixels). Cells with a clearly defined spherical and darker border (under bright field) were considered rounded. Morphology was calculated as rounded: $N_{rounded\ cells}/N_{total\ cells}$, subrounded: $N_{subrounded}/ N_{total}$ cells or other shapes: $[N_{total\ cells}-N_{rounded\ cells}- N_{subrounded}]/ N_{total\ cells.}$ Data were represented as the mean percentage ± SD.

Protein extraction and western blot analysis

GTCs were rapidly washed with ice-cold PBS. Cells were lysed on ice for 30–45 min in lysis buffer, and then the supernatant was collected after centrifugation for 10 min at 170 g at 4°C. Total protein concentration was quantified using BCA kits (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The samples were stored at −80 °C for subsequent use. Forty micrograms of lysate protein was separated by 12% SDS–PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA). Following preconditioning with 5% nonfat milk in TBST buffer, the membrane was incubated with primary antibodies against β-actin (1:1000, Tianjin Sanjian Biotech Co., Ltd., Tianjing, China) and GRP78 (1:500, Santa Cruz, USA) overnight at 4 °C. After washing with TBST containing 0.1% Tween 20, the membranes were incubated with the corresponding secondary antibody conjugated to HRP (1:2000, Zhongshan Golden Bridge Biotechnology, Nanjing, China) for one hour at room temperature. Finally, bands were visualized using a gel imaging system (Tannon Science and Technology Co. Ltd., Shanghai, China) and then digitized using Quantity One software (Bio–Rad Laboratories, Hercules, CA, USA).

Cell proliferation assay

Cells were plated into a 96-well plate (3×10^3 cells/well) and cultured for 24–96 h. MTT reagent (0.5 mg/mL, Sigma Aldrich) was added to the wells, and the cells were incubated for another 4 h at 37 °C. The supernatants were then removed, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO) (150 µL/well). The absorbance at 490 nm for each sample was measured using a plate reader (Model 680, Bio–Rad, Hercules, CA, USA).

Invasion assay

A total of 2×10^4 cells were serum-starved and then plated onto a BD Bio-Coat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA) 24-well plate with polycarbonate filters (8 µm pore size). The upper chamber contains medium without serum. The medium with 10% fetal bovine serum in the lower chamber served as a chemoattractant. The chambers were incubated for 24 h in 5% CO_2 at 37 °C. Then, the cells that did not migrate or invade through the pores were removed by cotton swabs. Finally, the cells that had migrated to the lower surface of the membrane were fixed and stained with crystal violet. Three random fields for each insert were counted under a microscope. Data are representative of three independent experiments.

Cell adhesion assay

Adhesion assays were performed in 96-well plates coated with a thin layer of Matrigel diluted with serum-free medium. Before the experiment, cells were starved for 24 h without serum. After being trypsinized and washed twice with serum-free medium, cells were allowed to attach for 2 h at 37°C, and unattached cells were washed 3 times with PBS. 10 µL (0.5 mg/mL) MTT reagent was added into each well. After 4 h incubation at 37 °C, the supernatants were removed, and the formazan crystals were dissolved in 150 µL/well DMSO. The absorbance at 490 nm for each sample was measured using a plate reader (Model 680, Bio–Rad, Hercules, CA, USA). The assay was performed in 5 technical and 3 biological replicas.

Hormone measurements

Cells were plated into 24-well plates at 1×10^5 cells/well and cultured with complete culture medium. After 24 h, the medium was replaced by serum-free medium. After 48 h, the supernatant was harvested and stored at −80 °C. Estradiol and progesterone were measured with commercial ELISA kits (Ji Yin Mei, Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Each sample was measured in triplicate.

Statistical analysis

All experiments were repeated at least 3 times. All data were expressed as the mean ± SD. Statistical analyses were performed using SPSS 17.0. Student's t-test was performed for two-group comparisons. Differences were considered significant when *p values* <0.05.

RESULTS

Grp78 was efficiently knocked out by using the CRISPR/Cas9 system

Lentiviruses carrying the lentiCRISPR vector with four different gRNA sequences were used to infect GTCs. Then, the protein level of GRP78 was detected by Western blot. The results showed that the gRNA-1 group had the lowest GRP78 expression (Fig. 1A). Then, the gRNA-1 group cells were selected with puromycin for 4 days. Two monoclonal cell lines, KO-1 and KO-2, with good knockout efficiency were obtained (Fig. 1B, C). To further verify the knockout efficiency of the KO-1 and KO-2 cell strains, Tm was added to the GTCs to stimulate GRP78 protein expression. The results showed that both cell lines

had detectable *Grp78* expression. The knockout efficiency of KO-1 was almost 100%, regardless of Tm addition (Fig. 1B, C). These results suggested that the KO-1 knockout cell line can be used for subsequent experiments. Sanger sequencing of PCR products from the KO-1 mutant genome generates double peaks that begin at the targeting site, indicating that indels were produced as expected (Fig. 1D).

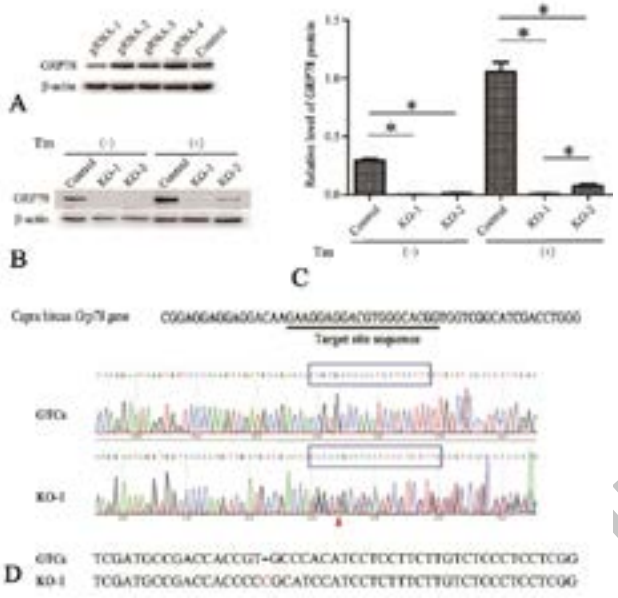

Fig. 1. CRISPR/Cas9 induces indels in the *Grp78* locus in GTCs. (A) Preliminary determination of the effect of gRNA (1–4) on GRP78 knockout in GTCs. (B-C) The expression of GRP78 in two knockout cell lines, KO-1 and KO-2. Tunicamycin (Tm) was added to the GTCs to stimulate GRP78 protein expression. (D) The position of the gRNA-1 target site and its sequence in the *Grp78* locus in Capra hircus (top). Representative Sanger sequencing results of the PCR amplicons from the GTCs and KO-1 cells. The blue box indicates the target site sequence, and the insertion is highlighted in red (middle and bottom). *p<0.05 versus control group.

Knockout of Grp78 altered GTC proliferation and invasion

By using the MTT assay, we found that knockout of *Grp78* significantly inhibited the proliferation of GTCs. Proliferation was reduced by approximately 35% at 72 hr and 27% at 96 h (Figs. 2A, 3A). The effect of *GRP78* knockout on cell invasion activity was also investigated. The results showed that *Grp78* knockout significantly increased the invasion rate of KO-1 cells compared with that of the control (Fig. 2B). Quantification of the invasion rate showed that cell invasion activity increased by 43% (Fig. 2C).

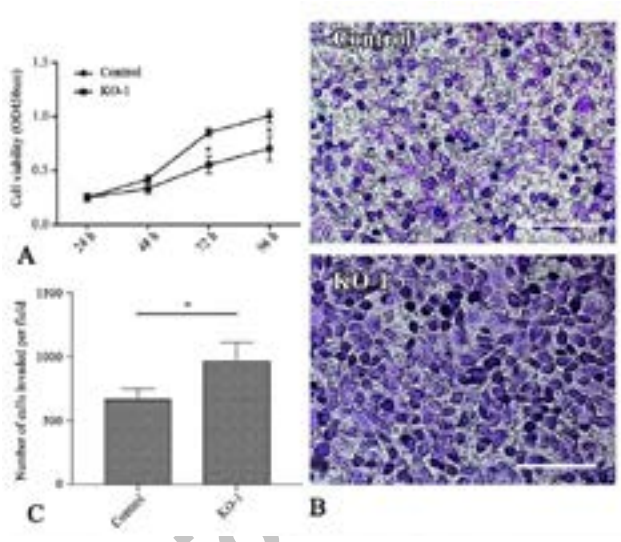

Fig. 2. Knockout of *Grp78* altered GTC proliferation and invasion. (A) The effect of *Grp78* knockout on the proliferation of GTCs. (B-C) The effect of *Grp78* knockout on the invasion potential of GTCs. Scale bar = 100 μm. *p<0.05 versus control group.

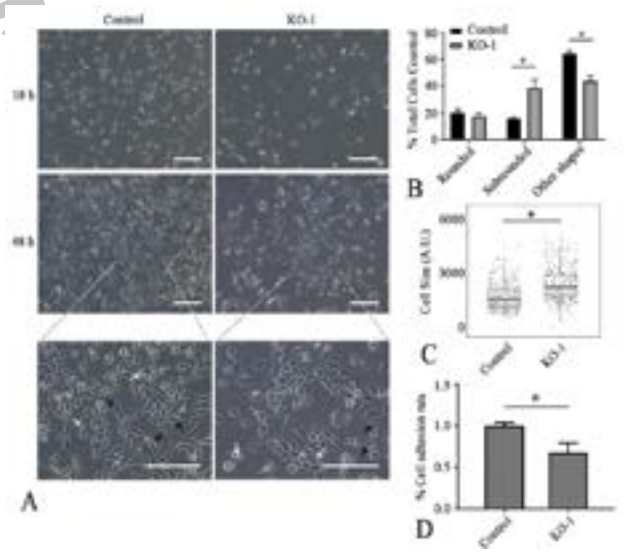

Fig. 3. Morphological and adhesion change of GTCs after knockout *Grp78*. (A) Cells were harvested at 48 hr post-transfection and re-seeded on a 24-well plate. Changes in morphology were analyzed at 18 hr and 48hr after seeding by phase-contrast microcopy. White arrows denote rounded cells, gray arrows denote subrounded cells, and black arrows denote the other shapes cells. (B) Percentage of three types of cells in the total cells observed. (C) Comparison of cell size between KO-1 cells and control cells. (D) The statistical graph of cell adhesion rates. Scale bar = 200 μm.*p<0.05 versus control group.

Knockout of Grp78 altered the morphology and adhesion properties of GTCs

Intriguingly, we found a dramatic change in the morphology of GTCs after *Grp78* was mutated. The cells changed from an outstretched shape to a subrounded configuration (Fig. 3A, B). Cell size measurements showed that the size of the GRP78 mutant was significantly increased compared with that of the control (Fig. 3C).

We then hypothesized that changes in morphology may affect the adhesion ability of GTCs. To test this hypothesis, we seeded control and KO-1 cells onto Matrigel-coated plates. Using a quantitative adhesion assay, we found that the attachment rate of KO-1 was reduced by 29% compared with that of the control (Fig. 3D).

Knockout of Grp78 disturbed the balance of hormone secretion by GTCs

Hormones secreted by trophoblast cells play important roles in reproductive function (Filant and Spencer, 2014), we measured the two main hormones estrogen and progesterone. The results showed that knockout of *Grp78* significantly decreased the secretion of these two hormones, with estrogen being reduced by 60% and progesterone being reduced by more than 92% (Fig. 4).

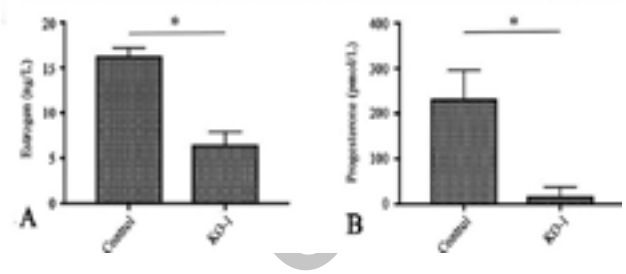

Fig. 4. Knockout of *Grp78* disturbed the endocrine balance of GTCs. *$p < 0.05$ versus control group.

DISCUSSION

Successful pregnancy is largely dependent on coordinated events taking place very early after implantation at the fetomaternal interface, in which trophoblast cells are key players (Al-Nasiry *et al*., 2006). Implantation involves attachment of the blastocyst trophectoderm to endometrial epithelial cells, followed by trophoblast invasion into the underlying endometrial stroma and eventual access to the maternal vasculature (Aplin and Ruane, 2017). This process involves the proliferation, adhesion and invasion of trophoblast cells. GRP78 plays an important role in the process of embryo implantation during early pregnancy (Liu *et al*., 2018; Luo *et al*., 2006). However, the role of GRP78 in maintaining the normal function of trophoblast cells has not been investigated directly. In the present study, we investigated the role of GRP78 in maintaining the functionality of GTCs using the CRISPR/Cas9 system. We found that knockout of GRP78 resulted in alterations in cell morphology and cell size. The mutant cells showed significantly increased invasion activity and decreased proliferation and adhesion activity, as well as hormone secretion.

The expression levels of GRP78 were positively correlated with proliferation in cancer cells and mammary epithelial cells (Liu *et al*., 2019; Luo *et al*., 2018). In the normal intestinal epithelium, knockout of Grp78 resulted in a loss of self-renewal capacity, accompanied by loss of crypt base columnar stem cells (Heijmans *et al*., 2013). These independent pieces of evidence support our conclusion that loss of GRP78 causes a significant change in the proliferation of GTCs.

Invasion is one of the most important characteristics of trophoblast cells. Several studies have found that GRP78 can affect cell invasion activity by regulating the RhoA/ROCK, JNK, and Wnt/β-catenin signaling pathways (Cultrara *et al*., 2018; Su *et al*., 2010; Xiong *et al*., 2019; Yin *et al*., 2017; Zhao *et al*., 2015). Hormone secretion is another important function of trophoblasts. Trophoblasts produce several pregnancy-specific hormones that play important roles in preparing the endometrium for implantation. Moreover, these hormones are also involved in regulating trophoblast invasion and migration (Halasz and Szekeres-Bartho, 2013; Poidatz *et al*., 2015; Robins, 2016). Some studies have confirmed that the expression of GRP78 is affected by estrogen and progesterone (Choi *et al*., 2018; Liu *et al*., 2018; Su *et al*., 2017; Xu *et al*., 2018). However, we showed that knockout of *Grp78* results in enormous estrogen and progesterone reduction. This result indicated that GRP78 in turn might regulate hormone secretion by GTCs.

In summary, our study provides evidence that GRP78 is directly involved in trophoblastic cell proliferation, adhesion, invasion and hormone secretion, which strongly suggests the importance of GRP78 in trophoblastic cells. However, further study is required to obtain a better understanding of the signaling crosstalk of GRP78 in trophoblasts.

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Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Al-Nasiry, S., Spitz, B., Hanssens, M., Luyten, C., and Pijnenborg, R., 2006. Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. *Hum. Reprod.*, **21**: 193–201. https://doi.org/10.1093/humrep/dei272

Aplin, J.D., and Ruane, P.T., 2017. Embryo-epithelium interactions during implantation at a glance. *J. Cell Sci.*, **130**: 15–22. https://doi.org/10.1242/jcs.175943

Arnaudeau, S., Arboit, P., Bischof, P., Shin-ya, K., Tomida, A., Tsuruo, T., Irion, O., and Clhen, M., 2009. Glucose-regulated protein 78: A new partner of p53 in trophoblast. *Proteomics*, **9**: 5316–5327. https://doi.org/10.1002/pmic.200800865

Baines, K.J., and Renaud, S.J., 2017. Transcription factors that regulate trophoblast development and function. *Prog. Mol. Biol. Transl. Sci.*, **145**: 39–88. https://doi.org/10.1016/bs.pmbts.2016.12.003

Chakraborty, S., and Ain, R., 2018. NOSTRIN: A novel modulator of trophoblast giant cell differentiation. *Stem Cell Res.*, **31**: 135-146. https://doi.org/10.1016/j.scr.2018.07.023

Chawengsaksophak, K., James, R., Hammond, V., Köntgen, F., and Beck, F., 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature*, **386**: 84–87. https://doi.org/10.1038/386084a0

Choi, J.Y., Jo, M.W., Lee, E.Y., Lee, D.Y., and Choi, D.S., 2018. Ovarian steroid dependence of endoplasmic reticulum stress involvement in endometrial cell apoptosis during the human endometrial cycle. *Reproduction*, **155**: 493–503. https://doi.org/10.1530/REP-17-0713

Cultrara, C.N., Kozuch, S.D., Ramasundaram, P., Heller, C.J., Shah, S., Beck, A.E., Sabatino, D., and Zilberberg, J., 2018. GRP78 modulates cell adhesion markers in prostate Cancer and multiple myeloma cell lines. *BMC Cancer*, **18**: 1263. https://doi.org/10.1186/s12885-018-5178-8

Dong, F., Huang, Y., Li, W., Zhao, X., Zhang, W., Du, Q., Zhang, H., Song, X., and Tong, D., 2013. The isolation and characterization of a telomerase immortalized goat trophoblast cell line. *Placenta*, **34**: 1243–1250. https://doi.org/10.1016/j.placenta.2013.09.009

Filant, J., and Spencer, T., 2014. Uterine glands: biological roles in conceptus implantation, uterine receptivity and decidualization. *Int. J. Dev. Biol.*, **58**: 107–116. https://doi.org/10.1387/ijdb.130344ts

Fradet, S., Pierredon, S., Ribaux, P., Epiney, M., Shin, Ya, K., Irion, O., and Cohen, M., 2012. Involvement of membrane GRP78 in trophoblastic cell fusion. *PLoS One*, **7**: e40596. https://doi.org/10.1371/journal.pone.0040596

Halasz, M., and Szekeres-Bartho, J., 2013. The role of progesterone in implantation and trophoblast invasion. *J. Reprod. Immunol.*, **97**: 43–50. https://doi.org/10.1016/j.jri.2012.10.011

Heijmans, J., van Lidth de Jeude, J.F., Koo, B.K., Rosekrans, S.L., Wielenga, M.C., van de Wetering, M., Ferrante, M., Lee, A.S., Onderwater, J.J., Paton, J.C., Paton, A.W., Mommaas, A.M., Kodach, L.L., Hardwick, J.C., Hommes, D.W., Clevers, H., Muncan, V., and van den Brink, G.R., 2013. ER stress causes rapid loss of intestinal epithelial stemness through activation of the unfolded protein response. *Cell Rep.*, **3**: 1128–1139. https://doi.org/10.1016/j.celrep.2013.02.031

Kawakami, T., Yoshimi, M., Kadota, Y., Inoue, M., Sato, M., and Suzuki, S., 2014. Prolonged endoplasmic reticulum stress alters placental morphology and causes low birth weight. *Toxicol. appl. Pharmacol.*, **275**: 134–144. https://doi.org/10.1016/j.taap.2013.12.008

Kusama, K., Bai, R., and Imakawa, K., 2018. Regulation of human trophoblast cell syncytialization by transcription factors STAT5B and NR4A3. *J. Cell Biochem.*, **119**: 4918-4927. https://doi.org/10.1002/jcb.26721

Liu, L., Zhang, Y., Wang, Y., Peng, W., Zhang, N., and Ye, Y., 2018. Progesterone inhibited endoplasmic reticulum stress associated apoptosis induced by interleukin-1β via the GRP78/PERK/CHOP pathway in BeWo cells. *J. Obstet. Gynaecol. Res.*, **44**: 463–473. https://doi.org/10.1111/jog.13549

Liu, Y., Wang, X., Zhen, Z., Yu, Y., Qiu, Y., and Xiang, W., 2019. GRP78 regulates milk biosynthesis and the proliferation of bovinemammaryepithelial cells through the mTOR signaling pathway. *Cell Mol. Biol. Lett.*, **24**: 57. https://doi.org/10.1186/s11658-019-0181-x

Luo, C., Xiong, H., Chen, L., Liu, X., Zou, S., Guan, J., and Wang, K., 2018. GRP78 Promotes Hepatocellular Carcinoma proliferation by increasing FAT10 expression through the NF-κB pathway. *Exp. Cell Res.*, **365**: 1–11. https://doi.org/10.1016/j.yexcr.2018.02.007

Luo, S., Mao, C., Lee, B., and Lee, A.S., 2006. GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early

mouse embryonic development. *Mol. Cell Biol.,* **26**: 5688–5697. https://doi.org/10.1128/MCB.00779-06

Park, H.J., Park, S.J., Koo, D.B., Lee, S.R., Kong, I.K., Ryoo, J.W., Park, Y.I., Chang, K.T., and Lee, D.S., 2014. Progesterone production is affected by unfolded protein response (UPR) signaling during the luteal phase in mice. *Life Sci*., **113**: 60–67. https://doi.org/10.1016/j.lfs.2014.07.033

Poidatz, D., Dos Santos, E., Gronier, H., Vialard, F., Maury, B., De Mazancourt, P., and Dieudonné, M.N., 2015. Trophoblast syncytialisation necessitates mitochondrial function through estrogen-related receptor-γ activation. *Mol. Hum. Reprod.,* **21**: 206–216. https://doi.org/10.1093/molehr/gau102

Roberts, R.M., Ezashi, T., and Das, P., 2004. Trophoblast gene expression: Transcription factors in the specification of early trophoblast. *Reprod. Biol. Endocrinol*., **2**: 47. https://doi.org/10.1186/1477-7827-2-47

Robins, J.C., 2016. Implantation: Trophoblast-endometrial interactions. *Semin. Reprod. Med.,* **34**: 3–4. https://doi.org/10.1055/s-0035-1570034

Russ, A.P., Wattler, S., Colledge, W.H., Aparicio, S.A., Carlton, M.B., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehls, M.C., Wilson, V., and Evans, M.J., 2000. Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature,* **404**: 95–99. https://doi.org/10.1038/35003601

Su, Q., Wang, Y., Yang, X., Li, X.D., Qi, Y.F., He, X.J. and Wang, Y.J., 2017. Inhibition of endoplasmic reticulum stress apoptosis by estrogen protects human umbilical vein endothelial cells through the PI3 Kinase-Akt signaling pathway. *J. Cell Biochem*., **118**: 4568–4574. https://doi.org/10.1002/jcb.26120

Su, R., Li, Z., Li, H., Song, H., Bao, C., Wei. J., and Cheng, L., 2010. *Grp78* promotes the invasion of hepatocellular carcinoma. *BMC Cancer*, **10**: 20. https://doi.org/10.1186/1471-2407-10-20

Takahashi, Y., Carpino, N., Cross, J.C., Torres, M., Parganas, E., and Ihle, J.N., 2003. SOCS3: An essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *EMBO J*., **22**: 372–384. https://doi.org/10.1093/emboj/cdg057

Trujillo-Alonso, V., Maruri-Avidal, L., Arias, C.F., and López, S., 2011. Rotavirus infection induces the unfolded protein response of the cell and controls it through the nonstructural protein NSP3. *J. Virol.,* **85**: 12594–12604. https://doi.org/10.1128/JVI.05620-11

Turco, M.Y., and Moffett, A., 2019. Development of the human placenta. *Development*, **146**. pii:dev163428. https://doi.org/10.1242/dev.163428

Wang, M., Wey, S., Zhang, Y., Ye, R., and Lee, A.S., 2009. Role of the unfolded protein response regulator GRP78/BiP in development, cancer, and neurological disorders. *Antioxid. Redox. Signal*., **11**: 2307–2316. https://doi.org/10.1089/ars.2009.2485

Xiong, H., Xiao, H., Luo, C., Chen, L., Liu, X., Hu, Z., Zou, S., Guan, J., Yang, D., and Wang, K., 2019. GRP78 activates the Wnt/HOXB9 pathway to promote invasion and metastasis of hepatocellular carcinoma by chaperoning LRP6. *Exp. Cell Res*., **383**: 111493. https://doi.org/10.1016/j.yexcr.2019.07.006

Xu, F., Ma, R., Zhang, G., Wang, S., Yin, J., Wang, E., Xiong, E., Zhang, Q., and Li, Y., 2018. Estrogen and propofol combination therapy inhibits endoplasmic reticulum stress and remarkably attenuates cerebral ischemia-reperfusion injury and OGD injury in hippocampus. *Biomed. Pharmacother*., **108**: 1596–1606. https://doi.org/10.1016/j.biopha.2018.09.167

Yin, Y., Chen, C., Chen, J., Zhan, R., Zhang, Q., Xu, X., Li, D., and Li, M., 2017. Cell surface GRP78 facilitates hepatoma cells proliferation and migration by activating IGF-IR. *Cell Signal*., **35**: 154–162. https://doi.org/10.1016/j.cellsig.2017.04.003

Zhao, G., Kang, J., Jiao, K., Xu, G., Yang, L., Tang, S., Zhang, H., Wang, Y., Nie, Y., Wu, K., Fan, D., Zhang, H., and Zhang, D., 2015. High expression of GRP78 promotes invasion and metastases in patients with esophageal squamous cell carcinoma. *Dig. Dis. Sci*., **60**: 2690–2699. https://doi.org/10.1007/s10620-015-3689-6