Dysfunction of Shh signaling impaired trophoblast motility and autophagy is involved in poor placentation of recurrent miscarriage

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Research

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Abstract

Background: In early pregnancy, the placenta anchors the conceptus and supports embryonic development and survival. This study aimed to investigate the underlying functions of Shh signaling on recurrent miscarriage, an serious disorder of pregnancy.

Methods: Immunofluorescence and immunohistochemistry were used to detect protein expression and its location in placental tissues. Quantitative real-time RT-PCR and Western blot analysis were performed to examine mRNA and protein levels, respectively. Lentiviruses expressing short hairpin RNA were used to knock down the target genes. Cell invasion and migration were performed by with or without Matrigel-coated transwell, respectively. Primary trophoblast migration was performed by villous explant assay. RNA-sequence was used to investigate the genes transcription profile. CCK-8 assay was used to evaluate cell viability. Flow cytometry was used to evaluate cell apoptosis.

Results: Our results showed that Shh and Gli2 were mainly located in cytotrophoblasts (CTBs), Ptch was mainly located in syncytiotrophoblasts (STBs), while Smo and Gli3 were expressed in both CTBs and STBs. Compared to the gestational age-matched normal human placenta, the expression of Shh was significantly decreased in recurrent miscarriage. Furthermore, inhibition of Shh signaling impaired motility of JAR cells via regulating the expression of Gli2 and Gli3 to decrease AKT Ser473 phosphorylation, elevate E-cadherin and VEGFA. Intriguingly, inhibition of Shh signaling also enhanced autophagy and autolysosome. Additionally, knockdown BECN1 reversed the effect of Gant61 on motility inhibition.

Conclusion: Our results indicated that dysfunction of Shh signaling impaired trophoblast motility, angiogenesis and activated autophagy in villous trophoblast, which would contribute to the pathophysiology of recurrent miscarriage.

Background

In mammals, after zygote dividing and developing into blastocyst, outer cells of blastocyst become polarization and firstly differentiate into trophoblast, which would further differentiate and form the placenta. Inner cells of blastocyst divide into inner cell mass, which would further differentiate and form the embryo proper. The human placenta serves as the fetomaternal material exchanging barrier and protective shield to give rise to fetus development. Physiologically, trophoblast progenitor cells differentiate into CTBs. CTBs either differentiate into invasive lineage to yields extravillous trophoblasts (EVTs), or undergo cell fusion to yields STBs[1]. EVT would further subdivide according to their anatomical location and degree of differentiation[2]. Invading the decidualized endometrium called interstitial EVTs, which play a pivotal role in implantation [3]. Invading and remodeling the spiral arteries called endovascular EVTs. Other subtypes have also been detected in uterine glands, veins, and lymphatics[4, 5]. The integrated proliferation and differentiation of all these trophoblast lineages are essential for normal placental development in a successful pregnancy. Impaired trophoblast development

is always associated with some severe pregnancy complications including miscarriage, preeclampsia, and intrauterine growth restriction[6–8].

Recurrent miscarriage (recurrent miscarriage) is a common pregnancy-related complication and defined as over two consecutive and unintentional miscarriages, which affects more than 5% of reproductive-aged women[9]. The pathogeny of recurrent miscarriage has remained as a complex context, at least including hereditary miscarriage, disorder of hormones, abnormal uterus and cervix anatomical conditions, autoimmune disorders, infections of uterus and cervix[10–15]. However, the signaling network that involved in recurrent miscarriage is not clearly understood. Recently study demonstrated that early miscarriage was mainly due to dysfunction of cytotrophoblast, including abnormal trophoblastic proliferation and reduction of endovascular EVT's penetration[16]. Low expression of MFN2 caused dysfunction of trophoblast cells, including aberrant activation of autophagy and mitochondrial damage, which in turn to be accounted for early unexplained miscarriage[17]. Activities of ERK and AKT signalings were significantly suppressed in the recurrent miscarriage patients, yet the downstream effectors of which remained unknown[18]. The miR-27a-3p/USP25 axis regulated trophoblast EMT process and might become a biomarker for recurrent miscarriage[19]. The expression of peroxiredoxin2 regulating trophoblast proliferation and apoptosis was decreased in recurrent miscarriage[20]. Enhancer of zeste homolog 2 (EZH2), known as an epigenetic factor that promoted trophoblast invasion, was also downregulated in recurrent miscarriage[21]. Thus, these findings indicated that initiation and progression of recurrent miscarriage is a complex context that involving in multiply signalings and steps dysfunctions on development of trophoblast and placentation.

Sonic hedgehog (Shh) together with Indian hedgehog (Ihh) and Desert hedgehog (Dhh) consist of the ligands of Hedgehog (Hh) signaling pathway, which plays pivotal roles in regulating cell proliferation, cell differentiation, organogenesis, and development, even involved in tumorigenesis and progression[22, 23]. Previous study had summarized and indicated that Hh signaling played important roles in regulating hematopoiesis, vasculogenesis and angiogenesis during embryogenesis and development[24, 25]. Moreover, Shh might crosstalk with TGF/SMAD signaling pathway to promoted G-CSF mobilized human CD34 + cell migration, proliferation and then differentiation into vascular cells during embryonic vascular development[26]. Our previous study found that Shh together with Gli2/3 was required for the proper development of placental and pregnant maintenance[27]. In addition, we also found that Hh signaling through GLI1/2 promoted EMT process of human trophoblast cells via transcriptional suppression of CDH1 gene[28]. However, the relationship between Shh and recurrent miscarriage, as well as the underlying functions of Shh on recurrent miscarriage, have not been investigated.

Autophagy is a lysosomal degradative process, which contributes to maintain development and homeostasis[29–31]. Recent studies have reported that Hh signaling can regulate autophagy in various cells, while functions of Hh signaling on autophagy were controversial. Inhibition of the Hh signaling can induce autophagy in Drosophila, HeLa cells, and human hepatocellular carcinoma cells[32, 33]. However, the Hh signaling acts as a positive regulator in autophagy of vascular smooth cells and hippocampal neurons[34, 35]. Additionally, the combined inhibition of Hh signaling and autophagy can overcome

chronic myeloid leukemia drug resistance, which was associated with PARP cleavage, CASP3 and CASP9 cleavage, and the BCR-ABL oncoprotein[36]. Other studies showed that the autophagy was highly activated in EVT and placenta, and its disruption associated with preeclampsia and intrauterine growth restriction[37–41]. However, the effect of autophagy on recurrent miscarriage still remains largely elusive.

In the present study, we investigated the potential role of Shh/Gli signaling and autophagy in recurrent miscarriage. Our results showed that Shh signaling was attenuated in the placenta of recurrent miscarriage patients, dysfunction of Shh/Gli impaired trophoblast migration and angiogenesis. Moreover, inhibition of Shh signaling enhanced autophagy, while inhibition of autophagy would reverse Gant-6 induced inhibition of trophoblast motility. Thus, these findings gave rise to an important implication for the pathological role of Shh signaling and autophagy in recurrent miscarriage and might provide potential targets for recurrent miscarriage therapy.

Material And Method

3.1 Preparation of placental tissues

Placental villi tissues were isolated from normal pregnant women who were voluntary to abortion by themselves (n = 10) between the age 27 and 40 years and the control group had an abortion at 49–82 days of gestation. Recurrent miscarriage patients (n = 10) between the ages of 24 and 37 years (mean age 32.2 ± 5.45 years) at 33 and 66 days of gestation (mean gestational age 44.2 ± 12.83 days) were recruited for this study. None of them had any risk factors such as genetic abnormalities (neither themselves nor their husbands), uterine malformation, thyroid dysfunction, anti-phospholipid antibody syndrome. All samples were stored at -80°C for further use or stored in 4% formaldehyde at room temperature overnight for immunohistochemistry analysis. The study protocol was approved by the ethics committee of the Sir Run Run Shaw Hospital, Zhejiang University School of Medicine.

3.2 First-trimester villous explant and Cell line culture

Villous explant cultures were captured from first-trimester (6–9 weeks of gestation) as described previously[62]. In brief, 8–10 explants (2–3 mm) from tips of the placental villi were dissected and explanted in 24-well culture dish pre-coated with 5 mg/ml Matrigel for 30 min at 37°C . Allowing the anchorage for 2 to 4 hours, tissues were carefully covered with 0.5 ml serum-free DMEM/F12 medium and incubated for next 24 hours. The explants with successful attachment would be selected for the treatment and photographed. The outgrowth of trophoblasts were measured with by Image J software from National Institutes of Health (<http://rsb.info.nih.gov/ij/download.html>)

JAR cells (a gift from Dr. Hai-Tao Pan, Shaoxing women and children's hospital) were used in this study. The complete growth medium for this cell line is adding 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin (Gibco by Life Technologies, USA) to the basic RPMI-1640 medium. This cell line was incubated at 37°C with 5% CO_2 .

3.3 Chemicals, Reagents and Oligonucleotides

Cyclopamine (Tocris,1623, USA), recombinant human Shh (R&D,1845-SH), chloroquine (Sigma, C6628), GANT61 (Selleck Chemicals,S8075), Lipofectamine 3000 (Thermo Fisher Scientific, L3000015), Gli2 shRNA1 (5'-GUACCAUUACGAGCCUCAUUC-3'), Gli2 shRNA2 (5'-CAACGCCCCCCCACCCGUAC-3'), Gli3 shRNA1 (5'-UUGAAGGUUGCACAAAGGC-3'), Gli3 shRNA2 (5'-AAGAGAUUAAACUGACUUU-3'), BECN1 shRNA1 (5'-GGATGACA GTGAACAGTTA-3'), BECN1 shRNA2 (5'-CCCGTGGAATGGAATGAGA-3').

3.4 Cell counting kit-8 assay

JAR Cells were seeded into 96-well plates at 1000 cells/well and cultured for 24 hours, then culture medium was replaced with fresh complete growth medium plus a final concentration of 5 μ M cyclopamine (Tocris,1623, USA) or 0.5 μ g/ml recombinant human Shh (R&D,1845-SH), complete growth medium plus equal volume alcohol was set as negative control. The maintenance medium was refreshed every two days with the same formulation as previous treatment. After the indicated timepoint treatment, 10 μ l cell counting kit-8 (Dojindo, Japan) solution was added to each well and then incubated for 1 hour, cellular vitality was measured by SpectraMax M5 (Molecular Devices, USA) according to the manufacturer's instructions.

3.5 Flow cytometry for Apoptosis assay

JAR Cells were seeded into 60 mm disks at 5×10^5 cells/well and cultured for 24 hours, culture medium was then replaced with fresh complete growth medium plus a final concentration of 5 μ M cyclopamine or 0.5 μ g/ml recombinant human Shh and culture for the next 24 hours, complete growth medium plus equal volume alcohol was set as negative control. At the end point of treatment, cells were gently detached by TrypLE™ Express Enzyme (1 $\times$) (Gibco by Life Technologies, USA). Cells were washed and resuspended with 100 μ l binding buffer, 5 μ l PE-conjugated Annexin V and 5 μ l 7-AAD were immediately added and incubated for 15 minutes in the dark at room temperature. After incubation, all samples were added another 400 μ l binding buffer and immediately quantified using the FACSCanto II flow cytometer (BD Biosciences, USA) according to the manufacturer's instructions.

3.6 Cell migration and invasion assay

For migration assay, JAR Cells were resuspended with 100 μ l basic RPMI-1640 medium and seeded into 24-well transwell polystyrene plates (COSTAR, USA) at 3×10^5 cells/well. For invasion assay, JAR Cells were seeded into 24-well transwell polystyrene plates which pre-coated matrigel (BD, USA). After transfecting with various shRNA (scramble shRNA, shGli2, shGli3 or shBeclin1), transfected cells were divided into three groups and add another 100 μ l basic RPMI-1640 medium plus a final concentration of 5 μ M cyclopamine or 0.5 μ g/ml recombinant human Shh to each well, basic RPMI-1640 medium plus equal volume alcohol was set as a negative control. The lower chambers were loaded fresh 600 μ l complete grown medium into each well. After culture for 24 hours, cells within inner insert were wiped by cotton sticks, migrated cells were fixed with 75% alcohol for 10 minutes at room temperature and then stained by 0.1% crystal violet. After washing with PBS twice, migrated cells were captured by microscope and each image was analyzed by Image J software.

3.7 Western blot

Western blot was performed as described previously[27]. Briefly, tissue or cellular samples were lysed on ice by using RIPA lysis buffer containing 1% PMSF (Beyotime, China). 40 µg total protein samples were separated to SDS-PAGE and then transferred onto a PVDF membrane. primary antibodies were used as following: anti-E-Cadherin (Cell Signaling, 3195), anti-VEGFA (Abcam, ab46154), anti-Sonic Hedgehog (Abcam, ab53281), anti-Gli2 (Abcam, ab2605556), anti-Gli3 (Abcam, ab6050), anti-p-AKT T308 (Cell Signaling, 4056), anti-p-AKT S473 (Cell Signaling, 4046), anti-pan-AKT (Cell Signaling, 4691), Beclin1 (Cell Signaling, 3495), anti-LC3B (Sigma-Aldrich, L7543). GAPDH (Proteintech, 600004-1-Ig) was used as internal standards. Immunofluorescent anti-rabbit and anti-mouse second antibody were purchased from LI-COR Bioscience (Lincoln, USA), and the signals were visualized with Odyssey Infrared Imaging System (Lincoln, USA). ImageJ software was used to quantify the immunoreactive bands.

3.8 Immunohistochemistry

For paraffin-embedded sections, samples were normally deparaffinized and rehydrated in xylene and a graded series of ethanol, antigen retrieval of samples were boiled in citric acid buffer (pH 6.0), and then incubated with 3% H₂O₂ to suppress the activity of endogenous peroxidase. Samples were further blocked in 10% normal serum with 1% BSA in TBS for 1 hour at room temperature, and then incubated with primary antibodies overnight at 4 °C. Then, the sections were stained with the HRP-conjugated secondary antibodies for 30 min, followed with incubation of diaminobenzidine (DAB) solution, and counterstained with hematoxylin. Primary antibodies used in immunohistochemistry were as following: anti-Sonic Hedgehog (Abcam, ab53281), anti-Cytokeratin 7 (Gene Tech, GM701802), anti-VEGFA (Abcam, ab51745), CD31 (Abcam, ab134168).

For frozen sections or cells grown on glass coverslips, samples were incubated in 0.3% Triton X-100 for 10 min, blocked in 5% goat serum for 1 hour at room temperature, and incubated with primary antibodies overnight at 4 °C. Then, the sections were incubated with fluorescent secondary antibodies for 1 hour at room temperature in the dark, mounted with a drop of mounting medium containing DAPI. The images were captured by Olympus BX53 fluorescence microscope, and then analyzed by ImageJ software. Primary antibodies used in this part were as follows: anti-Sonic Hedgehog (Abcam, ab53281), anti-Ptc1 (Abcam, ab109096), anti-Smo (Abcam, ab72130), anti-Gli1 (Abcam, ab92611), anti-Gli2 (Abcam, ab26056), anti-Gli3 (Abcam, ab6050), anti-Cytokeratin 7 (CK7), anti-11 β-HSD2 (Santa Cruz, sc-365529), anti-LC3B (Sigma-Aldrich, L7543), anti-LAMP1 (Abcam, ab25630).

3.9 RNA-seq

JAR Cells were seeded into 10 cm cell culture dish and treated with or without 5 µM Cyclopamine (Biomol International, USA) for 24 h. Total RNA of each group was extracted by TRIzol (Invitrogen) reagent. Then, samples were processed and performed RNA-seq by the Annoroad Gene Technology company (Beijing, China). The analysis platform was the Illumina HiSeq 2500 platform with a 101-bp paired-end sequencing strategy. The complete array dataset has been deposited into Gene Expression Omnibus (GEO accession number: GSE130367, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?>

acc=GSE130367). Gene Set Enrichment Analysis (GSEA, <http://gsea.org/>) was performed to identify the functions and associated enriched pathways of differentially expressed mRNAs.

3.10 Analysis of GFP-mCherry-LC3 puncta

For autophagosome maturation assays, JAR cells were transfected with GFP-mCherry-LC3 adenovirus vector (5×10^9 pfu/ml) more than 24 h and treated with or without Hh antagonist chloroquine (CQ) for 24 hours. As the GFP fluorescence is diminished in the acidic autolysosomes while mCherry can be visualized. GFP-mCherry-LC3 puncta were captured by Olympus BX53 fluorescence microscope, and then quantified ImageJ software as described[64].

3.11 Statistical analysis

All the numerous data are expressed as the mean $\pm$ S.E.M, and were analyzed by Student's t-test or one-way ANOVA (SPSS 13.0J software; SPSS, Inc., Chicago, IL, USA). Statistical significance was assessed at $P \leq 0.05$. Experiments were independently triplicated, and results were qualitatively identical. Representative experiments are shown.

Results

4.1 Shh signaling was attenuated in recurrent miscarriage

We first investigated the expression and cellular localization of Shh signaling core members in villous tissue from first-trimester placentas. 11 β -HSD2 served as a marker of STB layer and played important roles in fetus development[42]. Our results showed that both Shh and Smo were expressed in STB layer and CTB layer, but Shh mainly located in CTB layer. Otherwise, Ptch mainly located in STB layer (Fig. 1A). Compared to normal human placental villi (the healthy control), Shh was decreased in recurrent miscarriage patients' villous tissues (Fig. 1B, 1C). This result was also confirmed by immunoblotted Shh in villous tissues from the healthy control and recurrent miscarriage patients (Fig. 1D). Moreover, CK7, the marker of trophoblast, was decreased in recurrent miscarriage patients' villous tissues (Fig. 1E, 1F), and the thickness of CTB layer were significantly decreased in recurrent miscarriage patients while compared to the healthy control (Fig. 1E, 1G). Thus, Shh signaling was impaired in recurrent miscarriage patients' villous tissues.

4.2 Attenuating Shh signaling inhibited trophoblast motility and placental angiogenesis

To study the role of Shh signaling in trophoblast motility, matrigel-coated cell invasion and transwell cell migration models were performed. Our results showed that Smo antagonist cyclopamine (Cyc) significantly decreased 2-fold migration and 1.67-fold invasion in JAR cells while compared to control group ($p \leq 0.01$ and $p \leq 0.05$, respectively) (Fig. 2A-2C). We then investigated the effect of cyclopamine or recombinant human Shh (rShh) on JAR cells' viability. Our results showed that 5 μ M cyclopamine

0.5 µg/ml rShh had no effect on JAR cells' proliferation or apoptosis while compared to control group (Figure S1). Besides, cyclopamine significantly decreased outgrowth of invasive extravillous trophoblasts in first-trimester villous explant cultures seeded on collagen I (Fig. 2D, 2E). On the molecular event, cyclopamine significantly decreased phosphorylation of AKT S473 site, but not AKT T308 site (Fig. 2F). VEGF-A is predominantly important in vasculogenesis during placental development. Our results showed that cyclopamine significantly decreased VEGF-A, while significantly elevated E-cadherin expression in JAR cells (Fig. 2G). Moreover, VEGF-A and CD31 expression were significantly decreased in recurrent miscarriage, while VEGF-A was strongly expressed in trophoblast, and CD31 was strongly expressed in the fetal vessels in villous tissue from healthy controls ($p \leq 0.001$ and $p \leq 0.001$, respectively) (Fig. 2H-2K). These results indicated that downregulation of trophoblast motility might account for aberrant placental vessel regression in recurrent miscarriage patients.

4.3 Shh signaling regulated motility of JAR cells via Gli2 and Gli3

To identify downstream factors of Shh signaling on regulation in motility of JAR cells, we first investigated expression of Gli in normal human first-trimester placenta tissues. Our results showed that Gli1 was weakly detected in STB layer, while Gli2 was strongly located in CTB layer, Gli3 was strongly expressed in both STB layer and CTB layer (Fig. 3A). We previously study has showed that Hh regulated EMT in JEG3 cells through Gli1 and Gli2[43]. Herein, we successfully stably downregulated expression of Gli2 and Gli3 in JAR cells for further experiments (Fig. 3B, 3C). Gli2 knockdown significantly inhibited migration and invasion of JAR cells, while Gli3 knockdown alone or recombinant Shh treatment significantly promoted migration and invasion of JAR cells. Moreover, Gli2 knockdown would partly impair recombinant Shh-induced migration and invasion of JAR cells (Fig. 3D-3F).

4.4 Transcriptome Profiling of JAR treated with Cyclopamine

To further screen the cyclopamine-regulated target gene in JAR cells, we analyzed the transcriptome profile of JAR cells with or without cyclopamine treatment. RNA-seq data identified 1797 significant changing genes ($P \leq 0.05$, $q \leq 0.05$, fold change ≥ 2), and 876 genes were up-regulated, 921 genes were down-regulated (Fig. 4A). Gene Set Enrichment Analysis showed that hypoxia, apical junction and EMT as the top three significantly associated pathway, overall enrichment score of which were 0.47, 0.46 and 0.43, respectively. Normalized enrichment score of which were 2.88, 2.51 and 2.51, respectively (nominal p -value ≤ 0.001 , false discovery rate [FDR] ≤ 0.001) (Fig. 4B-4D).

4.5 Inhibition of Shh signaling induces autophagy and autolysosome

previous studies showed a close relationship between Shh signaling pathway and autophagy in normal development and tumorigenesis[32–34, 44], we reasoned that this relationship between Shh signaling and autophagy might involve in initiation and progression of recurrent miscarriage. Our results showed

that cyclopamine and Gant61 significantly elevated the ratio of LC3-II/LC3-I protein level in a dose-dependent manner, and this accumulation was enhanced by lysosomal inhibitor chloroquine (CQ) treatment (Fig. 5A, 5B). Gant61 increased the number of mCherry + GFP + yellow puncta (indicating co-localization of mCherry and GFP) in both CQ-treated and untreated JAR cells (Fig. 5C, 5D). Besides, the overlap of LC3B and LAMP1 puncta (the indicator for autophagosome-lysosome fusion) was increased in Gant61-treated JAR cells (Fig. 5E, 5F).

4.6 Downregulation BECN1 rescued the Gant61-induced inhibition of cell motility in JAR cells

To further investigate the potential role of autophagy on Gant61-induced inhibition of cell motility, we used siRNA to knockdown BECN1, the core autophagy molecule, which as a part of PI3K complex and regulated the localization of other autophagy proteins to phagophores[45, 46]. BECN1 was successfully knockdown in JAR cells (Fig. 6A). Downregulation BECN1 significantly reversed Gant61-induced inhibition of migration and invasion in JAR cells (Fig. 6B-6D). These data showed a critical role of BECN1 on regulating Gant61-induced inhibition of JAR cells' motility.

Discussion

Accumulating promising efforts have been made to improve the prognosis and treatment of unexplained recurrent miscarriages[47, 48]. However, the causes and pathophysiology of recurrent miscarriage remain largely elusive. Previous studies so far have focused on abnormal trophoblast development and placentation in recurrent miscarriage[13, 19–21, 23, 24]. Hh signaling was essential for hematopoiesis, vasculogenesis and angiogenesis during embryogenesis and development[24, 25]. Our previous studies have indicated that Hh signaling played pivotal roles in the development of placental and pregnant maintenance[27, 28]. Besides, in the clinic context, early miscarriage patients have several common traits, including deficient trophoblastic invasion, decreasing thickness of CTB layer, deficient myometrial spiral artery remodeling[16, 49, 50]. Thus, it rationally prompted us to further explore the underlying relationship between the Shh signaling and recurrent miscarriage. In the present study, our results uncovered a crosstalk between Shh signaling and autophagy on regulating trophoblast motility (Fig. 8). Thus this study suggested that dysfunction of Shh signaling inhibited the trophoblast motility through regulating autophagy and VEGFA, which subsequently resulted in recurrent miscarriage.

Our previous studies have indicated that Hh signaling was required for EMT process of human trophoblast cells, the pregnant maintenance, placental development[27, 28, 51–53]. Thus, in the present study, we firstly investigated the expression of Shh signaling core members in health human placental villi, our results showed that the Shh/Ptch/Smo/Gli2/Gli3 signaling axis preferred to be activated in CTB layer, and Shh protein was decreased in villous tissue of recurrent miscarriage patients while compared to the healthy control. Recently, the other study indicated that both previous miscarriages and single nucleotide polymorphisms (SNPs) rs3738880 in Gli2 were associated with anorectal malformations, though the relationship between miscarriages and SNP rs3738880 in Gli2 remains unknown[54]. Besides,

conditionally deleted of Smo in the mouse uterus impairs implantation and subsequent pregnancy loss[55].

During placental development, migration and invasion of trophoblast cells were essential for placental angiogenesis[3, 56]. In the molecular events, PAPP-A2 attenuated HTR8/SVneo trophoblast migration and invasion via decreasing expression of Gli1/2, Snail, Slug, N-Cadherin and Vimentin, while increasing expression of E-Cadherin and ZO-1[56]. Our previous study also showed that Gli1/2 induced expression of Snail, Slug and Twist1, while suppressed expression of E-Cadherin to promote the migration of JEG3 trophoblast cells[28]. Otherwise, The other study had shown that rosiglitazone increased phosphorylation of AKT to promote endothelial cell migration[57]. In the present study, our results further showed Shh-Gli2/Gli3 played an important role in JAR migration and invasion. Besides, our results showed that inhibition of Shh signaling would decrease phosphorylation of AKT while increase expression of E-cadherin in JAR cells. Thus, our results might provide an alternative mechanism that inactivation of Shh signaling attenuated migration and invasion of JAR cells via decreasing phosphorylation of AKT S473 site while increasing expression of E-cadherin. Moreover, It's reported that the inhibition of Hh pathway induced autophagy through downregulating the AKT-MTOR pathway[58]. In the present study, our results showed that there was no significantly change of autophagy-related genes upon cyclopamine treatment by using RNA-seq. However, inhibition of Shh signaling promoted autophagosome maturation. Besides, our results further showed that inhibiting autophagy by Beclin1 knockdown would reverse Gant61-induced the motility inhibition of JAR cells. It suggested that Shh signaling interplayed with autophagy in regulating trophoblast motility.

Accumulating studies have been reported that Hh signaling and VEGF-A were required for placental angiogenesis[24, 25, 59–61]. In the present study, our results also showed that inhibition of Shh signaling decreased the expression of VEGF-A in JAR cells. Additionally, we found that VEGF-A and CD31 were downregulated in villous tissue of recurrent miscarriage patients while compared to the health control. Thus, these results suggested poor vascular placentation in recurrent miscarriage patients.

In conclusion, we provided an alternative mechanism of Shh signaling in regulating trophoblast cells motility through AKT and VEGFA, which subsequently played an important role in placentation and vascularization in recurrent miscarriage patients. Besides, we found that Gant61 could induce autophagy and autolysosome in trophoblast cells, and Beclin1 responded to Gant61-induced the motility inhibition. Taken these together, our results firstly showed that Shh signaling interplayed with autophagy in human placenta, dysfunctions of which would account for initiation and progression of recurrent miscarriage. Therefore, restoration both of Shh signaling and autophagy might be a potential and promising therapeutical strategy to improve vascularization and penetrate maternal spiral arteries to overcome recurrent miscarriage in future.

Conclusion

Our results here showed that dysfunction of Shh signaling impaired trophoblast motility and angiogenesis in villous trophoblast to contribute to the pathophysiology of recurrent miscarriage, otherwise, Activation Shh signaling by recombinant Shh treatment, Gli3 knockdown or autophagy inhibition by BECN1 knockdown would improve trophoblast motility. Thus, our results provided a new promising strategy that combination targets of the Shh pathway and autophagy in recurrent miscarriage therapy.

Abbreviations

CCK-8: Cell counting kit-8; CTBs: Cytotrophoblasts; STBs: Syncytiotrophoblasts; EVT: Extravillous Trophoblasts; MFN2: Mitofusin-2; EMT: Epithelial-Mesenchymal Transition; EZH2: Enhancer of Zeste Homolog 2; Shh: Sonic hedgehog; Hh: Hedgehog; Ihh: Indian hedgehog; Dhh: Desert hedgehog; CQ: Chloroquine; Cyc: Cyclopamine; SNP: single nucleotide polymorphism.

Declarations

Ethical Approval and Consent to participate

The study protocol was approved by the ethics committee of the Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. Preparation of Placental villi tissues were acquired consents from normal pregnant women and recurrent miscarriage patients.

Consent for publication

All authors were consent for publication.

Availability of supporting data

The complete array dataset has been deposited into Gene Expression Omnibus (GEO accession number: GSE130367, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130367>).

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

SYZ conceived and designed the study. YBP, YDD, XMT, HYZ, MFL and YLZ performed the experiments, LLY, QQC, CW, XYJ and TZ collected and prepared the placental tissues, YBP and XNL analyzed the data. YBP prepared and submitted the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Figures

Figure 2

Shh signaling was impaired in recurrent miscarriage patients' villous tissues. (A) Double immunofluorescence staining of Shh, Ptch, Smo (red) and 11 β -HSD2 (green) in first-trimester villous tissue from healthy controls, nuclei were counterstained with DAPI (blue). Scale bars, 20 μ m. (B) Expression of Shh protein in control group and recurrent miscarriage (RM) group were detected by immunohistochemistry. Scale bars, 100 μ m. (C) Expression of Shh from (B) was analyzed and quantified

by Image J software. (D) Expression of Shh protein in control group and recurrent miscarriage group were detected by western blot. (E) CK7 staining in control group and recurrent miscarriage group were detected by immunohistochemistry. Scale bars, 100 μ m. (F) XExpression of CK7 from (E) was analyzed and quantified by Image J software. (G) Relative thickness of cytotrophoblast layer from (E) was analyzed and quantified by Image J software. $p < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Figure 4

Attenuating Shh signaling inhibited trophoblast motility and placental angiogenesis (A) The effect of cyclopamine on regulating migration and invasion of JAR cells. Scale bars, 200 μ m. (B) Migrated cell from (A) was quantified by Image J software. (C) Invaded cell from (A) was quantified by Image J software. (D) Motility of untreated and cyclopamine-treated villous explant cultures on collagen I. Scale bars, 500 μ m. (E) Outgrowth area from (D) was quantified by Image J software. (F) After with or without cyclopamine treatment, expression of p-AKT S473, p-AKT T308 and pan-AKT proteins were detected by western blot. (G) After with or without cyclopamine treatment, expression of E-Cadherin, VEGF-A and GAPDH proteins were detected by western blot. (H) VEGF-A staining in control group and recurrent miscarriage group were detected by immunohistochemistry. Scale bars, 100 μ m. (I) Expression of VEGF-A from (H) was analyzed and quantified by Image J software. (J) CD31 staining in control group and recurrent miscarriage group were detected by immunohistochemistry. Scale bars, 100 μ m. (K) Expression of CD31 from (J) was analyzed and quantified by Image J software. $p < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Figure 6

Shh signaling regulated motility of JAR cells via Gli2 and Gli3. (A) Double immunofluorescence staining of Gli1, Gli2, Gli3 (red) and 11 β -HSD2 (green) in normal human first-trimester villous tissues, nuclei were counterstained with DAPI (blue). Scale bars, 20 μ m. (B) Immunoblot analysis confirmed the Gli2 knockdown efficiency in sh-Scramble-JAR cells and sh-Gli2-JAR cells. (C) Immunoblot analysis confirmed the Gli3 knockdown efficiency in sh-Scramble-JAR cells and sh-Gli3-JAR cells. (D) After indicated

treatments, migration and invasion of JAR cells were measured in matrigel cell invasion and transwell cell migration assays. Scale bars, 200 μ m. (E) Migrated cell from (D) was quantified by Image J software, each group was normalized to “scramble + Ctrl” group. (F) Invaded cell from (D) was quantified by Image J software, each group was normalized to “scramble + Ctrl” group. $p^*<0.05$, $p^{**}<0.01$, $p^{***}<0.001$.

Figure 8

Transcriptome Profiling of JAR treated with Cyclopamine. (A) Heat map depicting hierarchical clustering in JAR cells under 5 μ M cyclopamine treatment for 24h . Red represents the high relative expression, while blue represents the low relative expression. (B) Gene Set Enrichment Analysis enriched "Hypoxia" pathway. Enrichment score of 0.47 and a normalized enrichment score of 2.88 (nominal p-value $\leq$ 0.001, false discovery rate $\leq$ 0.001). (C) Gene Set Enrichment Analysis enriched "Apical Junction" pathway. Enrichment score of 0.46 and an normalized enrichment score of 2.51 (nominal p-value $\leq$ 0.001, false discovery rate $\leq$ 0.001).). (D) Gene Set Enrichment Analysis enriched "EMT" pathway. Enrichment score of 0.43 and an normalized enrichment score of 2.51 (nominal p-value $\leq$ 0.001, false discovery rate $\leq$ 0.001).

Figure 10

Inhibition of Shh signaling induces autophagy and autolysosome. (A) after indicated treatments in JAR cells for 24 h, protein levels of LC3B and GAPDH were measured by western blot assays (B) Expression of LC3B/GAPDH from (A) was quantified by ImageJ software. (C, D) JAR cells were infected with GFP-mCherry-LC3 adenovirus vector (5×10^9 pfu/ml) more than 24 hours, followed with Gant61 (10 μ M) with or without chloroquine (CQ) for 24 hours, mCherry-positive GFP-negative (mCherry+ GFP-) puncta were

captured by microscope and quantified by ImageJ software . Scale bar, 50 μm . ** $P < 0.01$. (E, F) After treating with Gant61(10 μM), colocalization of LC3 and LAMP1 in JAR cells captured by microscope and quantified by ImageJ software. Scale bars, 20 μm . $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Figure 12

Downregulation BECN1 rescued the Gant61-induced inhibition of cell motility in JAR cells and schematic representation of the role of Shh signaling and autophagy in recurrent miscarriage disease. (A) JAR cells were transiently transfected with BECN1 siRNAs for 72 hours, the protein levels of BECN1 and GAPDH were detected by western blot. (B) After JAR cells were transiently transfected with BECN1 siRNAs for 48 h, cells were treated with or without 10 μM of Gant61 for another 24 h, migration and invasion of JAR cells were measured by matrigel cell invasion and transwell cell migration assays. Scale bars, 200 μm . (C) Migrated cells from (B) was quantified by Image J software. (D) Invaded cells from (C) was quantified by Image J software. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Figure 14

A working model for the role of Shh signaling and autophagy in recurrent miscarriage disease. Downregulation of Shh signaling is related to impaired trophoblast motility and poor placental vascular through autophagy and VEGFA. Shh, autophagy and VEGFA may provide new insight into the molecular mechanism of recurrent miscarriage, and these signaling pathway may serve as potential therapeutic targets for recurrent miscarriage treatments.

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