Activin A increases human trophoblast invasion by upregulating integrin β1 through ALK4

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Abstract
Activin A promotes human trophoblast invasion during the first trimester of pregnancy and is associated with preeclampsia and pregnancy-induced hypertension (PE/PIH) in naturally conceived pregnancies. However, whether integrin β1 mediates activin A-increased trophoblast invasion remains unknown and the evidence is limited regarding the predictive value of activin A for PE/PIH in women receiving in vitro fertilization (IVF) treatment. Here, we studied the role and underlying molecular mechanisms of integrin β1 in activin A-promoted invasion in immortalized (HTR8/SVneo) and primary human extravillous trophoblast (EVT) cells. A nest case-control study was designed to investigate the predictive/diagnostic value of activin A in IVF pregnancies. Results showed that integrin β1 expression increased after activin A treatment and knockdown of integrin β1 significantly decreased both basal and activin A-increased HTR8/SVneo cell invasion. SB431542 (TGF-β type I receptors inhibitor) abolished activin A-induced SMAD2/SMAD3 phosphorylation and integrin β1 overexpression. Activin A-upregulated integrin β1 expression was attenuated after the depletion of ALK4 or SMAD4 in both HTR8/SVneo and primary EVT cells. Furthermore, we found similar first-trimester activin A levels in IVF patients

**Abbreviations:** ActRI, activin type I receptor; ActRII, activin type II receptor; ALK, activin receptor-like kinase; ART, Assisted reproductive technology; ChIP, chromatin immunoprecipitation; ECM, extracellular matrix; ET, fresh embryo transfer; EVT, extravillous trophoblast; FBS, fetal bovine serum; FET, frozen embryo transfer; ITGB1, integrin β1; IVF, in vitro fertilization; mRNA, messenger RNA; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenyltetrazoliumromide; PBS, phosphate-buffered saline; PE, preeclampsia; PIH, pregnancy-induced hypertension; SEB, Smad-binding element; siRNA, small interfering RNA.
1 INTRODUCTION

Preeclampsia and pregnancy-induced hypertension (PE/PIH) refer to the development of hypertension with or without proteinuria after 20 weeks of gestation in previously normotensive women, which are serious pregnancy complications that adversely affect the health of both mothers and fetuses. The pathogenesis of PE/PIH has been wildly believed to be related to insufficient trophoblast invasion. In the physiological process of placentation, highly invasive extravillous trophoblasts (EVTs) which are derived from anchoring chorionic villi invade into the uterine wall, followed by modifying the uterine environment to provide sufficient oxygen and essential nutrients to the fetus. However, in pregnancy-related hypertensive disorders, the invasiveness of trophoblasts decreases, leading to unsuccessful decidual spiral arteries remodeling and deprived maternal oxygen supply. Hypoxic placentae then release antiangiogenic factors such as soluble fms-like tyrosine kinase 1 (sFlt-1) into maternal circulations, causing endothelial cell dysfunction and systemic inflammation, which lead to maternal clinical symptoms including hypertension and proteinuria. As a vital process in the establishment of pregnancy, the trophoblast invasion process is orchestrated by various growth factors, hormones, and cytokines. Thus, revealing the regulation factors and underlying molecular mechanisms regarding trophoblast invasion will provide insights into the development of predicting and therapeutic approaches for trophoblast invasion-dysfunction-related diseases.

Activin A, initially discovered as a gonadal protein stimulating the release of follicle-stimulating hormone (FSH), has been subsequently found to mediate a variety of biological activities in a wide spectrum of cells and tissues. In the human endometrium and placenta, the expression of activin A along with its receptors has been widely identified. Studies have shown that decidualized endometrium and placenta produce activin A which promotes outgrowth and invasion of trophoblast cells. However, the underlying molecular mechanism of the pro-invasive effect induced by activin A has not been fully revealed. Like other members of transforming growth factor-β (TGF-β) superfamily, the effects of activin A are mediated through canonical SMAD-dependent or SMAD-independent pathway activated by activin type I and type II receptors (ActRI/ActRII). In canonical SMAD-dependent pathway, activated ActRI/ActRII phosphorylates SMAD2/3 which then combines with SMAD4 to serve as a transcription factor regulating target gene expression. Molecules involving cellular adhesion such as N-cadherin (a cell-cell junction molecule) and MMP2 (ECM degradation molecule) have been reported to participate in activin A-promoted trophoblast invasion. However, neither of them fully decided the pro-invasive effect of activin A, suggesting a complex molecular regulatory network and the involvement of other molecules.

Integrins, composed of α and β subunits, are heterodimeric transmembrane receptors that mediate the connection and signaling transduction between cells and extracellular matrix (ECM), thereby regulating critical cellular functions such as proliferation, adhesion, migration, and invasion. Our RNAseq data demonstrate that the ECM-receptor interaction is the most significantly altered pathway in activin A pretreated HTR8/SVneo trophoblast cells. However, the roles of integrins in actin A-regulated trophoblast invasion have not been examined yet. During the invasion and differentiation of trophoblasts, a dynamic switching pattern of integrin expression has been identified. In situ immunocytochemical studies have shown that the expression of α1β1 and α5β1 integrins are significantly elevated in invasive and differentiating cytotrophoblasts of first-trimester human placentae, whereas α6 integrins are mainly characteristically expressed in the cytotrophoblast stem cells. The temporal and spatial homogeneity between the expression of integrin β1 and the development of invasive trophoblasts indicates that integrin β1 plays a vital role in trophoblasts invasion. Indeed, in vitro studies have demonstrated that integrin β1 mediates the trophoblast invasion and focal adhesion. However, the role of integrin β1 in the pro-invasive effect of activin A on trophoblast remains to be investigated. Interestingly, it has been reported that integrin β1 was responsible for epidermal growth factor (EGF)-mediated cell invasion in ovarian cancer. Therefore, we hypothesize that integrin β1 might be involved in activin A-induced trophoblasts invasion and might become a potential molecular target for the treatment of trophoblastic dysfunction-related diseases.
In addition to promoting trophoblasts invasion in early pregnancy, activin A has also been recognized as a potential predictive/diagnostic marker of hypertensive disorders in pregnancy.\textsuperscript{16,17} Increased serum activin A levels have been found in patients with PE/PIH as early as 11-13 weeks of gestation, which has been speculated that it is resulted from the body compensative response in homeostasis due to shallow trophoblast invasion during placentation.\textsuperscript{14,15} Therefore, first-trimester maternal serum activin A level has been considered as an indicator for early screening and diagnosis of PE/PIH since it is closely related to PE/PIH. It has been widely acknowledged that assisted reproductive technology (ART) serves as a risk factor for PE/PIH.\textsuperscript{18-20} However, whether maternal serum activin A levels have similar potential predictive/diagnostic value in pregnancies after in vitro fertilization (IVF) and embryo transfer remains unknown. Thus, it is of great significance to explore the predictive/diagnostic value of activin A for PE/PIH in women undergoing IVF treatment, which will facilitate the early screening and diagnosis of PE/PIH in this population.

The present study not only investigated the mediation role and the involved signaling pathways of integrin β1 in activin A-increased trophoblast invasion, but also explored the maternal serum activin A level in populations at high risk of PE/PIH (ie, patients receiving IVF treatment) to further explore the potential predictive/diagnostic value of activin A in IVF pregnancies. Our findings showed activin A significantly promoted the invasion of human trophoblast cells by upregulating integrin β1 through ALK4-activated SMAD2/3-SMAD4 signaling. Moreover, we found that first-trimester serum activin A level was similar among IVF pregnancies with or without subsequent PE/PIH, which was inconsistent with the findings in naturally conceived pregnancies.

2 MATERIALS AND METHODS

2.1 Cell culture

The HTR8/SVneo immortalized human EVT cell line was obtained from ATCC (CRL-3271, Manassas, VA, USA) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% (vol/vol) fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (HyClone, Logan City, UT, USA). The immortalized human endometrial stromal cells (HESCs) were kindly given by Prof. Haibin Wang (Xiamen University, Xiamen, China) and were incubated in phenol red-free DMEM/F-12 medium (Gibco, Grand Island, NY, USA) containing 10% charcoal-stripped fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were incubated at an atmosphere of 5% CO\textsubscript{2} and 95% humidity at 37°C. To avoid possible interference of factors from FBS, cells were starved in DMEM containing 0.1% (vol/vol) FBS for 24 hours prior to treatment.

2.2 Primary human EVT isolation and culture

Human EVT cells were isolated as described previously from explants of first-trimester chorionic villous (6-9 weeks of gestation).\textsuperscript{27} In brief, first-trimester villi tissues washed by Dulbecco’s Modified PBS (HyClone, Logan City, UT, USA) were minced into small pieces. The culture medium were DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The tissues were cultured for 3-4 days in flasks in the cell culture incubator and nonattached tissue pieces were then discarded. After further culture of villous tissue fragments for 5-7 days, EVT cells were outgrown from villous tissue fragments. EVTs were then purified by differential trypsin digestion and adhesion method. The purity of isolated EVT cultures was identified by immunofluorescence staining for EVT-specific marker HLA-G (human leukocyte antigen G; Exbio, Vestec, Czech Republic), which showed 99% positive staining of our purified primary EVTs. Cellular morphology of primary EVTs in culture with or without siRNA treatments were observed through the inverted microscope. The images of EVT outgrowth and cellular morphology and immunofluorescence staining on primary EVTs were displayed in Supplementary Figure S1. All patients provided informed written consents and all procedures regarding human EVT cells were approved by the School of Medicine Ethics Board of Shandong University.

2.3 RNA sequencing and data analysis

The HTR8/SVneo cells treated with or without activin A treatment (N = 3) were sent to Xiuyue Biology (Jinan, China) for RNA extraction, cDNA library construction and sequencing. For RNA quality examination, RNA purity was assessed by kiaok5500Spectrophotometer (Kiaao, Beijing, China) and integrity and concentration of RNA were tested by the RNA Nano 6000 Assay Kit from Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Sequencing libraries were prepared by NEBNext Ultra RNA Library Prep Kit for Illumina (#E7530L, NEB, USA) following the manufacturer’s instructions and 2 µg RNA for each sample was employed as input material. The library RNA concentrations were quantified by Qubit RNA Assay Kit in Qubit 3.0 and subsequently diluted to 1 ng/µL. Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to measure insert size and StepOnePlus Real-Time PCR System was employed.
for the quantification of insert size. Then, cBot cluster generation system was used to conduct the clustering of the index-coded samples based on HiSeq PE Cluster Kit v4-cBot-HS (Illumina). Subsequently, on Illumina platform, we sequenced the libraries and produced reads of 150 bp paired-end. Gene expression levels were quantitatively estimated by FPKM method, and differentially expressed mRNA was confirmed. According to the differentially expressed mRNA profile, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis was conducted. Raw sequencing data are available at Sequence Read Archive (SRA) database (PRJNA640458).

2.4 | Antibodies and reagents

The antibodies employed in this study were purchased from Abcam (Cambridge, MA): anti-Integrin beta 1 antibody [EPR16895], anti-Smad3 (phospho S423 + S425) antibody [EP823Y]; Cell Signaling Technology (Beverly, MA): Smad2 (L16D3) mouse mAb, Phospho-SMAD2 (Ser465/467) (138D4) rabbit mAb, SMAD3 (C67H9) rabbit mAb, SMAD4 (D3M6U) rabbit mAb; Santa Cruz Biotechnology (Santa Cruz, CA): α-Tubulin (B-5-1-2). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-antibody IgG were obtained from Zsbio (Beijing, China). The TGF-β type I receptor inhibitor SB431542 (#S4317) was provided by Sigma Aldrich. ACVR1B (ALK4) Taqman primers (Hs00244715_m1) and ITGB1 Taqman primers (Hs01127536_m1) were obtained from Thermo Fisher. Recombinant Human/Mouse/Rat Activin A Protein was purchased from R&D (Minneapolis, MN).

2.5 | Matrigel-coated transwell invasion assay

Transwell invasion assay was used to measure cell invasiveness. 24-well transwell inserts (pore size 8 μm; BD Biosciences) were coated with 45 μL of growth factor-reduced Matrigel (1 mg/mL, BD Biosciences). Cells were seeded to the inserts in 250 μL DMEM medium with 0.1% (vol/vol) FBS, and 750 μL medium with 10% (vol/vol) FBS was added to the lower chambers. After incubation for 40 hours, noninvading cells were removed from the upper chambers and cells invading to the lower side were fixed with cold methanol (−20°C). Cell nuclei were then stained with Hoechst 33258 (Sigma-Aldrich) and counted three fields per insert using a fluorescence microscope and ImageJ software. Triplicate microscopic fields were counted per insert and at least three independent experiments were conducted.

2.6 | MTT assay

MTT assay was performed as described previously. HTR8/SVneo cells were seeded into 96-well plates after interventions and incubated for 40 hours, and then, MTT was added into wells and incubated for 4 hours. Subsequently, culture supernatant was discarded and DMSO was added into each well. The absorption values were detected at 490 nm by a microplate reader (Bio-Rad, Hercules, CA).

2.7 | Reverse transcription quantitative real-time PCR (RT-qPCR)

Based on manufacturer’s instructions, total RNA of the cells was extracted with TRIzol Reagent (Invitrogen). About 2 μg of total RNA was employed for reverse transcription using Prime Script RT reagent Kit (Takara, Shiga, Japan). RT-qPCR was conducted by SYBR Premix Ex Taq (Takara) based on the manufacturer’s instructions and conducted on the Roche LightCycle 480 (Roche, Penzberg, Germany) with 96-well optical reaction plates. TaqMan gene expression assays (Applied Biosystems) was used to detect the mRNA levels of ALK4 and ITGB1. The primers information used was as follows: Integrin β1-F, 5ʹ-TGGACAATGTGCACCTGGAA-3ʹ and Integrin β1-R, 5ʹ-AGCTCCTTGTAAACAGGCTGAA-3ʹ; GAPDH-F, 5ʹ-GCACCGTCAAGGCTGAGAAC-3ʹ and GAPDH-R 5ʹ-TGGTGAAGACGCCAGTGGA -3ʹ. Agarose gel electrophoresis and dissociation curve analysis of PCR products were used to validate the specificity of assays. The performances of assay were confirmed by evaluating amplification efficiencies using the means of calibration curves. The mRNA expression was measured by a mean value using the comparative Cq method of which GAPDH was indicated as the reference gene. Each sample was detected in triplicate and at least three independent experiments were conducted.

2.8 | Western blot

Western blotting was performed as described previously. In brief, cells were harvested and lysed by RIPA buffer with protease inhibitor (Solarbio). The concentrations of protein samples were determined using the BCA protein assay kit (Thermo Scientific). An amount of 30 μg cellular protein was separated by SDS-polyacrylamide gel electrophoresis, and then, electro-transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA), which were blocked with 5% nonfat milk or 5% BSA (Sigma) for 1 hour and incubated, respectively, with primary antibodies against α-Tubulin (1:1000), integrin β1 (1:2000), SMAD2 (1:1000), phospho-SMAD2 (1:1000), SMAD3 (1:1000), phospho-SMAD3
(1:2000), or SMAD4 (1:1000) overnight at 4°C. After incubation with HRP-conjugated secondary antibodies for 1 hour at room temperature, the bands of interest were tested by Pro-lighting HRP agent (Thermo Fisher). α-tubulin was set as the loading control. At least three independent experiments were conducted and semi-quantitative analysis of each band was performed by ImageJ software.

### 2.9 Small interfering RNA (siRNA) transfection

Based on the manufacturer’s instructions (Dharmacon, Lafayette, CO), HTR8/SVneo cells were transfected with 20 nM ON-TARGETplus SMARTpool siRNA (si-ITGB1, si-ALK4, si-SMAD4) or 20 nM NON-TARGETING Control pool siRNA (Dharmacon, Lafayette, CO) for 48 hours using Opti-MEM and Lipofectamine RNAiMAX. RT-qPCR or Western blot was employed to assess transfection efficiency.

### 2.10 Immunohistochemistry

All clinical tissues were obtained with informed consent from each participant. First-trimester chorionic villi and decidual tissue were obtained from a woman who received a voluntary artificial abortion in Qilu Hospital and postpartum placental tissues were collected from normal and PE puerperae from Qilu Hospital of Shandong University. The study was conducted under the approval of the ethics committee of Shandong University and the ethical principles in the declaration of Helsinki were followed. The inclusion criteria for this cohort and women with a history of smoking, hypertension, kidney disease, or cardiovascular disease were excluded. Subsequently, these patients were divided into two groups based on whether they developed PE/PIH: IVF with normal pregnancies (N = 59) and IVF with subsequent PE/PIH (N = 60). Then, subgroup analyses for different embryo transfer types in IVF treatment were conducted and these patients were further divided into the following four groups: fresh embryo transfer (ET) with normal pregnancies (N = 29), ET with subsequent PE/PIH (N = 30), frozen embryo transfer (FET) with normal pregnancies (N = 30), and FET with subsequent PE/PIH (N = 30). Serum activin A levels in the above groups at 11-13 weeks of pregnancy were detected using ELISA assay kit (R&D, Minneapolis, MN) based on the manufacturer’s instructions. Briefly, 200 µL of Activin A biotinylated antibody were added into all wells for 15 minutes incubation with subsequently two washes. Then, 100 µL Assay Diluent and 100 µL of sample or standard were added into each well and cultured

ELISA assay was performed to measure the serum concentration of activin A of patients at 11-13 weeks of gestation after IVF treatment. This is a nest case-control study based on an ongoing prospective cohort conducted at the Reproductive Hospital Affiliated to Shandong University from 2003. Baseline characteristics of all participants were recorded before pregnancy including age, weight, blood pressure, parity, lifestyle, and history of diseases, and were followed up throughout the pregnancy. Fasting serum was collected at 11-13 weeks of gestation and immediately stored in a serum bank at −80°C. Follow-up questionnaires were employed to confirm the occurrence of PE/PIH. Overall 119 primiparas who underwent IVF treatment and got successful delivery were randomly selected from this cohort and women with a history of smoking, hypertension, diabetes, or cardiovascular disease were excluded. 

Staining intensity (SI) and the percentage of positive cells (PP) using immunoreactive score (IRS). IRS = SI × PP. SI was scored as 0 points for stained negative; 1 points for stained weak; 2 points for stained moderate; 3 points for stained strong. PP was assigned as 0 point for 0%; 1 point for 0%-25%; 2 points for 25%-50%; 3 points for 50%-75%; 4 points for 75%-100%.

First-trimester maternal blood sampling and ELISA assay

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for 3 hours. Then, wells were washed again for six times, and 200 µL of Activin A Conjugate were added for 1-hour incubation at room temperature. Next, wells were washed for six times with subsequent substrate solution added into each well. After 30 minutes of incubation, 50 µL of stop solution were added. The final absorbance value was obtained by subtracting the reading at 540 nm from the reading at 450 nm on a microplate reader (Bio-Rad, Hercules, CA).

2.12 Statistical analysis

All data are expressed as mean ± SEM from at least three independent experiments. Student’s t test was used to identify statistical differences between two groups. Mann-Whitney test was used to compare differences of maternal serum activin A levels. GraphPad Prism was employed for statistical analysis. Means were considered significantly different if $P < .05$ and columns without letters in common are significantly different.

3 RESULTS

3.1 Activin A increases integrin β1 expression in HTR8/SVneo cells

To explore the molecular mechanism underlying activin A-upregulated HTR8/SVneo cell invasion, we first investigated

FIGURE 1 Activin A induces integrin β1 expression in HTR8/SVneo cells. A, Kyoto Encyclopedia of Genes Genomes (KEGG) pathways enrichment analysis for the transcriptome profiles of HTR8/SVneo cells after activin A exposure showed the most significant enrichment was the ECM-receptor interaction pathway as ranked by the logP value. B, Volcano plot of transcriptome profiles of HTR8/SVneo cells treated with or without activin A for 24 hours. C, The expression level of ITGB1 mRNA in HTR8/SVneo cells increased in a time-dependent manner after activin A intervention of 1, 3, 6, 12, and 24 hours. D, The expression level of integrin β1 protein in HTR8/SVneo cells increased after activin A intervention of 24 and 48 hours. The representative figure is shown in upper panel and the quantification results are presented in lower panel. Data are illustrated as mean ± SEM of at least three independent experiments and columns without letters in common are significantly different ($P < 0.05$).
the transcriptome profiles of HTR8/SVneo cells treated with or without activin A. HTR8/SVneo cells were intervened with activin A (50 ng/mL, which is comparable to physiopathological concentration) for 24 hours, and then, harvested for detection of transcriptome profiles. Results showed that significantly differentially expressed genes were primarily enriched in extracellular matrix-receptor interaction (ECM) pathway, suggesting the involvement of the ECM pathway in activin A-induced effects (Figure 1A). As a key molecule in the ECM pathway that mediates cell-ECM adhesion, the expression of integrin β1 (ITGB1) was significantly increased after 24 hours of activin A treatment in the mRNA level (Figure 1B). To verify that activin A can increase integrin β1 expression in trophoblast cells, HTR8/SVneo cells were incubated with activin A at 50 ng/mL in a time-dependent pattern, and immortalized human endometrial stromal cell line HESCs were used as control. Consistent with the above results, SYBR RT-qPCR revealed that activin A significantly increased ITGB1 mRNA expression in a time-dependent manner in HTR8/SVneo cells but not in HESCs (Figures 1C and S2A). Similarly, Western blot analysis demonstrated that 24 hours of activin A treatment significantly elevated protein levels of integrin β1 in HTR8/SVneo cells rather than in HESCs (Figures 1D and S2B), which indicate a specific inducing effect of activin A on integrin β1 in human trophoblast cells.

### 3.2 Upregulation of integrin β1 mediates activin A-increased HTR8/SVneo cell invasion

To examine whether the upregulation of integrin β1 is involved in activin A-increased HTR8/SVneo cell invasion, si-RNA mediated ITGB1 knockdown was performed prior to activin A treatment. Our results showed that si-RNA mediated knockdown of ITGB1 attenuated both basal and activin A-upregulated integrin β1 expression in both mRNA and protein levels (Figure 2A,B). Importantly, ITGB1 knockdown significantly reduced both basal and activin A-mediated HTR8/SVneo cell invasion by 80.1% and 67.7% (Figure 2C,D). MTT assay showed no obvious effect of activin A on cell proliferation at the concentrations of 50 ng/mL, but the proliferation rate of HTR8/SVneo cells with or without activin A treatment decreased by 37.3% after ITGB1 knockdown (P < .05) (Supplementary Figure S3A). Moreover, we further investigated the role of integrin β1 in the pro-invasive effect of activin A in primary human first-trimester EVTs. Consistent with the results of HTR8/SVneo cells, TaqMan RT-PCR showed that activin A increased the mRNA levels of ITGB1 in primary EVTs, and siRNA-mediated knockdown of ITGB1 significantly reduced the invasion of primary EVTs by 79.1% (Figure 2E,F). Meanwhile, MTT assay showed that the proliferation rate of EVTs decreased by 10.4% after ITGB1 knockdown (P < .05) (Supplementary Figure S3B). Hence, these results indicate that integrin β1 is both involved in trophoblasts invasion and viability.

### 3.3 Activin A increases integrin β1 expression through ALK4

In order to identify the specific TGF-β type I receptor involved in activin A-induced integrin β1 expression, HTR8/SVneo cells were pretreated with SB431542, a specific inhibitor of TGF-β type I receptors (ALK4, 5, and 7) followed by activin A treatment. Results showed that pretreatment with SB431542 for 1 hour completely abolished the effect of activin A on integrin β1 mRNA (Figure 2G) and protein (Figure 2H,I) levels. Moreover, si-RNA mediated ALK4 knockdown was conducted before activin A treatment to determine whether ALK4 mediates activin A-increased integrin β1 expression. TaqMan RT-PCR showed that ALK4 knockdown attenuated activin A-induced ITGB1 upregulation in HTR8/SVneo cells (Figure 3A), which was confirmed by Western blot at the protein level (Figure 3B). Importantly, Western blot showed consistent results in human primary EVT cells (Figure 3E).

### 3.4 Activin A increases integrin β1 expression through ALK4-activated SMAD2/3-SMAD4 signaling in human trophoblast cells

Subsequently, the involvement of SMAD-dependent signaling pathway in activin A-mediated integrin β1 upregulation was investigated. Western blot showed that SMAD2/3 phosphorylation increased significantly after 30 or 60 minutes treatment of activin A, and SB431542 pretreatment completely abolished activin A-induced SMAD2/3 phosphorylation in HTR8/SVneo cells (Supplementary Figure S4). Moreover, HTR8/SVneo and human primary EVT cells were transfected with si-RNA targeting common SMAD4 to validate the role of SMAD-dependent signaling in activin A-induced integrin β1 expression. The results showed that knockdown of common SMAD4 completely abolished the activin A-induced integrin β1 overexpression at mRNA (Figure 3C) and protein levels in HTR8/SVneo cells (Figure 3D). Similarly, the crucial role of SMAD4 to activin A-induced integrin β1 upregulation was confirmed by western blot in primary EVT cells (Figure 3E). These results indicate the canonical SMAD signaling pathway mediates activin A upregulated integrin β1 expression.

### 3.5 The integrin β1 expression in first-trimester maternal-fetal interface and postpartum placentae with or without PE

Immunohistochemistry was conducted to investigate integrin β1 expression patterns in first-trimester chorionic villi and corresponding maternal decidua tissue. Results showed
that integrin β1 is highly expressed in cytotrophoblast cells rather than in endometrial stromal cells (Figure 4A), indicating that integrin β1 is specifically expressed in first-trimester column cytotrophoblasts, which are precursors of EVT cells. The reduction of trophoblasts invasiveness during implantation is believed to be involved in the pathogenesis of PE. Therefore, integrin β1 expression levels were compared between normal and preeclamptic postpartum placentae (6 control vs 6 cases) by immunohistochemical analysis. However, no significant difference was observed between the two groups (Figure 4B,C). To further investigate the mRNA levels of integrin β1 in normal and preeclamptic postpartum placentae, TaqMan RT-PCR was conducted in another 11 postpartum placental samples (5 control vs 6 cases) (Figure 4D), showing that the mRNA level of integrin β1 was similar between postpartum normal and preeclamptic placentae, which were consistent with the result of immunohistochemistry.

3.6 | First-trimester maternal serum activin-A levels in women undergoing IVF with or without subsequent PE/PIH

Previous studies have indicated that activin-A could serve as a potential candidate marker to predict the occurrence...
of PE/PIH in naturally conceived pregnancies. To explore the clinical predictive significance of activin A for PE/PIH in women undergoing IVF treatment, we performed a nest case-control study to analyze the difference of first-trimester (11-13 weeks) maternal serum activin A level among IVF patients with or without subsequent PE/PIH. No significant difference was observed in baseline characteristics between different groups (Supplementary Table S1). Compared with IVF patients with normal pregnancies, there was a slight but not significant increase of first-trimester serum activin A levels in IVF patients with subsequent PE/PIH (Figure 5A). In subgroup analysis, first-trimester serum activin A levels were slightly but not significantly higher in ET pregnancies with subsequent PE/PIH (Figure 5B) and no statistically significant difference was observed in first-trimester maternal serum activin A levels among FET pregnancies with or without subsequent PE/PIH (Figure 5C).
4 | DISCUSSION

The present study provides important insights into the molecular mechanisms underlying the pro-invasive effect of activin A on trophoblast invasion as well as the predictive/diagnostic value of activin A for hypertensive disorders in IVF pregnancies. Our results first demonstrate that integrin β1 is essential for activin A-promoted trophoblast invasion, and ALK4 mediates the effect of activin A upregulated integrin β1 expression through activating SMAD2/3-SMAD4 pathway. The proposed signaling model was displayed in Figure 6. In brief, activin A binds to the extracellular ligand-binding domain of ActRII and activates ActRI (ALK4). Activin A-activated
FIGURE 6  Proposed signaling model of integrin β1 mediating activin A-promoted trophoblast invasion. Our findings identify an essential cellular molecular pathway which mediates the role of activin A in promoting human trophoblast invasion. After the binding of activin A to the extracellular ligand-binding domain of ActRII on trophoblast cells, ActRII is subsequently recruited and ActRI (ALK4) is phosphorylated. Activin A-activated ALK4 then phosphorylates SMAD2/3 to form heterotrimeric SMAD complexes with SMAD4. The SMAD complexes translocate from cytoplasm into the nucleus where they serve as transcriptional factors to regulate the expression of integrin β1, which is essential for activin A upregulated human trophoblast cell invasion in the first trimester of pregnancy.

ALK4 phosphorylates SMAD2/3 to form heterotrimeric SMAD complexes with SMAD4, which serve as transcriptional factors to regulate the expression of integrin β1 to promote trophoblast cell invasion. Moreover, the expression of integrin β1 in normal and preeclamptic postpartum placentas has been described. Importantly, we also demonstrate that first-trimester maternal serum activin A level is comparable in IVF patients with or without subsequent PE/PIH, suggesting that the predictive/diagnostic value of activin A for hypertensive disorders in pregnancy may differ between IVF and naturally conceived pregnancies.

ECM is a highly dynamic extracellular scaffold assembled by multiple macromolecules combining with each other and cell adhesion receptors, which play a vital role in various cellular functions including growth, adhesion, migration, and signaling. Based on transcriptome profiles of HTR8/SVneo cells, activin A treatment regulates the transcription of a variety of genes, most of which are related to the ECM-receptor interactions. Integrin β1 is the critical transmembrane cell adhesion molecule in the ECM signaling pathway and has been demonstrated to take part in the regulation of embryo implantation. About 12 members of integrin β1 all bind to different ECM molecules, including collagen, laminin, fibronectin, tenasin C, and vitronectin. During embryo implantation, trophoblasts invade the tissue of the uterine wall with the increased expression of α1β1 collagen/laminin receptor. This expression pattern indicated that integrin β1 is essential for the invasiveness of trophoblast. Here, we found integrin β1 was upregulated by activin A treatment and mediated activin A-increased trophoblast cell invasion. Notably, ITGB1 depletion slightly attenuated the proliferation of trophoblast cells, suggesting that integrin β1 is involved in cell viability. However, the viability rate reduced by integrin β1 knockdown is lower than the invasion rate reduced by integrin β1 knockdown, which validated the pro-invasive effect of integrin β1 we observed is independent of its cell viability effect.

Activin A signaling is triggered by interactions with heterotetrameric complexes of activin receptors (ActRII/ActRII). Activin A directly binds to the extracellular ligand-binding domain of ActRII and activates ActRII to recruit and phosphorylate ActRI. Among seven variants of activin receptor-like kinase (ALK), only ALK2 and ALK4 bind to activin A with high affinity. SB431542 is a specific inhibitor of ALK4/5/7, and pretreatment with SB431542 completely abolished activin A-increased integrin β1 expression in trophoblasts, indicating that ALK2 is not involved in this process. Then, we demonstrated that activin A-increased integrin β1 expression was mediated through the ALK4-activated SMAD2/3-SMAD4 signaling pathway, which is consistent with previous studies showing that ALK4 plays an essential role in activin A-mediated trophoblast cell apoptosis and invasion. In SMAD-dependent signaling pathway, activin A-activated ActRI phosphorylates SMAD2/3 to form heterotrimeric SMAD complexes with SMAD4. The complexes translocate from cytoplasm into the nucleus where they serve as transcriptional factors to regulate the expression of multiple genes. In this study, we demonstrated that the SMAD2/3 signaling pathway could be stimulated by activin A treatment and depletion of SMAD4 completely abolished activin A-increased integrin β1 expression, indicating that SMADs play a critical role in the regulation of activin A-induced integrin β1 upregulation. It has been reported that there is a Smad-binding element (SBE) located on the promoter area of integrin β1. Chromatin immunoprecipitation (ChIP) assay demonstrated that Smad3 could directly bind to the promoter of integrin β1 and luciferase activity assay showed that Smad3 functionally promotes the transcriptional activity of integrin β1. This evidence indicated a direct SMADS-dependent regulation on activin A-upregulated integrin β1 expression.

Insufficient trophoblast invasion has been considered responsible for the development of PE. PE is one of the most common complications during pregnancy, which is characterized by maternal hypertension, proteinuria, and accompanied IUGR, which adversely affects the health of both mothers and infants. Poorly invasive trophoblasts unsuccessfully remodel maternal spiral arteries, which...
lead to limited utero-placental circulation capacity, placental oxidative stress, and eventually PE.\textsuperscript{1,42} Considering the essential role of integrin β1 in trophoblast invasion, we tested integrin β1 expression in postpartum placentas tissues of PE patients and normal pregnancies to study the association of integrin β1 and PE. Although some studies have reported that integrin β1 expression was lower in pre-eclamptic placentas, we found that integrin β1 expression in pre-eclamptic postpartum placentas was similar to their normal controls, which was consistent with the study of Divers et al who demonstrated similar expression patterns of integrin β1 between third-trimester placenta tissues from patients with severe PE and those from normal pregnancy.\textsuperscript{43-45} Given that the volume of cytotrophoblasts only accounts for 15% of the total volume at term and integrins have a switching pattern of expression, we speculated that invasion-related integrin β1 expression may be specific for first-trimester and hard to be traced in the late stage of pregnancy.\textsuperscript{46}

Activin A is produced by invasive trophoblasts and endometrial cells during pregnancy.\textsuperscript{47,48} Studies demonstrated that activin A concentrations were higher in the maternal serum of women with PE/PIH, indicating the association of altered maternal serum activin A levels with these diseases.\textsuperscript{49-51} In a nest case-control study, researchers prospectively investigated first-trimester serum activin A concentrations in 304 pregnancies and followed up the development of PE. In women subsequently developing PE, serum activin A levels increased as early as 11 to 13+6 weeks of gestation.\textsuperscript{31} Similar elevation of the first-trimester activin A level has also been observed in patients with subsequently PIH.\textsuperscript{52} Although the increased serum activin A levels in PE/PIH patients may appear contradictory to the pro-invasive effect of activin A, it is worth noting that the pro-invasive effect of activin A on trophoblast cells is strictly limited to a certain period of early pregnancy at 6-8 and 8-10 weeks rather than 10-12 weeks gestation.\textsuperscript{11} Therefore, it remains elusive to what extent the excessive activin A after 10 weeks of pregnancy contributes to PE/PIH development and it has been proposed that the increased serum activin A is reflecting disease manifestation or a part of the adaptive compensatory response to adverse placental conditions.\textsuperscript{53} Due to the close relationship between activin A and PE/PIH, first-trimester maternal serum activin A level has the potential to serve as a risk indicator for early clinical screening of PE/PIH. It has been widely believed that the risk of PE/PIH is increased in women treated with assisted reproductive technology, especially in frozen embryo transfer (FET) pregnancies.\textsuperscript{54-56} However, evidence is limited on the predictive value of first-trimester serum activin A for PE/PIH in women receiving IVF treatment. In this study, we have found that first-trimester activin A level in IVF pregnancies with subsequent PE/PIH is comparable to those in normal IVF pregnancies despite a slightly higher activin A level has been detected in IVF pregnancies developing PE/PIH, suggesting the difference between IVF and naturally conceived pregnancies should be noticed when using activin A to screen for hypertensive disorders in pregnancy in clinical practice. However, the possibility of activin A as a suitable biomarker for PE/PIH cannot be excluded considering the relatively small sample size used in our study. Therefore, future studies with large sample size are definitely warranted.

In conclusion, after activin A binding to ALK4, the SMAD2/3-SMAD4 signaling pathway is activated and the expression of integrin β1 is upregulated, which plays a key role in trophoblast invasion. Moreover, the predictive/diagnostic value of first-trimester maternal activin A levels for hypertensive disorders in pregnancy may differ between natural conceptions and IVF pregnancies and future studies with large sample size are needed. Our findings will benefit the understanding of molecular mechanisms of trophoblast invasion and shed light on the development of therapeutic and predictive/diagnostic approaches for trophoblast-dysfunction-related diseases.

ACKNOWLEDGMENTS

We thank the patients and staff from Reproductive Hospital Affiliated to Shandong University and Department of Obstetrics and Gynecology at the Qilu Hospital of Shandong University for providing the samples used in this study.

CONFLICT OF INTERESTS

No conflict of interest was declared.

AUTHOR CONTRIBUTIONS

YL designed the study. SQZ and YL performed the experiments, analyzed the data and wrote the paper. LLC conducted maternal serum collection and clinical follow-up. YLB collected first-trimester placental villi for primary EVT isolation. ZYL, P.C.K.L, JLM, and YL revised the manuscript. All authors have critically reviewed and approved the final manuscript.

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SUPPORTING INFORMATION
Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Zhu S, Li Z, Cui L, et al. Activin A increases human trophoblast invasion by upregulating integrin β1 through ALK4. The FASEB Journal. 2021;35:e21220. https://doi.org/10.1096/fj.202001604R