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HGF promotes HTR-8/SVneo cell migration through activation of MAPK/PKA signaling leading to up-regulation of WNT ligands and integrins that target β-catenin

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Abstract

Inadequate migration and invasion of the trophoblast cells during embryo implantation is one of the reasons for pregnancy-related complications such as intrauterine growth restriction and preeclampsia. In the present study, relevance of WNT ligands and integrins associated with hepatocyte growth factor (HGF)-mediated migration of HTR-8/SVneo trophoblastic cells has been investigated. Treatment of HTR-8/SVneo cells with HGF led to a dose-dependent increase in their migration. RT-PCR studies revealed a significant increase in the transcripts of *WNT4, WNT11, ITGA2*, and *ITGAV*, which was further confirmed at protein level by Western blotting. HGF treatment also led to increased expression of integrin α2β1 and αVβ5 in HTR-8/SVneo cells. Silencing of *WNT4, WNT11, ITGA2*, and *ITGAV* by siRNA led to a significant decrease in HGF-mediated migration of cells. Treatment of cells with HGF led to activation of mitogen-activated protein kinases (MAPK) and protein kinase A (PKA) signaling pathways. Inhibition of MAPK/PKA, by selective inhibitors, led to decrease in the expression of above WNT ligands and integrins. Silencing of *WNT4*/*WNT11* led to concomitant decrease in the expression of *ITGA2* and *ITGAV* and *vice versa*. HGF treatment also led to significant increase in β-catenin expression, a downstream target of both WNT ligands and integrins. Silencing of β-catenin led to decrease in HGF-mediated migration. β-catenin expression was also down-regulated in *WNT4*/*WNT11*/*ITGA2*/*ITGAV* silenced cells suggesting a possible cross-communication of WNT ligands and integrins via β-catenin. These studies have established the significance of WNT4/WNT11 as well as ITGA2/ITGAV during HGF-mediated migration of HTR-8/SVneo trophoblastic cells.

Keywords Cell migration · β-catenin · Hepatocyte growth factor · Integrins · Trophoblast · WNT ligands

Introduction

Embryo implantation is a multifactorial process, which requires synchrony between the implanting blastocyst and the receptive endometrium. The extravillous trophoblast (EVT) cells differentiated from cell column of anchoring

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villi invade and migrate into maternal decidua to remodel the uterine spiral arteries [1, 2]. Any impairment or failure in EVT migration and invasion during spiral arteries remodeling may lead to pregnancy-related complications like preeclampsia and intrauterine growth restriction (IUGR) [3, 4]. EVT cells are associated with extracellular matrix (ECM) through cell surface integrins. Integrin switching occurs during EVT cells differentiation, migration, and invasion in response to alteration of ECM components. Studies have shown that in the differentiating invasive cytotrophoblasts, integrin α6β1 is down-regulated whereas integrin α5β1 and α1β1 are up-regulated [5, 6]. Further, the invasive interstitial trophoblast cells of preeclamptic placenta failed to down-regulate ITGB4 integrin subunit and were unable to up-regulate ITGA1 integrin subunit, as compared to normal pregnancy placenta [7]. Likewise, studies by immunohistochemistry showed that invading cytotrophoblast cells of preeclamptic placenta were unable to up-regulate the expression of integrin αVβ3 [8]. Moreover, insulin-like growth factor-1 (IGF-1)-mediated migration of EVT cells also depends on the activation of integrin αVβ3, which was localized in the focal adhesion of the cells with other proteins [9]. The cytotrophoblast cells present at the base of column are positive for ITGA2 integrin subunit [10]. Integrin α2β1 has been known to be involved in the migration of melanoma cells [11], but its role in trophoblast migration is still unknown. Even though the integrin switching has been known to occur during trophoblast migration, the role of growth factors and cytokines regulating this switching process needs further investigations.

The process of trophoblast migration and invasion is controlled by cytokines and growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF), interleukin family (IL-6, IL-11), and granulocyte macrophage colony stimulating factor (GM-CSF) secreted by diverse cell types at the feto-maternal interface by modulating different cell adhesion molecules and ECM proteins [12, 13]. During placental development, HGF is secreted by syncytiotrophoblast (STB), EVT, endothelial and mesenchymal cells, while its receptor is mainly expressed on trophoblast cells [14]. HGF is a cytokine with potent angiogenic activity. It binds to transmembrane receptor, c-MET, leading to proliferation and migration of endothelial and smooth muscle cells thereby enabling formation of blood vessels [15]. Knockout studies have shown that mice mutant for either *HGF* or *c-MET* were unable to survive in utero due to inadequate placentation, while mRNA level of HGF was down-regulated in IUGR placentae [16, 17].

HGF induces trophoblast migration through up-regulation of homeobox transcription factors like HLX and HLX1, which in turn lead to an increase in the expression of matrix metalloproteinases like MMP2 and MMP9 [18, 19]. HGF also activates phosphatidylinositol-3-kinase (PI-3-K) and mitogen-activated protein kinase (MAPK) signaling pathways and up-regulates inducible nitric oxide synthase (iNOS) expression which further stimulates trophoblast motility [20]. HGF is also shown to regulate WNT signaling pathway in mammary epithelial cells [21, 22]. In mice, during epithelial morphogenesis, HGF-induced signaling pathways regulate the expression of *WNT7B* isoforms [23]. Expression of 14 WNT ligands and 8 frizzled receptors has been reported in the first trimester human placenta [24]. However, functions of individual WNT ligands have not been studied with respect to trophoblast migration except, WNT3A. The trophoblast migration was activated by WNT3A through canonical WNT signaling *via* up-regulation of MMP-2 secretion [25]. However, we still do not know how various WNT ligands are activated and regulated by different growth factors and cytokines secreted by trophoblast cells. Thus, it would be interesting to delineate the WNT ligands involved in trophoblastic cell migration mediated by HGF. We also hypothesized that regulation of trophoblast migration by HGF may involve co-operative participation of both WNT ligands and integrins. Thus in the present study, we have investigated the role of HGF in trophoblastic cell migration with respect to expression of different WNT ligands and integrins. In addition, we have also studied HGF-mediated activation of MAPK and protein kinase A (PKA) signaling pathways and effect of their inhibition on the expression of WNT ligands and integrins. HTR-8/SVneo cells derived from human first trimester placental explant cultures, immortalized by SV40 large T antigen, that closely resemble physiological phenotype of isolated first trimester EVT cells were used in the present study [26]. This cell line has been extensively used to study trophoblast migration and invasion [27–29]. Furthermore, *WNT4, WNT11, ITGA2*, and *ITGAV* were silenced and their effect on HGF-mediated trophoblast cell migration was studied. In addition, correlation between the expression of WNT ligands and integrins involving the common downstream target like β-catenin during HGF-mediated migration of HTR-8/SVneo cells was also investigated.

Materials and methods

Cell line and culture conditions

HTR-8/SVneo cells (kindly provided by Prof. P. K. Lala, Queen's University, Kingston, ON, Canada) were maintained in Dulbecco's modified Eagle medium (D1152-10L, Sigma-Aldrich Inc., USA) and Ham's Nutrient Mixture F12 medium (N3520-10L, Sigma-Aldrich Inc., USA) in 1:1 ratio along with 10% heat-inactivated fetal bovine serum (10270, FBS; Gibco®, USA) and supplemented with

antibiotic–antimycotic cocktail containing penicillin (100 units/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL) (1674049, MP Biomedicals, USA) at 37 °C under humidified atmosphere containing 5% CO_2.

Wound-healing migration assay

In-vitro scratch wound assay was performed to assess directional cell migration [30]. HTR-8/SVneo cells (0.8×10^6 cells/plate) were grown to form monolayer in 60×15 mm culture plate. Confluent HTR-8/SVneo cell monolayer was incubated with 5 µM mitomycin-C (M0503, Sigma–Aldrich Inc., USA) for 2 h to inhibit cell proliferation, followed by extensive washing. Subsequently, cells were scratched in horizontal as well as in vertical directions parallel to the diameter of the culture plate using 200-µL pipette tips. Plates were further washed with plain medium to remove detached cells and fresh medium containing 1% FBS and recombinant human HGF (PHG0254, Gibco®, USA) was added to the plates. Thereafter, images of cells from different regions at 0 h were taken using fluorescent phase contrast microscope (Nikon Instruments Inc., Melville, NY, USA) and cells were further incubated at 37 °C in 5% CO_2. To measure the area of wound closure, images were taken at specified times and cell frontiers bordering the wounds were traced using ImageJ software (US National Institute of Health). The percent migration was calculated based on the equation $\{(Wi - Wz)/Wi\} \times 100$, where Wi is the area of wound at $t = 0$ h and Wz is the area of wound closure after 24 h. These cells were further used to study the expression of different WNT ligands and integrins by qRT-PCR and Western blotting.

Transwell migration assay

HTR-8/SVneo cell migration assay was performed using 8-µm filter pore-size transwell insert in 24-well cell culture plates [31]. DMEM + Ham's F12 medium (300 µL/well) supplemented with 1% FBS with or without optimized concentration of HGF was added to the lower chamber containing transwell inserts. The HTR-8/SVneo cells (1×10^5 cells/150 µL) suspended in the same medium were seeded in the upper chamber of the transwell with and without HGF. After 24 h of incubation, medium from lower chamber was aspirated, while excess cells and medium from the upper chamber of transwell were removed using moist cotton swab. Cells were fixed with chilled methanol for 7–10 min at 4 °C. After subsequent washing with PBS, the membrane was stained with Hoechst 33342 nuclear dye (0.2 µg/mL; H3570, Life Technologies, USA) for 5 min at 37 °C and cells on the lower surface of the membrane were counted under fluorescent phase contrast microscope (Nikon Instruments Inc., USA).

Quantitative reverse transcription polymerase chain reaction

HTR-8/SVneo cells harvested after wound-healing migration assay were used to study relative expression levels of transcripts encoding various genes. The total RNA was isolated from these cells using Ribo-Zol™ reagent (N580, AMERSCO®, USA) according to the manufacturer's protocol. Purity and quantity of extracted RNA was analyzed by NanoDrop 3300 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA). RNA samples were treated with DNase1 (EN0525, Fermentas International Inc., Canada) at 37 °C for 30 min as per manufacturer's protocol, followed by heat inactivation at 65 °C for 5 min. Complementary DNA (cDNA) was synthesized using 5 µg of the isolated RNA, oligo (dT) 18 primer (SO132, Fermentas International Inc., Canada), random hexamer primer (SO142, Fermentas International Inc., Canada) dNTP mix (18427088, Fermentas International Inc., Canada), RiboLock RNase Inhibitor (EO0381, Fermentas International Inc., Canada), and Maxima reverse transcriptase enzyme (EP0742, Fermentas International Inc., Canada) according to the manufacturer's instructions. Subsequently, quantitative polymerase chain reactions for various WNT ligands, integrins, and β-catenin were carried out in duplicates in 20 µL reaction mixture containing Maxima™ SYBR green master mix (2X; K0222, Fermentas International Inc., Canada), synthesized cDNA (diluted 3 times), and gene-specific primers (Supplementary Table S1). Quantitative polymerase chain reaction was carried out in Stratagene Mx3005P (Agilent Technologies Inc., Santa Clara, CA, USA). The cycle profile for target gene amplification involved initial denaturation for 10 min at 95 °C, followed by 40 cycles of target amplification involving 15 s at 95 °C and 60 s at primer-specific annealing temperature (Supplementary Table S1). Finally, a dissociation curve analysis was carried out at a temperature range of 60–95 °C for 20 min. A single peak in the dissociation curve analysis confirmed gene-specific amplification. *18S* rRNA was run in parallel to normalize average threshold cycle (Ct) values. Ct values were used to calculate relative ΔCt values for each experimental set. These relative ΔCt values were used to determine the fold change in expression between the groups. To check the specificity of primers used for the respective WNT ligands and integrins, 2 µL cDNA was used as template to run a polymerase chain reaction (PCR) and amplified products were resolved on 2% agarose gel along with 0.1–1 kb DNA ladder (SM0241, Fermentas International Inc., Canada) (Supplementary Fig. 1). Size of the amplicons was cross-checked with the data available in NCBI (National Centre for Biotechnology Information) using Primer Blast. In addition, analyses of nucleotide sequences of the amplified

PCR products (sequencing outsourced to Xcleris Labs Ltd. Gujarat, India) were performed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the respective mRNA transcript sequences (NCBI database), which confirmed the specificity of the used primers.

Gene silencing by siRNA

HTR-8/SVneo cells (0.2×10^6/well) were seeded in 6-well culture plates in DMEM + Ham's F-12 medium with 10% FBS. Next day, cells were transfected with control siRNA, siRNA for *WNT4, WNT11, ITGA2,* and *ITGAV* (sc-37007, sc-41110, sc-50360, sc-29371, and sc-29373, respectively, SantaCruz Biotechnology Inc., USA) and *β-catenin* (6225S, Cell Signaling Technology®, USA) using lipofectamine2000 (11668-019, LifeTechnologies, USA) and Opti-MEM® medium (31985070, Gibco®, USA). Cells were washed once and 800 µL of fresh Opti-MEM® medium was added into each well. *WNT4, WNT11, ITGA2*, control siRNA (optimized concentration of 40 pmol), and *ITGAV* and β-catenin (optimized concentration of 25 pmol) were mixed with Opti-MEM® medium to make a total volume of 185 µL. In separate tube, 5 µL of lipofectamine2000 was mixed with 10 µL Opti-MEM® medium and incubated for 5 min at room temperature. Both solutions were mixed and incubated for 20 min at room temperature. The mixed solutions were added carefully drop by drop in respective wells and after 6 h of incubation at 37 °C under humidified condition of 5% CO_2, complete medium was added to the cells. After 48 h of transfection, *WNT4*/*WNT11*/*ITGA2*/*ITGAV* and *β-catenin* silenced cells were used for either wound-healing assay or transwell migration assay, respectively. In addition, these silenced cells were also processed for qRT-PCR and Western blotting.

Western blotting

Preparation of whole cell lysate

After wound-healing migration assay, HTR-8/SVneo cells were lysed for 10 min in cell lysis buffer (20 mM Tris–HCl, 10% glycerol, 0.2 mM EDTA, 0.137 M NaCl, 1% NP-40) supplemented with complete protease and phosphatase inhibitor cocktail (05892791001 and 4906845001, Roche Diagnostic, USA). This was followed by three rapid freeze and thaw cycles to ensure the complete cell lysis. Cell lysates were centrifuged at 12,000×*g* for 10 min at 4 °C and the respective supernatants were collected. In addition, the respective culture supernatants were collected and further concentrated using 30 kDa membrane centrifugal filter unit (UFC903024, Millipore, Germany).

Preparation of nuclear and cytoplasmic fraction

For β-catenin expression, after wound-healing assay, cells were harvested in ice-cold PBS containing 1 mM EDTA. The cell pellet was suspended in cytoplasmic extraction buffer (1 M HEPES-KOH pH 7.9, 3 M KCl, 0.5 M EDTA, 10% NP-40). After three cycles of vortexing (3 min each) and incubation on ice for 1 min, cell suspension was centrifuged for 5 min at 10,000×*g* at 4 °C and supernatant thus obtained represented the cytoplasmic extract. The pellet was dissolved in the nuclear extraction buffer (1 M Tris pH 7.5, 3 M KCl, 0.5 M EDTA) followed by three cycles of rapid freeze–thaw. Nuclear fraction was obtained by spinning it at 10,000×*g* for 5 min. The amount of protein in whole cell lysate, culture supernatant, and nuclear and cytoplasmic fractions was quantitated by bicinchoninic acid colorimetric assay (BCA) using BSA as standard (23225, Thermo Fisher Scientific, USA).

Procedure

Cell lysate/culture supernatant/cytoplasmic fraction/nuclear fraction (40 µg/lane) were resolved by 0.1% SDS-10% polyacrylamide gel electrophoresis (SDS–PAGE). The resolved proteins were transferred to the nitrocellulose membrane by wet transfer method. After transfer of proteins on to membrane, it was blocked with 5% BSA in TBST (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20; pH-7.4) for 1 h at room temperature. Further, the membrane was incubated at 4 °C overnight with an optimized dilution of antibodies against WNT4 (1:750), WNT11 (1:500), ITGA2 (1:500), ITGAV (1:500) (sc-5214, sc-50360, sc-9089 and sc-10719, SantaCruz Biotechnology Inc., USA), non-phosphorylated active β-catenin (1:1000), TATA-binding protein (TBP) (1:1000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2000) (19807S, 12578S, and 5174S, Cell Signaling Technology®, USA) in TBST containing 5% BSA. After subsequent washings with TBST, membrane was further incubated with horseradish peroxidase (HRP)-conjugated anti-goat antibody (1:10,000) (sc-2020, SantaCruz Biotechnology Inc., USA) or anti-rabbit antibody (1:3000) (32460, Thermo Scientific Inc., USA) for 1 h at room temperature in TBST containing 5% BSA. After subsequent washings, blots were developed using Immobilon chemiluminescent substrate (WBKLS0500, Millipore Corp. Germany). Pictures of the chemiluminescent blots were taken by FluorChem E system (ProteinSimple, SJ, California,

USA). Intensity of bands on Western blots was quantitated using ImageJ software (http://rsb.info.nih.gov/ij/).

Indirect immunofluorescence

HTR-8/SVneo cells (0.3×10^5) were cultured in 24-well plates on coverslips. After overnight incubation, cells were serum starved for 6 h and then treated with HGF (50 ng/mL) for 24 h. Coverslips were washed with PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl and pH-7.4) and blocked in 3% BSA in PBS for 30 min. The primary antibodies against integrin α2β1 (1:500) (ALX-805-030-C100, Enzo Life Sciences Inc., USA), αVβ1 (1:100), αVβ3 (1:100), and αVβ5 (1:100) (bs-2016R, bs-1310R and bs-1356R, Bioss Antibodies™, USA) in 1% BSA in PBS were added to respective wells separately, and incubated for 1 h at room temperature. Cells were washed thrice with PBS and incubated with either goat anti-mouse IgG (1:1000) or anti-rabbit (1:1000) secondary antibody conjugated with Alexa Fluor 488 (A11001 and A11008, Invitrogen Corporation., USA), diluted in PBS, and supplemented with 1% BSA for 45 min at room temperature. Cells were washed three times with PBS and fixed for 10 min in freshly prepared 4% formaldehyde. After subsequent washings, coverslips containing fixed cells were mounted on glass slide using ProLong® Diamond Antifade containing DAPI (P36962, Invitrogen Corporation, USA). Cells were examined under fluorescent phase contrast microscope (Nikon Instrument Inc., USA) and images were captured and processed using Image proplus software (Media cybernetics, USA).

MAPK and PKA signaling pathways

HTR-8/SVneo cells (0.2×10^6) were seeded in 6-well culture plates and allowed to adhere overnight. Monolayer of cells was serum starved for 6 h before treatment with HGF (50 ng/mL) for 10, 30, and 60 min. After each time point, the medium was aspirated and cells were harvested in cell lysis buffer to prepare whole cell lysate. The cell lysates were processed for Western blot employing primary antibodies against p44/42 MAPK (1:1000), Phospho-p44/42 MAPK (1:1000), PKA C-α (1:1000), and Phospho-PKA C (Thr197) (1:1000) (9101S, 9101S, 5842S, 5661S, Cell Signaling Technology®, USA) essentially as described above.

To perform, signaling pathways inhibition studies, after serum starvation, monolayer of HTR-8/SVneo cells was treated with MAPK inhibitor, UO126 (10 µM) (9903, Cell Signaling Technology®, USA), and PKA inhibitor, H89 dihydrochloride (10 µM) (19-141, Sigma-Aldrich Inc., USA) for 2 h. Monolayer of cells was scratched and washed with plain medium to remove detached cells and further processed as in wound-healing migration assay as described above in the presence or absence of HGF (50 ng/mL) and U0126/H89 inhibitor. After 24 h of incubation, cells were processed to prepare cell lysates, which were used to perform Western blots as described above.

Statistical analysis

All experiments were performed at least three times and results were expressed as mean ± standard error of the mean (s.e.m). Statistical analyses were performed using one-way ANOVA and $p \leq 0.05$ was considered as statistically significant.

Results

HGF-mediated migration of HTR-8/SVneo trophoblast cells is associated with increased expression of WNT4 and WNT11

In scratch wound-healing migration assay, treatment of HTR-8/SVneo trophoblastic cells with varying concentration of HGF led to dose-dependent increase in their migration. At 10, 20, and 50 ng/mL of HGF, a significant increase in the HTR-8/SVneo cell migration by 1.8-, 2.1-, and 2.9-fold, respectively, was observed as compared to the untreated control (Fig. 1a, b). Since maximum fold change in migration was observed at 50 ng/mL of HGF, this concentration was chosen to carry out further experiments. To discern the relevance of WNT ligands in HGF-mediated migration of trophoblastic cells, expression profile of the transcripts for *WNT2B, WNT3, WNT4, WNT5A, WNT7B, WNT10B*, and *WNT11* in HTR-8/SVneo cells treated for 24 h with HGF (50 ng/mL) was examined. *WNT2B* expression was found to be decreased by ~5-fold in both HGF-untreated as well as HGF-treated cells after 24 h as compared to 0 h control (Fig. 2a). No significant changes in the expression of *WNT3* and *WNT10B* were observed. *WNT5A* expression was increased in HGF-untreated cells by ~5-fold, while no significant change in expression was observed in HGF-treated cells as compared to 0 h control. A significant increase in the *WNT7B* expression of ~3.5– and ~7-fold was observed in the HGF-untreated and HGF-treated cells, respectively, as compared to 0 h control (Fig. 2a). However, maximum increase in the expression of *WNT4* (~110-fold) and *WNT11* (~29-fold) was observed in the HGF-treated HTR-8/SVneo cells after 24 h (Fig. 2a). As compared to baseline, at 24 h the transcript profiles of both *WNT4* and *WNT11* were also significantly up-regulated by ~29- and 12-fold, respectively, in the untreated cells; however, their expression was lower as compared with the HGF-treated counterpart at the same time point (Fig. 2a). Since, *WNT4* and *WNT11* expression was highest among all the WNT ligands analyzed, their protein expression was further studied by Western blotting. A

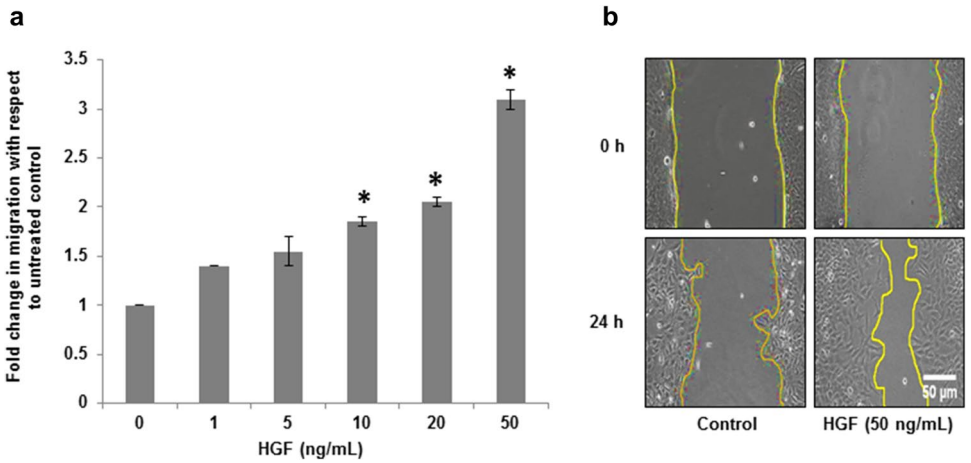

Fig. 1 HGF-mediated migration of HTR-8/SVneo cells. HTR-8/SVneo cells were cultured to form monolayer followed by creation of wound by scratching with pipette tips and subsequently treated with varying concentrations of HGF for 24 h. The fold change in the migration was calculated based on the area of wound closure after 24 h of HGF treatment. The results are shown as mean ± *s.e.m* of three independent experiments (**a**). Representative images at 0 and 24 h with and without HGF (50 ng/mL) treatment are appended in **b**. Scale bar represents 50 µm. *$p \leq 0.05$ was considered statistically significant as compared with untreated control

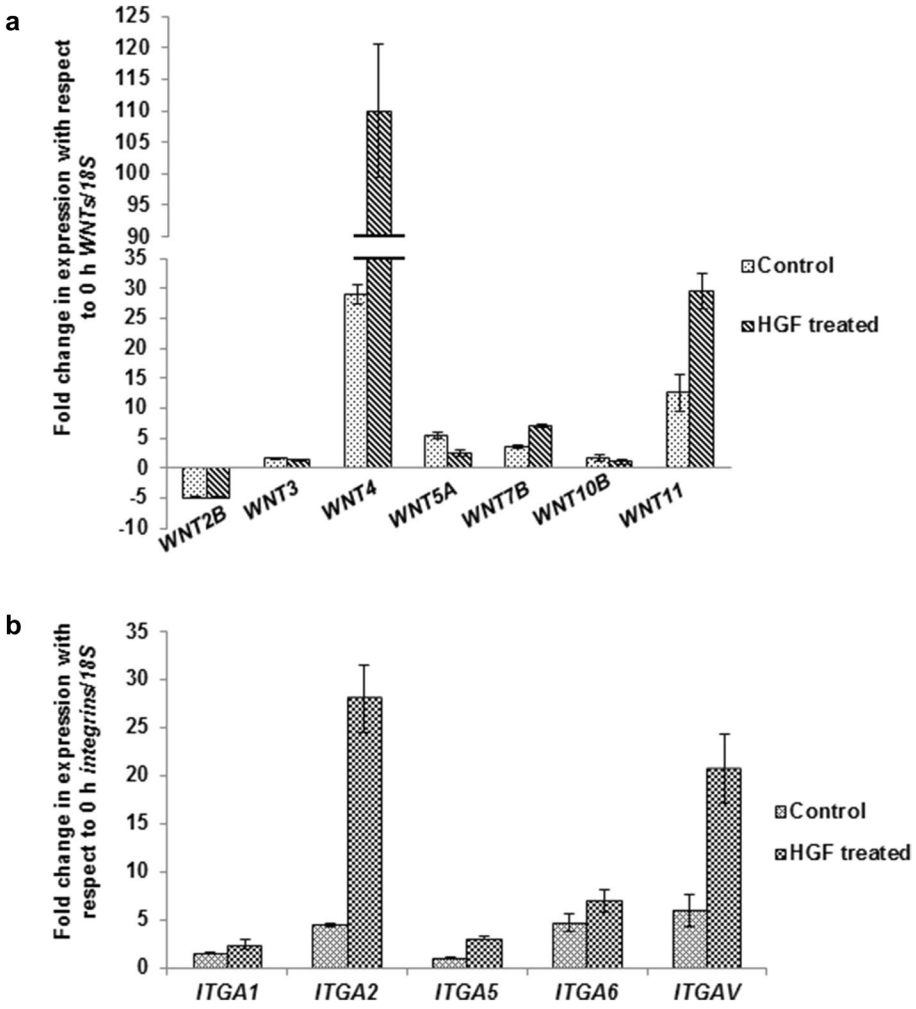

Fig. 2 Transcript levels of different *WNT* ligands and integrins in HGF-treated HTR-8/SVneo cells. In a scratch wound-healing assay, HTR-8/SVneo cells were treated with HGF (50 ng/mL) for 24 h followed by analyses of the *WNT2B, WNT3, WNT4, WNT5A, WNT7B, WNT10B, WNT11, ITGA1, ITGA2, ITGA5, ITGA6*, and *ITGAV* transcripts by qRT-PCR as described in "Materials and methods". Each bar represents relative ΔCt values after normalization with the *18S* rRNA, and expressed as mean ± *s.e.m* of three independent experiments performed in triplicates

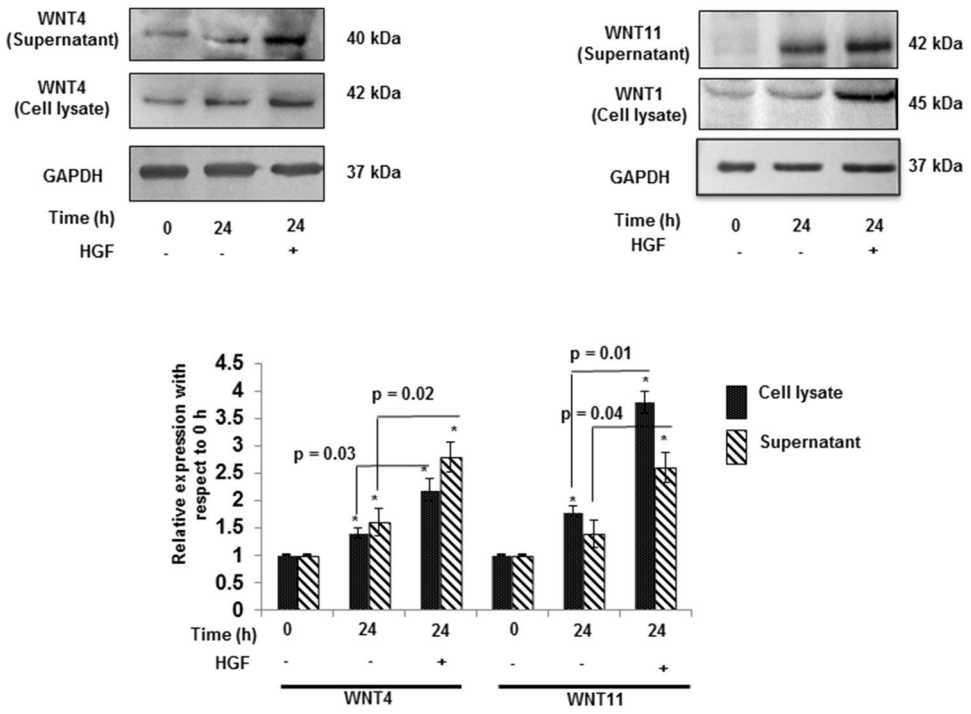

Fig. 3 Expression profile of WNT4 and WNT11 in HTR-8/SVneo cells treated with HGF. HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. After treatment with HGF, culture supernatant was collected and cells used to prepare cell lysate. Subsequently, culture supernatant and cell lysates were used to determine WNT4 and WNT11 expression by Western blotting as described in "Materials and methods". Values are expressed as mean ± *s.e.m* of band intensity of three independent experiments. Representative Western blot profiles of WNT4 and WNT11 protein in cell lysate and culture supernatant at 0 h and after 24 h with/without HGF (50 ng/mL) treatment are also appended. GAPDH was used as a loading control. $*p \leq 0.05$ was considered statistically significant as compared with 0 h

significant increase ($p < 0.05$) in WNT4 expression both in cell lysate as well as culture supernatant was observed at 24 h as compared to 0 h with/without treatment of HGF (Fig. 3). Similarly, a significant increase ($p < 0.05$) of WNT11 in cell lysate was also observed in HGF-untreated/treated cells at 24 h as compared to 0 h. However, expression of WNT11 was increased significantly in the culture supernatant of only HGF-treated cells as compared to 0 h control (Fig. 3). Interestingly, HGF treatment for 24 h led to a significant increase in WNT4 and WNT11 in cell lysate (WNT4; $p = 0.03$, WNT11; $p = 0.01$) as well as in cell culture supernatant (WNT4; $p = 0.02$, WNT11; $p = 0.04$) as compared to 24 h untreated group (Fig. 3).

Expression of ITGA2 and ITGAV was also up-regulated in HGF-treated HTR-8/SVneo cells

Further, to determine the role of integrins in HGF-mediated migration of trophoblastic cells, expression profile of the transcripts for *ITGA1, ITGA2, ITGA5, ITGA6*, and *ITGAV* in HTR-8/SVneo cells treated for 24 h with HGF (50 ng/mL) was studied. No significant changes in the expression of *ITGA1* and *ITGA5* were observed in HGF-treated HTR-8/SVneo cells as well as untreated cells as compared to 0 h baseline. While *ITGA6* expression was significantly up-regulated by ~ 4.7- and ~ 7.0-fold in HGF-untreated and HGF-treated cells, respectively, as compared to 0 h control. However, maximum increase in the expression of *ITGA2* (~ 28 fold) and *ITGAV* (~ 20 fold) was observed in the HGF-treated HTR-8/SVneo cells after 24 h (Fig. 2b). The transcript profiles of both *ITGA2* and *ITGAV* were also significantly up-regulated by ~ 4.5- and ~ 6-fold, respectively, in the untreated cells as compared to 0 h baseline, but their expression was lower as compared with the HGF-treated counterpart (Fig. 2b). Expression of ITGA2 and ITGAV by Western blotting revealed that only ITGAV levels were significantly ($p < 0.05$) increased at 24 h as compared to 0 h with and without HGF treatment, whereas ITGA2 levels were significantly increased only in HGF-treated cells (Fig. 4). Interestingly, the levels of both the integrins were significantly higher in HGF-treated (ITGA2: $p = 0.02$; ITGAV: $p = 0.03$) *versus* the HGF-untreated cells at 24 h (Fig. 4). However, treatment of HTR-8/SVneo cells with HGF did not result in any significant increase in the transcript encoding *ITGB1, ITGB3, ITGB4,* and *ITGB5* as compared to untreated cells

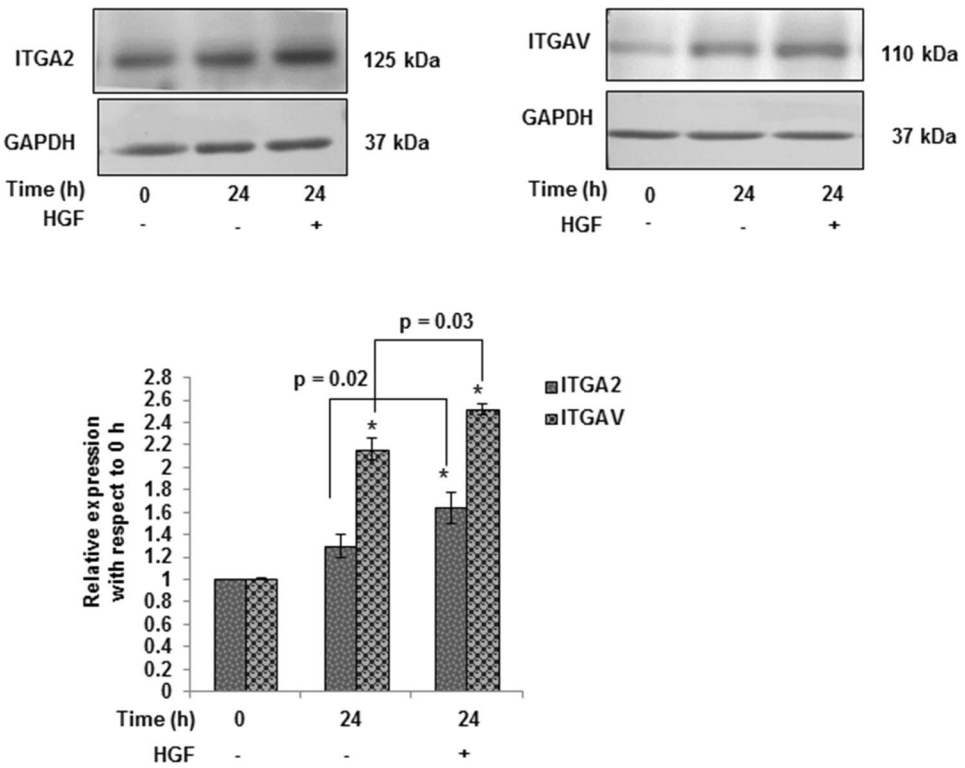

Fig. 4 Expression profile of ITGA2 and ITGAV in HTR-8/SVneo cells treated with HGF. HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. Subsequently, cell lysates were prepared to determine ITGA2 and ITGAV expression by Western blotting as described in "Materials and methods". Values are expressed as mean ± *s.e.m* of band intensity for three independent experiments. GAPDH was used as a loading control. Representative blots at 0 h and after 24 h in the presence and absence of HGF are appended alongside the graph. $*p \leq 0.05$ was considered statistically significant as compared with 0 h

(Supplementary Fig. 2). Indirect immunofluorescence studies revealed higher expression of α2β1 and αVβ5 in HTR-8/SVneo cells treated with HGF as compared to untreated control (Fig. 5a, b). However, no significant changes in the expression of αVβ1 and αVβ3 subsequent to treatment with HGF were observed.

Silencing of *WNT4* and *WNT11* reduced HGF-mediated migration of HTR-8/SVneo cells

To study the role of WNT4 and WNT11 in HGF-mediated trophoblastic cell migration, HTR-8/SVneo cells were silenced for *WNT4* and *WNT11* using siRNA as described in "Materials and methods". These silenced cells were treated with HGF (50 ng/mL) for 24 h. The qRT-PCR analysis showed ~ 67% decrease in *WNT4* and *WNT11* transcript levels in HGF-treated siRNA-transfected cells as compared to the HGF-treated control siRNA-transfected cells (Fig. 6a, d). Further, a significant decrease in the basal levels (without HGF treatment) of *WNT4* and *WNT11* transcript in siRNA-transfected cells as compared to control siRNA-transfected cells was also observed. Moreover, silencing of WNT4 and WNT11 also led to a significant decrease in WNT4 and WNT11 at protein level in siRNA-transfected HTR-8/SVneo cells as compared to control siRNA-transfected cells after treatment with HGF (Supplementary Fig. 3). Interestingly, silencing of *WNT4* and *WNT11* led to a significant decrease in the HGF-mediated as well as basal migration (without treatment with HGF) of HTR-8/SVneo cells (Fig. 6b, c, e, f).

Silencing of *ITGA2* and *ITGAV* led to decrease in the HGF-mediated migration of HTR-8/SVneo cells

In addition to *WNT4* and *WNT11, ITGA2* and *ITGAV* were also silenced using siRNA as described in "Materials and methods". The qRT-PCR analysis of *ITGA2* and *ITGAV* silenced cells revealed ~ 71% decrease in the respective transcripts in HGF-treated siRNA-transfected cells as compared to HGF-treated control siRNA-transfected cells (Fig. 7a, d). A significant decrease in the *ITGA2* ($p = 0.03$) and *ITGAV* ($p = 0.04$) transcripts was also observed in the HGF-untreated siRNA-transfected cells as compared to control siRNA-transfected cells (Fig. 7a, d). Significant decrease in ITGA2 and ITGAV at protein level in cells transfected with respective siRNA at basal as well as after HGF treatment was observed by Western blotting (Supplementary Fig. 3). Further, a significant decrease in cell migration was also observed in cells silenced for *ITGA2* in both HGF-untreated ($p = 0.01$) and HGF-treated ($p = 0.002$) cells as compared to their respective controls (Fig. 7b, c). Silencing of *ITGAV* in HTR-8/SVneo cells led to the loss in their adherence ability. Hence, the effect of *ITGAV* silencing on the migration of HTR-8/SVneo cells was investigated using transwell migration assay instead of wound-healing migration assay. A significant decrease in HTR-8/SVneo cell migration was

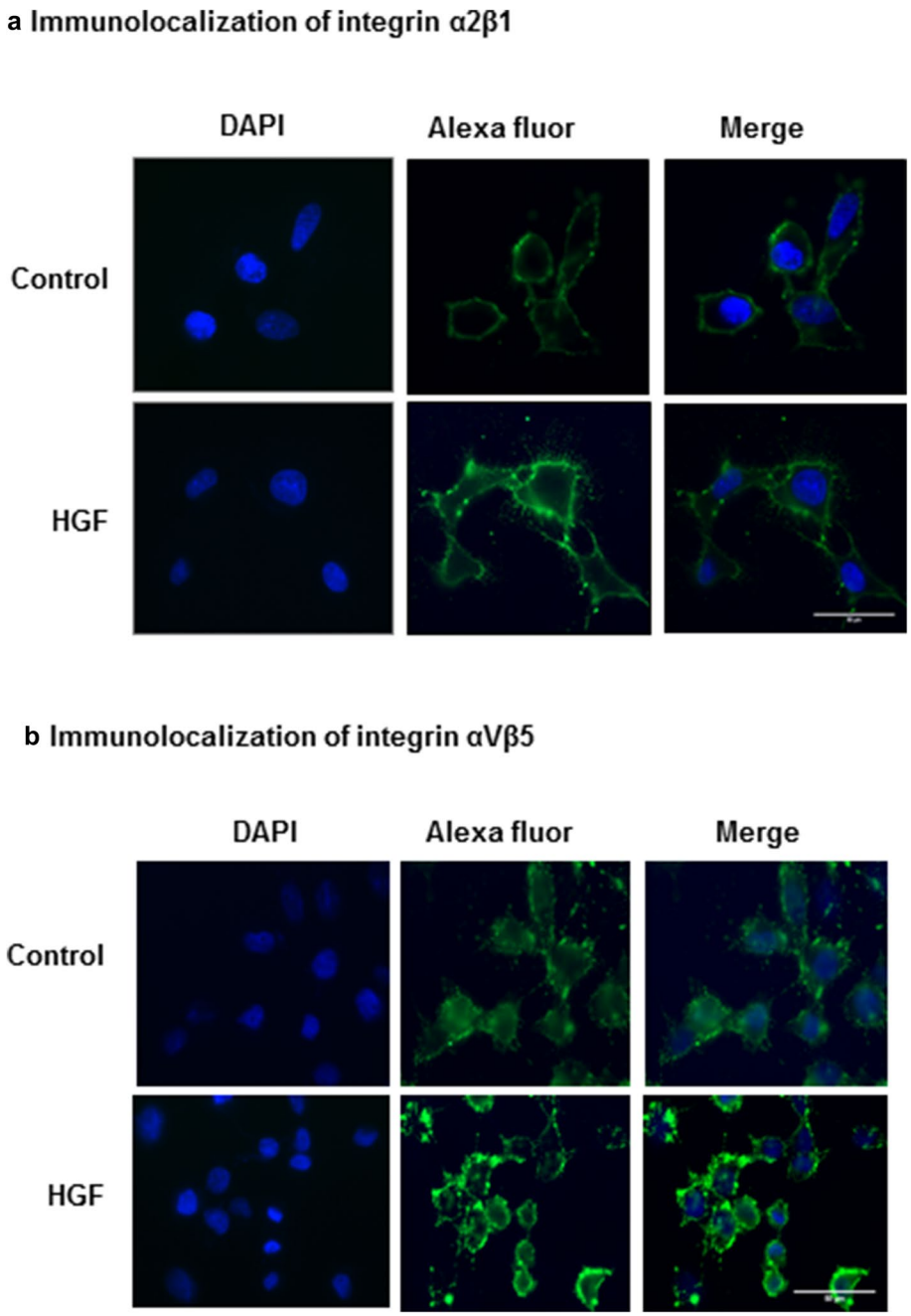

Fig. 5 Immunofluorescent profile of integrin α2β1 and αVβ5 in HTR-8/SVneo cells treated with HGF. HTR-8/SVneo cells were cultured on cover slips and treated with HGF (50 ng/mL) for 24 h followed by immunolocalization (green) of α2β1 and αVβ5 as described in "Materials and methods". The nuclei were stained by DAPI (blue). Cells were fixed in formaldehyde and examined under fluorescent microscope. Images were compiled by proplus software. Scale bar represents 50 µm. (Color figure online)

observed in *ITGAV* silenced cells as compared to control siRNA-transfected cells on treatment with HGF ($p = 0.003$). Interestingly, a significant ($p = 0.02$) decrease in the basal migration of *ITGAV* siRNA-transfected HTR-8/SVneo cells was also observed as compared to control siRNA-transfected cells without any treatment with HGF (Fig. 7e).

Treatment of HTR-8/SVneo cells with HGF led to activation of MAPK and PKA signaling pathways

To delineate which signaling pathways may be involved in up-regulation of WNT4, WNT11, ITGA2, and ITGAV expression, HTR-8/SVneo cells were treated with HGF (50 ng/mL) for 0, 10, 30, and 60 min and processed for analysis of the ERK½ and PKA phosphorylation by Western blotting. Treatment of HTR-8/SVneo cells with HGF (50 ng/mL) led to an increase in the phosphorylation of both ERK½ and PKA (Fig. 8). Time kinetics analysis revealed increase in phosphorylation of ERK1 (~2.8-fold) and ERK2 (~2.2-fold) at 10 min after HGF treatment followed by gradual decrease till 60 min (Fig. 8a). Similarly, an increase in the phosphorylation of PKA (~1.6-fold) was also observed at 10 and 60 min after HGF treatment as compared to 0 min control (Fig. 8b).

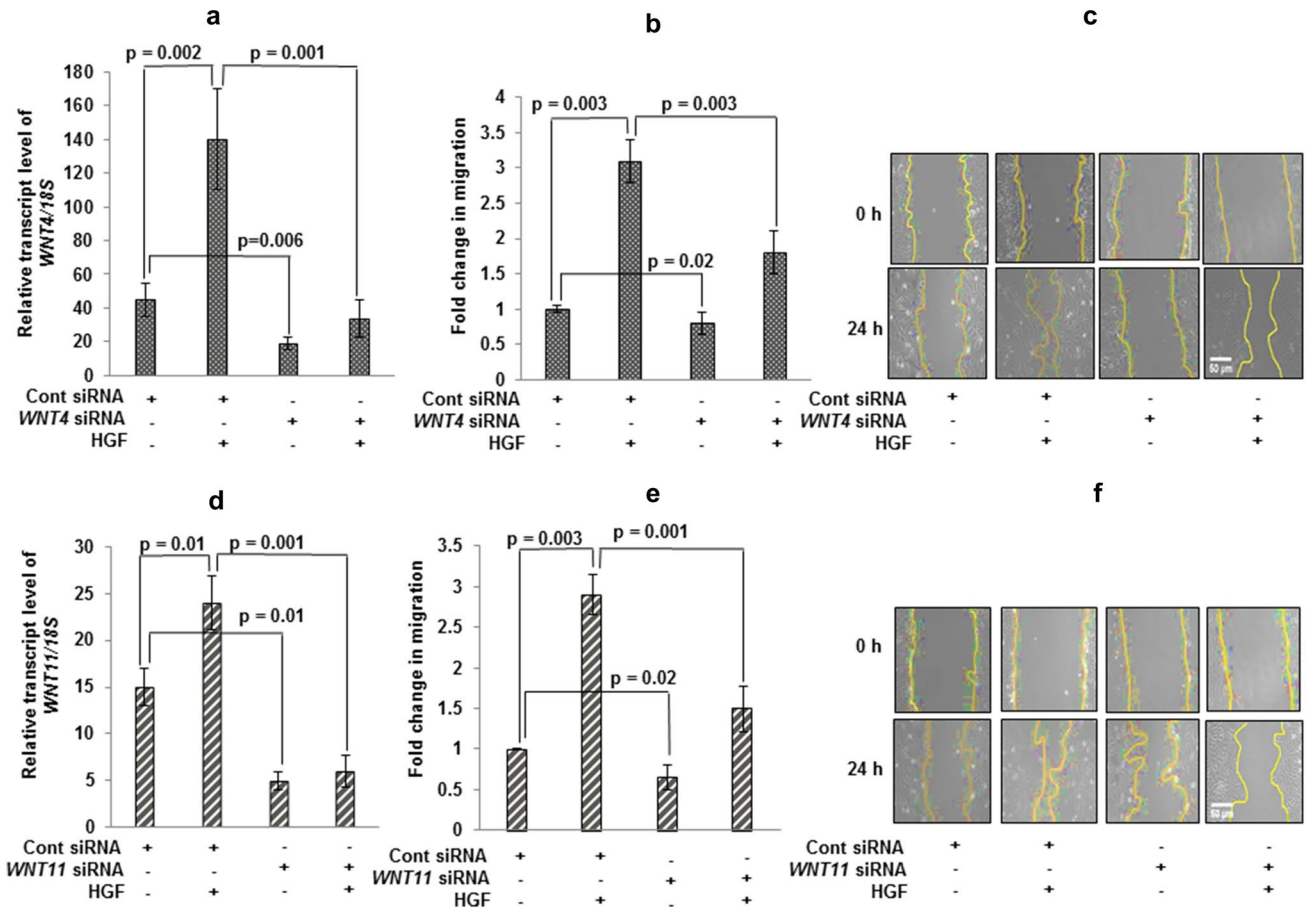

Fig. 6 Effect of *WNT4* and *WNT11* silencing on HTR-8/SVneo cell migration. HTR-8/SVneo cells were transfected with *WNT4, WNT11,* and control siRNA and subsequently used to study their migration by scratch wound-healing migration assay as described in "Materials and methods". **a** and **d** show the expression of *WNT4* and *WNT11* at transcript levels on treatment with/without HGF (50 ng/mL) for 24 h to confirm silencing of *WNT4* and *WNT11*. Each bar represents relative expression after normalization with *18S* rRNA, expressed as mean ± *s.e.m* of three independent experiments. **b** and **e** show fold change in migration of cells transfected with *WNT4, WNT11,* and control siRNA, respectively, subsequent to treatment with HGF (50 ng/mL) for 24 h, as compared to 0 h control. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative images are appended in (**c**) and (**f**). Pictures were taken at 0 and 24 h. Scale bar represents 50 µm

Inhibition of MAPK and PKA signaling pathways led to decrease in HGF-induced expression of WNT ligands and integrins

To investigate if activation of MAPK and PKA signaling pathways by HGF are also involved in increased expression of WNT ligands and integrins, HTR-8/SVneo cells were pre-treated with pharmacological inhibitors for MAPK (U0126) and PKA (H89) signaling pathways and studied for subsequent HGF-mediated increase in expression of WNT ligands and integrins as described in "Materials and methods". Inhibition of MAPK pathway led to significant decrease in expression of WNT11 and ITGA2 (WNT11, $p = 0.003$; ITGA2, $p = 0.04$) in cells treated with HGF as compared to untreated counterparts (Fig. 9b, c). On the other hand, no significant changes in the expression profiles of WNT4 and ITGAV were observed in U0126-pre-treated and HGF-induced cells under similar experimental conditions (Fig. 9a, d). Similarly, inhibition of PKA signaling led to significant decrease in expression of WNT4/WNT11 (WNT4, $p = 0.04$; WNT11, $p = 0.007$) ligands and ITGA2/ITGAV (ITGA2, $p = 0.002$; ITGAV, $p = 0.007$) in HTR-8/SVneo cells treated with HGF as compared to cells that were not pre-treated with H89 but subsequently treated with HGF (Fig. 10a–d).

Silencing of *WNT4*/*WNT11* and *ITGA2*/*ITGAV* revealed cross-talk in HTR-8/SVneo cells

It was further investigated whether silencing of *WNT4* and *WNT11* would have any effect on the expression of *ITGA2* and *ITGAV* and *vice versa*. Interestingly, significant decrease in the transcript levels of both *ITGA2* ($p = 0.03$) and *ITGAV*

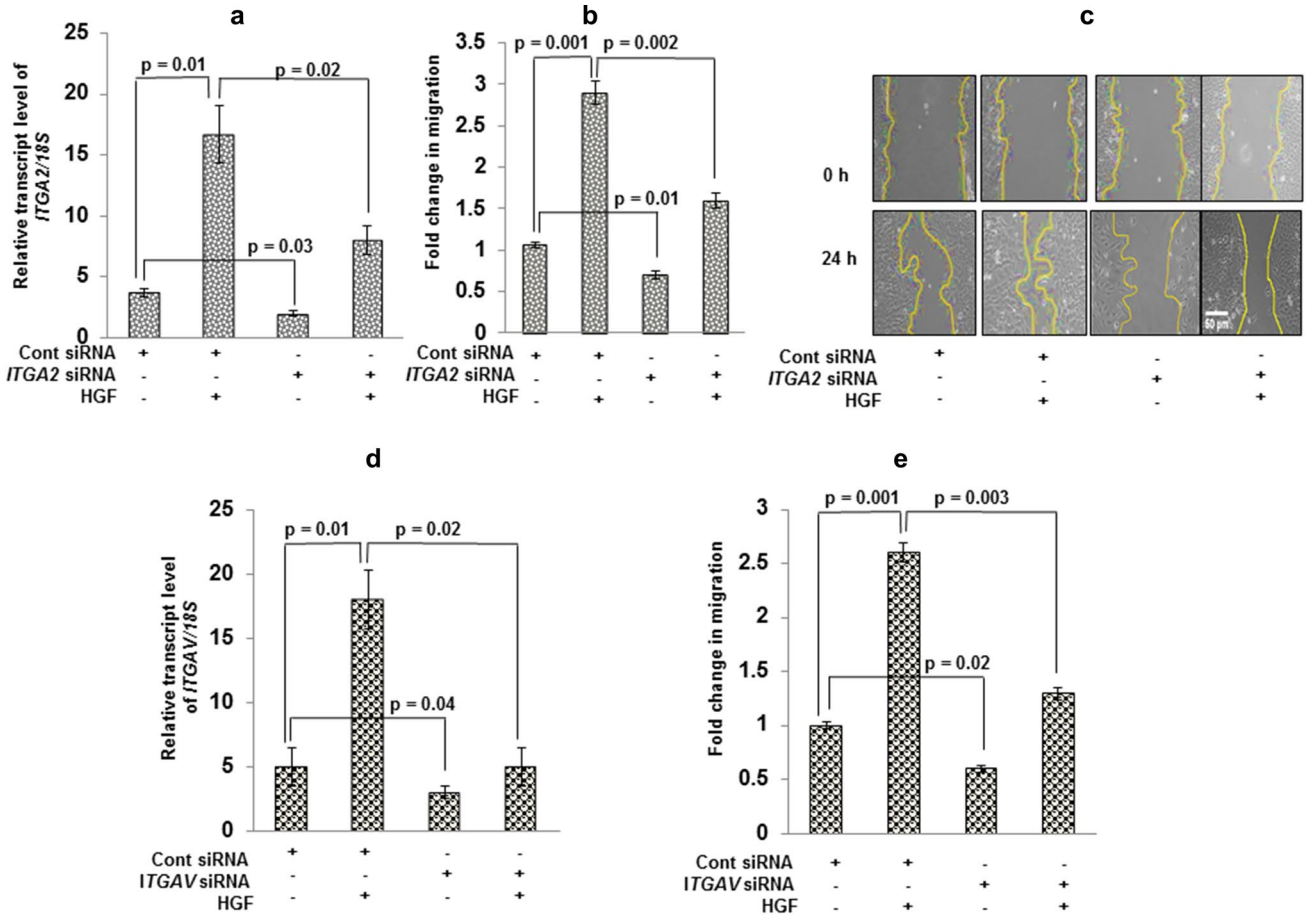

Fig. 7 Effect of *ITGA2* and *ITGAV* silencing on HTR-8/SVneo cell migration. HTR-8/SVneo cells were transfected with *ITGA2, ITGAV*, and control siRNA and subsequently used to study their migration by scratch wound-healing migration (ITGA2*)* and transwell migration (ITGAV) assay as described in "Materials and methods". **a** and **d** show the expression of *ITGA2* and *ITGAV* at transcript levels on treatment with/without HGF (50 ng/mL) for 24 h to confirm silencing of *ITGA2* and *ITGAV*. Each bar represents relative expression after normalization with *18S* rRNA expressed as mean ± *s.e.m* of three independent experiments. **b** and **e** show fold change in migration of cells transfected with *ITGA2, ITGAV*, and control siRNA, respectively, subsequent to treatment with HGF (50 ng/mL) for 24 h. Values are expressed as mean ± s.e.m of three independent experiments. Representative images for ITGA2 silenced cells are appended in (**c**). Pictures were taken at 0 h and 24 h. Scale bar represents 50 µm

($p = 0.01$) expression was observed in *WNT4* silenced cells as compared to the control siRNA-transfected cells after HGF treatment for 24 h (Fig. 11a, b). In case of *WNT11* silenced cells, no significant difference in the expression of *ITGA2* was observed as compared to control siRNA-transfected cells treated with HGF (Fig. 11c). However, the expression of *ITGAV* ($p = 0.01$) was found to be significantly reduced in HGF-treated *WNT11* silenced cells as compared to HGF-treated control siRNA-transfected cells (Fig. 11d).

Further, expression of *WNT4* and *WNT11* was also analyzed after silencing of *ITGA2* and *ITGAV*. A significant decrease in the expression of both *WNT4* ($p = 0.01$) and *WNT11* ($p = 0.04$) in *ITGA2* silenced HTR-8/SVneo cells as compared to control siRNA-transfected cells after treatment with HGF for 24 h was observed (Fig. 12a, b). Similarly, a significant decrease in the expression of *WNT4* ($p = 0.001$) and *WNT11* ($p = 0.04$) was also observed in case of ITGAV silenced HTR-8/SVneo cells as compared to control siRNA-transfected cells after HGF treatment (Fig. 12c, d).

β-catenin may be involved in HGF-mediated increase in HTR-8/SVneo cell migration

HGF/c-MET, WNT, and integrin signalings are known to stabilize the β-catenin, which further translocate to nucleus to activate target genes. To examine the same, we observed significant ($p = 0.003$) increase in *β-catenin* transcript levels in HGF (50 ng/mL)-treated HTR-8/SVneo cells as compared to untreated control after 24 h (Fig. 13a). In addition, a significant increase of β-catenin at protein level was also observed both in cytoplasmic (~ 1.7-fold) as well as in nuclear (~ 2-fold) fractions of

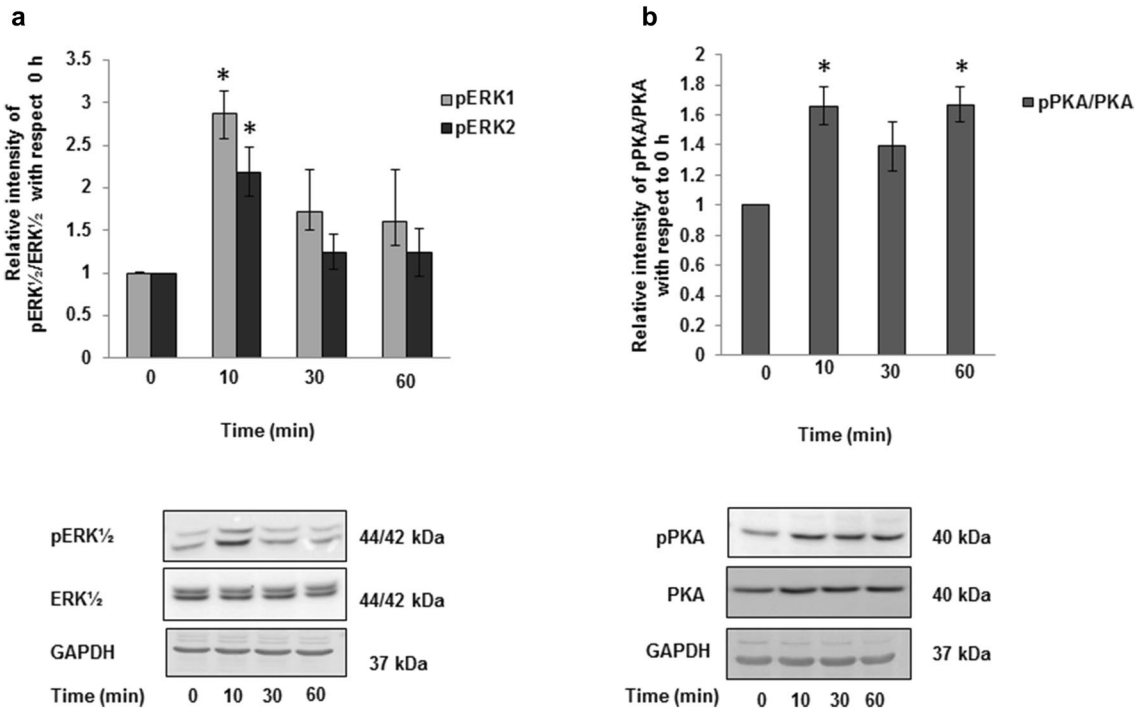

Fig. 8 Activation of MAPK and PKA signaling pathways by HGF in HTR-8/SVneo cells. HTR-8/SVneo cells were treated with HGF (50 ng/mL) for varying time periods (10, 30 and 60 min) followed by Western blot analysis to determine activation of ERK½ and PKA as described in "Materials and methods". **a** represents the densitometric plot showing the relative increase in phosphorylated ERK½ (p-ERK½) in HGF-treated cells with respect to untreated control as compared to total ERK½. **b** represents the densitometric plot showing the relative increase in phosphorylated PKA (p-PKA) in HGF-treated cells with respect to untreated control as compared to PKA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously developed as loading control for each experimental set. The data is expressed as fold change with respect to untreated control and values are shown as mean ± s.e.m of at least three experiments. Representative blots of p-ERK½, ERK½, p-PKA, PKA, and GAPDH are appended alongside in **a** and **b**. *$p \leq 0.05$ with respect to untreated control

cells treated with HGF (Fig. 13b). To link the stabilization of β-catenin with trophoblast migration, HTR-8/SVneo cells were silenced for *β-catenin* by siRNA and subsequently used in wound-healing assay in the presence or absence of HGF as described in "Materials and methods". Silencing of β-catenin was confirmed by Western blotting (Supplementary Fig. 4). A significant decrease ($p = 0.01$) in HTR-8/SVneo cell migration was observed in *β-catenin* silenced cells as compared to control siRNA-transfected cells on treatment with HGF, whereas no significant change in basal migration was observed (Fig. 13c, d). Since β-catenin has been known to be a common target for both WNT ligands and integrin signaling, we further studied its expression in HTR-8/SVneo cells silenced for WNT4/WNT11 and ITGA2/ITGAV. A significant decrease in the expression of β-catenin was observed in both WNT4 and WNT11 (WNT4, $p = 0.006$; WNT11, $p = 0.005$) as well as in ITGA2 and ITGAV (ITGA2, $p = 0.001$; ITGAV, $p = 0.02$) silenced cells as compared to control siRNA-transfected cells treated with HGF for 24 h. However, no significant changes in the expression of β-catenin were observed in control and target-specific siRNA-transfected cells in the absence of treatment with HGF under similar experimental conditions (Fig. 14).

Discussion

Cytokines and growth factors secreted at fetal–maternal interface play a crucial role during implantation by regulating trophoblast cell migration/invasion and differentiation. Impairment in EVT migration can lead to pregnancy-related complications like preeclampsia and IUGR. In this study, we have used HTR-8/SVneo, an immortalized cell line derived from first trimester villous explant, transfected with PSV3neo vector encoding the simian virus 40 large T antigens to study trophoblast migration [26]. This cell line exhibits characteristic molecules of EVTs like expression of cytokeratins 18 and 8, human placental lactogen (hPL), human chorionic gonadotropin (hCG), human leukocyte antigen G (HLA-G), and type IV collagenase. In addition, HTR-8/SVneo cells produce no tumors when injected into nude mice subcutaneously [26, 32], thus is a suitable *in-vitro* model for studying the biology of trophoblast.

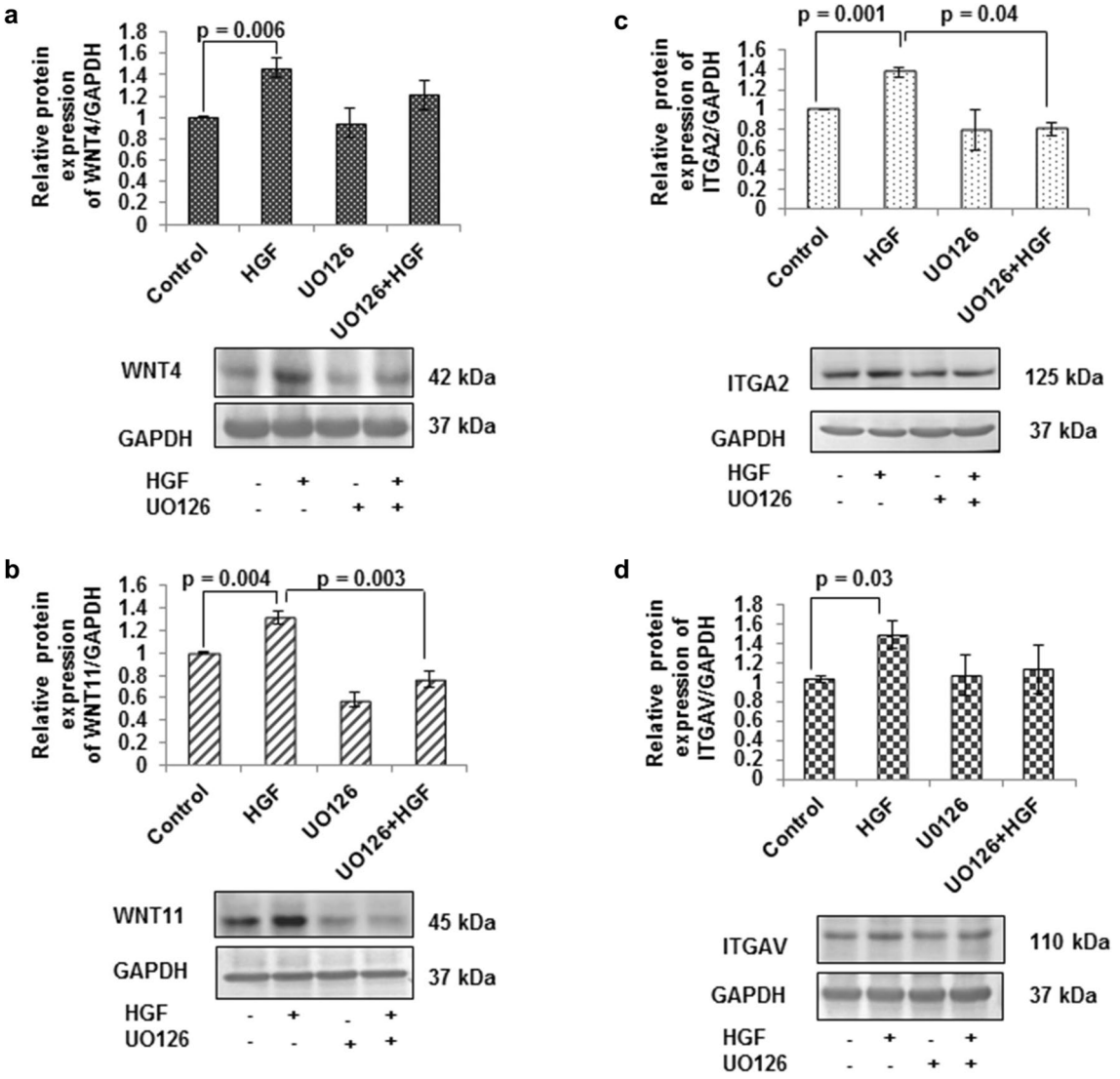

Fig. 9 Effect of inhibition of MAPK signaling on HGF-induced expression of WNT ligands and integrins in HTR-8/SVneo cells. HTR-8/SVneo cells with or without pre-treatment of UO126 for 2 h were subsequently treated with HGF (50 ng/mL) for 24 h and assessed for expression of WNT4, WNT11, ITGA2, and ITGAV by Western blot as described in "Materials and methods". **a** and **b** represent the densitometric profile of WNT4 and WNT11, respectively, in HGF-treated, U0126-pre-treated, and U0126-pre-treated cells subsequently treated with HGF as compared to untreated cells after normalization with GAPDH. Similarly, **c** and **d** represent the densitometric profiles of ITGA2 and ITGAV, respectively. Data are shown as mean $\pm$ *s.e.m* of three independent experiments. Representative blots were appended below each panel, respectively. $p \leq 0.05$ between U0126-pre-treated and U0126-untreated cells under similar experimental conditions was considered statistically significant

In the present study, HTR-8/SVneo cells were treated with HGF, which is highly expressed in human placenta to study trophoblast migration. We have observed dose-dependent increase in trophoblast cell migration on treatment with HGF (Fig. 1) as reported by others [19, 20], which might be due to the fact that HGF also acts as scatter factor [15]. HGF (50 ng/mL) concentration used in the present study may be in physiological range as its level in plasma of pregnant women at 15–20th week of gestation was approximately 670 ng/mL [33]. In amniotic fluid of pregnant women at 20–29th week of gestation HGF levels of ~ 45–50 ng/mL has been reported [34].

WNT ligands, a family of secreted glycoproteins, have been explored for their role in placental biology. Human first trimester placenta have been shown to express fourteen WNT ligands, namely *WNT1, WNT2, WNT2B, WNT3, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT9B, WNT10A, WNT10B*, and *WNT11* [24]. Among all the WNT ligands known to be expressed in human placenta, mRNA expression of *WNT2B, WNT3, WNT4, WNT5A, WNT5B,*

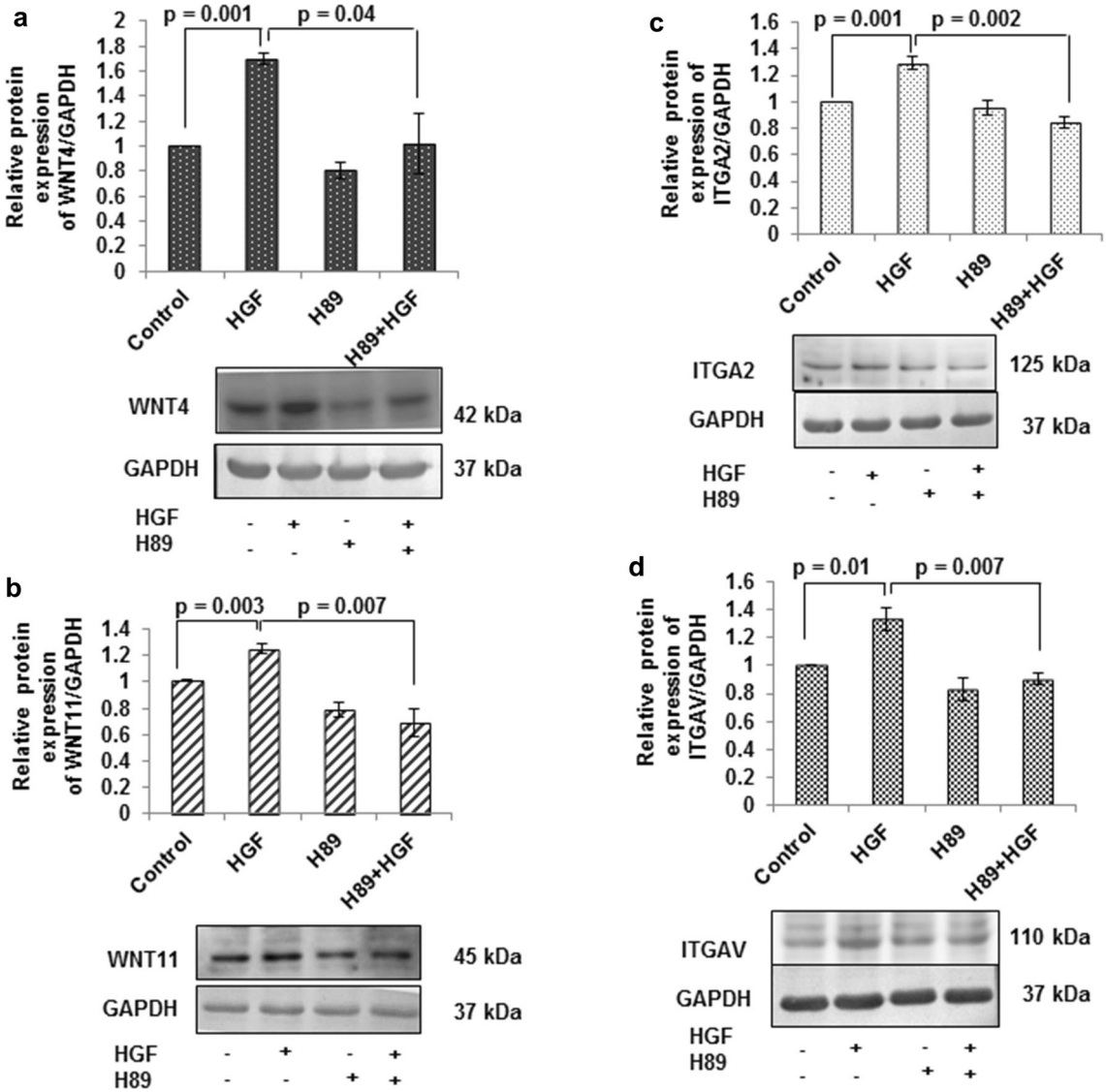

Fig. 10 Effect of inhibition of PKA signaling on HGF-induced expression of WNT ligands and integrins in HTR-8/SVneo cells. HTR-8/SVneo cells with or without pre-treatment of H89 for 2 h were subsequently treated with HGF (50 ng/mL) for 24 h and assessed for expression of WNT4/WNT11 and ITGA2/ITGAV by Western blot as described in "Materials and methods". The densitometric graphs show relative expression of WNT4 (**a**), WNT11 (**b**), ITGA2 (**c**), and ITGAV (**d**) after normalization with GAPDH. Data are shown as mean ± *s.e.m* of three independent experiments. Representative blots have been shown below each panel, respectively. $p \leq 0.05$ between H89-pre-treated and H89-untreated cells under similar experimental conditions was considered statistically significant

WNT7B, WNT9B, and *WNT11* was highly up-regulated in HTR-8/SVneo cells [24]. In this context, the above WNT ligands were chosen to study their role in HTR-8/SVneo cell migration. Treatment of HTR-8/SVneo cells with HGF led to an increase in WNT4 and WNT11 both at the transcript as well as protein levels. Lower fold increase in WNT4 and WNT11 at protein level as compared to their respective transcript may be due to post-transcriptional/post-translational regulatory mechanisms. For example, in case of *Xenopus* Wnt8 (Xwnt8), the presence of a 40 nucleotide regulatory element negatively regulates adenylation and thereby represses translation [35]. Moreover, distinct expression pattern of different isoforms of WNT ligands has also been reported like *Xwnt8* in case of *Xenopus* and *WNT2B* isoforms in different human cancer lines [36, 37]. These alternately generated isoforms might undergo translation on different polysomes, thus resulting in variable expression pattern. In addition, post-translation modifications like glycosylation and acylation also regulate WNT protein stability and secretion [38, 39]. Silencing of *WNT4* and *WNT11* by respective siRNA led to a decrease in cell migration by ~30% and ~40%, respectively, as compared to

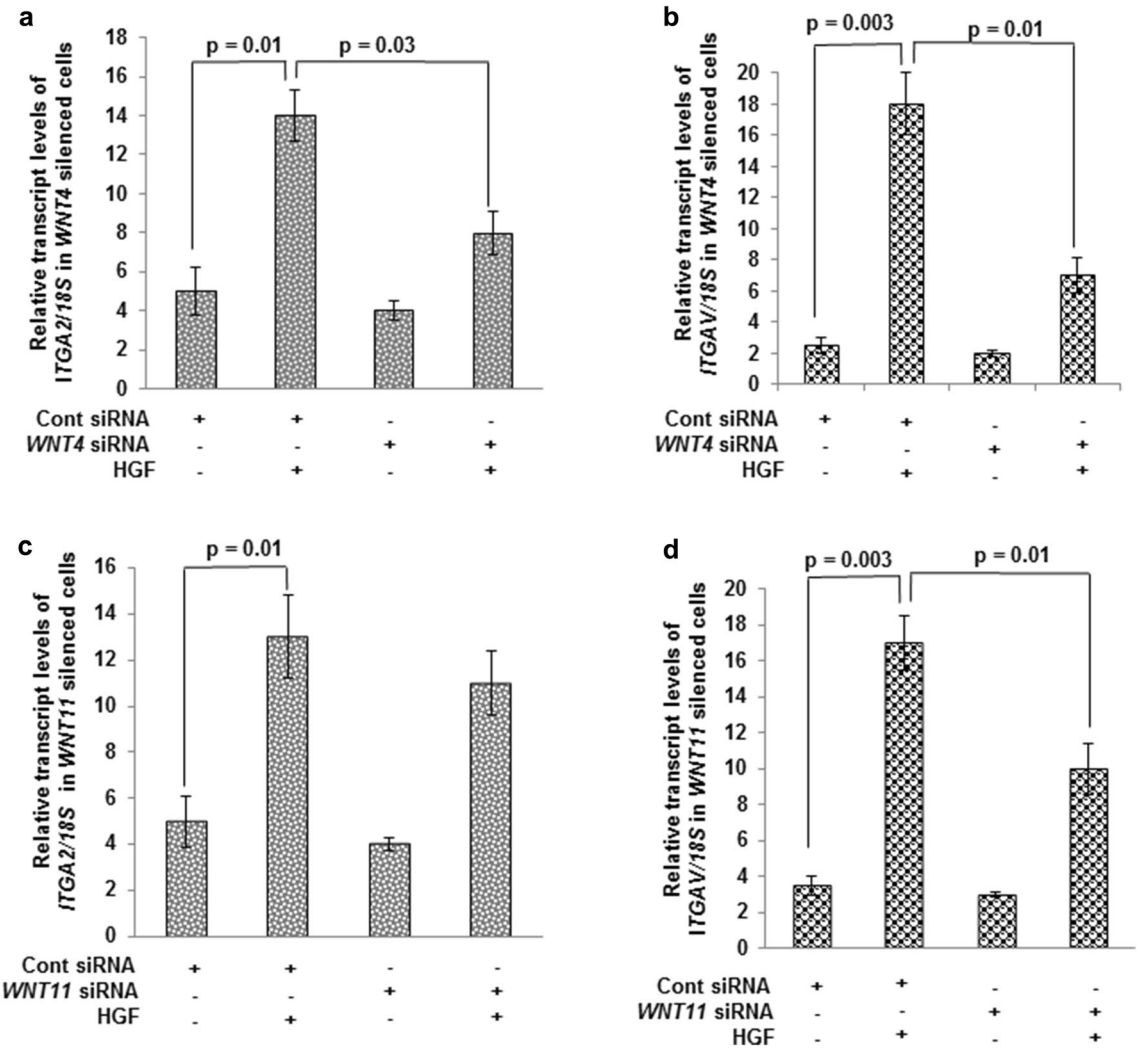

Fig. 11 Effect of *WNT4* and *WNT11* silencing on the expression of *ITGA2* and *ITGAV*. *WNT4* and *WNT11* silenced HTR-8/SVneo cells were treated with HGF (50 ng/mL) for 24 h. Subsequently, total RNA was isolated and *ITGA2* and *ITGAV* transcript levels were determined by qRT-PCR. **a** and **b** compare *ITGA2* and *ITGAV* transcript levels between control siRNA-transfected and *WNT4* silenced cells with/without HGF treatment. **c** and **d** compare *ITGA2* and *ITGAV* transcript levels between control siRNA-transfected and *WNT11* silenced cells after treatment with/without HGF. Each bar represents relative expression after normalization with *18S* rRNA, expressed as mean ± *s.e.m* of three independent experiments. $p < 0.05$ between control and WNT4/WNT11 silenced cells was considered statistically significant

control siRNA-transfected HTR-8/SVneo cells on treatment with HGF. In addition, significant reduction in migration of HTR-8/SVneo cells in *WNT4* and *WNT11* silenced cells in the absence of HGF treatment was also observed, suggesting these WNT proteins may have a role in basal as well as HGF-mediated migration of HTR-8/SVneo cells (Fig. 6). The siRNAs against WNT4 and WNT11 employed in the present study have been used previously to silence WNT4 in human epithelial cells BEAS-2B [40] as well as WNT11 in hepatocellular carcinoma cells [41]. Previously, only WNT3A has been reported to promote trophoblast migration and invasion [25]. Till now, there is no information pertaining to the role of WNT4 and WNT11 in trophoblast migration. However, studies done in $WNT4^{-/-}$ knockout XX gonads in mice showed decrease in endothelial cell migration and coelomic formation during mammalian gonad development [42]. Similarly, WNT4 signal was required for the initiation and elongation of mullerian duct in mice [43]. Mouse embryonic fibroblast cells (NIH3T3) overexpressing WNT4 have higher migration rate as compared to the control cells, thereby suggesting its role in cell migration [43]. On the other hand, WNT11-conditioned medium promotes the migration of intestinal epithelial cells (IEC6) via activating the non-canonical (β-catenin independent) pathways through activation of protein kinase C and Ca^{2+}/Calmodulin-dependent protein kinase I [44]. Similarly, up-regulation of WNT11

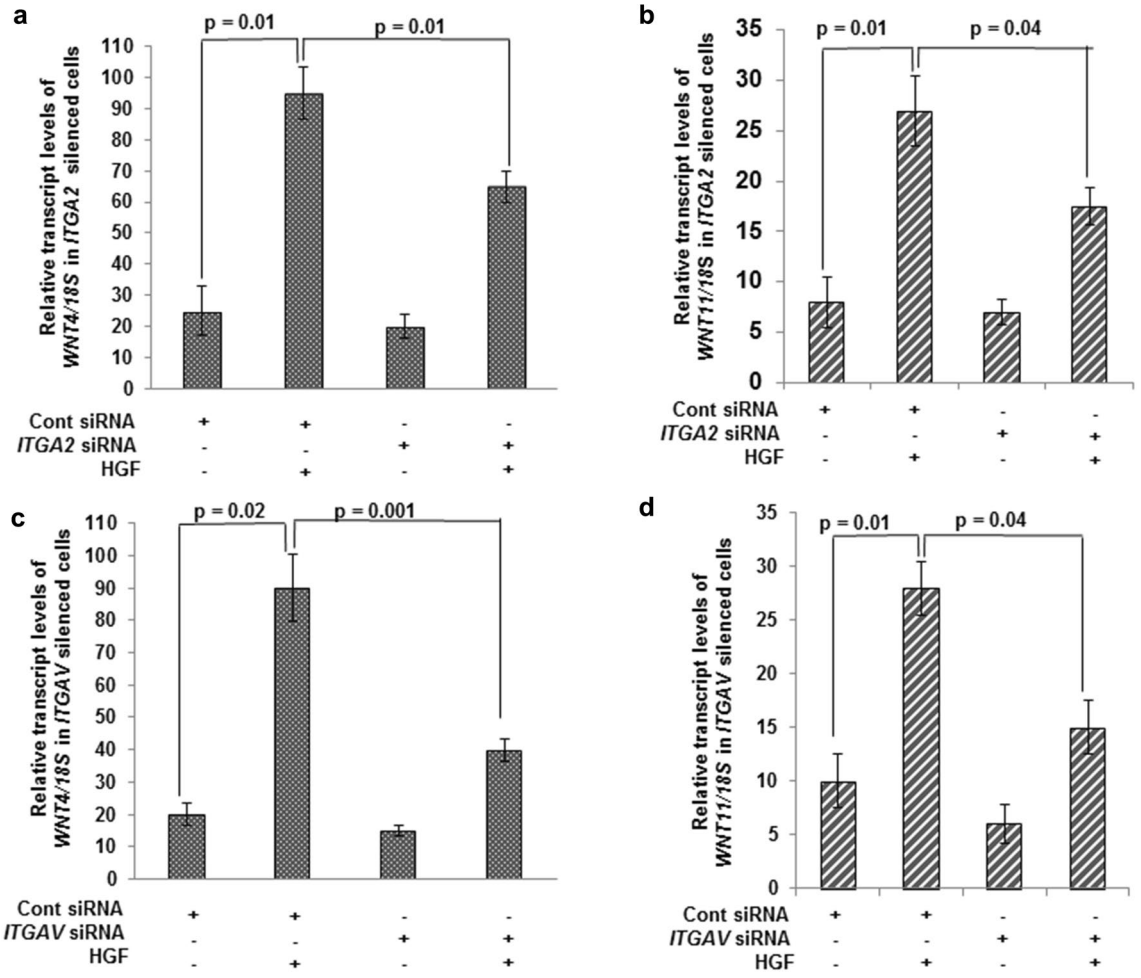

Fig. 12 Effect of *ITGA2* and *ITGAV* silencing on the expression of *WNT4* and *WNT11*. *ITGA2* and *ITGAV* silenced HTR-8/SVneo cells were treated with HGF (50 ng/mL) for 24 h. Subsequently, total RNA was isolated and *WNT4* and *WNT11* transcript levels were determined by qRT-PCR. **a** and **b** compare *WNT4* and *WNT11* transcript levels between control siRNA-transfected and *ITGA2* silenced cells in HGF-untreated and HGF-treated cells. Whereas, **c** and **d** compare *WNT4* and *WNT11* transcript levels in control siRNA-transfected and *ITGAV* silenced cells in HGF-untreated and HGF-treated cells. Each bar represents relative expression after normalization with *18S* rRNA, expressed as mean ± *s.e.m* of three independent experiments. $p < 0.05$ between control and *ITGA2*/*ITGAV* silenced cells was considered statistically significant

by transcription factor complex, ERRα/β-catenin, increases the migration of cancer cells in an autocrine manner [45].

Integrins are heterodimeric transmembrane glycoproteins, and the majority of them are receptors for ECM proteins. They consists of non-covalently associated α and β subunits. These subunits have a large extracellular domain, a transmembrane segment, and a cytoplasmic tail [46]. EVT cells communicate with ECM through cell adhesion molecules such as integrins. Moreover, switching of integrins was mediated by various cytokines like transforming growth factor-β (TGF-β), insulin-like growth factor-2 (IGF2), and insulin-like growth factor binding protein-1 (IGFBP1) during trophoblast migration [47]. Treatment of epithelial, endothelial, and colon cancer cells with HGF led to activation of integrins, while integrin clustering activates c-met receptor of HGF suggesting a cross-talk between the two signaling pathways [48]. In this context, we further studied the expression of various integrins reported to be expressed in HTR-8/SVneo cells subsequent to treatment with HGF (Fig. 2). Among all the integrins studied, a significant increase in the expression of ITGA2 and ITGAV both at transcript and protein levels was observed on treatment with HGF. Higher fold change of ITGA2/ITGAV on treatment with HGF at transcript level as compared to protein level was observed. Similar observations with respect to integrins have been reported previously in breast cancer cell line T47-D and in human trabecular meshwork (HTM) cells [49, 50]. It can be due to better stability and increased half-life of mRNA transcript [51]. The other possible reason could be the promoter sites at 5′ UTR regions, which act as

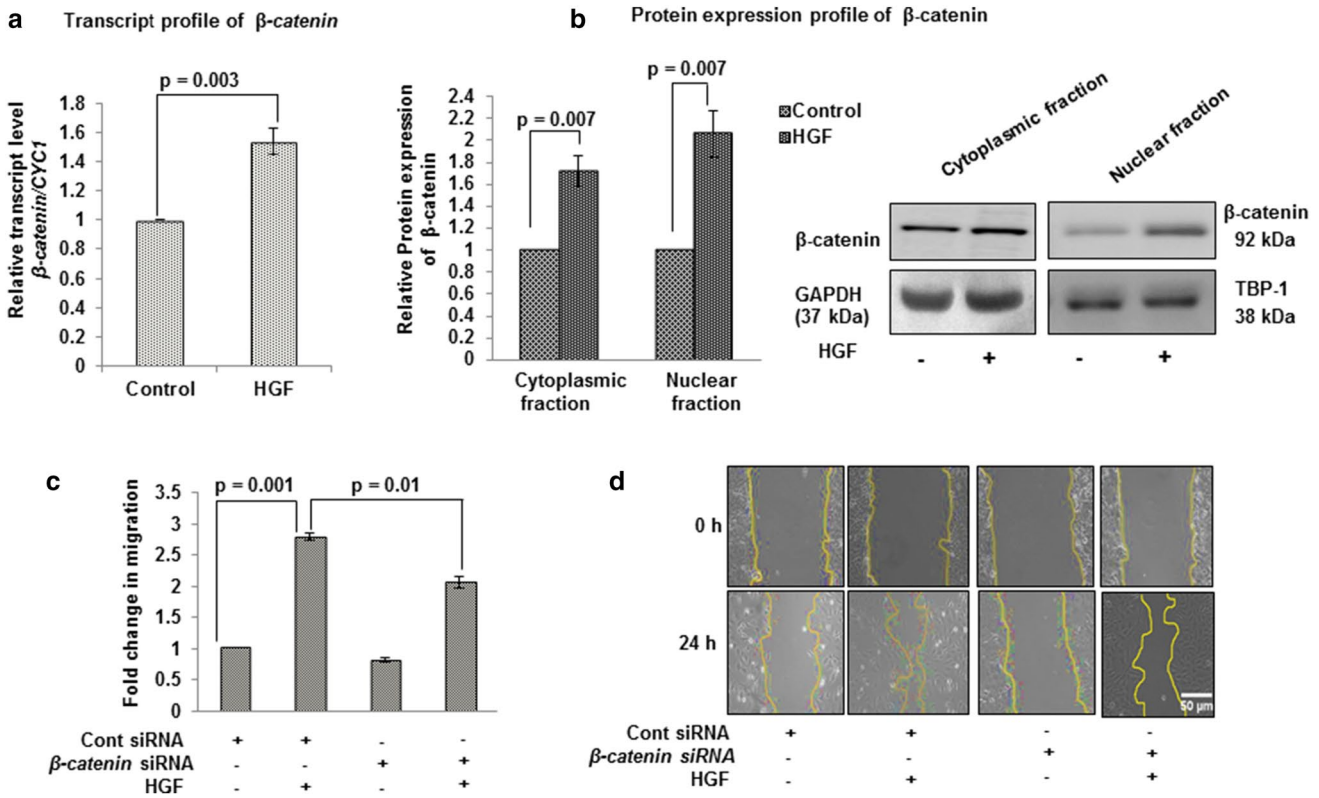

Fig. 13 Expression of β-catenin in HGF-treated HTR-8/SVneo cells and effect of its silencing on migration. HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. The transcript level of *β-catenin* was measured by qRT-PCR; protein level was measured in both cytoplasmic and nuclear fractions by Western blotting as described in "Materials and methods". **a** shows transcript profile of *β-catenin*, and the bars represent relative expression after normalization with cytochrome C1 (*CYC1*) as loading control, expressed as mean ± *s.e.m* of three independent experiments. **b** shows densitometric graph for β-catenin, expressed as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside. GAPDH/TBP was used as cytoplasmic/nuclear internal loading controls, respectively. HTR-8/SVneo cells were transfected with *β-catenin* and control siRNA. After 48 h of transfection, cells were subsequently used to study their migration by scratch wound-healing migration assay as described in "Materials and methods". **c** shows fold change in migration of cells transfected with *β-catenin* and control siRNA, respectively, on subsequent treatment in the presence and absence of HGF (50 ng/mL) for 24 h. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative images are appended alongside in **d**. Scale bar represents 50 µm

translational control for the integrin transcripts [52]. No significant changes in the expression profiles of *ITGB1, ITGB3, ITGB4*, and *ITGB5* were observed (Supplementary Fig. 2). Treatment of first trimester trophoblast cells with estradiol and IL-1α also did not show any increase in *ITGB1* [53]. None the less, expression of β chain is ubiquitous and critical for survival of the embryo [54]. The extracellular matrix ligands bind to integrin receptor through I domain of α chain in association with β chain to activate downstream signaling [55]. Silencing of ITGA2 and *ITGAV* by siRNA led to significant decrease in cell migration after treatment with HGF (Fig. 7). The siRNA employed against ITGAV in the present study has been previously used in human kidney epithelial cell line TCL-598 [56]. Further studies presented herein revealed that treatment of HTR-8/SVneo cells with HGF led to increased expression of α2β1 and αVβ5. Studies in pancreatic cancer cell lines and human melanoma cells have documented the role of α2β1 in their migration on ECM substrate [57, 58]. Clinical studies have shown a decrease in immunostaining of ITGAV in cytotrophoblast cells of preeclamptic placenta [59]. HGF treatment also promotes the αVβ3 clustering via activating focal adhesion components [60] (Fig. 15). However, the present study showed increased expression of αVβ5 without any significant changes in the expression of αVβ1 and αVβ3 (Fig. 5).

HGF binds to c-MET receptor leading to phosphorylation at Tyr (1349) and Tyr (1356) of its β-subunit, which acts as docking sites for Src homology 2 domain-containing signal transducers. It results in activation of signaling pathways like Ras, PI3-kinase, phospholipase-Cγ, and MAPK [61] (Fig. 15). HGF is known to regulate trophoblast migration through up-regulation of (iNOS) via MAPK signaling pathway and also by activation of cAMP-induced PKA signaling pathway through Rap1 [20, 62]. In addition, HGF

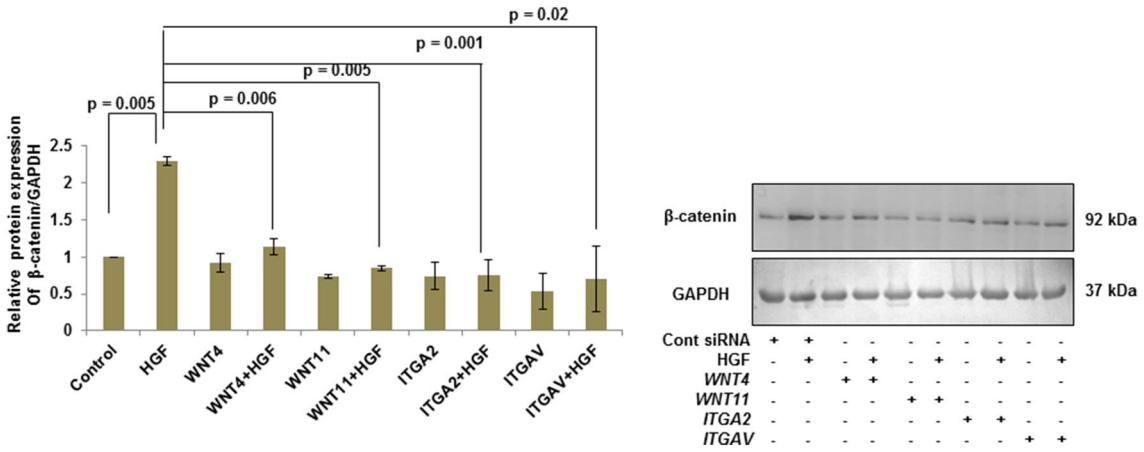

Fig. 14 Effect of silencing of *WNT4/WNT11/ITGA2/ITGAV* on expression of β-catenin in HTR-8/SVneo cells treated with HGF. HTR-8/SVneo cells silenced for *WNT4, WNT11, ITGA2, ITGAV* and those transfected with control siRNA were treated with HGF for 24 h. Subsequently, cell lysates were prepared and processed for Western blotting to check the expression of β-catenin. Each bar represents the relative expression of β-catenin after normalization with GAPDH. Values are expressed as mean ± *s.e.m* of three independent experiments. $p \leq 0.05$ between control siRNA -transfected cells and *WNT4/WNT11/ITGA2/ITGAV* silenced cells after treatment with HGF were considered as statistically significant

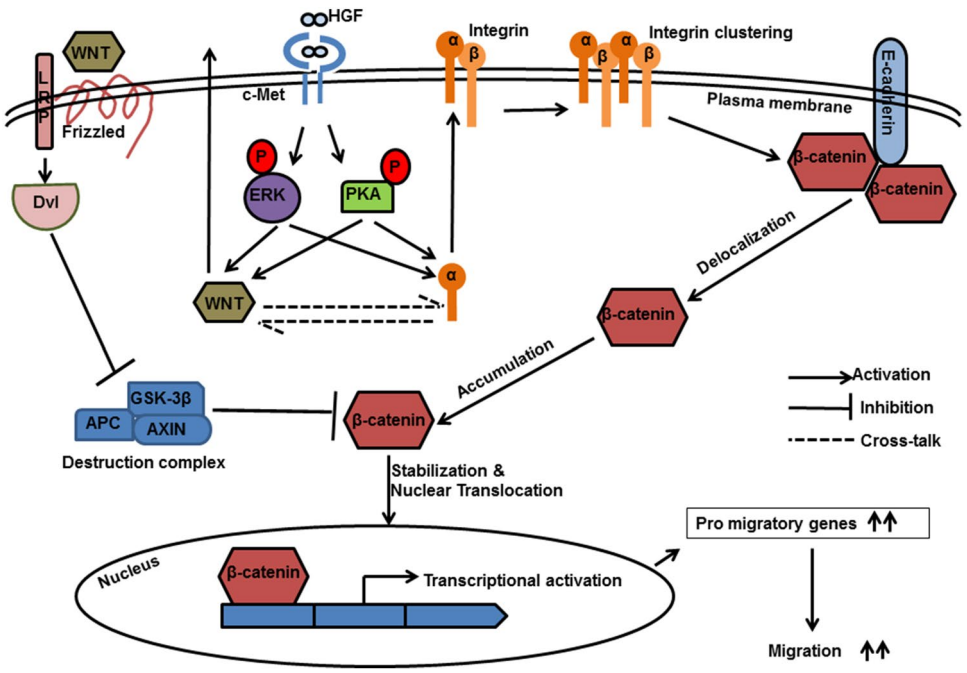

Fig. 15 The mechanistic model for HGF-mediated HTR-8/SVneo cell migration. HGF regulates WNT and integrin expression via activation of ERK/PKA signaling pathways after binding to its c-Met receptor. Cross-communication between WNT ligands and integrin is also shown. Integrin translocates to plasma membrane and undergoes integrin clustering leading to delocalization of E-cadherin-β-catenin complex and thereby accumulation of β-catenin. Simultaneously, WNT ligands are secreted outside the cells and bind to LRP-Frizzled receptor complex, leading to stimulation of downstream Dvl protein which prevents recruitment of destruction complex (GSK-3β/Axin/APC), and hence protects β-catenin from degradation. The active form of β-catenin translocates to nucleus and promotes transcription of genes that may be involved in promoting migration. Therefore, β-catenin might be the common denominator for the WNT and integrin pathways during HGF-mediated HTR-8/SVneo cell migration

also activates NF-kB and JNK signaling, during migration of glioma and brain's endothelial cells [63, 64]. We have also observed phosphorylation of ERK½ and PKA in HTR-8/SVneo cells after treatment with HGF (Figs. 8, 15). Previously, activation of ERK½ and PKA has been shown with respect to WNT signaling [65, 66]. We have

also observed a decrease in HGF-induced WNT4/WNT11 expression in HTR-8/SVneo cells pre-treated with inhibitor of PKA signaling, suggesting its role in regulation of WNT ligand expression. On the other hand, HTR-8/SVneo cells pre-treated with UO126 and subsequent treatment with HGF showed decrease in expression of WNT11 ligand and does not affect WNT4 expression. The MAPK inhibition studies done in mesenchymal progenitor cells also showed decrease in WNT7A expression on subsequent treatment with TGF-β [67]. Similarly, we also observed significant decrease in ITGA2 expression in U0126-treated cells and reduced expression of both of ITGA2 and ITGAV in H89-treated cells on subsequent treatment with HGF. Interestingly, ERK½ signaling is also known to regulate ITGA2 expression in Madin Darby Canine kidney (MDCK) cells treated with HGF [68].

Since HGF/c-Met and integrins signaling regulates various cellular processes like migration, invasion, and differentiation [48] very little is known about cross-talk between WNT and integrin signaling. Therefore, the next major question was to study the link between WNT ligands and integrins in HGF-mediated HTR-8/SVneo cell migration. We studied the effect on *ITGA2* and *ITGAV* expression profile in cells silenced for *WNT4* and *WNT11* during cell migration on treatment with HGF. The qRT-PCR analysis showed that in *WNT4* silenced cells, expression of *ITGA2* transcript was significantly decreased (~ 35%) whereas silencing of *WNT11* has no significant decrease in its expression (Fig. 11). On the other hand, *ITGAV* expression was significantly compromised in both *WNT4* and *WNT11* silenced cells. These observations indirectly link HGF to integrin expression through WNT ligands during HTR-8/SVneo cell migration. Simultaneously, we also studied the expression of *WNT4* and *WNT11* in cells silenced for *ITGA2* and *ITGAV*. The expression of *WNT4* and *WNT11* was significantly reduced in cells silenced for both *ITGA2* and *ITGAV* (Figs. 11, 12). These observations give rise to a new paradigm in WNT and integrin signaling pathway, as results suggest their interdependence. There are few reports on the regulation of WNT expression by integrin signaling like in case of epithelial morphogenesis, where integrin α3β1 regulates *WNT7B* expression via HGF receptor [23].

Stiff ECM leads to increased expression of β-catenin by the activation of integrin/focal adhesion kinase (FAK) pathway. Accumulated β-catenin binds to WNT1 promoter leading to its increase expression. The integrin-activated β-catenin/WNT pathway regulates mesenchymal stem cell differentiation and primary chondrocyte phenotype maintenance [69]. Further, it has been suggested that integrin ligation and FAK activation synergize with WNT signaling through a Grb2-rac-jnk-c-jun pathway [70]. On the other hand, there is a report suggesting β1 integrin regulation by WNT3A *via* integrin-linked kinase (ILK) protein in the migration and adhesion of vascular smooth muscles cells [71]. The above studies have suggested the role of various interlinking proteins like β-catenin, FAK, and ILK between WNT and integrin signaling pathways. HGF stabilizes the β-catenin through c-MET via GSK-mediated phosphorylation of LRP receptor in renal epithelial cells [72]. We also observed increase in expression and nuclear localization of β-catenin in HGF-treated HTR-8/SVneo cells (Fig. 13). Further, siRNA-mediated silencing of β-catenin showed decrease (~ 30%) in HTR-8/SVneo cell migration as compared to control siRNA-transfected cells after treatment with HGF (Fig. 13). Partial decrease in the HGF-mediated migration of HTR-8/SVneo cells subsequently to silencing of β-catenin by siRNA may be due to fact that HGF can also activate other downstream transcription factors like ZEB1, snail, and slug as observed in various cancer cell lines [73, 74], which may be involved in HGF-mediated increase in migration of these cells. Interestingly, silencing of either *WNT4*/*WNT11* as well as *ITGA2*/*ITGAV* led to decreased expression of β-catenin in HTR-8/SVneo cells treated with HGF, suggesting thereby that β-catenin may be a common downstream target for WNT ligands and integrins (Figs. 14, 15). It is possible that interdependence of these pathways may involve other downstream denominators or may act independently.

Conclusion

In summary, we have demonstrated that WNT4, WNT11, ITGA2, and ITGAV play an important role in HGF-mediated increase in HTR-8/SVneo cell migration (Fig. 15). Further, HGF regulates the expression of WNT ligands and integrins via activation of MAPK and PKA signaling pathways. In addition using siRNA, a cross-talk between WNT ligands and integrins has been proposed which may occur through β-catenin, a downstream target, activated by WNT ligands and integrin signaling (Fig. 15). This study used transformed HTR-8/SVneo trophoblast cell line and it is imperative to confirm main experimental findings using primary extravillous trophoblast cells.

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Author contributions PC, SKG, and GSB were involved in project conception and experimental design. PC carried out the experiments.

PC, SKG, SSM, GSB, and RCS interpreted the data thus obtained and were involved in writing the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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