ROLE OF HEPATOCYTE GROWTH FACTOR AND SIGNALING PATHWAYS IN TROPHOBLASTIC CELL MIGRATION UNDER NORMAL AND HYPOXIC CONDITION

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By

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DECLARATION

I, **Piyush Chaudhary** hereby declare that the thesis entitled **"ROLE OF HEPATOCYTE GROWTH FACTOR AND SIGNALING PATHWAYS IN TROPHOBLASTIC CELL MIGRATION UNDER NORMAL AND HYPOXIC CONDITION"** is an authentic research work carried out by me under the guidance of Dr. G. Sunil Babu, Assistant Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow and co-guidance of Dr. Satish Kumar Gupta, Emeritus Scientist, National Institute of Immunology, New Delhi and Prof. R. C. Sobti, Emeritus Professor, Panjab University, Chandigarh. The research work is original, and no part of this work has been submitted for any other degree or diploma.

I also declare that the thesis is essentially free from all kinds of plagiarism.

All the above given information is true to the best of my knowledge.

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CERTIFICATE

This is to certify that the thesis entitled "**ROLE OF HEPATOCYTE GROWTH FACTOR AND SIGNALING PATHWAYS IN TROPHOBLASTIC CELL MIGRATION UNDER NORMAL AND HYPOXIC CONDITION**" submitted by Mr. **Piyush Chaudhary** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other University.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow, satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulation-1999* as amended in *2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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Abbreviations

ATP- adenosine triphosphate

BCA- bicinchoninic acid

BSA- bovine serum albumin

CAM- cell adhesion molecule

cDNA- complementary DNA

CSF-1- colony stimulating factor-1

CTBs- cytotrophoblast cells

CXCL- C-X-C motif chemokines ligand

CYC1- cytochrome C1

DAB- 3, 3′-diaminobenzidine

Dkk- dickkopf-related protein

dNTP- deoxyribonucleotide triphosphate

E-cadherin- epithelial-cadherin

ECM- extracellular matrix

enEVTs- endovascular cytotrophoblast cells

ELISA- enzyme-linked immunosorbent assay

EMT- epithelial to mesenchymal transition

ERK1/2- extracellular signal-regulated kinase ½

FBS- fetal bovine serum

FF-folicular fluid

FGF-fetal growth restriction

g- gravity

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

GM-CSF- granulocyte-macrophage colony stimulating factor

GPCR- G protein-coupled receptor

Grb-2- growth factor receptor-bound protein 2

h- hour

hCG- human chorionic gonadotropin

HGF- hepatocyte growth factor

hPL- human placental lactogen

HRP- horseradish peroxidase

iEVTs- interstitial cytotrophoblast cells

IFN-γ- interferon gamma

IGFBP1- insulin-like growth factor binding protein 1

IGF-II- insulin growth factor II

IL-6/8/10/11/12- interleukin-6/8/10/11/12

ITGA-integrin alpha chain

ITGB-integrin beta chain

IUGR- intrauterine growth restriction

IVF- *in vitro* fertilization

JAK-STAT- janus kinase-signal transducer and activator of transcription

LIF- leukemia inhibitory factor

LRP- lipoprotein receptor

M- molar

MAPK- mitogen-activated protein kinase

mg/mL- milligram per milliliter

min- minute

miRNA- micro ribonucleic acid

mM- millimolar

MMP- matrix metalloproteinase

mRNA- messenger ribonucleic acid

mTOR- mammalian target of rapamycin

N-cadherin- neural-cadherin

ng- nanogram

ng/mL- nanogram per milliliter

nm- nanometer

nM- nanomolar

ºC- degree centigrade

PAI- plasminogen activator inhibitor

PBS- phosphate buffered saline

PE- preeclampsia

pg/mL- picogram per milliliter

PI3K- phosphoinositide-3 kinase

PKA- protein kinase A

QC- quality check

RT-qPCR- quantitative reverse transcription polymerase chain reaction

RTK- receptor tyrosine kinase

SDS-PAGE- sodium dodecyl sulfate-polyacrylamide gel electrophoresis

sec- second

SHP2- src homology region 2 (SH2)-containing protein tyrosine phosphatase

SMAD5- mothers against decapentaplegic homolog 5

STBs- syncytiocytotrophoblast cells

TBP- TATA-binding protein

TCF- T-cell factor

TGF-β- transforming growth factor-beta

TIMP- tissue inhibitor of metalloproteinase

TNF-α- tumor necrosis factor-alpha

uNK- uterine natural killer

uPA- urokinase plasminogen activator

VEGFA- vascular endothelial growth factor A

VSMC- vascular smooth muscle cells

WNT- wingless-type *MMTV* integration site family

μL- microliter

μM- micromolar

Introduction

Cell migration is an evolutionary conserved mechanism which involves movement of cells from one place to another. The process of cell migration plays a crucial role in development and functioning of single to multicellular organisms and also in normal and disease processes which include various events of embryogenesis, wound healing, immune response and cancer metastasis. Though these migratory events involve different cell types, it is believed that all these migratory cells follow the four basic steps; polarization, protrusion, adhesion, and retraction (Vicente-Manzanares *et al*., 2005). In humans, the first event of cellular migration occurs during blastocyst implantation and continues throughout the embryonic development and in later stages of life. The utmost importance of cellular migration has been seen during the implantation of embryo and placental development (Reig *et al*., 2014).

During the process of implantation and placentation, cytotrophoblast cells undergo proliferation and differentiation into various trophoblast lineages. Among them, syncytiotrophoblast (STB) arise from fusion of cytotrophoblast cells and perform placental functions like exchange of nutrients, gases and secretion of hormones like human chorionic gonadotrophin (hCG), human placental lactogen (hPL) and various growth factors, crucial for fetal growth and placental development. Whereas, interstitial extravillous trophoblast (iEVT) and endovascular extravillous trophoblast (enEVT) are derived from extravillous trophoblast (EVT) lineage (Aplin, 1991; Ji *et al*., 2013). The iEVT cells invade the inner third of myometrium and interact with uterine NK cells to modulate the maternal immune response for successful implantation of blastocyst (Oreshkova *et al*., 2002). The enEVTs invade the spiral arteries and migrate into its lumen to replace endothelial cells into low resistance high capacity uteroplacental arteries that provide nutrition to developing embryo (Krüssel *et al*., 2003; Bischof and Irminger-Finger, 2005). Besides, migratory and invasive properties, these cells also have the capacity to recognize, modify and stimulate the behavior of other cell types at the maternal-fetal interference. Any defects in migration and invasion of these cells either during the invasion of myometrium or during the spiral arteries remodeling, may lead to

pregnancy complications like preeclampsia and intrauterine growth restriction (IUGR) (Lim *et al*., 1997; Kaufmann *et al*., 2003; Matthiesen *et al*., 2005). Likewise, excessive invasion of maternal endometrium by trophoblastic cells can cause placenta accrete, increate and percreta (Seckl *et al*., 2010; Wortman and Alexander, 2013). While, insufficient spiral artery remodeling due to aberrant trophoblast invasion is also one of the reason for late sporadic miscarriages in pregnant women (Ball *et al*., 2006). Thus, it is imperative to discover the factors and molecular mechanisms associated with trophoblast migration and invasion that will help in better understanding how they contribute to normal and abnormal placentation.

The process of trophoblastic cells migration and invasion is controlled by plethora of cytokines and growth factors secreted by diverse cell types at the fetal-maternal interface. During the implantation process, interlukin-6 (IL-6; Jovanovic and Vicovac, 2009), leukemia inhibitory factor (LIF; Suman and Gupta, 2014), hepatocyte growth factor (HGF; Kauma *et al*., 1999), epidermal growth factor (EGF; Xie *et al*., 2015; Malik *et al*., 2017), CXCL16/CXCR6 (Huang *et al*., 2006), CXCL12/CXCR4 (Ren *et al*., 2012) etc. are known as positive regulator as they promote trophoblast migration and invasion. On the other hand, transforming growth factor-beta (TGF-β; Graham *et al*., 1994), interferon-gamma (IFN-γ; Lash *et al*., 2006; Verma *et al*., 2018), tumour necrosis factor-alpha (TNF-α; Bauer *et al*., 2004; Huber *et al*., 2006), IL-10 (Rooth and Fisher, 1999; Hanna *et al*., 2000), IL-12 (Karmakar *et al*., 2004) and kisspeptin (Bilban *et al*., 2004; Hiden *et al*., 2007; Francis *et al*., 2014) are known to inhibit trophoblast invasion. The fine balance between the positive and negative regulators of trophoblast migration and invasion is essential for the successful pregnancy.

In the present study, the role of HGF in the migration and invasion of trophoblast cells has been investigated. HGF has been identified as a cellular growth factor, mitogen and morphogen (induction of multicellular tissue-like structure) which is known to stimulate the organ development and tissue regeneration (Gherardi and Stoker, 1990; Nakamura and Mizuno, 2010). In non-pregnant women, HGF is secreted by cells like fibroblasts,

Kupffer cells, macrophages and endothelial cells of mesenchymal origin (Kinoshita *et al*., 1989; Noji *et al*., 1990). While during pregnancy, both the placenta as well as amniotic membrane produce and secrete HGF (Wolf *et al*., 1991; Clark *et al*., 1996). HGF is also known to have a physiological role in fetal growth, development and differentiation of placenta (Selden *et al*., 1990; Uehara *et al*., 1995). Previous findings have suggested the importance of HGF in pregnancy, as both HGF mRNA and protein expression levels are reduced in the preeclamptic placenta (Furugori *et al*., 1997). In addition, HGF knockout mice show embryo lethality due to inadequate placentation (Uehara *et al*., 1995). HGF has also been reported to stimulate invasiveness and motility of EVT cells (Kauma *et al*., 1999) suggesting its role during trophoblast migration and invasion. Besides this, increased expression of HGF is also reported in serum and follicular fluid (FF) in polycystic ovarian syndrome (PCOS) patients (Sahin *et al*., 2013). Similarly, considerable amount of HGF is also reported in FF and granulosa cells of women undergoing in-vitro fertilization (IVF) treatment (Osuga *et al*., 1999). HGF has an important role in pregnancy and decrease in its levels can lead to pregnancy complications like preeclampsia and IUGR. Hence, it is imperative to study the molecular mechanism associated with HGF-mediated trophoblast migration and invasion.

Cytokines and growth factors, on binding to their specific receptors, trigger the activation of various downstream signaling pathways. Among them, Janus kinase/signal transducer and activation of transcription (JAK/STAT), mitogen activated protein kinase (MAPK), Phosphoinositide 3-Kinase PI3K/AKT, cyclic-AMP/protein kinase A signaling (cAMP/PKA) and WNT signaling pathways are important in trophoblast migration and invasion (Gupta *et al*., 2016). Among the MAPK families, extracellular signal-regulated kinases (ERK) are reported to be active in iEVTs throughout pregnancy (Ichikawa *et al*., 1998). ERK activation by various growth factors and cytokines in various trophoblast cell lines also gives evidence in favor of its role in the migration and invasion. Phosphorylated form of ERK½ is significantly higher in EVTs from normal pregnancy as compared to EVTs in placental bed biopsies from women with preeclampsia (Moon *et*

al., 2008). In mice knockout for ERK isoforms, placental insufficiency and implantation failure is observed suggesting its role in placental development and pregnancy (Hatano *et al*., 2003). PI3K/AKT signaling is associated with a variety of cellular processes including cell growth, proliferation, migration, and survival. Studies in mice report that AKT isoforms are widely expressed in all types of trophoblast lineages and vascular endothelial cells. Targeted gene disruption of AKT leads to hypertrophy or structural abnormalities in placenta, due to loss of proliferative CTBs (Yang *et al*., 2003). In presence of HGF, via activation of cAMP, PKA signaling modulate adhesion and migration of trophoblast cells (Chen *et al.*, 2013). Although, fourteen WNT ligands have been known to be expressed in placental tissue (Sonderegger *et al*., 2007), but their role in trophoblast migration has not been studied extensively. However, higher number of WNT signaling associated β-catenin-positive nuclei in EVTs are detected in placentae of complete hydatidiform mole (CHM) suggesting that aberrant WNT signaling could contribute to abnormal invasion in this pregnancy disorder (Pollheimer *et al*., 2006). HGF also function as pleotropic cytokine and on binding to c-met receptor, it activates downstream signaling pathways in various cancer cell lines (Vande Woude *et al*., 1997). Thus, it would be interesting to decipher the role of various signaling pathways associated with HGF-mediated migration of trophoblastic cells and their cross communication in regulation of pro-migratory genes.

Besides signaling pathways, during cell migration, intracellular molecules and transmembrane proteins are also involved, which can sense the outside micro environment, responds to signals from extracellular matrix (ECM) and thus modulate the cell behavior. Integrins are a diverse family of transmembrane glycoproteins that form heterodimeric receptors for ECM proteins. These receptors can form at least 25 distinct pairings of its 18 α-subunits and 8 β-subunits, with each pairing being specific for a unique set of ligands (Van der Flier and Sonnenberg, 2001). During EVT cells differentiation, migration and invasion, integrin switching occurs in response to alteration of ECM components. Studies have shown that during differentiation of invasive

cytotrophoblasts, expression of integrin α6β1 is down-regulated, whereas α5β1 and α1β1 are up-regulated (Damsky *et al*., 1992; 1994). Failure in switching of integrins during EVT cells invasion can lead to preclampsia, as observed in preeclamptic placenta in which iEVTs cells failed to regulate the expression of αVβ3 (Zhou *et al.*, 1997). In addition, insulin like growth factor (IGF-1) mediated migration of EVT cells also depends on the activation of integrin αVβ3, which is localized adjacent to focal adhesion proteins (Irving and Lala, 1995). Although, it has been known that integrin switching is the integral part of trophoblast migration, but how the presence of different cytokines and growth factors influence this process needs further investigations. Apart from cell surface integrins, trophoblast cell migration and invasion into maternal endometrium involves degradation of ECM by matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) (Shimonovitz *et al*., 1994; Staun-Ram *et al*., 2004). The activity of MMPs and uPA are regulated by tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitor (PAI) respectively. The activity of MMPs is regulated by TIMPs by binding to MMPs in 1:1 stoichiometric ratio and thereby modulating their proteolytic activity (Visse and Nagase, 2003). The integrity of ECM is determined by MMP/TIMP ratio and hence a balance between MMPs and TIMPs is crucial. MMPs and TIMPs play a crucial role in placental functions, therefore it has been suggested that spatial and temporal alterations in placental MMPs and TIMPs activities might be contributing to preterm delivery (Demir-Weusten *et al*., 2007; Mayor-Lynn *et al*., 2011). Several studies have also reported altered MMP and TIMP expression in fetal membranes, amniotic fluid and cervical tissues (Weiss *et al*., 2007; Nishihara *et al*., 2008; Menon and Fortunato, 2004). Thus the relevance of MMPs and TIMPs in HGF-mediated increase in migration/invasion of trophoblastic cells has also been investigated in the present thesis.

During pregnancy, a correlation exists between oxygen concentration and placental development (Sharashenidze *et al*., 2017). Oxygen is a key regulator for trophoblast differentiation, proliferation and migration during placental development (Ji *et al*., 2013).

During first trimester of pregnancy, low partial pressure of O_2 of 18-40 mmHg was measured in intervillous space (Rooth *et al*., 1961) and endometrial tissue (Rodesch *et al*., 1992). It rises to 60-80 mm Hg in the beginning of second trimester, after remodeling of spiral arteries and increased placental perfusion (Jauniaux *et al*., 2006). Failure in EVT cells migration and invasion into spiral arteries leads to premature onset of maternal placental circulation leading to oxidative stress and pregnancy complications preeclampsia and IUGR (Poston and Raijmakers, 2004). Placenta responds to hypoxia by activation of hypoxia inducible factors (HIFs), which are key regulators of placental vascularization and invasion. HIFs are heterodimeric transcription factors consisting of HIF-α (HIF-1α, HIF-2α and HIF-3α) and HIF-1β. HIF-1α and HIF-2α mRNA and proteins are expressed in placenta and trophoblast (Highet *et al*., 2015), while multiple HIF-3α mRNA isoforms have been detected in the placenta tissue, but not evaluated in trophoblast cells (Maynard *et al*., 2003). Deciphering the molecular interactions between HGF and signaling pathways and how it regulates HIFs expression will help in better understanding of trophoblastic cell migration and invasion under hypoxic conditions.

Several trophoblastic cell lines like SGHPL-5, HTR-8/SVneo, ACH-3P (First trimester primary trophoblast transformed cell lines) and JEG-3, JAR (Choriocarcinoma cell lines) have been employed to study trophoblast migration and invasion. HTR-8/SVneo cell line was derived from first trimester trophoblast cells, transfected with PSV3neo vector encoding the simian virus 40 large T antigens (Graham *et al*., 1993). HTR-8/SVneo cells also express trophoblast stem/progenitor cell transcription factor; Caudal type homeobox2 (CDX2), also present on trophoblast cells (Weber *et al*., 2013). This cell line exhibits characteristic similar to EVTs, like expression of cytokeratin 18 and 8, hPL, hCG, cluster of differentiation 9 (CD9), and type IV collagenase. HTR-8/SVneo cells when grown on matrigel, express human leukocyte antigen G (HLA-G), similar to EVTs (Kilburn *et al*., 2000). Moreover, in EVT and HTR-8/SVneo cells, similar intermediate signaling molecules of Smad family are activated in the presence of TGF-β, while other choriocarcinoma trophoblastic cell lines like JEG-3 and JAR failed to show similar

response due to loss of Smad3 protein (Xu *et al*., 2001). Similarly, after IL-11 treatment, GRP78 is the common target protein in EVT as well as in HTR-8/SVneo cells (Sonderegger *et al*., 2011). In addition, IL-11 treatment led to decrease in EVT growth as well as HTR8-SVneo invasion (Sonderegger *et al*., 2011; Suman *et al*., 2012). Besides this, HTR-8/SVneo cells do not form tumor when injected subcutaneously into nude nice (Graham *et al*., 1993; Shiverick *et al*., 2001). From these studies, it is reasonable to conclude that HTR-8/SVneo cells respond similarly to the physiological ligands as primary trophoblast cells and thus it is a suitable *in-vitro* model to study trophoblast biology.

Taking the above into account, in the present thesis, the role of HGF and associated signaling pathways during migration and invasion has been investigated using pathway inhibitors. In addition, the relevance of WNT ligands and integrins and their cross-talk has also been deciphered. To understand the relevance of hypoxia, the expression of various MMPs and TIMPs responsible for trophoblast migration/invasion along with transcription factors was also studied.

Review of Literature

2.1 Embryo Implantation

In human pregnancy, successful embryo implantation requires a bilateral fine-tuned communication between the competent blastocyst and a receptive endometrium during a specific window of time during the menstrual cycle. The uterus is receptive for implantation only for a short window of time called the "*window of implantation"* or "*window of receptivity*". In humans, window of implantation starts between 6–10 days after ovulation during the mid-secretory phase of menstrual cycle (Blesa *et al*., 2014; Donaghay and Lessey, 2007; Psychoyos 1973). During this period, endometrium undergoes a series of morphological, physiological and biochemical changes, which transforms the endometrial cells into the decidua. The endometrial epithelial and stromal cells under the influence of increased level of progesterone cease to proliferate and undergo differentiation (Dockery *et al*., 1988). On the other hands, glandular epithelial cells gain the secretory potential and starts producing several kinds of cytokines and growth factors (Tabibzadeh *et al*., 1995; Cullinan *et al*., 1996). At the same time, due to excessive infiltration of leukocytes into endometrium, stromal cells also undergo decidualization. Meanwhile, the fertilized ovum also undergoes several round of division to form blastocyst. After attaining blastocyst stage, the embryo arrives at the endometrium and the process of implantation begins.

The process of implantation consists of three main stages: apposition, adhesion, and invasion (Hertig *et al*., 1956; Lindenberg 1991). Apposition is the first initial connection between the blastocyst and the endometrium, during which the human blastocyst using microvilli finds a location to implant, guided by the pinpodes (micro-protrusions) on the maternal endometrium. Following apposition, the attachment phase initiates a stronger physical connection between the blastocyst and the endometrium along with orientation of the embryonic pole towards endometrium. The whole surface of the blastocyst has the potential to initiate the process of apposition and attachment with the endometrium

(Vinatier and Monnier 1990; Sharma and Kumar 2012). During apposition and attachment stages of implantation, receptor-ligand interactions established the communication between the blastocyst and the endometrium. For example, L-selectin a protein expressed on the surface of trophoblast cells mediates apposition of the blastocyst via interacting with carbohydrate ligands of the uterine epithelial cells (Genbacev *et al*. 2003). Likewise pairing of integrin αvβ3 and its ligand osteopontin, both of which are spatially and temporally expressed on surface of endometrial epithelium, also mediates blastocyst-endometrial communication (Apparao *et al*., 2001; Lessey 2002). Since integrin αvβ3 is also expressed on the surface of trophoblastic cells; it is likely that integrin αvβ3 and osteopontin actively engaged in trophoblast endometrial recognition during attachment (Reddy and Mangale 2003).

After adhesion, blastocyst proceeds for the final stage called "invasion" which starts with penetration of trophoblastic cells (STBs & EVTs) into of the uterine endometrium to reach till maternal blood vessels. In human pregnancy, the blastocyst is completely embedded within the endometrial stroma after 8-10 days of fertilization and entry site is covered by endometrial epithelial cells (Bischof and Campana 1996).

2.2 Trophoblast differentiation

The trophectoderm of the blastocyst is the first cell lineage that exhibits a highly differentiated function during embryonic development. In humans, highly proliferative, undifferentiated primitive cytotrophoblast cells which are derived from the trophectoderm, give rise to differentiated trophoblast cells through two general pathways: villous pathways and extravillous pathways (Fig. 2.1).

In the villous pathway, mononucleated cytotrophoblasts fuse to form multinucleated STBs, which form the syncytial layer and cover the placental villous tree. Later on, these cells are involved in secretion of pregnancy related hormones such as hCG and progesterone, gaseous exchange and nutrients transport across the maternal–fetal

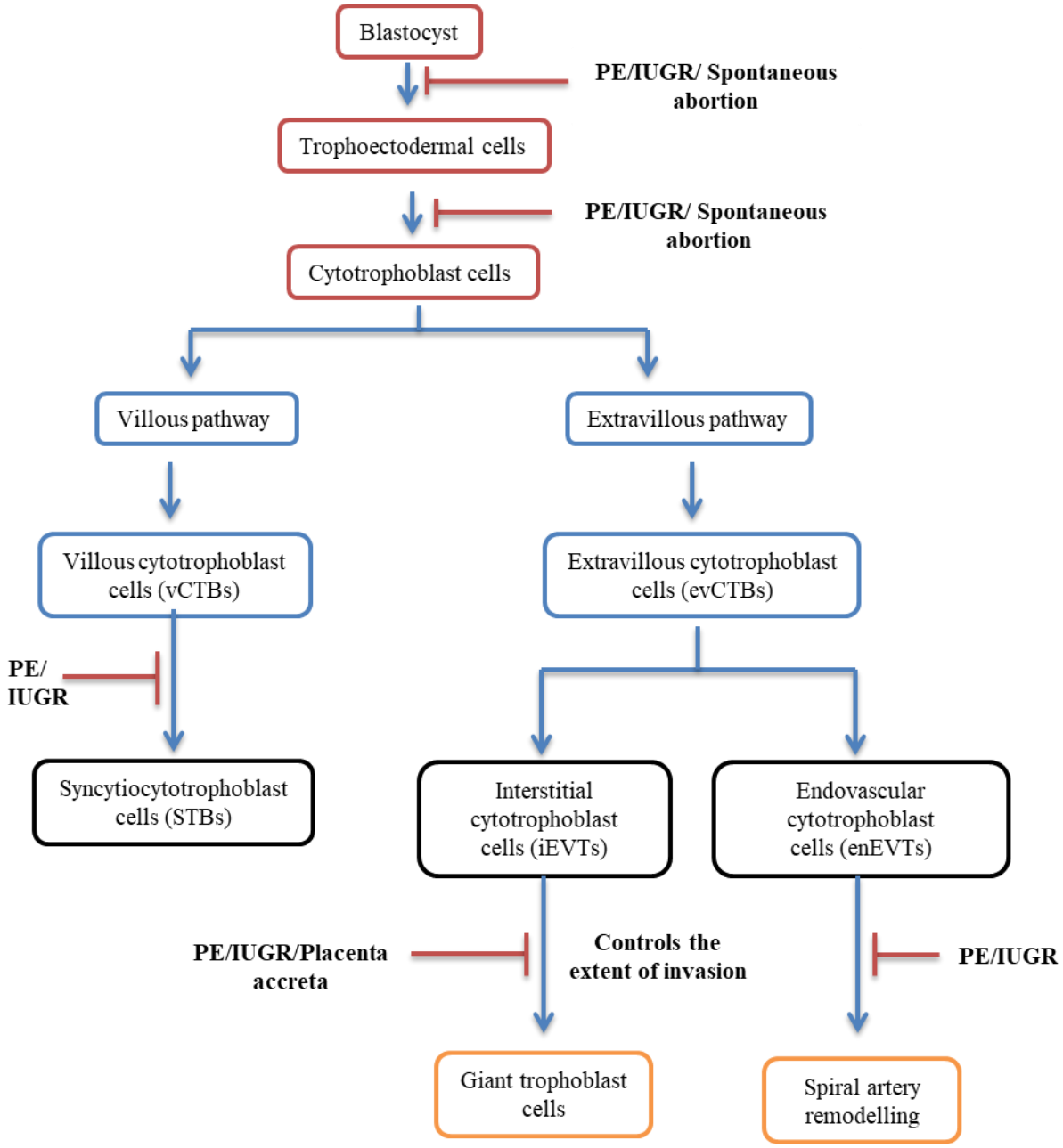

Fig. 2.1 Schematic representation of the stages of trophoblast differentiation and their significance in the establishment of pregnancy. The diagram shows the development of trophoblast lineages. If there is a failure during any decisive step it might lead to development of pregnancy related complications. Very early stage aberrations may result in pregnancy complications such as, PE/IUGR/spontaneous abortions. If the villous pathway is affected, it may result in PE/IUGR. In case, if the extravillous pathway is affected, it may result in an IUGR/PE/placenta accreta.

interface (Kaufmann *et al.,* 2003). The microvilli present on the syncytial layer further increase the surface area and absorption capacity (Teasdale and Jean-Jacques, 1985). Since, STBs are in close contact with the maternal blood, they must exhibit a degree of immune tolerance, which is achieved by lack of expression of the HLA protein (Nakamura, 2009). STBs are non-proliferative cells, due to this they continually replenished throughout pregnancy via the fusion of the underlying cytotrophoblast cells layer (Kar *et al*., 2007). Interestingly, during STB formation, cell fusion occurs exclusively between the cytotrophoblast and the overlaying syncytium and not between neighboring cytotrophoblast cells. In addition to the formation of floating villi, it is the establishment of anchoring villi, which serve to attach the placenta to the uterine wall and to create the degree of placental perfusion that is necessary to sustain the growing fetus.

In extravillous pathway, cytotrophoblasts cells proliferate to form anchoring villi that attach to the uterine wall (Red-Horse *et al*., 2004; Knofler, 2010). From the cell columns of the anchoring villi, EVTs arise by detaching from the placental villi and starts migrating into the decidua. During the migration process, EVT cells exit cell cycle and begin to lose their cell-cell contacts. On reaching near decidual ECM, they further differentiate into iEVTs and enEVTs. The iEVTs migrate and invade into the deep layer of the maternal endometrium and even into the inner third of the myometrium, thereby anchoring the fetus to the mother (Knofler, 2010). The iEVTs further differentiate into small spindle shaped cell with ovoid nuclei present in early pregnancy and big irregular shaped nuclei in advanced stage of pregnancy. enEVTs gain endothelial-like functions and express various vascular adhesion molecules (Zhou *et al*.,1997) and further invade spiral arteries and modified them into low-resistance, high-capacity utero-placental arteries that provide the increased blood flow towards the placenta and is needed to meet the requirements of the growing fetus (Lyall, 2006). Both iEVTs and enEVTs of the EVT lineage play an important role in reducing loss of vascular smooth muscle cells (VSMCs) and endothelial cells for established blood flow from endometrium to embryo (Kaufmann *et al*., 2003). Any aberration in the trophoblast differentiation, invasion or remodeling of

spiral arteries lead to development of preeclampsia, IUGR or placenta accrete, thus it is important to study the basic biology of trophoblastic cells migration and invasion (Lim *et al*., 1997; Matthiesen *et al*., 2005; Seckl *et al*., 2010; Wortman and Alexander, 2013).

2.3 Role of chemokines, cytokines and growth factors regulating blastocyst implantation

During the first trimester, in the absence of maternal blood supply, placental growth is regulated by plethora of cytokines and growth factors derived from trophoblast cells, uterine stromal and glandular cells, myometrial cells, endothelial cells, villous mesenchymal cells and various immune cells at the maternal-fetal interface (Knofler and Pollheimer, 2012). The importance of these cytokines, chemokines have been revealed by knockout studies done in animal models. Among them knockout of IL-11, LIF, IL-6, HGF, EGF, IFN-γ, TGF-β and CSF-1 leads to implantation failure, whereas knockdown of others like IL-1, IL-10, IL-15, GM-CSF and TNF-α resulted in decrease in reproductive efficacy (Makrigiannakis and Minas, 2007; Guzeloglu-kayisli *et al*., 2009). Besides classical growth factors, chemokines are also secreted from CTBs and decidual cell type's likes macrophages and fibroblasts present at fetal-maternal interface (Jokhi *et al*., 1997; Hannan and Salamonsen, 2007). However, their respective receptors are mostly identified on CTBs (Drake *et al*., 2004). Chemokines such as CX3CL1, CCL14, CCL4 (Hannan *et al*., 2006), CXCL16 (Huang *et al*., 2006) or CCL21 (Red-Horse *et al*., 2005) are known to stimulate trophoblast migration or invasion (Fig. 2.2). Interestingly, the action of chemokines CCL12, CCL16 and CCL21 was regulated by binding of ephrin ligand to its receptors EBHB and this ligand-receptor complex further regulate not only chemokine-mediated invasion but also uterine spiral arteries remodeling (Red-Horse *et al*., 2005). Cytokines, chemokines and growth factors can act as a pro-invasive factors (IL-8, IL-6, EGF, HGF, LIF and LIF) and also can be act as anti-invasive factor (TGF-β, TNF-α and IFN-γ). The fine balance of both pro- and anti-invasive factors is essential for the successful embryo implantation as well as the placental development as they regulate

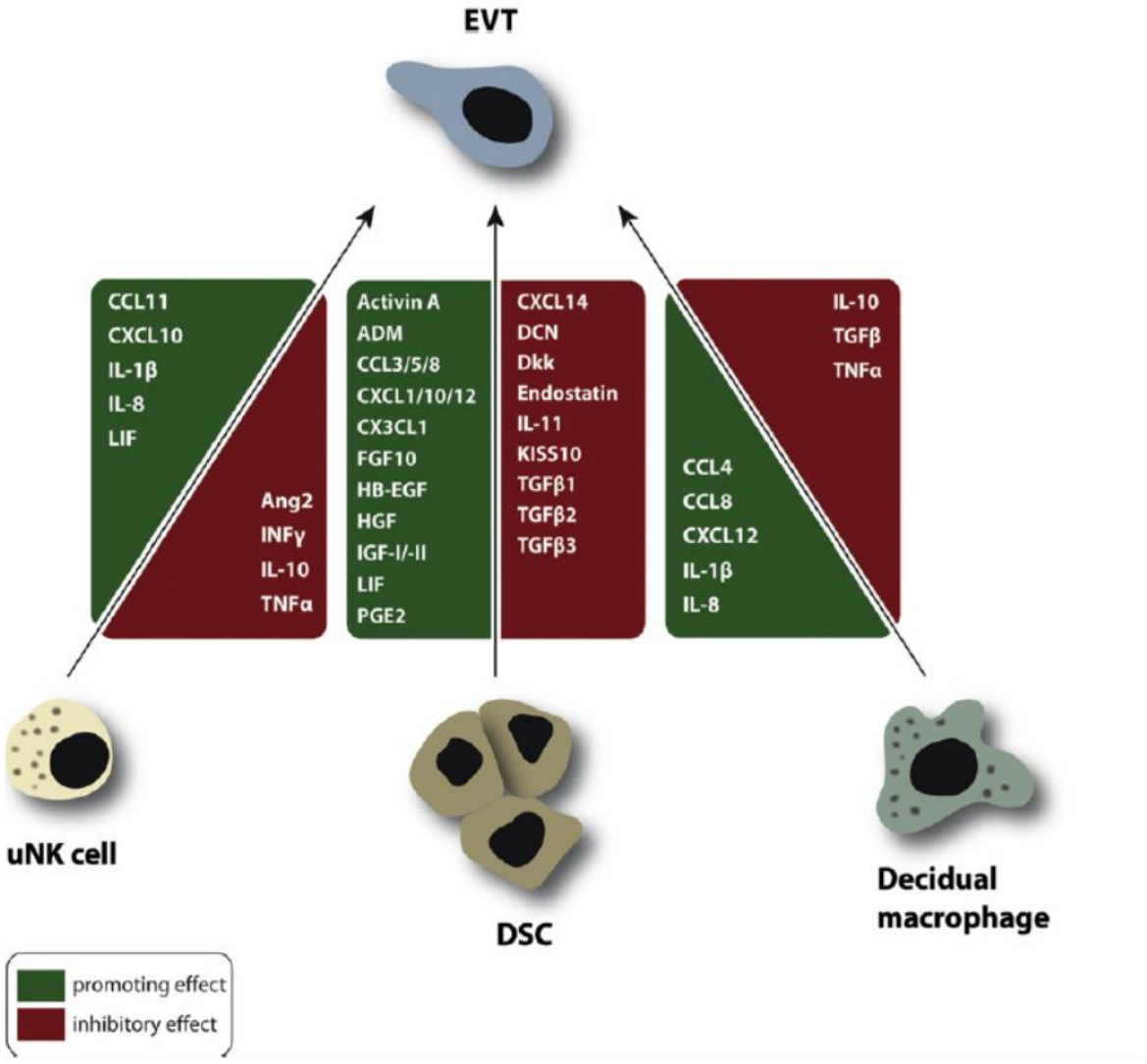

Fig. 2.2 Cytokines, chemokines and growth factors involved in EVT cell migration. The influence of decidual cell types on interstitial trophoblast invasion and migration. Predominant cell types of the maternal decidua, uterine natural killer (uNK) cells,decidual stromal cells (DSC) and decidual macrophages produce soluble factors promoting and inhibiting trophoblast motility. Source: Knöfler and Pollheimer, 2012.

the process of trophoblast migration and invasion (Knofler 2010, Gupta *et al*., 2016). These factors not only facilitate but also regulate the activity of invasive proteinases through activation of various downstream signaling cascades.

2.4 Relevance of HGF in pregnancy

HGF is a cellular growth factor synthesized as a pro-protein. It is composed of 728 amino acid (aa) with a molecular weight of 84 kDa. It consists of one α chain containing four kringle domains and one β chain with a structure similar to serine-protease enzyme, but do not have proteolytic activity. HGF is also a multifunctional cytokine, on binding to c-met receptor, activate various downstream signaling pathways, by which it regulates cell growth, morphogenesis, differentiation, migration and invasion (Nakamura *et al*., 2011). In human placenta, HGF is mainly secreted by trophoblast cells (STBs & EVTs) and endometrium cells (endothelial cells and villous mesenchymal cells), whiles its receptor, c-Met, and is mainly expressed in trophoblast cells and villous endothelial cells (Kauma *et al*., 1997; Trovato *et al*., 2002; Yang *et al*., 2012). In general, the level of HGF remains constant in placental tissue (first, second, and third trimester); however, a steady rise in its level is seen in maternal serum till *term*. The concentration of HGF is reported to be higher in second trimester both in amnion and amniotic fluid as compared to third trimester, which is in contrast to other cytokines (EGF, TNF-α, IL-1, IL-6) present in amniotic fluid, which increase in concentration towards *term* (Horibe *et al*., 1995). HGF is essential for fetal and placental development, as mice knockout for *HGF* gene showed embryo lethality at early third trimester, due to reduction in labyrinthine trophoblast cells number and poor vascularization (Uehara *et al*., 1995). In humans, reduced expression of HGF as well as c-met is reported in IUGR placentae (Li *et al*., 1996; Somerset *et al*., 1998a; 1998b). Clinical studies have shown that high HGF concentration in serum of pregnant women who were overweight/obese is associated with risk of early gestation diabetes mellitus (GDM) and further suggest that serum HGF level can act as a biomarker for detection of early GDM (Dishi *et al*., 2015). In addition, low level of circulating HGF

is reported in pregnant women with small gestational age (SGA) infants as compared to appropriate gestation age (AGA) infants, suggesting a significant role of maternal serum HGF in fetal growth during pregnancy (Aoki *et al*., 1998).

HGF has been shown to stimulate trophoblastic cells migration, invasion and endothelial cell tubulogenesis (Grant *et al*., 1993; Kauma *et al*., 1999; Saito *et al*., 1995; Dokras *et al*., 2001; Fitzgerald *et al*., 2005) via activation of various downstream signaling pathways like Rho/Rac, PI3K and ERK/MAPK (Jiang *et al*., 2005; Rosario and Birchmeier, 2003). In addition, on stimulation with HGF, activated MAPK further activates cGMP signaling pathways, which increase nitric oxide (NO) production during trophoblast cells migration and invasion (Ayling *et al.*, 2006; Cartwright *et al*., 2002). Moreover, transcription factors like HLX and HLX1, which mainly expressed in EVTs, have also been shown to be the downstream target of HGF during EVTs invasion (Liu *et al*., 2012; Rajaraman *et al*., 2010). HGF has been shown to be a mediator of dNK cells-mediated EVT cells migration (Fraser *et al*., 2012), suggesting that it might help in the immune recognition between dNK and EVT. Besides HGF, truncated form of c-Met, which released from membrane as a soluble protein (s-Met) influence trophoblast cell invasion, by disrupting HGF/c-met signaling on binding to HGF (Wajih *et al*., 2002; Yang *et al*., 2012). HGF also regulate trophoblast cell invasion through self-feedback loop which involves downregulation of c-Met expression through receptor proteolysis involving ADAM (a disintegrin and metalloproteinase) proteins (Yang *et al*., 2012).

2.5 Role of WNT ligands in trophoblast and placenta

Wingless ligands are the family of hydrophobic cysteine rich secreted glycoproteins, which are functionally involved in organ development, tissue homeostasis as well as various cellular processes like cell proliferation, cell death and differentiation (Mikels and Nusse, 2006). There are 19 WNT (WNT1, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A,

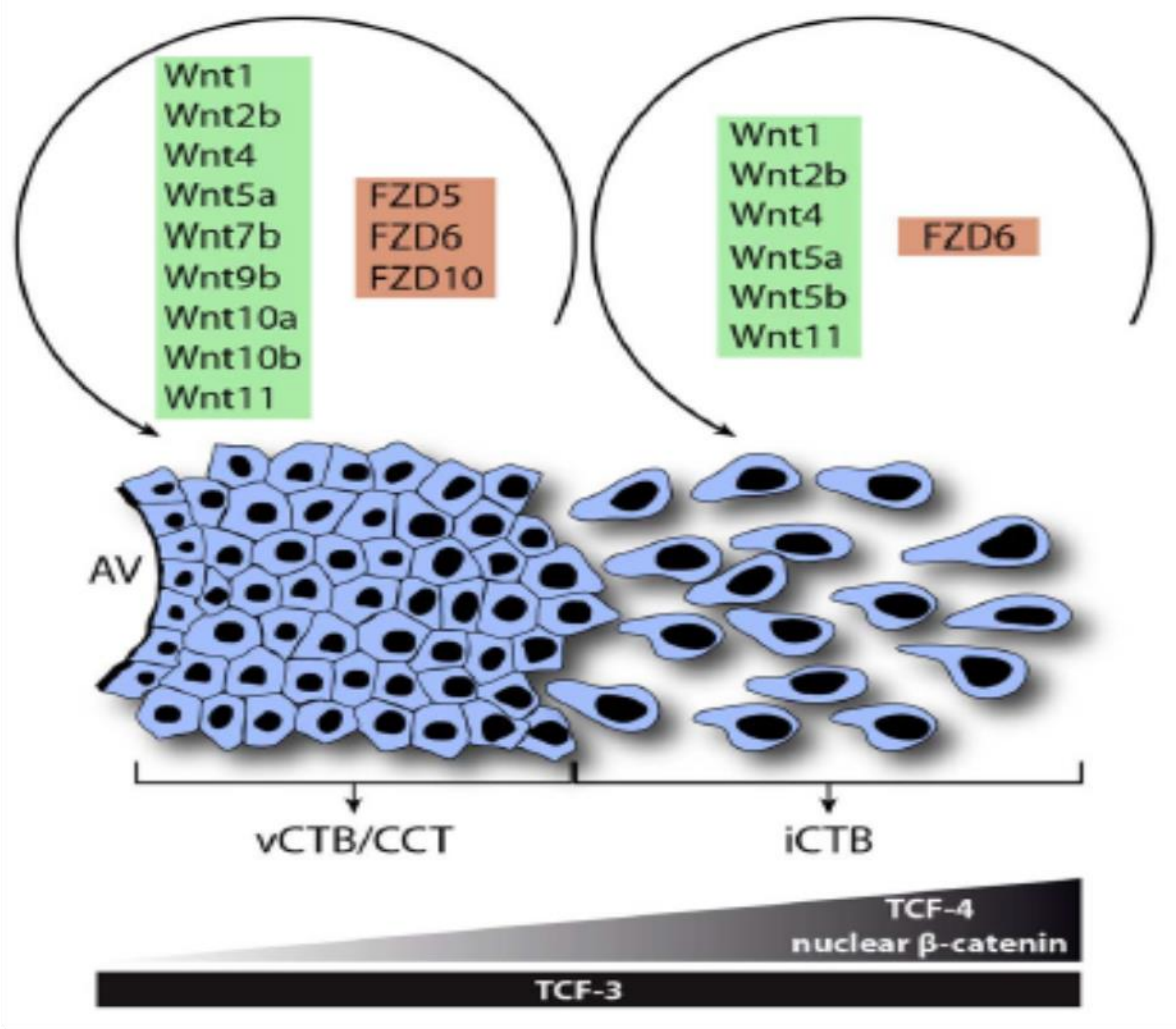

Fig. 2.3 Model system for the role of Wnt signaling in function and differentiation of the human anchoring villus. Wnt ligands and Fzd receptors expressed in vCTBs/CCTs and EVTs are shown. During EVT formation, Wnt7b, Wnt9b, Wnt10a, Wnt10b, Fzd5, and Fzd10 are down-regulated suggesting a role in trophoblast proliferation. However, interstitial cytotrophoblasts (iCTBs) up-regulate TCF-4 and nuclear β-catenin to promote trophoblast motility and possibly EVT differentiation. Source: Knöfler and Pollheimer, 2013.

WNT9B, WNT10A, WNT10B, WNT11, WNT16) and 10 frizzled (FZD) receptors (FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10) identified in mammals (Wodarz & Nusse, 1998) and among them 14 WNT ligands and 8 FZD receptors are known to be expressed in first trimester placental tissue (Sonderegger *et al*., 2007). The mRNAs of these 14 WNT ligands are detected in the villous trophoblast epithelium. In particular, abundantly (*WNT1*, *WNT2B*, *WNT4*, *WNT7B*, *WNT10A*, *WNT10B*, *WNT11*), moderately (*WNT5A*, *WNT9B*) and partially (*WNT2*, *WNT3*, *WNT5B*, *WNT6*, *WNT7A*) expressed WNT ligands are known to be present in first trimester CTBs (Fig. 2.3). WNT1, WNT7B, WNT10A, and WNT10B are reported to be down regulated from first trimester onwards till *term*, suggesting their role during early pregnancy (Sonderegger *et al*., 2007; Knofler and Pollheimer, 2013). While in case of FZD receptors, FZD1, FZD3, FZD5, FZD6, FZD7, and FZD10 are reported in CTBs of first trimester placenta. However, FZD2 and FZD4 are reported to be only expressed in villous mesenchymal cells (Knöfler & Pollheimer, 2013). Similarly, in another previous study, RT-qPCR analysis in CTB also revealed the temporal expression of *WNT4*, *WNT5A*, *WNT7B*, *WNT10A*, *WNT10B*, β-catenin and WNT antagonists, secreted frizzled related proteins (SFRP2 & 5) in response to the protease activate receptor1 (PAR1) activation during the invasion process (Grisaru-Granovsky *et al*., 2009). WNT ligands are also expressed in endometrium; the role of endometrial expressed WNT ligands WNT2, WNT4, WNT5A, WNT7A, WNT8B, and WNT3 has been suggested to affect the trophoblast function in a paracrine manner (Tulac *et al*., 2003). Besides this, WNT4 under the stimulatory effect of bone morphogenic protein2 (BMP2) has been reported to regulate human primary endometrial stromal cells (HESCs) differentiation through the canonical pathway, suggesting that it might act as a regulator of decidulization during implantation (Li *et al*., 2013). Moreover, the role of WNT6 has also been reported in stromal cell proliferation and differentiation (Wang *et al*., 2013). In addition, the gene disruption studies in mice revealed that different WNT ligands like WNT4, WNT5A, and WNT7A and associated β-catenin are also critical for uterine development (Miller and

Sassoon, 1998; Vainio *et al*., 1999; Mericskay *et al*., 2004; Arango *et al*., 2005). Pregnancy related hormones like progesterone and estrogen has also been shown to induce WNT11, WNT4 and WNT7B expression in ovariectomized mice, suggesting that they can also play role during decidulization and implantation (Hayashi *et al*., 2009). WNT ligands can exert their effect by activating multiple intracellular signaling cascades, including the β-catenin-dependent and -independent pathways and can regulate both the signaling at the same time. For example, WNT5A secreted from trophoblast cell lines and primary trophoblast cultures, act through non-canonical pathways as it does not involve TCF/β-catenin-dependent transcription, but at the same time inhibit the downstream canonical signaling pathway in trophoblast cells (Sonderegger *et al*., 2007).

Although the expression and role of different WNT ligands have been explored in menstrual cycle, endometrial tissue and mouse trophoblast differentiation and implantation, none of the individual WNTs and FZDs has been studied in the context of human trophoblast migration and invasion so far. Till now, only WNT3A has been reported to regulate the trophoblast migration and invasion through canonical WNT and AKT signaling pathways (Sonderegger *et al*., 2010). Keeping this in view, the relevance of different WNT ligands during HTR-8/SVneo cell migration has been investigated in present thesis.

2.6 Roles of different signaling pathways in trophoblast migration and invasion

During the early pregnancy, various lineage of trophoblastic cells and endometrium secrete different cytokines, chemokines and growth factors for successful implantation of blastocyst and placental development. Cytokines, chemokines and growth factors act either in the autocrine or paracrine manner to alter the expression of different effector proteins, which regulate the process of invasion/migration/proliferation/differentiation of the trophoblast cells. In addition, at the same time multiple signaling pathways work together or individually to control these biological processes. However, the process of

trophoblast migration and invasion differs from the migration/invasion of cancer cells as it is finely regulated. During pregnancy, signaling pathways can either promote or inhibit both the migration and invasion. The role of different signaling pathways during trophoblast migration and invasion has been reviewed by various studies (Pollheimer and Knofler, 2005; Knofler, 2010; Gupta *et al*., 2016) and have been depicted in (Fig. 2.4).

2.6.1 Mitogen-activated protein kinases (MAPK) signaling pathway

The family of MAPK protein comprises four large groups namely enzymes-extracellular regulated kinases (ERKs), ERK5, c-Jun N-terminal kinases (JNKs) and different p38 MAPKs. Binding of ligand to receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCR) of the MAPK kinase kinase family activates Ras which further binds to Raf and phosphorylates MAPK kinase which subsequently phosphorylates MAPK. Activated MAPK further phosphorylates different proteins including transcription factors which regulate various biological responses (Zhang and Liu, 2002; Pullikuth and Catling, 2007; Cargnello and Roux, 2011). MAPK/ERK signaling pathway plays an important role in pregnancy by regulating invasion and migration of trophoblast cells. *ERK5* and *p38a* knockout mice show defects in placenta formation, angiogenesis and embryonic lethality (Regan *et al*., 2002; Sohn *et al*., 2008). The expression of phosphorylated ERK in invasive extravillous trophoblast cells shows its importance during pregnancy as its abberrant expression has been observed in cases of PE and recurrent miscarriages (Ismail *et al*., 2017). In HTR-8/SVneo cells, IGF-II and IGFBP-I activate ERK1/2 and increase their invasion and migration which can be blocked by treatment of MEK inhibitor PD98059 (Gleeson *et al*., 2001; McKinnon *et al*., 2001). Further, treatment of SGHPL-4 with HGF leads to an increase in their motility by activating ERK1/2 but not by p-38 MAPK pathway (Cartwright *et al*., 2002). Studies suggest that there may be a cross-talk between STAT and ERK1/2 pathways which regulates trophoblast invasion (Suman and Gupta, 2014; Malik *et al*., 2017). In addition, EGF activates MAPK and PI3K signaling pathways and promotes migration of HTR-8/SVneo cells by increasing the level of

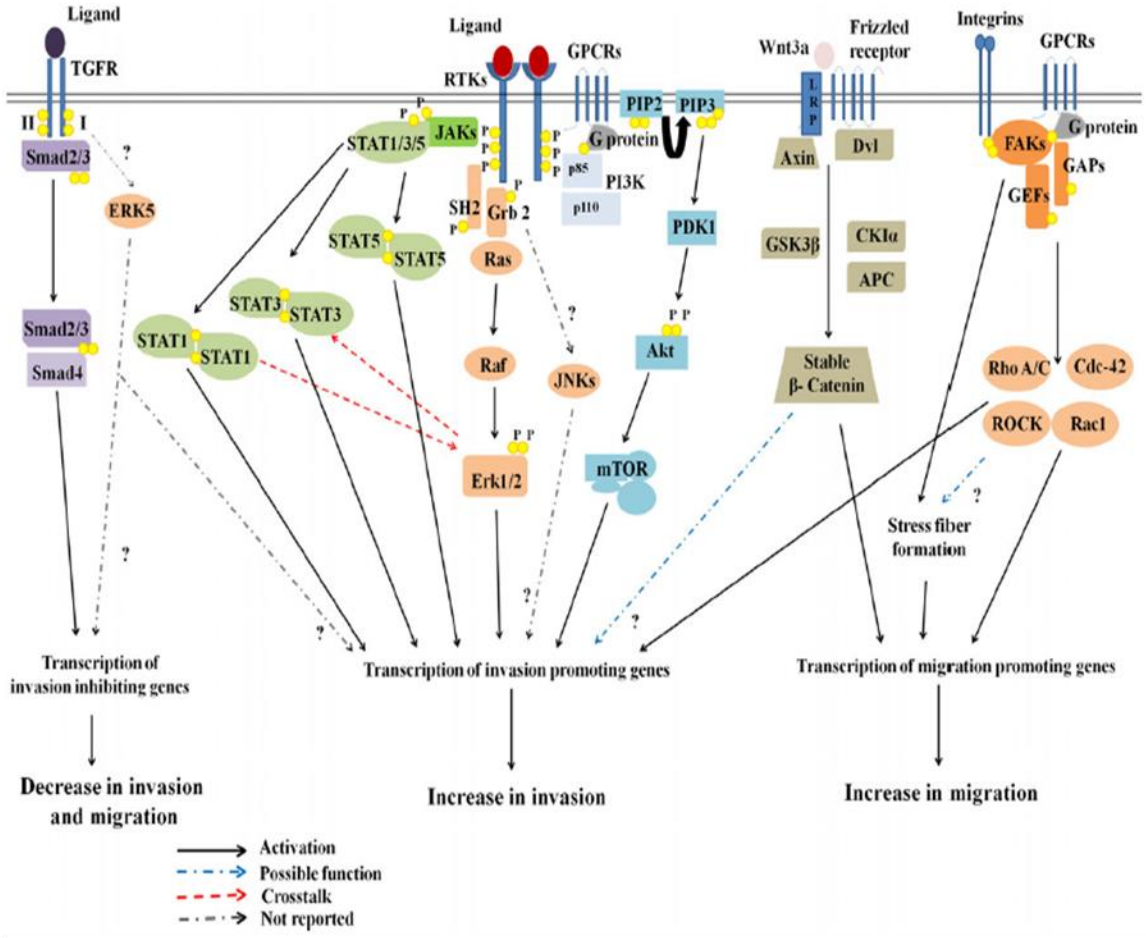

Fig. 2.4 Schematic representation of the signaling pathways activated during invasion and migration of trophoblast cells. Variety of growth factors and cytokines are known to stimulate Akt, Erk½, STAT1, STAT3, and STAT5 during the process of trophoblast invasion. It has been shown that Erk½ and STAT1/STAT3 control each other's phosphorylation suggesting a cross-talk between them. There is also involvement of JNK and ERK5 in placental development but their role in trophoblast invasion and migration has not been elaborated. Activation of Smad2/3 by TGFR is known to inhibit trophoblast invasion. Wnt3a promotes trophoblast cell migration and up-regulates MMP2 expression, showing its role in invasion. FAK activation by various growth factors leads to RhoA/Rac1/cdc42/ROCK activation and stress fiber formation which increases migration and invasion of trophoblast cells (Reproduced from Gupta *et al*., 2016). Various abbreviation used are JNK-c-Jun N-terminal kinases; FAK-Focal adhesion kinase; STAT-Signal transducer and activator of transcription; MMP2-Matrix metalloproteinase; TGFR-Transforming growth factor receptor; ERK-Extracellular signal-regulated kinases.

MMP9 and TIMP1 (Qiu *et al*., 2004). JEG-3 cells stimulated with LIF activate both STAT3 and ERK1/2 signaling pathways (Suman and Gupta, 2014). HTR-8/SVneo cells treated with prostaglandin E2 and endothelin promote invasion through activation of the ERK1/2 pathway (Chakraborty *et al*., 2003; Nicola *et al*., 2008a). In SGHPL-5 cells, activation of ERK1/2 and AKT signaling pathways promote migration and invasion by up-regulating the expression of hCG and MMP2 (Prast *et al*., 2007). Overexpression of epidermal growth factor-like domain 7 (EGFL 7) in JEG-3 cells promotes invasion and migration by activating MAPK and PI3K signaling pathways without affecting their proliferation (Massimiani *et al*., 2015). The non-glycosylated form of nephroblastoma overexpressed (CCN3) protein increases migration of JEG-3 cells by activating ERK1/2 and AKT signaling pathways. This study also showed that glycosylated and non-glycosylated form of CCN3 is involved in maintaining a balance of trophoblast proliferation, migration and invasion by activating different signaling pathways (ERK1/2, AKT and NOTCH1; Wagener *et al*., 2012). In addition, a study done in HTR-8/SVneo cells showed that treatment with sphingosine-1-phosphate (S1P) increases invasion of these cells by activating MEK-ERK pathway, which further regulates the level of MMP2 and MMP9 (Yang *et al*., 2014).

2.6.2 Phosphoinositide 3-kinase (PI3K)/AKT signaling pathway

PI3K/AKT signaling pathway is known to be involved in the regulation of different cellular events like proliferation, invasion, migration, cell growth, and survival (Manning and Cantley, 2007). Binding of ligand to RTKs or GPCRs leads to membrane recruitment of p85 and p110 subunits of PI3K, respectively. Further, PI3K phosphorylates phosphatidylinositol-4,5-bis-phosphate (PIP2) at 3'position of its inositol ring and converts PIP2 into PIP3. Activation of PIP3 promotes recruitment of AKT at the membrane by phosphoinositide-dependent kinase 1 (PDK1), which further phosphorylates a wide range of proteins like mammalian target of rapamycin (mTOR). There are three isoforms of AKT: AKT1/PKBa, AKT2/PKBb, and AKT3/PKBc which

have 80% homology at the protein level. Activation of mTOR regulates protein translation by phosphorylating ribosomal S6 kinases and contributes to cell cycle progression (Hemmings, 1997). AKT is known to regulate differentiation and development of placenta/trophoblast cells. Homozygous deletion of *AKT1* in mice shows reduced number of proliferative trophoblast cells (Yang *et al*., 2003). IGF-II promotes migration of HTR-8/SVneo cells by regulating PI3K/AKT signaling pathway (Qiu *et al*., 2004; 2005). Treatment of SGHPL-4 cells with HGF promotes migration by activating PI3K pathway, inhibition of PI3K signaling pathway results in the down-regulation of basal as well as HGF-induced migration of these cells (Cartwright *et al*., 2002). Interestingly, treatment of SGHPL-5 and EVTs with hCG activates ERK1/2 and AKT signaling pathways which also regulate invasion and migration by promoting secretion of MMP2 (Prast *et al*., 2007). In another study, EGF activates MAPK and AKT signaling pathways and increases expression of MMP9 and TIMP1 in HTR-8/SVneo cells (Qiu *et al*., 2004). Inhibition of both the signaling pathways is necessary for downregulating the expression of MMP9 and TIMP1 in EGF treated HTR-8/SVneo cells (Qiu *et al*., 2004). Wnt3A activates canonical WNT and PI3K/AKT signaling pathways through different receptors and upregulates migration of primary EVTs and SGHPL-5 cells by increasing the expression of MMP2 (Sonderegger *et al*., 2010). Low expression of transforming acidic coiled-coil protein3 (TACC3) in preeclamptic patients and HTR-8/SVneo cells shows decrease in proliferation, invasion and migration due to inhibition of p-AKT/AKT signaling pathway (Zhu *et al*., 2016). HOX transcript antisense RNA (HOTAIR) activates Yin Yang 1 (YY1) *via* PI3K/AKT signaling pathway in *ex vivo* explants culture model and promotes invasion and migration of trophoblast cells by up-regulating the expression of MMP2. This study suggests that HOTAIR is a potential therapeutic target for the patients of recurrent miscarriage (Zhang *et al*., 2017a). Further, decreased expression of selenocysteine insertion binding protein 2 (SECISBP2) inhibits invasion/migration, proliferation and hormone secretion (β hCG) in JEG-3 and JAR cells due to inhibition of PI3K/AKT and ERK1/2 signaling pathway (Li *et al*., 2017). The study performed in

HTR-8/SVneo cells also showed the role of a polycyclic aromatic hydrocarbons, benzo[a]pyrene-7,8-diol-9,10-epoxide(BPDE), which inhibits trophoblast invasion and migration *via* downregulating FAK/SRC/PI3K/AKT/eNOS/MMP2 pathway (Wang *et al*., 2017).

2.6.3 Wnt signaling pathway

Wingless (Wnt) are a family of secreted glycoproteins which are known to be involved in tumorigenesis and embryonic development (Gordon and Nusse, 2006). Binding of Wnt to its receptor frizzled (FZD), a GPCR, and many co-receptors like lipoprotein receptor-related protein (LRP-5/6), receptor-like tyrosine kinase, or receptor tyrosine kinase-like orphan receptor (Ror) activates downstream signaling (Rao and Kuhl, 2010). Binding of WNT to receptor FZD/LRP-5/6 inhibits β-catenin destruction complex consisting of Axin, synthase kinase-3β (GSK-3β), casein kinase1a (CK1a) and adenomatous polyposis coli (APC). Inhibition of destruction complex by dishevelled protein (Dvl) stabilizes cytosolic levels of β-catenin and translocates the dephosphorylated form of β-catenin into the nucleus. Nuclear β-catenin together with lymphoid enhancer binding factor 1 (LEF-1) and TCF transcription factor family promote transcription of different genes like *c-myc*, *MMP7*, *MT1-MMP* and *cyclin D1* (Li *et al*., 2005).

The canonical Wnt signaling pathway is involved in the differentiation of EVTs and also promotes their migration by inducing the expression of pro-migratory genes (Knofler and Pollheimer, 2013). Wnt canonical ligand WNT3A and non-canonical ligands WNT5A and WNT10B regulates expression of CD44 and MMP9; hence, controls the invasion of EVTs (Takahashi *et al*., 2013). In preeclamptic patients, the appearance of long noncoding RNA, SPRY4 intronic transcript 1 (SPRY4-IT1), increases which contribute to the defects in spiral artery remodelling *via* activation of Wnt/β-catenin signaling pathway (Zuo *et al.*, 2016). Knockdown of *LRP6* in HTR-8/SVneo cells is reported to reduce the proliferation, invasion, migration and tube formation; however, it promotes the

apoptosis of these cells. It is also associated with decrease in the levels of MMPs, VEGF and placental growth factor (PIGF), and inhibits activation of Wnt/β-catenin signaling pathway (Li *et al*., 2018). The Wnt dependent β-catenin canonical signaling pathway has been reported to play a role in EVT differentiation, migration through transcription factor TCF4, which further promote the expression of pro-migratory genes (Meinhardt *et al*., 2014). The expression levels of Dickkopf-related protein1 (Dkk1) and sFRP4 are reported to be higher, whereas the levels of WNT2 and β-catenin expression were decreased in preeclamptic placentae as compared with normal placental tissues, in the third trimester of women undergo cesarean section (Zhang *et al*., 2013a, b). In the oxidative stress, β-catenin activation led to decrease in generation of intracellular reactive oxygen species (ROS), which leads to a significant increase in HTR8/SVneo cells invasion and also the outgrowth and migration in villous explants, suggesting its role in preeclampsia arise due to abnormal placental hypoxia (Zhuang *et al*., 2015). The RT-qPCR, Western blotting and immunohistochemistry analysis reveals lower expression of β-catenin in the placental tissue of women suffering from placenta accrete and previa as compared to normal placental tissue sample (Han *et al*., 2019). The ability of different trophoblastic cell lines and primary explant cultures treated with recombinant Dkk1 showed decrease in migration and invasion, suggesting that canonical WNT ligand expression in EVT exerts autocrine effect (Sonderegger *et al*., 2010)

2.6.4 Focal adhesion molecules (FAKs) and Rho/ROCK signaling pathway

FAK signaling can be activated by integrin clustering, growth factors, and stimulus from GPCR. FAK has two prominent domains: a central kinase domain, which binds to integrin and activates other nontyrosine receptor kinase; and c-terminal domain containing focal adhesion targeting sequences, which bind integrin related proteins paxillin and talin (Mitra *et al*., 2005). The FAK signaling is initiated after its phosphorylation at Tyr-397 residue that create the binding site for Src-family members and other adaptor proteins, which further activate kinase cascades. FAK also

phosphorylate Rho guanine nucleotide exchange factors (GEFs) and Rho GTPase-activating protein (GAPs), which are the positive and negative regulators of Rho family GTPases such as Rho, Rac, and Cdc-42. Rho, Rac, and Cdc-42 further trigger downstream effector molecules such as Rho-associated kinases (ROCKs) and p21-activated kinases (PAKs), which are known to regulate cytoskeleton protein and transcription of various genes (Mitra *et al*., 2005). FAK phosphorylation at Tyr-397 is crucial during invasion, as downregulation of FAK activation decreases cell migration and MMP-2 activity (Ilic *et al*., 2001). CD98 heavy chain is expressed on EVT cells that promote avb3 integrin-dependent signaling resulting in FAK phosphorylation, which is associated with their invasive property (Kabir-Salmani *et al*., 2008). The recent studies have shown that FAK-Src signaling plays a crucial role in cyclosporine A-induced trophoblast migration and invasion by upregulating the MMP-2 and MMP-9 activity and downregulating E-cadherin expression in human primary trophoblasts and JEG-3 cells (Tang *et al*., 2012). RhoA and ROCK are expressed in the cytoplasm of the cytotrophoblast cells and are involved in the assembly of the stress fibers; inhibition of Rho-ROCK signaling using Y27632-a selective ROCK inhibitor reduces the trophoblast migration (Shiokawa *et al*., 2002). Various growth factors and cytokines have been known to increase trophoblast motility by activating Rho-ROCK signaling pathway. Both IGF-I and IGF-II increase the migration of human EVT cells. IGF-I primarily acts through IGF type I receptor (IGF1R) and IGF-II by both IGF1R-dependent and IGF1R-independent pathways. IGF1R-mediated EVT migration involved RhoA and RhoC, whereas IGF1R-independent pathway required ROCKs, suggesting involvement of different members of Rho GTPase family during IGFII-mediated EVT migration (Shields *et al*., 2007). Further involvement of Rho GTPases such as Rho and Rac1 is important during IGFBP1-induced EVT migration (Saso *et al*., 2012). However, under these experimental conditions, involvement of RhoA and Cdc-42 was not established. During the prostaglandin E2 stimulated migration of HTR-8/SVneo cells, as well as villous

explants from first-trimester placentae, the involvement of Rac1 and Cdc-42 were demonstrated (Nicola *et al*., 2008b).

2.6.5 Janus kinases/signal transducers and activators of transcription (JAK/STAT) signaling pathway

The JAK/STAT pathway is known to control various biological processes like cell growth, proliferation, invasion, migration, differentiation and apoptosis (Leonard and O'Shea, 1998; Mui, 1999). JAK/STAT pathway was first discovered as mediator of IFN-γ-dependent gene responses (Darnell *et al*., 1994). STAT proteins consist of a six domains structure- an oligomerization domain, a coiled-coil domain, a DNA-binding domain (determines the DNA binding specificity), a linker and SH2 domain (helps in receptor binding and dimerization), and a transcription domain. The transcription domain contains a conserved serine residue except in STAT2 and STAT6. All six STAT proteins are encoded by different genes and made up of about 750 to 848 amino acids (90–155 kDa). STATs are present in the latent form in the nucleus until activated by different cytokines, growth factors, extracellular ligands and hormones. They have tyrosine residue in SH2 domain which is essential for dimerization (at position tyr701 in STAT1 and tyr705 in STAT3). For maximal transcriptional activity, phosphorylation at c-terminus serine residue is also crucial in STAT1, STAT3 and STAT5. Binding of cytokine to its receptor, bring the JAK protein monomers together, facilitating the cross-phosphorylation of JAKs and cytoplasmic domain of the receptor. These phosphorylated domains can bind to proteins having phosphotyrosine binding (PTB) or SH2 binding domain (like STATs) and phosphorylate them on their serine and tyrosine residues. After activation, STATs dissociate from the ligands and form hetero- or homo-dimers and enter into the nucleus, where they bind to the specific promoter region of the targeted genes, and regulate their expression (Lim and Cao, 2006). Study performed in human EVTs treated with IL-11 showed activation of STAT3 which further regulates migration of these cells (Paiva *et al*., 2009). JAK/STAT1 pathway also regulates endothelial activation of human

umbilical vein endothelial cells (HUVEC) after treatment with IFN-γ by promoting the level of Eph receptor B4 (EPHB4) protein (Liu *et al*., 2016). Further, primary EVTs cells treated with human placental growth hormone (hPGH) activates STAT5 and increases their invasion (Lacroix *et al*., 2005).

2.6.6 TGF-β superfamily signaling pathway

The members of TGF-β superfamily (consist of more than 40 members) regulate different biological processes such as immune response, matrix synthesis, cell proliferation, differentiation, apoptosis, invasion and migration (Nagaraj and Datta, 2010; Worthington *et al*., 2012). There are three types of TGF-β isoforms in mammals: TGF-β1, TGF-β2 and TGF-β3, which are coded by different genes and signal through, type I and type II Ser/Thr receptor tyrosine kinases known as activin receptor-like kinases (ALKs). All the members of TGF-β family bind to specific combination of type I and type II ALKs. Type II receptor phosphorylates the type I receptor which further activates smad down-stream (R-smad: smad2/3; co-smad: smad4) proteins. Activated R-smad binds to co-smad and then enters into the nucleus, and regulates transcription of different genes (Chang *et al*., 2002). The aberrant expression of TGF-β has been observed in cases of preeclamptic women, which show its importance in the development of the placenta (Caniggia *et al*., 1999). Further, the study performed in explants culture of first-trimester chorionic villi have shown that activin-A promote the growth of CTBs and also up-regulates the expression of MMP2 and MMP9. This study also suggested the importance of activin-A in the formation of CTB column during placentation (Caniggia *et al*., 1997). Further, constant active expression of ALK7 or overexpression of Nodal (member of TGF-β superfamily) down-regulates the invasion and migration ability of HTR-8/SVneo cells (Nadeem *et al*., 2011). Overexpression of Nodal in placental explant cultures down-regulates the growth of explants and also inhibits migration of EVTs. The decrease in the migration is associated with an increase in the level of TIMP1 and loss in the expression of MMP2 and MMP9, which shows the relevance of Nodal/ALK7 in human implantation

(Nadeem *et al*., 2011). In HTR-8/SVneo cells, overexpression of smad ubiquitination regulatory factor (Smurf2) promotes invasion and migration *via* down-regulating the expression of TGF-β type I receptor (Yang *et al*., 2009).

2.6.7 cAMP- dependent signaling pathways

cAMP pathway is activated by ligand(s) binding to their specific GPCRs. It triggers a conformational change within the receptor that is transmitted to an attached intracellular heteromeric G-protein complex leading to activation of its α subunit by exchanging GDP for GTP, which in turn activates adenylyl cyclase (AC) to convert adenosine triphosphate (ATP) to cAMP. Increased intracellular levels of cAMP lead to activation of various signaling mediators, such as PKA and exchange protein activated by cAMP (Epac) (Meinkoth *et al*., 1993; Bos, 2006).

2.6.7.1 *cAMP/PKA signaling*: Several factors such as hCG and calcitonin gene related protein act in either an autocrine or paracrine manner to increase intracellular levels of cAMP, promoting syncytia formation (Shi *et al*., 1993; Green *et al*., 2006). Mostly, biological effects of cAMP are mediated via PKA. There are two forms of PKA, type I and II. These differ in the nature of their cAMP-binding regulatory subunits, RI(a/b) and RII(a/b). However, they have common catalytic subunits Ca, Cb, and Cc. Messenger RNAs of only RIa, RIIa, Ca, and Cb were detected in the cytotrophoblasts (Lohmann and Walter, 1984). Moreover, PKA catalytic subunit transfection was sufficient to increase BeWo cell fusion (Knerr *et al*., 2005) and inhibition of PKA catalytic subunit by H89 inhibitor-impaired cell fusion (Keryer *et al*., 1998). Most of the studies related to PKA signaling have been done with respect to trophoblast cell fusion or syncytia formation. However, in response to HGF, increase in cAMP level activate PKA signaling, which further up regulate the expression of Rap1 and integrinβ-1, leading to increased MMP9 expression and trophoblast cell migration (Chen, 2014).

2.7 Role of transcription factors during trophoblast migration and invasion

Various transcription factors are known to be expressed in the cell columns and EVTs, some of which are known to be altered in pregnancy associated complications like IUGR. These transcription factors regulate proliferation, differentiation of CTBs in the cell columns and invasive potential of the non-proliferative EVTs. According to their functional relevance, they are also differentially expressed in CTBs or EVTs (Knofler & Pollheimer, 2012). Trophoblast proliferation is promoted by H2.0-like homeobox (HLX), HIF-1α, and the winged helix protein Storkhead box 1 (Stox1) in hypoxic environment prior to 10 weeks of gestation, but they prevent EVT differentiation into invasive phenotype if oxygen levels remain low after 10 weeks (Caniggia *et al.*, 2000a; Van Dijk & Oudejans, 2011; Murthi *et al.*, 2011). HIF-1α and Stox1 might be involved in the pathogenesis of preeclampsia. HIF-1α is known to control TGFβ3, an inhibitor of EVT differentiation, which is elevated in placentae of women suffering from this preeclampsia (Caniggia *et al.*, 2000a). Downregulation of TGFβ3 is shown to restore the invasive capacity of preeclamptic villi *in vitro*, suggesting that HIF-1α - mediated oxygen sensing could be affected in preeclampsia. Further, Stox1 has been identified as a susceptibility gene for familial preeclampsia in a Dutch population (Van Dijk & Oudejans, 2011). Similarly, inhibitor of DNA binding 2 (Id2) acts as a negative regulator of trophoblast invasiveness (Janatpour *et al.*, 2000). Downregulation of Id2 expression during differentiation leads to increased activity of differentiation-promoting basic helix-loop-helix (bHLH) genes in EVTs (Meinhardt *et al.*, 2005). Ligand-dependent activation of proliferator-activated receptor γ (PPARγ) controls invasion-promoting genes such as hCG and interferes with trophoblast motility (Fournier *et al.*, 2008). In contrast, glial cells missing 1 (GCM1) and TCF-4 are activated in non-proliferating EVTs and increase trophoblast migration and invasion (Pollheimer *et al*., 2006; Baczyk *et al*., 2009). Nuclear localization of TCF-4 and β-catenin (co-activator of TCF-4) is observed in EVTs; and CHM placentae display elevated expression of β-catenin contributing to trophoblastic hyperplasia and increased local invasion (Pollheimer *et al.*, 2006).

2.8 Effector proteins involved in trophoblast migration and invasion

The fate of trophoblast migration and invasion seems to be determined through the expression of integrins in the surrounding decidual matrix and the ability of the trophoblast to produce MMPs. The migration and invasion of EVTs is spatially and temporally regulated by three major factors:

1. Polar degeneration of ECM in the direction of migration by proteolytic enzymes such as MMPs and PA.

2. Inhibition of ECM degradation and restraining the invasion by specific inhibitors of proteases like TIMPs and PAI.

3. Binding of trophoblast cells to the ECM through differential expression of adhesion molecules like integrins, cadherins, tetraspanins etc.

The expression of these effectors of migration and invasion are controlled by various transcription factors activated by the cytokines and growth factor-mediated signaling pathways. This section summarizes the molecules and the transcription factors involved in the process of trophoblast migration and invasion.

2.8.1 Integrin

The cell adhesion proteins consist of four families: integrins, cadherins, immunoglobulin superfamilies, and CD44 proteins and selectins in one group (Albelda and Buck, 1990). Generally, the function of adhesion proteins is to enable the cells to recognize each other as well as sense the ECM components. Integrins are the principal adhesion molecules comprise of two heterodimeric transmembrane glycoproteins; α chain of approximately 1,000 amino acids (aa) and β chain of approximately 750 aa (Meredith *et al*., 1996). The combination of α (20 identified) and β (8 identified) subunits can form a family of different integrins that adhere to various ECM components. An integrin can interact with

different ECM components, and at the same time a ECM protein can be recognized by several integrins (Hynes 1992). The α subunit regulates the ligand attachment and extracellular conformation, while β subunit, whose intracellular tail domain (40–60 aa) is associated with various cytoskeletal proteins, can communicate in-out signaling for various cellular processes i.e. the cytoskeletal organization, proliferation, migration and cellular differentiation (Kuhn and Eble, 1994). The expression of integrin α1 and α4 subunits is known to be expressed in the endometrial epithelium during the luteal phase of the menstrual cycle. The β3 subunit mainly expressed during the 20^{th} day of cycle, and interacted with α1 and α4 subunit, during embryo implantation. Thus integrin α4β3 is suggested to be an excellent marker during the time of implantation window. At the same time αV and β1 subunits are also present, thereby leading to assembly and formation of heterodimers αVβ3, αVβ1, α4β1 and α1β1. Integrin αVβ3 binds to the Arg-Gly-Asp (RGD) sequences, which initiates attachment of trophoblast with the uterine epithelium through cell to cell interaction. The αVβ3 integrin is simultaneously present on both trophoblastic cells and cells of the uterine epithelium, suggesting that its expression would be crucial during time of implantation. In addition, the clinical studies suggested that the absence of integrin αVβ3 expression on the endometrial lining might act as a marker of poor endometrial receptivity (Lessey *et al.,* 1992, 1994a). During the phases of implantation, when the embryo attaches to the endometrium lining, the expression of a new set of integrin subunits α2, α6 and α7 occurs, which further confirmed that the blastocyst has acquired the capacity to be implanted. During the invasion of blastocyst integrin α6β1 and α7β1 of the blastocyst binds to the laminin component of ECM present in the basement membrane of endometrial epithelium (Merviel *et al*., 2001). During the early pregnancy, villous cytotrophoblast is positive for α6 integrin subunit, while EVT cells expressed integrin α5 subunit. The same study also suggested that the expression of a α5 subunit on EVT cell is correlated with the appearance of the invasive phenotype during trophoblastic cell migration. The expression of integrin α6 and α2 subunits are localized on to the glandular epithelium of the endometrium and their expression is up

regulated during the secretory phase of menstrual cycle but became low after decidualization of endometrium. However, the expression of integrin α5 subunit in stromal cells, dramatically increased after decidualization of endometrium (Bischof *et al*., 1993)

During the trophoblastic cell migration and invasion, as the cells at the distal end of cell column loose contact with the basement membrane and progressively come into contact with various ECM components secreted by the decidua, the expression of integrin α6β4 is lost, while the expression of integrin α1β1, αVβ1 and αVβ3 increased, whereas the expression of integrin α5β1 remain constant (Burrow *et al*., 1995). Trophoblast cells on reaching at the ECM of decidua; they stop proliferating and become invasive in nature (Armant *et al*., 1986; Aplin and Charlton, 1990). This phenomenon of differential expression of integrins during the migration process is called the integrin switching, and is also observed in tumor cells (Keely *et al*., 1998). The integrin switch marks the transition from a proliferative (α6β4 and α5β1) to an invasive (α1β1) phenotype (Birchmeier and Birchmeier, 1994; Damsky *et al*., 1994). The expression of various α subunits in the iEVT have been observed, but in case of β chain, only β1 subunit is expressed and form the dimers like α5β1, α1β1 and α6β1 (Damsky *et al*., 1992; Burrows *et al*., 1993). It has been reported in previous studies (Zhou *et al*., 1993, 1997b) that in pregnancies complicated due to preeclampsia, there is no decrease in expression of β4 subunit (α6β4) and no increase in expression of α1 subunit (α1β1) in iEVTs isolated from preeclamptic placentae as compared to normal pregnancy placentae. In another study, EVTs isolated from IUGR placenta, showed low expression of α2β1, α3β1 and α5β1 as compared to cells derived from normal pregnancies (Zygmunt *et al*., 1997).

2.8.2 Matrix degrading enzymes and their inhibitors

During the process of trophoblast invasion in the first trimester, number of factors have been shown to regulate the synthesis, activation, and/or secretion of MMPs and TIMPs at

the maternal-fetal interface, including a variety of cytokines, chemokines, growth factors, hormones, and oxygen tension (Bischof and Campana, 2000; Bischof *et al*., 2000). MMPs also known as a matrixins are a family of 23 zinc and calcium-dependent endopeptidases, known to degrade different components of ECM. MMPs are divided into different group's like gelatinases (MMP2 and MMP9), collagenases (MMP1, MMP8 and MMP13), stromelysins (MMP3, MMP7, MMP10, MMP11 and MMP12), membrane-anchored MMPs (MMP14, MMP15, MMP16 and MMP17) and other MMPs based on their preferences for the substrate. These MMPs are made up of a common domain structure: a pro-domain, a catalytic domain, a hinge region and a hemopexin domain; and share high protein sequence homology. The membrane-type matrix metalloproteinases (MT-MMP) are further classified into two groups-those anchored by transmembrane domain followed by cytoplasmic domain and the others anchored with the glycosylphosphatidylinositol (GPI)-anchor lacking a cytoplasmic domain (Malemud, 2006). Most MMPs are secreted as inactive pro-enzymes (zymogens) which get activated in the extracellular compartment except MMP11 and MT-MMPs. MMPs are involved in the control of many physiological processes like blastocyst implantation, endometrial cycling, mammary gland morphogenesis, wound healing, angiogenesis, apoptosis and nerve growth. In addition to this, MMPs are also involved in several pathological conditions like rheumatoid arthritis, gastric ulcer, cancer metastasis and invasion (Malemud, 2006; Kanth and Reddy, 2014).

The trophoblast cells and the cells which are present at the fetal-maternal interface like uNK cells, endometrial stromal cells secrete MMPs (except MMP10 and MMP25 present only in EVTs; (Curry and Osteen, 2003; Anacker *et al*., 2011). The gelatinase activity of MMP2 and MMP9 which cleave collagen IV (the main component of the basement membrane) plays the most important role during trophoblast invasion. The expression of MMP2 dominates during early pregnancy (6-8 weeks) while the expression of MMP9 rises in the late first trimester (9-12 weeks) of gestation (Xu *et al*., 2000; Staun-Ram *et al*., 2004). However, *MMP2* and *MMP9* knockout mice are fertile and only show partial

effects on the pregnancy (Itoh *et al*., 1997; Dubois *et al*., 2000). Other then MMP2 and MMP9, the expression of pro-MMP3 and active MMP13 and MMP23 is down-regulated during the course of pregnancy; however, the proforms of MMP8, -19 and -23 and active forms of MMP9, 10, 12, 15, 16, 26, 28 and pro- and active- forms of MMP14 are up-regulated towards the end of gestation. Differential expression of MMPs has also been demonstrated before and after parturition (Tsatas *et al*., 1999; Merchant *et al*., 2004a). Moreover, aberrant MMP expression has also been reported in IUGR and preeclampsia (Merchant *et al*., 2004a; Myers *et al*., 2005).

The activity of MMPs is tightly controlled physiologically by TIMPs. TIMP-1 inhibits all the MMPs in their activated form, preferentially binding MMP-9 in both the latent and active form (Cross *et al.*, 1994). TIMP1, 2 and 3 are expressed by decidua in early pregnancy and thus together with MMPs play a pivotal role in controlling the invasion process (Hurskainen *et al.*, 1996). Trophoblast behavior and function is also affected by ECM components. Trophoblasts grown in presence of fibronectin, laminin and vitronectin secrete more MMP9 than in presence of Collagen I or IV, whereas MMP2 and MMP14 were not affected (Xu *et al.*, 2000).

The presence of serine proteases like uPA, tissue-type plasminogen activator (tPA) and the inhibitors of plasminogen activator, PAI-1 and PAI-2 have been reported to be present in the human placenta (Floridon *et al*., 1999; Aflalo *et al*., 2004). The function of plasminogen activator is to convert plasminogen into active form of serine protease plasmin, which can directly degrade non-collagenases component of ECM and also degrades collagen *via* activation of collagenases (Littlefield, 1991). The presence of PAI balances the activity of plasminogen activator (Nordengren *et al*., 2004). Instead of degradation of ECM directly, plasminogen activator may also contribute in the proteolytic activation of MMPs (Zhang and Nothnick, 2005).

2.9 Role of hypoxia in trophoblast migration and invasion

Hypoxia during normal development regulates cellular differentiation, immune defense and tissue. During the initial stages of pregnancy, a physiological hypoxia is essential for placental growth and development. Hypoxia plays a crucial role in fetal development; it is involved in different embryonic processes like placentation, angiogenesis, and hematopoiesis (Webster and Abela, 2007; Dunwoodie, 2009). During early first trimester, the hypoxia is maintained by EVTs which plug the lumen of spiral arteries, prevent maternal blood flow into intervillous spaces and thereby maintaining a low O_2 environment (Genbacev *et al*., 1997). Besides normal physiological hypoxia, non-physiological intrauterine hypoxia also developed during pregnancy, which has been classified into three sub-types according to its origin (Kingdom and Kaufmann, 1997): (1) **Pre-placental**: Mother and fetus both are hypoxic (i.e., high-altitude, cyanotic maternal heart disease) (2) **Utero-placental**: When maternal oxygenation is normal but the placental fetal unit are hypoxic (i.e., preeclampsia, placental insufficiency); (3) **Post-placental**: When only the fetus is hypoxic (fetal diseases, cord blood compression). Out of these, utero-placental hypoxia occurs due to placental insufficiency, failure in the timing and incapacity of the placenta to supply adequate O_2 and nutrients to the fetus, leading to disruption in developmental process of fetus. There are various reasons for improper fetal development such as deficient implantation, impaired maternal spiral arteries remodeling, and placental vascular diseases (Chaddha *et al*., 2004; Norwitz, 2006). In addition, utero-placental hypoxia is also a reason for pregnancies associated complications like IUGR, gestational hypertension, and preeclampsia (van Patot *et al*., 2012), which occur due to insufficient migration and shallow invasion of EVT. Thus relevance of hypoxia in relation to trophoblast cell migration and invasion has been investigated in the present thesis.

Earlier in vitro studies have reported that hypoxia inhibits the invasion of primary trophoblast cells (Genbacev *et al*., 1996; Genbacev, 1997), but recent studies using

trophoblast cell lines such as HTR-8/SVneo and JEG3 cells show that genes related to cell migration and invasion are highly expressed, and in vitro cell migration and invasion were up-regulated under hypoxic conditions (Lash *et al*., 2007; Wang *et al*., 2015). The embryo responds to intrauterine hypoxia by means of specific cytokines and its associated downstream signaling pathways that can modify target gene expression through oxygen sensitive transcription factor like HIFs. Although, HIF-1α is constitutively expressed throughout pregnancy (Pringle *et al*. 2010), its abnormal expression has been reported in preeclamptic women (Caniggia and Winter, 2002). In the frirst trimester, HIF-1α and HIF-2α have been shown to localized in STB, villous CTB and feto-placental vascular endothelium (Rajakumar and Conrad, 2000: Genbacev *et al*., 2001). HIF-1α plays an important role during trophoblastic cells migration and invasion by regulating expression of certain genes like uPAR (Meade *et al*., 2007) and TGF-β3 (Nishi *et al*., 2004). In general, expression of HIF-1α is primarily regulated by changes in oxygen concentration; however, during pregnancy different cytokines and hormones also regulate HIF-1α under different physiological conditions (Pringle *et al*. 2010). Further, placentas from Arnt−/−, Hif-1α−/− or Hif-2α−/− embryos exhibit defective trophoblast invasion and placental vascularization (Cowden Dahl *et al*., 2005). Due to shallow trophoblastic cell invasion and incomplete spiral arteries remodeling, persistent hypoxia occurs in preeclamptic placenta, which resulted in continued HIF-1α expression, leading to imbalance in circulating levels of soluble VEGF receptor-1 (sFLT-1) and soluble endoglin proteins (Rajakumar *et al*., 2004; Tal, 2012). Hypoxia induced HIF-1α and HIF-2α regulate transcriptional activity of invasion promoting genes like MMP-1, LIM and SH3 Protein1 (LASP1) (Highet *et al*., 2015; Fang *et al*., 2017). Impaired trophoblast fusion and biochemical differentiation has been observed under hypoxic conditions in isolated cytotrophoblasts and forskolin stimulated BeWo cells (Alsat *et al*., 1996; Hu *et al*., 2007). This reduced fusion is associated with reduced syncytin-1, GCMa and ASCT2 expression (Wich *et al*., 2009).

Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Acrylamide, N, N'-methylene bisacrylamide, ammonium persulfate (APS), bovine serum albumin (BSA), chloroform, glycine, isopropanol, methanol, sodium dodecyl sulfate (SDS) and tris were purchased from Sisco Research Laboratories (SRL), New Delhi, India. Agarose, acetic acid, bromophenol blue, β-mercaptoethanol, calcium chloride ($CaCl_2$), Coomassie brilliant blue-R250, dimethyl sulfoxide (DMSO), diethylpyrocarbonate (DEPC), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), gelatin, casein, nonidet P-40, paraformaldehyde, phenylmethane sulfonyl fluoride (PMSF), sodium bicarbonate ($NaHCO_3$), zinc chloride ($ZnCl_2$), N,N,N',N'-tetramethylethylenediamine (TEMED), tween-20, TRI reagent, triton X-100 and trypsin were purchased from Sigma Aldrich Inc., Missouri, USA. Glycerol, sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH) and hydrogen chloride (HCl) were purchased from Merck Limited, Mumbai, India. 3,3'-diaminobenzidine (DAB) was purchased from Amresco Inc, Pennsylvania, USA. Collagen type 1, Geneticin (G418) sulfate and ethanol were purchased from Himedia Laboratories, Mumbai, India.

3.1.2 Kits

Bicinchoninic acid (BCA) assay kit for protein estimation was purchased from Pierce, Illinois, USA. Gel extraction kit was purchased from Advanced Microdevices PVT. LTD, Haryana, India.

3.1.3 Cell line and culture media

HTR-8/SVneo cells (EVT cells isolated from first trimester villous explant and transfected with SV40 large T antigen), were kindly provided by Prof. P. K. Lala,

Queen's University, Kingston, ON, Canada. Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium were purchased from Sigma Aldrich Inc. Fetal bovine serum (FBS) was purchased from Gibco®, LifeTechnologies, California, USA. The antibiotic-antimycotic cocktail was ordered from MP Biomedicals, California, USA. Opti-MEM™ medium was procured from Invitrogen Corporation, California, USA.

3.1.4 Cell culture materials

Tissue culture flasks (T-25 and T-75), tissue culture plates (6-, 12-, 24- well) and culture dishes (60 x 15 mm & 35 x 15 mm), cell scrapper, serological pipettes and transwell insert with 8 µm membranes pore size for 24-well cell culture plates were purchased from Greiner Bio-One, Frickenhausen, Germany. Glass slides and coverslips were purchased from Blue star, Polar Industrial Corporation, Mumbai, India.

3.1.5 Transfection reagents, siRNAs, primers and enzymes

Lipofectamine2000 and RNAiMAX transfection reagent were obtained from Invitrogen Corporation. Oligonucleotides for different genes were custom made by Sigma Aldrich Inc. and Eurofins Genomics, Bengaluru, India. siRNA for *WNT4*, *WNT11*, *ITGA2*, *ITGAV* and *HIF-1α* were purchased from Santa Cruz Biotechnology Inc., Texas, USA. *β-catenin* siRNA was procured from Cell Signaling Technology, Massachusetts, USA. Random hexamer, Oligo$(dT)_{18}$ primer, deoxyribonucleotide triphosphates (dNTP) mix, DNase1, Maxima Reverse transcriptase, Ribolock RNase inhibitor and Maxima™ SYBR green qPCR master mix (2X) were purchased from Fermentas International Inc., Ontario, Canada.

3.1.7 Molecular weight markers

PINK Plus Pre-stained protein ladder for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was ordered from GeneDireX, Keelung, Taiwan. GeneRuler 100 bp DNA ladder was purchased from ThermoScientific Inc., Massachusetts, USA.

3.1.6 Antibodies and conjugates

Mouse monoclonal antibodies against vimentin was purchased from Sigma Aldrich Inc. Rabbit monoclonal antibodies for total AKT, p-AKT (Thr308), PKA, p-PKA, HIF-1α, β-catenin, MMP2, MMP3, TBP1 and rabbit polyclonal antibodies against p44/42 MAPK (ERK½) and phospho- p44/42 MAPK (ERK½) were purchased from Cell Signaling Technology Inc. Goat polyclonal antibody against WNT4 and rabbit polyclonal antibodies against WNT11, ITGA2 and ITGAV were purchased from Santa Cruz Biotechnology Inc. Mouse monoclonal against α2β1 was purchased from Enzo Life Sciences Inc., New York, USA. Rabbit polyclonal antibodies against αVβ1, αVβ3 and αVβ5 were purchased from Bioss Antibodies™, Massachusetts, USA. Mouse monoclonal antibody against E-cadherin and rabbit polyclonal antibodies against TIMP1 and TIMP3 were purchased from Cloud-Clone Corp., Houston, USA. Rabbit polyclonal antibodies against MMP1 and MMP9 were purchased from Abcam Inc., Cambridge, UK. Horseradish peroxidase (HRP) conjugated goat anti-rabbit, goat anti-mouse and donkey anti-goat antibodies were purchased from Pierce, Illinois, USA. Alexa Fluor 488-labelled goat anti-rabbit IgG (H+L) and Alexa Fluor 488-labelled rabbit anti-mouse IgG (H+L) antibodies were obtained from Invitrogen Corporation.

3.1.8 Others

Recombinant hepatocyte growth factor (HGF) was purchased from Gibco®. Mitomycin-C, Brij-35 solution and H89 dihydrochloride (PKA inhibitor) were obtained from Sigma Aldrich Inc. Complete protease and phosphatase inhibitor cocktail tablets were purchased from Roche diagnostic, Mannheim, Germany. Growth factor reduced (GFR) matrigel matrix was purchased from Becton and Dickinson (BD) Biosciences, Massachusetts, USA. U0126 (MAPK inhibitor) and LY294002 (PI3K inhibitor) were ordered from Cell Signaling Technology Inc. The nitrocellulose membrane (pore size 0.22 and 0.45 µm) was purchased from Advanced Microdevices PVT. LTD. Whattman sheets were procured from GE healthcare, Illinois, USA. Hoechst 3342 nuclear dye and Diamond Antifade

with DAPI were purchased from Life Technologies. Immobilon Western chemiluminescent HRP substrate was obtained from Millipore, Massachusetts, USA**.**

3.2 Methods

3.2.1 Cell culture

HTR-8/SVneo cells were cultured in DMEM and Ham's F-12 medium in 1:1 ratio supplemented with 10% heat inactivated FBS and antibiotic-antimycotic cocktail [penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (0.25 µg/mL)] at 37°C in a humidified chamber containing 5% CO_2. The cells were passaged at 70-80% confluency and maintained in G418 (75 µg/mL) after every 3rd passage to restrict the growth of un-transfected HTR-8/SVneo cells with SV40 large T antigen. For various experiments under hypoxic conditions, cells were incubated at 37°C in humidified chamber containing 93% N_2, 2% O_2 and 5% CO_2.

3.2.2 Cell migration and invasion assays

3.2.2.1 ***Scratch wound migration assay*****:** Cell migration was studied by *in vitro* scratch wound assay. HTR-8/SVneo cells (0.2 x 10^6 cells/well) cultured in 6-well culture plate were grown to form monolayer in humidified chamber containing 5% CO_2 at 37°C. Monolayer of HTR-8/SVneo cells was treated with 5 µM mitomycin-C for 2 h to inhibit cell proliferation. Subsequently, cells were scratched with 200 µL pipette tips to create wounds in horizontal as well as in vertical directions parallel to the diameter of the culture plate. Wells were washed extensively with plain medium to remove detached cells and fresh medium containing 1% FBS and recombinant human HGF was added to wells. Cells were imaged from different regions at 0 h using phase contrast microscope (Nikon Instruments Inc., New York, USA) and incubated under normoxic (20% O_2) conditions in humidified chamber containing 5% CO_2 at 37°C for 24 h. To study cell migration under hypoxia, cell culture plates were incubated in hypoxic (2% O_2) chamber containing 5% CO_2 in humidified conditions at 37°C for 24 h after wound imaging. To

measure the area of wound closure, images were taken from the same area and cells bordering the wounds were traced using ImageJ software (http: //rsb.info. nih.gov/ij/). The percentage migration was calculated from the equation {(Wi-Wz)/Wi} x 100; where Wi is the area of wound at t = 0 h and Wz is the area of wound closure after 24 h. The cell culture supernatants were collected in separate tubes and further used to identify secreted proteins by Western blotting. In addition, these cells were also used to study relative transcript level of different genes by RT-qPCR and proteins level by Western blotting.

3.2.2.2 ***Transwell migration assay*****:** HTR-8/SVneo cells migration assay was also performed using transwell insert with 8 µm pore size filter membrane in 24-well cell culture plates. DMEM + Ham's F-12 medium (1:1 ratio) (300 µL/well) supplemented with 1% FBS with or without HGF (50 ng/mL) was added to the lower chamber containing transwell inserts. The HTR-8/SVneo cells ($1x10^5$ cells/150 µL) suspended in the same medium were seeded in the upper chamber of the transwell in the presence or absence of HGF. After 24 h incubation, medium from lower chamber was aspirated, and excess cells and medium from the upper chamber of transwell were removed using moist cotton swab. Cells were fixed in chilled methanol for 7-10 min at 4°C. After subsequent washing with phosphate buffer saline (PBS 50 mM, pH-7.4), the membrane was stained with Hoechst 33342 nuclear dye (0.2 µg/mL) for 5 min at 37°C. After staining, the membrane of transwell insert was cut and mounted with the lower surface of the membrane facing up on the microscope slides in immersion oil. The cells present on the lower surface of the membrane were counted under fluorescent phase contrast microscope (Nikon Instruments Inc.). The number of cells of untreated control was taken as one and fold change was calculated by dividing number of cells on membrane of treated transwell insert by the number of cells on untreated control transwell insert membrane.

3.2.2.3 ***Matrigel matrix invasion assay*****:** The Matrigel matrix was prepared by adding 50 µl (1µg/mL) of growth factor reduced matrigel matrix on the upper surface of the

transwell insert of 8 µm pore size filter kept in a 24-well cell culture plate and incubated overnight at 37˚C under humidified atmosphere to form a semisolid gel-like matrix. Culture medium (300 µl, 1:1 DMEM + Ham's F12 supplemented with 1% FBS) was added to the lower chamber of all the wells with the transwell insert. Following which, cells (0.1×10^6 cells/transwell) suspended in reduced serum (1%) medium were seeded in the upper chamber over the Matrigel matrix in a total volume of 150 µl. HGF (50 ng/mL) was added in the upper and lower chambers. Cells were allowed to invade for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions (according to experimental need) at 37˚C. Non-invading cells along with the Matrigel remaining in the upper chamber of the transwell inserts were carefully removed using moist cotton swab. Transwell inserts were washed with 50 mM PBS, pH 7.4 and cells were fixed for 10 min in chilled methanol. Fixed cells were washed with PBS and stained with 0.2 µM Hoechst 3342 nuclear dye for 10 min. After PBS wash, the membranes were cut and placed on a slide and cells counted on the whole membrane under oil immersion in a fluorescent phase contrast microscope (Nikon Instruments Inc.). The number of cells of untreated control was taken as one and fold change was calculated by dividing number of cells on membrane of treated transwell insert by the number of cells on untreated control transwell insert membrane.

3.2.3 Immunolocalization

3.2.3.1 ***Immunolocalization of integrins*****:** HTR-8/SVneo cells (0.3×10^5/well) were seeded in 24-well plates on coverslips and incubated under normoxia (20% O_2) at 37ºC in humidified conditions containing 5% CO_2. 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, pH-7.4) and blocked in 3% BSA in PBS for 30 min. The primary antibody against integrin α2β1 was diluted at 1:500, while antibodies against αVβ1, αVβ3 and αVβ5 were diluted at 1:100 dilutions each in PBS supplemented with 1% BSA and added to the respective wells separately, and incubated for 1 h at room temperature (RT). Cells were washed thrice with PBS and

incubated with either goat anti-mouse IgG at a dilution of 1:1000 or anti-rabbit IgG at a dilution of 1:1000 secondary antibody conjugated with Alexa Fluor 488 diluted in PBS and supplemented with 1% BSA for 45 min in dark at RT. After incubation, cells were washed three times with PBS and immediately 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. Cells were examined under fluorescent phase contrast microscope (Nikon Instrument Inc.) and images were captured and processed using Image proplus software (Media cybernetics, Maryland, USA).

3.2.3.2 ***Immunolocalization of HIF-1α*****:** HTR-8/SVneo cells (0.3 x 10^5/well) were seeded on coverslips in 24-well plate and incubated overnight under normoxic (20% O_2) conditions at 37°C in humidified atmosphere of 5% CO_2. Next day, after serum starvation for 6 h in normoxic conditions, cells were incubated under hypoxia (2% O_2) in presence of HGF (50 ng/mL) for 24 h at 37ºC. After treatment, cells were fixed in chilled methanol for 10 min at 4°C. Subsequently, coverslips were washed with PBS and blocked in 3% BSA in PBS for 30 min at RT. The primary antibody against HIF-1α in PBS (diluted 1:100) containing 1% BSA was added to wells and incubated for overnight at 4°C. Cells were washed thrice with PBS, followed by incubation with anti-rabbit secondary antibody conjugated with Alexa Fluor 488 diluted (1:500) in PBS and supplemented with 1% BSA for 45 min at RT. After extensive washings, coverslips containing fixed cells were mounted on a glass slide using ProLong® Diamond Antifade containing DAPI (Invitrogen Corporation). Cells were examined under fluorescent phase contrast microscope (Nikon Instrument Inc.) and images were captured and processed using Image proplus software (Media cybernetics).

3.2.4 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 (1:1 ratio) supplemented with 10% FBS and incubated at 37ºC in

humidified atmosphere containing 5% CO_2. Next day, cells were transfected with optimized concentration of control siRNA or *WNT4/WNT11/ITGA2/ITGAV/β-catenin/HIF-1α* using lipofectamine2000 or RNAiMAX and Opti-MEM® medium. In brief, cells were washed once and 750 µL of fresh Opti-MEM® medium was added into each well. Further, the optimized concentration of above mentioned siRNAs were mixed with Opti-MEM® medium to make a total volume of 125 µL. In separate tube, lipofectamine2000 (7.5 µL) or RNAiMAX (9 µL) was mixed with Opti-MEM® medium to make a final volume of 125 µL and incubated for 5 min at RT. After incubation, both solutions were mixed and incubated for 10 min at RT. The mixed solutions were added carefully drop by drop in the respective wells and after 6 h of incubation under normoxic conditions at 37°C in humidified condition of 5% CO_2, complete medium was added to the cells. After 48 h of transfection, silenced cells were used for either wound healing assay or transwell migration assay, qRT-PCR and Western blotting.

3.2.5 Quantitative Reverse Transcription Polymerase chain reaction (RT-qPCR)

3.2.5.1 ***Total RNA isolation*****:** In different set of experiments, HTR-8/SVneo cells (0.2 x 10^6/well) were grown overnight in 6-well culture plate in humidified atmosphere containing 20% O_2 and 5% CO_2 at 37ºC. Next day, cells were serum starved for 6 h under same conditions, followed by treatment with HGF (50 ng/mL) for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions. After treatment, cells were lysed using Tri reagent and stored at -80ºC. The cells lysate was thawed and passed through pipette several times and incubated for 5 min at RT. Further, 200 µL of chloroform was added into each tube and shaken vigorously for 20-30 sec and incubated for 15 min at RT. After incubation, the cell fraction was centrifuged at 12000 x g for 15 min at 4ºC. Following centrifugation, the clear aqueous phase was collected carefully in a separate tube and 500 µL of isopropanol was added and mixed gently using 1 mL pipette and incubated for 15 min at RT. The fraction was again centrifuged at 12000 x g for 25 min at 4ºC. After centrifugation, supernatant was discarded and pellet was washed with 75% ethanol and spin at 7500 x g for 10 min at 4ºC. Subsequently, ethanol was discarded

carefully and tubes were allowed to dry for 20 min. The pellet containing RNA was dissolved in diethyl pyrocarbonate (DEPC) treated water. The total RNA content was quantified using NanoDrop 3300 spectrophotometer (Thermo Scientific, Denver, USA). Subsequently, isolated RNA was treated with DNase1 (1U/µL) for 30 min at 37ºC, followed by treatment with EDTA (50 mM) and heat inactivated at 65ºC for 5 min in a water bath. Thereafter, isolated RNA was used to prepare complementary DNA (cDNA).

3.2.5.2 ***Complementary DNA (cDNA) preparation***: To prepare cDNA, 4.5 µg of RNA was added to reaction mixture containing Oligo $(dT)_{18}$ primer, random hexamer (100 pmol each) and dNTP mix (0.5 mM) in a microcentrifuge tube and further incubated at 65ºC for 5 min followed by immediate chilling on ice. The cDNA strand synthesis was carried out by addition of RiboLock RNase inhibitor (20 U) and reverse transcriptase (200 U) in a RT buffer (1X) (Fermentas Inc.) to the above mixture and further incubated at 25ºC for 10 min followed by 30 min at 50ºC. The reaction was terminated by heating at 85ºC for 5 min.

3.2.5.3 ***Quantitative polymerase chain reaction (qPCR)*:** The qPCR was performed in duplicates in 20 µL reaction for analysis of expression profile of various *WNT* ligands, integrins, *β-catenin*, *MMPs*, *TIMPs* and *HIF-1α*. The nucleotide sequences of the primers used are given in Table 3.1. The qPCR was carried out in Stratagene Mx3005P (Agilent Technologies Inc., California, USA) using the reaction mixture comprising of Maxima SYBR green master mix (1X) (Fermentas Inc.), synthesized cDNA (diluted 5 times) and gene specific primers (0.1 µM). The cycle profile for target gene amplification: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and primer specific annealing temperature (Table 3.1) for 60 sec. Finally, a melting curve analysis was carried out at a temperature range of 60 to 95°C for 20 min. A single peak in the melting curve analysis confirmed gene-specific amplification. The fold changes in expression (relative expression) of genes were calculated from relative Ct values using ΔΔCt (Ct; cycle threshold) method after normalized with *18S* rRNA/*CYC1* (cytochrome

*C*1) /*TOP1* (DNA topoisomerase type I), which acted as loading control in the same sample.

3.2.6 Analysis of cellular signaling and activated downstream molecules by Western blotting

3.2.6.1 ***Preparation of whole cell extract*****:** HTR-8/SVneo cells obtained post-wound healing migration assay 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 (Roche Diagnostics). In different set of experiments, HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate in DMEM + Ham's F-12 medium supplemented with 10% FBS and incubated at 37°C for overnight under normoxic (20% O_2) conditions in a humidified chamber containing 5% CO_2. Next day, cells were starved of serum for 6 h and then treated with HGF (50 ng/mL) for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions to study the expression of various genes following HGF stimulation. Thereafter, cells were lysed in cell lysis buffer and stored at -80ºC till used. In another set of experiments, after serum starvation of HTR-8/SVneo cells under normoxia (20% O_2), cells were treated for 10, 30 and 60 min in the presence and absence of HGF (50 ng/mL) under both normoxic (20% O_2) as well as hypoxic (2% O_2) conditions to study the activation of various downstream signaling pathways (ERK, PKA & PI3K). After each time point, the medium was aspirated and cells were lysed in lysis buffer mentioned above. Cell suspensions from all set of experiments were subjected to three rapid freeze-thaw cycles to ensure complete cell lysis. These cell lysates were further centrifuged at 12,000 × g for 10 min at 4°C, supernatants thus obtained containing cellular proteins were collected and stored at -70°C till used.

3.2.6.2 ***Preparation of nuclear and cytoplasmic fractions***: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate in DMEM + Ham's F-12 medium supplemented with 10% FBS and incubated at 37°C for overnight under normoxic (20%

O_2) conditions in a humidified chamber containing 5% CO_2. Next day, cells were starved of serum for 6 h and then treated with HGF (50 ng/mL) for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions. After treatment, cells were harvested in ice-cold PBS containing 1 mM EDTA. Immediately, cell suspension was centrifuged at 5000 x g for 5 min at 4ºC. The cells pellet obtained after centrifugation was suspended in cytoplasmic extraction buffer (1 M HEPES-KOH pH-7.9, 3 M KCl, 0.5 M EDTA, 10% NP-40) and lysed by cycle of vortexing for 3 min and incubation on ice for 1 min for three times. Subsequently, cell lysate was centrifuged for 5 min at 10,000 x g at 4°C and supernatant thus obtained represented the cytoplasmic extract. The residual pellet was dissolved in the nuclear extraction buffer (1 M Tris pH 7.5, 3 M KCl, 0.5 M EDTA) followed by rapid freeze-thaw (three times) in liquid nitrogen. Nuclear fraction was collected by centrifugation at 10,000 x g for 5 min. The protein content in whole cell lysate, cytoplasmic & nuclear fractions was quantitated by bicinchoninic acid colorimetric assay (BCA) using bovine serum albumin (BSA) as standard.

3.2.6.3 ***Western blotting*****:** For protein gel electrophoresis, whole cell lysate/culture supernatant/cytoplasmic fraction/nuclear fraction (40 µg/lane) was solubilized in gel loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 1% bromophenol blue, 5% β-mercaptoethanol) by boiling for 10 min. Subsequently, lysates were resolved by 0.1% SDS-10% polyacrylamide gel electrophoresis (SDS–PAGE) at 25 mA till all the protein bands in the molecular weight markers were clearly resolved. The resolved proteins were then transferred to the nitrocellulose membrane (0.22 µm or 0.45 µm) by wet transfer method at 280 mA for 3 h at 4ºC. After transfer of proteins on to membrane, the membrane was blocked in 5% BSA in PBS (50 mM; pH 7.4) for 1 h at RT. 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), non-phosphorylated active β-catenin (1:1000), TATA-binding protein1 (TBP1) (1:1000), MMP1 (1:1000), MMP2 (1:1000), MMP3 (1:1000), MMP9 (1:1000), HIF-1α (1:1000), TIMP1 (1:1000), TIMP3 (1:1000), ERK½ (1:1000), p-ERK½ (1:1000), PKA (1:1000), p-

PKA (1:1000), Akt (1:1000), p-Akt (Thr308) (1:1000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) in tris-buffered saline-Tween (TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH-7.4) containing 5% BSA. After subsequent washings with TBST, membrane was further incubated with horseradish peroxidase (HRP) conjugated anti-goat antibody (1:10000), anti-rabbit antibody (1:3000) and anti-mouse antibody (1:5000) respectively for 1 h at RT in TBST containing 5% BSA. After subsequent washings, blots were developed using Immobilon chemiluminescent substrate. Pictures of the chemiluminescent blots were taken by FluorChem E system (ProteinSimple, California, USA). Alternatively, blots were also developed by DAB (1 mg/mL) in PBS with H_2O_2 (0.03%). Intensity of bands on Western blots were quantitated using ImageJ software (http: //rsb.info. nih.gov/ij/).

3.2.6.4 *Zymography*

HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in 6-well culture plate and incubated overnight under normoxic (20% O_2) conditions in a humidified chamber containing 5% CO_2 at 37°C. After serum starvation for 24 h in above conditions, cells were treated with HGF (50 ng/mL) for 24 h in plain medium either under normoxic (20% O_2) or hypoxic (2% O_2) conditions. To measure activity of secreted matrix metalloproteases (MMPs), culture medium was collected, concentrated (lyophilized) and proteins were resolved in 8% polyacrylamide gels containing 0.1% casein/collagen/gelatin. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h to remove SDS and then incubated in incubation buffer containing 50 mM Tris-HCl (pH-7.5), 150 mM NaCl, 10 mM $CaCl_2$ and 0.5 mM $ZnCl_2$ for gelatin and 50 mM Tris-HCl (pH-7.5), 150 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij 35 for collagen and casein at 37°C for 36 h to allow proteolysis of the respective substrate. After incubation, gels were stained in 0.5% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for 3 h followed by washing with destaining solution (40% methanol and 10% acetic acid in water). Proteolytic activities appeared as clear band on gel against dark blue background. Area of substrate degradation was measured by ImageJ software.

3.2.7 Inhibition of PKA signaling pathways by H89

HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plates and grown to form a monolayer under normoxic conditions in humidified atmosphere of 5% CO_2 at 37ºC. Monolayer of cells was serum starved for 6 h in plain medium (DMEM + Ham's F-12) and then treated with PKA inhibitor, H89 dihydrochloride (10 µM) for 2 h (according to manufacturer's protocol) to inhibit phosphorylation of PKA. After 2 h of H89 treatment, 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 previously in presence or absence of HGF (50 ng/mL) and H89 inhibitor. In addition, after 24 h of incubation, these cells were processed to prepare cell lysates, to study the changes in expression of WNT ligands and integrins.

3.2.8 Inhibition of MAPK signaling pathways by U0126

HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plates and grown to form a monolayer under normoxic conditions in humidified atmosphere of 5% CO_2 at 37ºC. Monolayer of cells was serum starved for 6 h in plain medium and then treated with MAPK inhibitor, U0126 (10 µM) for 2 h (according to manufacturer's protocol) to inhibit phosphorylation of ERK½. After 2 h of U0126 treatment, 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 previously in presence or absence of HGF (50 ng/mL) and U0126 inhibitor. After 24 h of incubation, these cells were processed to prepare cell lysates, to study the changes in the expression of WNT ligands and integrins. For various experiments under hypoxia, after treating the monolayer of cells with UO126, the cells were processed for wound healing assay in presence and absence of HGF (50 ng/mL) for 24 h under hypoxic (2% O_2) conditions at 37ºC in 5% CO_2.

To study the effect of MAPK inhibition on cell invasion, HTR-8/SVneo cells were seeded in 6-well culture plates and cultured under normoxia (20% O_2) at 37ºC in 5% CO_2. Next

day, cells were serum starved for 6 h and subsequently treated with UO126 (10 µM) for 2 h. After 2 h treatment, cells were used for matrigel matrix invasion assay in the presence and absence of HGF (50 ng/mL) either in normoxic (20% O_2) or hypoxic (2% O_2) conditions (according to experimental needs) in presence of UO126 for 24 h.

In another experiment, after UO126 treatment, cells were incubated in presence and absence of HGF (50 ng/mL) for 24 h under hypoxic conditions and subsequently processed for Western blotting to check the expression of HIF-1α and ERK½ phosphorylation.

3.2.9 Inhibition of PI3K signaling pathways by LY294002

HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plates and grown under normoxic (20% O_2) conditions at 37ºC in 5% CO_2 to form a monolayer. Monolayer of cells was serum starved for 6 h in plain medium and then treated with PI3K inhibitor, LY294002 (50 µM) for 2 h (according to manufacturer's protocol) to inhibit phosphorylation of Akt. After 2 h of LY294002 treatment, 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 previously in presence or absence of HGF (50 ng/mL) and LY294002 inhibitor under hypoxic (2% O_2) conditions.

To study the effect of PI3K inhibition on cell invasion, HTR-8/SVneo cells were seeded in 6-well culture plates and grown under normoxia (20% O_2) at 37ºC in 5% CO_2. Next day, cells were serum starved for 6 h and subsequently treated with LY294002 (50 µM) for 2 h. After 2 h, cells were used for matrigel matrix invasion assay in the presence and absence of HGF (50 ng/mL) either in normoxic (20% O_2) or hypoxic (2% O_2) conditions (according to experimental needs) for 24 h. In different set of experiment, after LY294002 treatment, cells were incubated in presence and absence of HGF (50 ng/mL) for 24 h under hypoxic conditions and subsequently processed for Western blotting to check the expression of HIF-1α and Akt phosphorylation.

3.2.10 Statistical analysis

All experiments were done at least three times and results were expressed as arthematic 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.

Table 3.1 Primers sequences for confirming gene expression by RT-qPCR

Gene	Primer sequences (5'-3')	Annealing temperature
WNT2B	F:5'AAGATGGTGCCAACTTCACCG 3' R:5'CTGCCTTCTTGGGGGCTTTGC 3'	60°C
WNT3	F:5'TGAACAAGCACAACAACGAG 3' R:5'CAGTGGCATTTTTCCTTCC 3'	59°C
WNT4	F:5'TACCTGGCCAAGCTGTCGTC 3' R:5'CCCGAGTCCCTTGCGTCAC 3'	58°C
WNT5A	F:5'GGGAGGTTGGCTTGAACATA 3' R:5'GAATGGCACGCAATTACCTT 3'	60°C
WNT7B	F:5'GGGCCACCTGCTGAAGGAGAA 3' R:5'TTGACGAAGCAGCACCAGTGGAA 3'	57°C
WNT10B	F:5'GGGGTGGCTGTAACCATGAC 3' R:5'TTTTCAGTTACCACCTGGCG 3'	58°C
WNT11	F:5'GTAAGTGCCATGGGGTGTCT 3' R:5'GCTTCCAAGTGAAGGCAAAG 3'	60°C
ITGA1	F:5'GTCCTGCCCTGCGAACCAGC 3' R:5'CATGTCTTCCACCGGGCCGC 3'	57°C
ITGA2	F:5'CGGTTATTCAGGCTCACCGA 3' R:5'GCTGACCCAAAATGCCCTCT 3'	58°C
ITGA5	F:5'CGCAGCTCTGCTTCCTCGGG 3' R:5'GCTGTGGCCACCTGACGCTC 3'	59°C
ITGA6	F:5'TGCAGGCACTCAGGTTCGAGTGA 3' R:5'AGCATGGTATCGGGGAACACTGTCA 3'	60°C
ITGAV	F:5'GCTCCATCTTCAGTGGCCTT 3' R:5'TTGGCAGACAATCTTCAAGCA 3'	60°C
18S	F:5'GGAGAGGGAGCCTGAGAAAC 3' R:5'CCTCCAATGGATCCTCGTTA 3'	60°C
ITGB1	F:5'ATCAGACGCGCAGAGGAG 3' R:5'TGGTTGTAAATTCATCTTTTCCGC 3'	60°C
ITGB3	F:5'CAGTGCCTGGCTGTGAGCCC 3' R:5'CGAATCATCTGGCCGGAGCCG 3	60°C
ITGB4	F:5'ACTACAACTCACTGACCCGC 3' R:5'CTCCTCCCGTGTTCCCAGAT 3'	58°C
ITGB5	F:5'CGGCTCGCAGGTCTCAA 3' R:5'GGAGCACCAGGCACATTTTG 3'	58°C
CTNNB1 (β-catenin)	F:5'GTCTGAGGAGCAGCTTCAGT 3' R:5'TGGTAGTGGCACCAGAATGG 3'	58°C
CYC1	F:5'AGAGTTTGACGATGGCACCCC 3' R:5'GCCTCCCAACCCTTTTACCTT 3'	58°C
MMP1	F:5'CCCATCGGCCCACAAACCCC 3' R:5'AGCAGCTTCAAGCCCATTTGGCA 3'	60°C

MMP2	F:5'ACCGCAAGTGGGGCTTCTGC 3' R:5'CGTGGCCAAACTCGTGGGCT 3'	59°C
MMP3	F:5'TTGGCCCATGCCTATGCCCC 3' R:5'ACAGGCGGAACCGAGTCAGG 3'	60°C
MMP9	F:5'CCGGCATTCAGGGAGACGCC 3' R:5'TGGAACCACGACGCCCTTGC 3'	60°C
TIMP1	F:5'TGACATCCGGTTCGTCTACA 3' R:5'GTTTGCAGGGGATGGATAAA 3'	57°C
TIMP2	F:5'GATGCACATCACCCTCTGTG 3' R:5'GTGCCCGTTGATGTTCTTCT 3'	58°C
TIMP3	F:5'CTGACAGGTCGCGTCTATGA 3' R:5'AGTCACAAAGCAAGGCAGGT 3'	58°C
TIMP4	F:5'TGGGTGAGGCATGCAGCTGC 3' R:5'GGGTCTGCACTGGCCGGAAC 3'	57°C
HIF-1α	F:5'CTGAGAGGTTGAGGGACGGA 3' R:5'GACGTTCAGAACTTATCCTACCATT 3'	58°C
TOP1	F:5'AGCCTCAGCCGTTTCTGGAG 3' R:5'GCTTCGATCTGGGAATCGTTGT 3'	58°C

Results and Discussion

Chapter – I

HGF-mediated activation of signaling pathways during trophoblastic cells migration: Role of WNT ligands and integrins

Background

Embryo implantation in humans begins with the adherence of blastocyst to the uterine epithelium. During this process, when cytotrophoblast cells differentiating into EVT undergo a partial epithelial to mesenchymal transition (EMT) and gain the capacity to migrate and invade (E Davies *et al*., 2016). EMT involves loss of interaction with basement membrane along with ECM degradation and remodeling through active participation of integrins, MMPs, cadherins and growth factors respectively (Lamouille *et al*., 2014). Failure in EVTs migration and invasion, due to any impairment in EMT process may lead to pregnancy-related complications like preeclampsia and IUGR. (Lim *et al*., 1997; E Davies *et al*., 2016). What control the trophoblastic cells (EVTs) migration is yet not fully understood? The process is complex and it involves both molecular and cellular interactions.

The migration of trophoblast cells is spatially and temporally regulated by presence of various cytokines and growth factors, secreted by diverse cell types present at feto-maternal interface. HGF is one of such growth factor, which promotes the migration and invasion of trophoblastic cells and also involved in cancer cell's metastasis (Vande Woude *et al*., 1997; Cartwright *et al*., 2002). The importance of HGF comes into limelight when its levels were found to be downregulated in IUGR placentae (Somerset *et al*., 1998a, 1998b). Besides growth factors, importance of WNT ligands and associated proteins has also been documented in previous study, where expression of WNT2 and β-catenin is reported to be downregulated in preeclamptic women (Zhang *et al*., 2013). In human, 14 out of 19 WNT ligands have been reported in first trimester placental tissue (Sonderegger *et al*., 2007). However, the role of individual WNT ligands is not yet explored in trophoblast biology.

Thus, in this chapter an attempt has been made to understand the molecular basis of trophoblastic cells migration and its associated signaling pathways activated during HGF mediated increase in trophoblastic cells migration. For this purpose, HTR-8/SVneo cells

were used as experimental model as these cells closely resemble to EVTs. The relevance of WNT ligands and integrins and their regulation by different signaling pathways has also been addressed. Further, the connecting link between the WNT ligands and integrins and their cross-talk has also been deciphered by using siRNA, to explain the molecular circuit during HGF-mediated HTR-8/SVneo cell migration.

Results

Effect of HGF on the migration of HTR-8/SVneo trophoblastic cells

To study the effect of HGF on HTR-8/SVneo cells migration, scratch wound migration assay was performed. Treatment of HTR-8/SVneo cells with varying concentration of HGF (1, 5, 10, 20 and 50 ng/mL) led to dose dependent increase in cell migration (Fig. 4.1). Significant increase in cell migration was observed at 10 (~1.8 fold), 20 (~2.1 fold) and 50 (2.9 fold) ng/mL respectively, as compared to untreated HTR-8/SVneo cells (Fig. 4.1). To eliminate the possibility that the observed increase in migration of HTR-8/SVneo cells is not due to cell proliferation, cells were pre-treated with mitomycin-C prior to use in scratch wound assay as described in *Materials and Methods*. Since maximum fold change in migration was observed at 50 ng/mL of HGF, this concentration was chosen to carry out further experiments.

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

WNT ligands, a family of secreted glycoproteins has been known to be involved in various cellular processes like cell proliferation, differentiation, migration/invasion along with tissue differentiation and organ development (Nusse and Clevers, 2017). During human pregnancy, 14 out of 19 WNT ligands have been shown to be expressed in first trimester placenta (Sonderegger *et al*., 2007). To discern the relevance of various WNT ligands in HGF mediated migration of HTR-8/SVneo cells, total RNA was isolated from the cells treated in the presence and absence of HGF (50 ng/mL) for 24 h post-wound

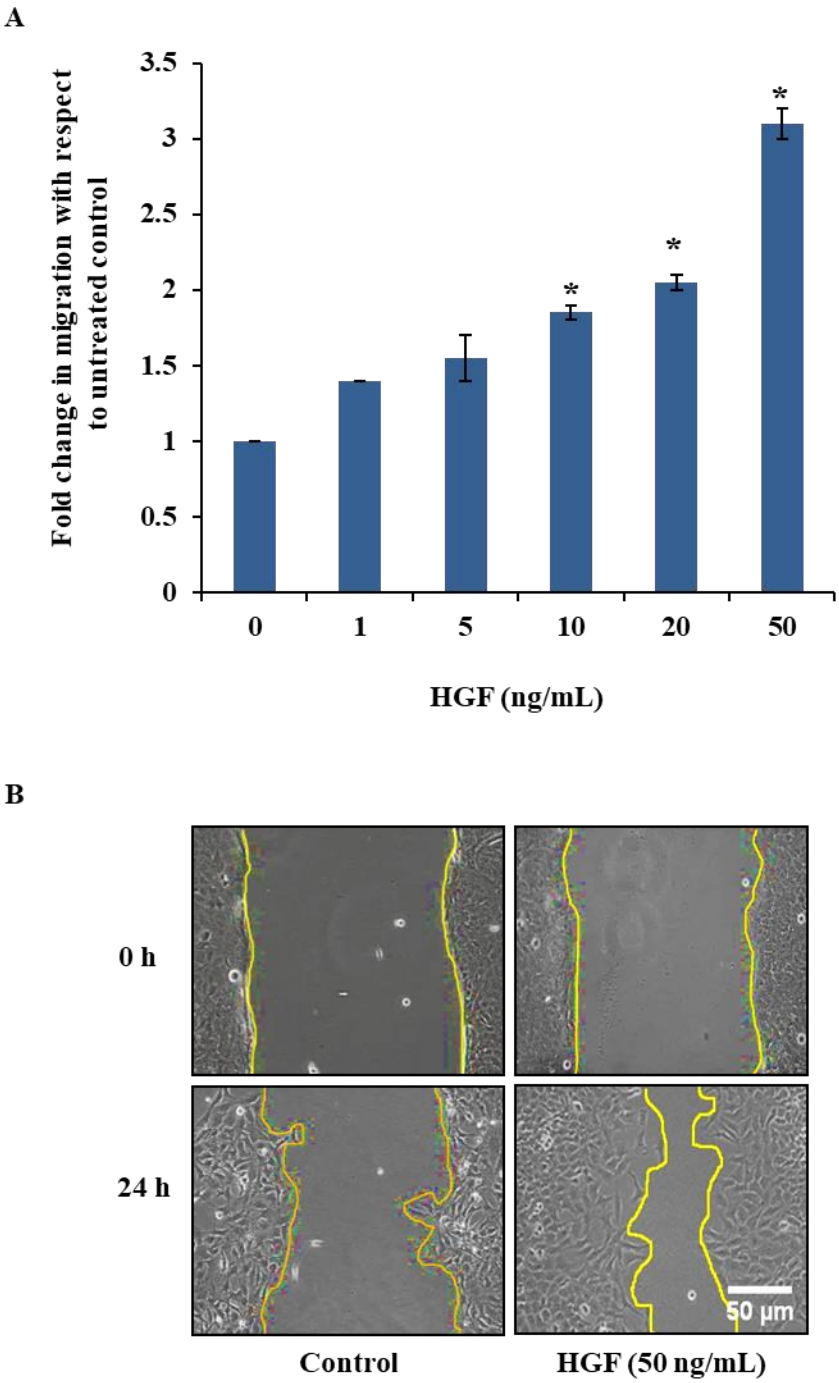

Fig. 4.1 Effect of HGF on the migration of HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6) were seeded in cell culture dishes (35 x 15 mm) and grown under humidified conditions with 5% CO_2 at 37ºC. Cells were allowed to form monolayer followed by treatment with mitomycin-C for 2 h to inhibit cell proliferation. Subsequently, wound was created in monolayer by scratching with pipette tips. After extensive washings, cells were treated with varying concentrations (0, 1, 5, 10, 20, 50 ng/mL) 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 as described in *Materials and Methods*. **Panel A** shows the fold change in migration after treatment with HGF for 24 h as compared to untreated HTR-8/SVneo cells. The results are shown as mean ± *s.e.m* of three independent experiments. **Panel B** shows representative images at 0 and 24 h with and without HGF (50 ng/mL) treatment. Scale bar represents 50 µm. * denotes $p \leq 0.05$ as compared with untreated control, which was considered statistically significant.

healing assay. Isolated RNA was converted into cDNA, to study the expression profile of the transcripts for *WNT2B*, *WNT3*, *WNT4*, *WNT5A*, *WNT7B*, *WNT10B* and *WNT11* by qPCR as described in *Materials and Methods*. The expression of *WNT2B* was found to be significantly decreased by ~5 fold both in untreated as well as HGF treated cells after 24 h as compared to 0 h control (untreated) cells (Fig. 4.2). No significant changes in the expression of *WNT3* and *WNT10B* were observed, whereas in case of *WNT5A*, significant increase in expression (~ 5 fold) was observed only in untreated cells as compared to 0 h (Fig. 4.2). On the other hand, significant increase in transcript levels of *WNT7B* by ~3.5 fold in untreated and ~7 fold in HGF treated cells was observed, respectively, as compared to 0 h. However, maximum increase in the expression was observed in *WNT4* (~ 110 fold) and *WNT11* (~ 29 fold) in cells treated with HGF for 24 h. In addition, as compared to baseline (0 h), at 24 h the transcript levels of *WNT4* and *WNT11* were also increased significantly by ~29 and ~12 folds respectively in HGF untreated cells; however, their expression was lower as compared with the HGF-treated counterpart at same point (Fig. 4.2).

Since, expression of WNT4 and WNT11 was highest among all the WNT ligands studied above, their expression was further studied at protein level by Western blotting as described in *Materials and Methods*. A significant increase in WNT4 expression in cell lysate was observed in untreated as well as HGF-treated HTR-8/SVneo cells by ~1.4 and ~2.2 fold respectively, as compared to 0 h (control) (Fig. 4.3). Similarly, the expression of WNT11 was also found to be increased significantly by ~1.8 fold without HGF treatment and ~3.8 fold with HGF treatment after 24 h. Since, WNT ligands are secretory proteins, their expression in cell culture supernatant was also studied by Western blotting. Significant increase ($p < 0.05$) in the expression of WNT4 and WNT11 was observed in supernatant of cells treated with HGF for 24 h (Fig. 4.3). However, expression of only WNT4 was significantly up regulated in untreated cells at 24 h as compared to 0 h. Interestingly, on comparing the expression of WNT4 or WNT11 between HGF treated and untreated cells, significant increase in WNT4 and WNT11 in cell lysate (WNT4; $p =$

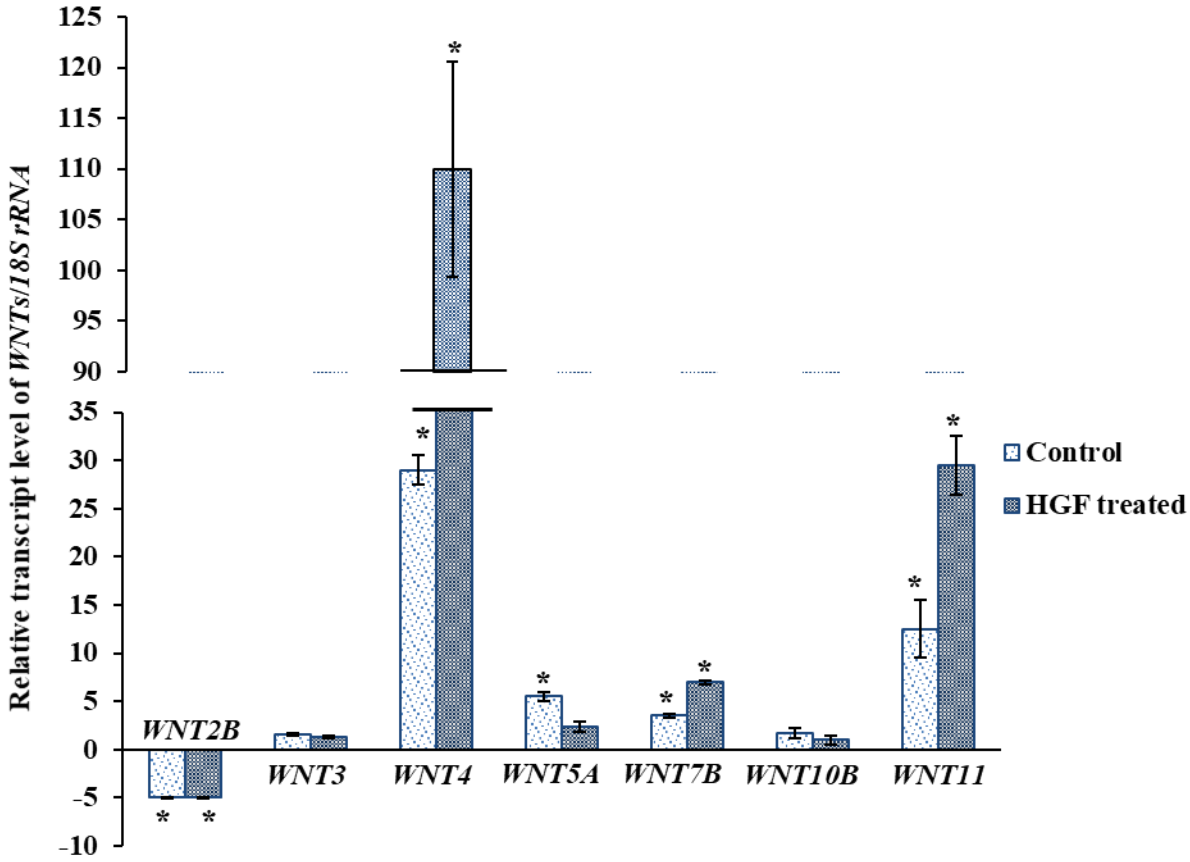

Fig. 4.2 Transcript levels of different *WNT* ligands in HGF treated HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate and grown under humidified conditions with 5% CO_2 at 37°C. Cells were allowed to form monolayer and processed for scratch wound assay for 24 h in the presence and absence of HGF (50 ng/ mL) for 24 h. Further, total RNA was isolated and processed for cDNA preparation followed by RT-qPCR analysis as described in *Materials and Methods*. The bar graph shows the relative expression of *WNT2B, WNT3, WNT4, WNT5A, WNT7B, WNT10B* and *WNT11* at 24 h in HGF treated and untreated HTR-8/SVneo cells as compared to untreated control at 0 h. Each bar represents relative expression after normalization with *18S* rRNA as an internal control. The values are shown as mean ± *s.e.m* of three independent experiments performed in duplicates. * denotes $p \leq 0.05$ with respect to untreated control at 0 h.

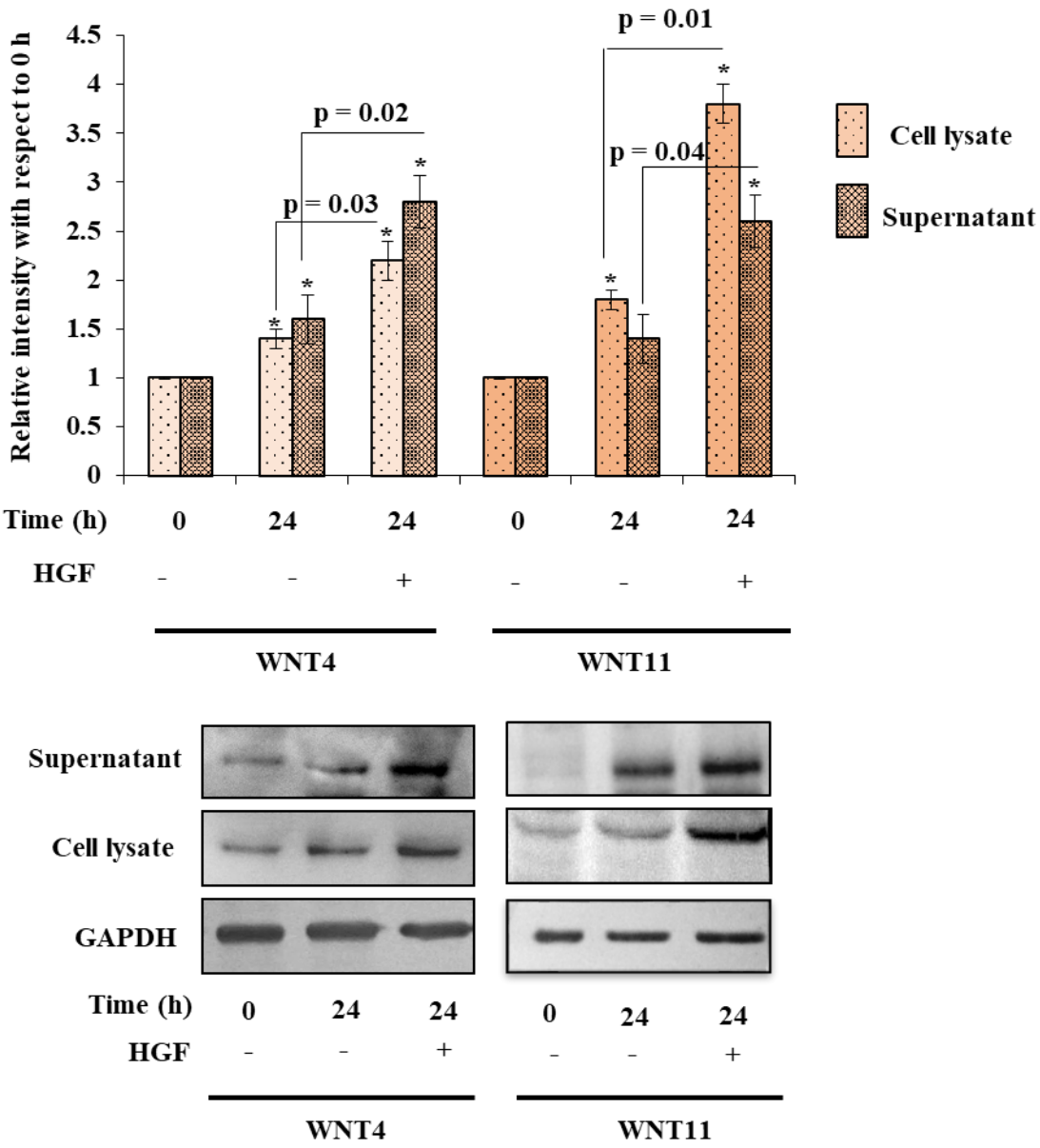

Fig. 4.3 Expression profile of WNT4 and WNT11 in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. After treatment with HGF, culture supernatant was collected and concentrated, while cells were used to prepare cell lysate. Subsequently, proteins in culture supernatant and cell lysates were separated on SDS–PAGE to determine WNT4 and WNT11 expression by Western blotting as described in *Materials and Methods*. The bar graph shows the densitometric analysis of WNT4 and WNT11 protein at 0 h and 24 h with and without HGF treatment. 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 appended below. GAPDH was used as a loading control. * denotes $p \leq 0.05$ as compared to cells at 0 h which was considered statistically significant.

0.03, WNT11; p = 0.01) as well as in cell culture supernatant (WNT4; p = 0.02, WNT11; p = 0.04) was observed in HGF treated as compared to 24 h untreated cells (Fig. 4.3).

Treatment of HTR-8/SVneo cells with HGF resulted in increased expression of ITGA2 and ITGAV

Integrins are family of diverse heterodimeric transmembrane glycoproteins, which act as receptors for ECM proteins. They comprise of non-covalently associated α and β subunits. Integrin switching occurs during EVTs differentiation, migration and invasion in response to alteration of ECM components (Burrows *et al*., 1996). To determine the role of integrins in HGF-mediated migration of HTR-8/SVneo cells, expression level of the transcripts for *ITGA1*, *ITGA2*, *ITGA5*, *ITGA6* and *ITGAV* was studied in the cells incubated for 24 h in presence and absence of HGF (50 ng/mL) as described in *Materials and Methods*. 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 control (Fig. 4.4A). On the other hand, significant increase in expression of *ITGA6* by ~4.7 and ~7 folds were observed in untreated and HGF treated cells, respectively, as compared to cells at 0 h. However, maximum increase in expression was observed in *ITGA2* (~28 fold) and ITGAV (~20 fold) in HTR-8/SVneo cells treated with HGF for 24 h. Similarly, increase in expression of *ITGA2* (~4.5 fold) and *ITGAV* (~6 fold) were also observed in cells incubated for 24 h without HGF treatment, as compared to 0 h baseline (Fig. 4.4A). Nevertheless, expression of *ITGA2 and ITGAV* in untreated cells was significantly lower than the HGF-treated counterpart.

In addition to α chains, the expression of various β chains like *ITGB1, ITGB3, ITGB4* and *ITGB5* was also checked at transcript levels in untreated as well HGF treated cells after 24 h. No significant changes were observed in any of the β chain integrin neither in the untreated nor in the HGF treated cells (Fig. 4.4B). These observations suggest that β-chains of integrins may behave differently or have some different functions as compared to α chains, in response to HGF treatment in HTR-8/SVneo cells.

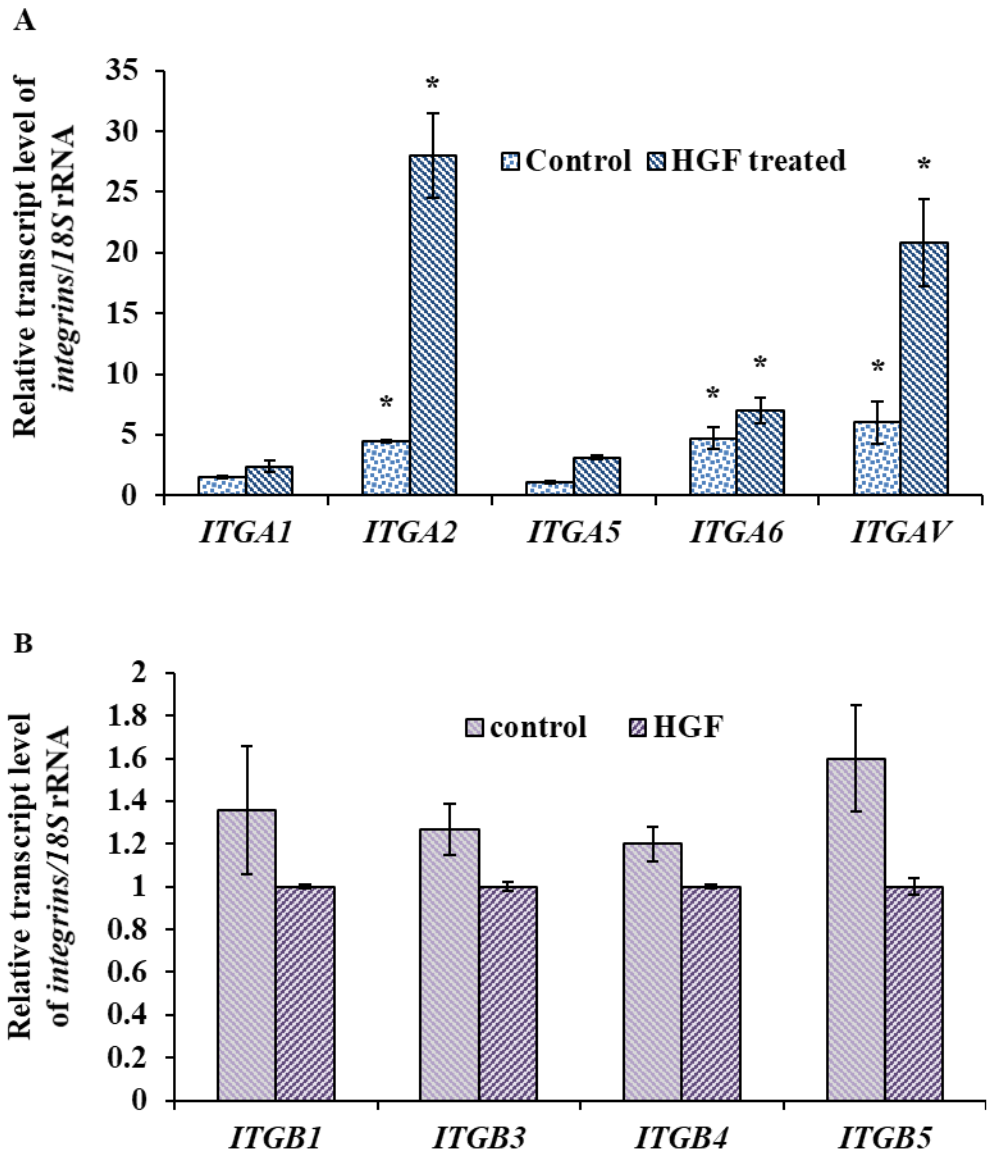

Fig. 4.4 Transcript levels of different integrins in HGF treated HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate and grown under humidified conditions with 5% CO_2 at 37ºC. Cells were allowed to form monolayer and processed for scratch wound assay for 24 h in presence and absence of HGF (50 ng/mL) for 24 h. Further, total RNA was isolated and processed for cDNA preparation followed by RT-qPCR analysis as described in *Materials and Methods*. The bar graphs show the relative expression of *ITGA1*, *ITGA2*, *ITGA5*, *ITGA6* and *ITGAV* in **Panel A** and *ITGB1*, *ITGB3*, *ITGB4* and *ITGB5* in **Panel B** at 24 h in HGF treated and untreated HTR-8/SVneo cells as compared to untreated control at 0 h. Each bar represent relative expression after normalization with *18S* rRNA used as an internal control. The values are shown as mean ± *s.e.m* of three independent experiments. * denotes $p \leq 0.05$ as compared with untreated control cells at 0 h, which was considered statistically significant.

Apart from transcript level, the expression of ITGA2 and ITGAV was also checked at protein level by Western blotting. Treatment of HTR-8/SVneo cells with HGF (50 ng/mL) for 24 h led to significant increase in up regulation of ITGA2 (~1.7 fold) and ITGAV (2.5 fold), as compared to 0 h control (Fig. 4.5). However, increase in the expression of untreated cells after 24 h was observed only in case of ITGAV, as compared to 0 h baseline. Moreover, the expression level of both the integrins were significantly higher in HGF-treated (ITGA2: $p = 0.02$; ITGAV: $p = 0.03$) HTR-8/SVneo cells as compare to untreated counterpart (Fig 4.5).

Integrins α and β chains combine with each other to form ligand binding domain, which communicate with ECM proteins to sense the external environment and further transduce the signal inside the cell. The α2 chain combines to only β1 chain, while αV can combine with three different β chain partners (β1, β3 & β5). To study which combination is activated or up regulated in HGF mediated migration of HTR-8/SVneo cells, indirect immunofluorescence was done on the cells treated in the presence and absence of HGF as described in *Materials and Methods*. Integrin α2β1 appeared green as it was stained with Alexa-488 conjugated secondary antibody and was restricted to the cell membrane (Fig. 4.6A). In addition, higher intensity of α2β1 was observed in HGF treated HTR-8/SVneo cells as compared to untreated control at 24 h. Similarly, cells treated with HGF for 24 h also showed increase in the expression of αVβ5 (green), as compared to cells without HGF treatment (Fig. 4.6B). However, no significant changes in the expression of αVβ1 and αVβ3 subsequent to treatment with HGF were observed (Fig. 4.7).

siRNA mediated *WNT4* silencing led to decrease in HGF-mediated migration of HTR-8/SVneo cells

To establishe the importance of WNT4 during HGF-mediated trophoblastic cells migration, WNT4 was knockdown by using siRNA mediated gene silencing approach to further study its effect on migration process. The silencing was confirmed by RT-qPCR and Western blotting and silenced cells were further used in scratch wound migration

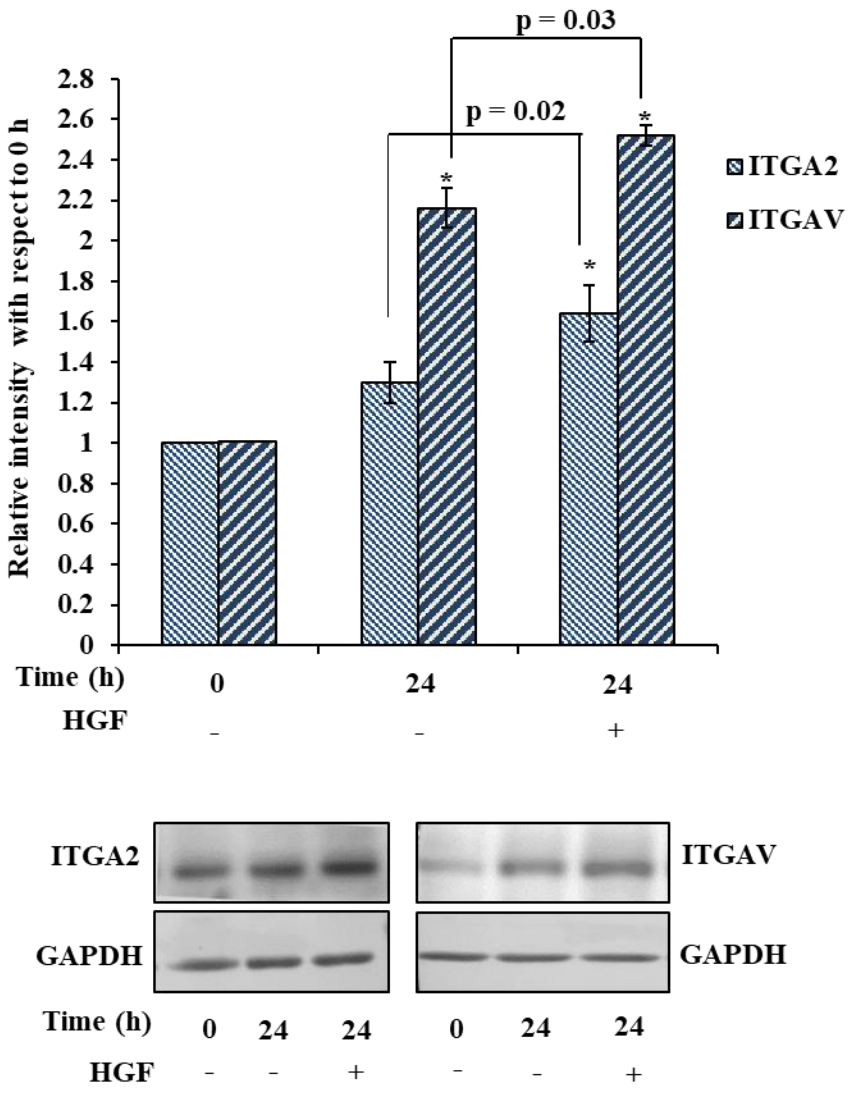

Fig. 4.5 Expression profile of ITGA2 and ITGAV in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. Subsequently, cell lysates were prepared to determine ITGA2 and ITGAV expression levels by Western blotting as described in *Materials and Methods*. The bar graph shows the densitometric profile of ITGA2 and ITGAV protein after treatment with and without HGF (50 ng/mL). Values are expressed as mean ± *s.e.m* of band intensity for three independent experiments as compared to cells at 0 h. GAPDH was used as a loading control. Representative blots at 0 h and after 24 h in presence and absence of HGF are appended below the graph. * denotes $p \leq 0.05$ as compared with 0 h, which was considered statistically significant.

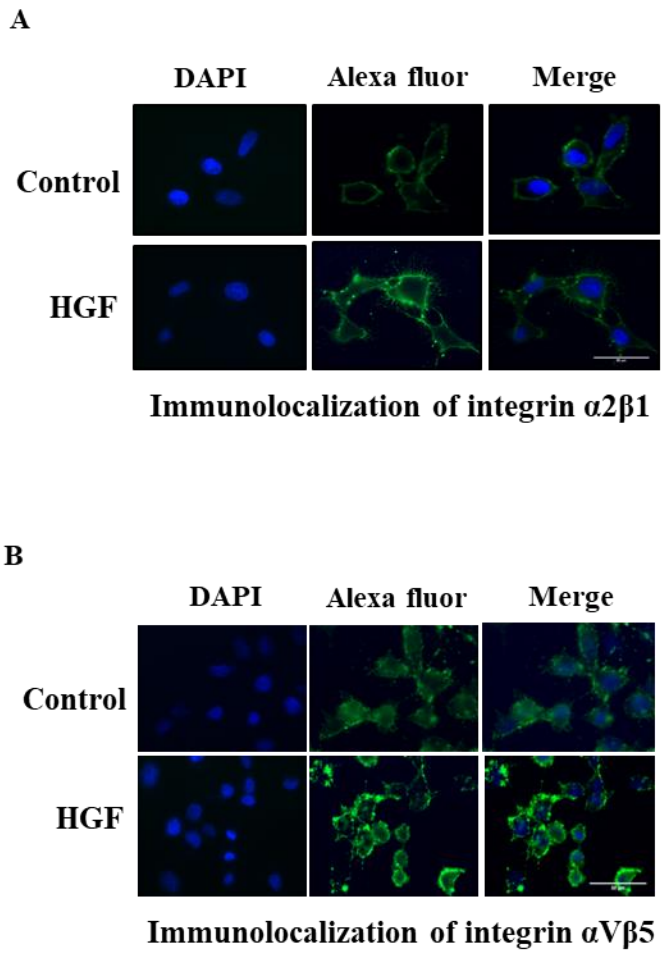

Fig. 4.6 Immunofluorescence profile of integrin α2β1 and αVβ5 in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells (0.3 x 10^5/well) were cultured in 24-well plates in humidified conditions with 5% CO_2 at 37°C. Next day, cells were serum starved and then treated with HGF (50 ng/mL) for 24 h. Coverslips with cells were incubated with primary antibodies against α2β1 and αVβ5 and subsequently processed for indirect immunoflorsecence as described in *Materials and Methods*. **Panel A** represents the immunolocalization studies of α2β1 (green) in control (untreated) as well as HGF-treated cells. **Panel B** shows the staining profile of αVβ5 (green) in control and HGF-treated cells at 24 h. The nuclei were stained using DAPI (blue). Cells were fixed in formaldehyde and examined under fluorescent microscope. Images were compiled by proplus software. Scale bar represents 50 μm.

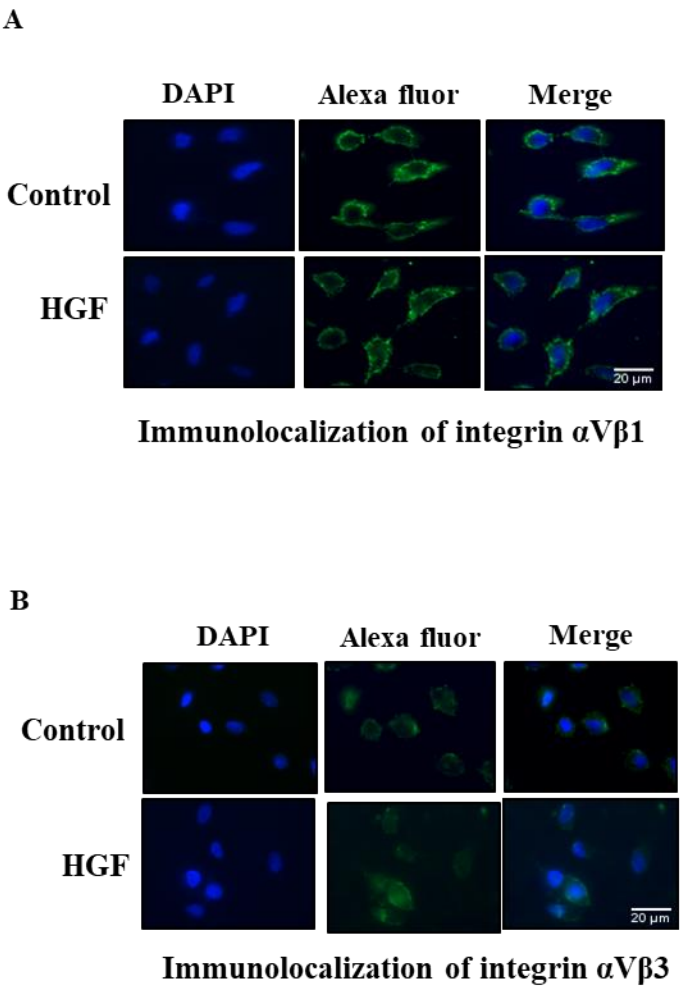

Fig. 4.7 Immunofluorescence profile of integrin αVβ1 and αVβ3 in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells (0.3 x 10^5/well) were cultured in 24-well plates in humidified conditions with 5% CO_2 at 37°C. Next day, cells were serum starved and then treated with HGF (50 ng/mL) for 24 h. Coverslips with cells were incubated with primary antibodies against αVβ1 and αVβ3 and subsequently processed for indirect immunoflorsecence as described in *Materials and Methods*. **Panel A** represents the immunolocalization studies of αVβ1 (green) in control (untreated) as well as HGF-treated cells. **Panel B** shows the staining profile of αVβ3 (green) in control and HGF-treated cells at 24 h. The nuclei were stained using DAPI (blue). Cells were fixed in formaldehyde and examined under fluorescent microscope. Images were compiled by proplus software. Scale bar represents 20 µm.

assay to assess cell migration as described in *Materials and Methods*. As expected, a significant ($p = 0.002$) increase in the WNT4 transcript levels were observed in control siRNA transfected cells treated with HGF (50 ng/mL) as compared to untreated cells. Significant decrease (~67%; $p = 0.001$) in expression of *WNT4* transcript levels were observed in HGF treated *WNT4* siRNA transfected cells as compared to HGF treated control siRNA-transfected cells. In addition, significant decrease (~65%; $p = 0.006$) at basal level of *WNT4* transcript was also observed in *WNT4* siRNA transfected cells as compared to control siRNA transfected cells without HGF treatment (Fig. 4.8A). Similarly, reduced expression of WNT4 at protein level was also observed in HGF-treated *WNT4* silenced cells ($p = 0.03$) as compared to control siRNA transfected cells treated with HGF. Significant ($p = 0.04$) reduction in WNT4 levels were also observed in HTR-8/SVneo cells transfected with WNT4 siRNA without any treatment with HGF as compared to HGF untreated control siRNA transfected cells (Fig. 4.8B). After confirmation of silencing, *WNT4* silenced cells and control siRNA transfected cells were used in scratch wound migration assay. Interestingly, significant decrease (~44%; $p = 0.003$) in migration was observed in *WNT4* silenced cells as compared to control siRNA transfected cells after treatment with the HGF (50 ng/mL) for 24 h. Moreover, the decrease in basal migration (without HGF treatment) was also observed in HTR-8/SVneo cells transfected with *WNT4* siRNA, which was statistically significant ($p = 0.02$) as compared to control counterpart (Fig. 4.9). These results suggest that WNT4 has a crucial role in HGF mediated trophoblastic cells migration.

WNT11 knockdown abrogates the effect of HGF on the migration of HTR-8/SVneo cells

Besides WNT4, the expression of WNT11 was highest among the WNT ligands studied during HGF-mediated migration of HTR-8/SVneo cells. To associate the relevance of WNT11 during trophoblastic cells migration, HTR-8/SVneo cells were silenced for *WNT11* using siRNA. The silencing of WNT11 at the transcript and protein levels was confirmed by RT-qPCR and Western blotting respectively. Silenced cells were further

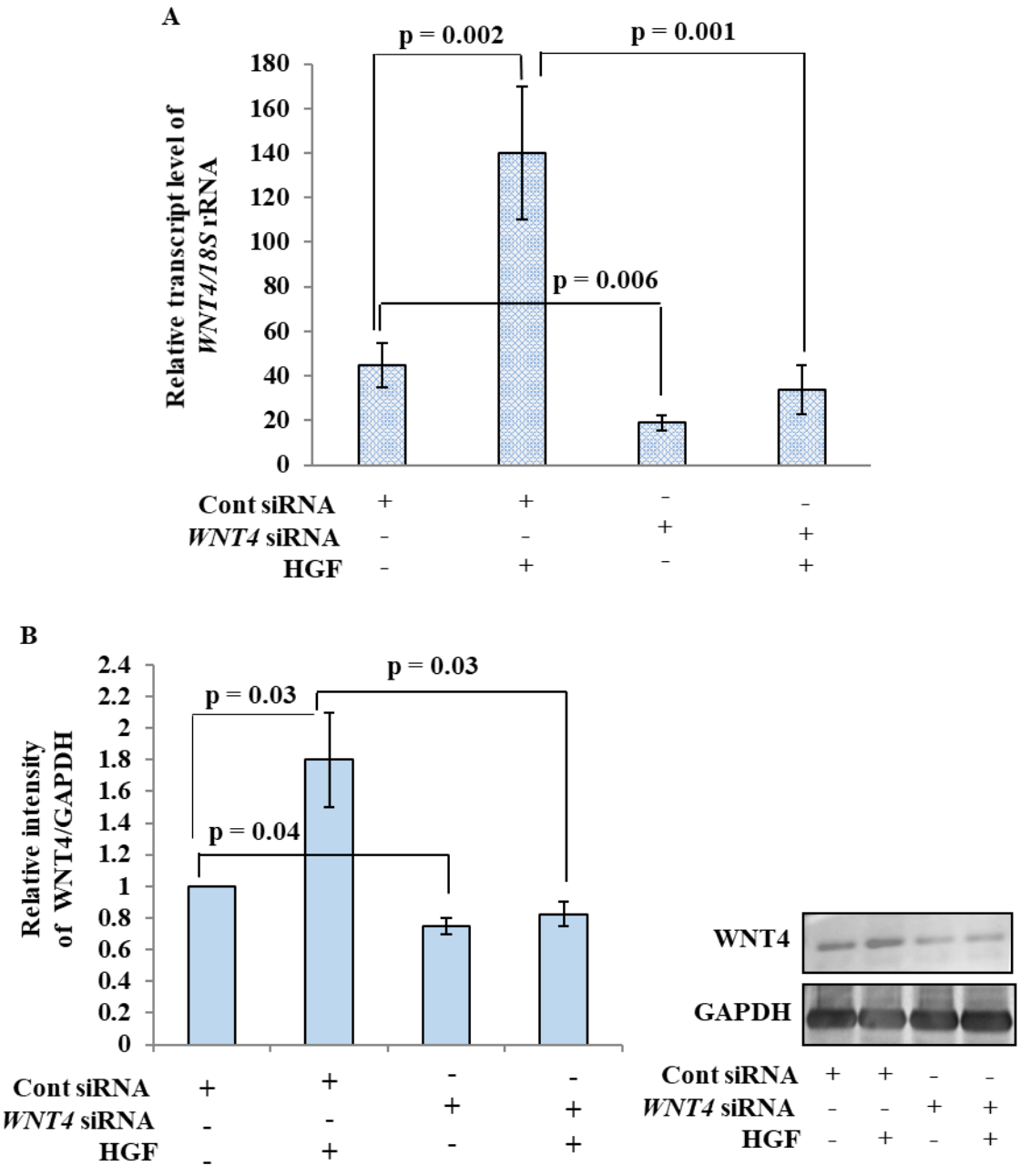

Fig. 4.8 Confirmation of WNT4 silencing by RT-qPCR and Western blotting in HTR-8/SVneo cells after HGF treatment: HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in 6-well culture plate and grown under humidified atmosphere with 5% CO_2 at 37ºC. Next day, cells were transfected with control and *WNT4* siRNA respectively. After 48 h of transfection, cells were treated with and without HGF for 24 h as described in *Materials and Methods.* Silencing of WNT4 was confirmed by RT-qPCR and Western blotting. **Panel A** represents the expression profile of *WNT4* at the transcript levels in control and *WNT4* silenced cells after treatment with and without HGF (50 ng/mL) for 24 h. Each bar represents relative expression with respect to untreated control at 0 h after normalization with *18S* rRNA and values are expressed as mean ± *s.e.m* of three independent experiments. **Panel B** shows the densitometric profile of WNT4 at protein level by Western blotting in control and *WNT4* silenced cells respectively after treatment with and without HGF for 24 h. Each bar represents relative intensity of WNT4 with respect to untreated control siRNA silenced cells after normalised with GAPDH. Representative Western blot profiles of WNT4 and GAPDH are appended on the right side of **Panel B**. Values shown are mean ± *s.e.m* of three independent experiments.

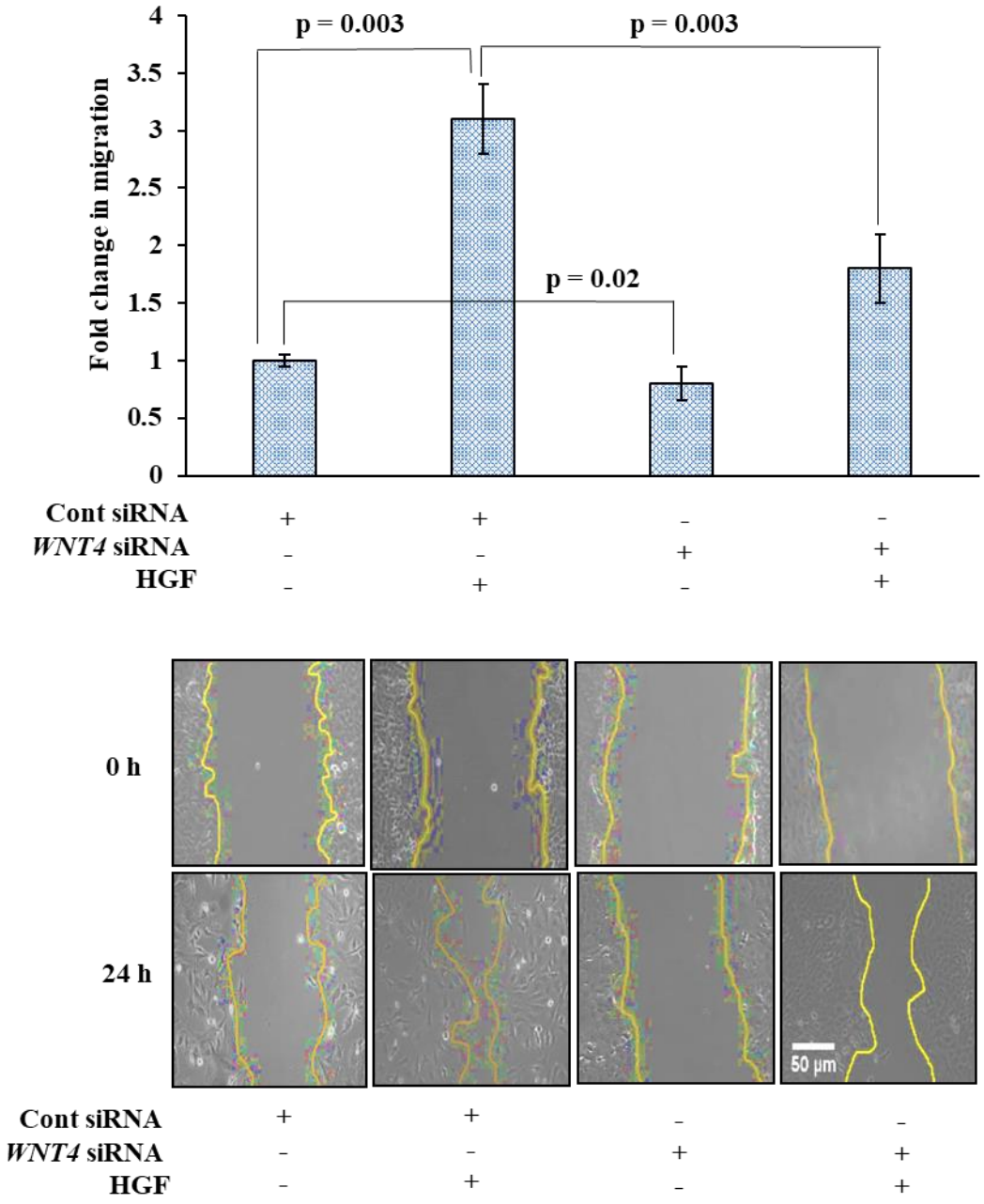

Fig. 4.9 Effect of WNT4 silencing on the HGF-mediated migration of HTR-8/SVneo cells: HTR-8/SVneo cells were transfected with *WNT4* and control siRNA and subsequently used to study their migration by scratch wound healing migration assay as described in *Materials and Methods*. Each bar represents the fold change in migration of cells transfected with control and *WNT4* siRNA respectively subsequent to treatment with and without HGF (50 ng/mL) for 24 h, as compared to untreated control siRNA silenced cells. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative images are appended below. Pictures were taken at 0 and 24 h. Scale bar represents 50 µm.

used in scratch wound migration assay to assess the effect of WNT11 silencing on the cell migration as described in *Materials and Methods*. RT-qPCR analysis of HTR-8/SVneo cells transfected with *WNT11* siRNA and subsequently treated with HGF (50 ng/mL) for 24 h showed a significant decrease (~66%; p = 0.001) in the levels of WNT11 transcripts as compared to control siRNA transfected cells treated with HGF. Similarly, substantial decrease (p = 0.01) in *WNT11* transcripts has also been observed at basal level in *WNT11* siRNA transfected cells as compared to control siRNA transfected cells in absence of treatment with HGF (Fig. 4.10A). At protein level, a significant (p = 0.02) decrease in WNT11 expression was also observed in HGF treated *WNT11* siRNA transfected cells as compared to HGF treated control siRNA transfected cells (Fig. 4.10B). Further, a significant (p = 0.04) decrease in the levels of WNT11 were also observed in *WNT11* siRNA transfected cells in absence of treatment with HGF as compared to untreated control siRNA transfected cells. After confirmation of silencing, *WNT11* silenced cells and control siRNA transfected cells were used in scratch wound migration assay. Significant (p = 0.001) decrease in migration by ~44% was observed in *WNT11* silenced HTR-8/SVneo cells, as compared to control siRNA transfected cells after treatment with the HGF (50 ng/mL) for 24 h. Moreover, the decrease in basal migration (without HGF treatment) was also observed in HTR-8/SVneo cells transfected with *WNT11* siRNA, which was statistically significant (p = 0.02) as compared to control counterpart (Fig. 4.11).

ITGA2 silenced HTR-8/SVneo cells showed reduced HGF-mediated trophoblastic cells migration

During the cell migration, integrins are best known as the mediators between the ECM and the actin cytoskeleton of the cell. The integrin-mediated cell migration is dependent on the various factors like density of ligand, ligand-binding affinity and concentration of receptor-ligand along with associated downstream signaling pathways**.** Although, the cells present at the base of cell column of first trimester placenta are positive for ITGA2 (Albelda, 1993), but its role in trophoblast migration is still unknown. Since the

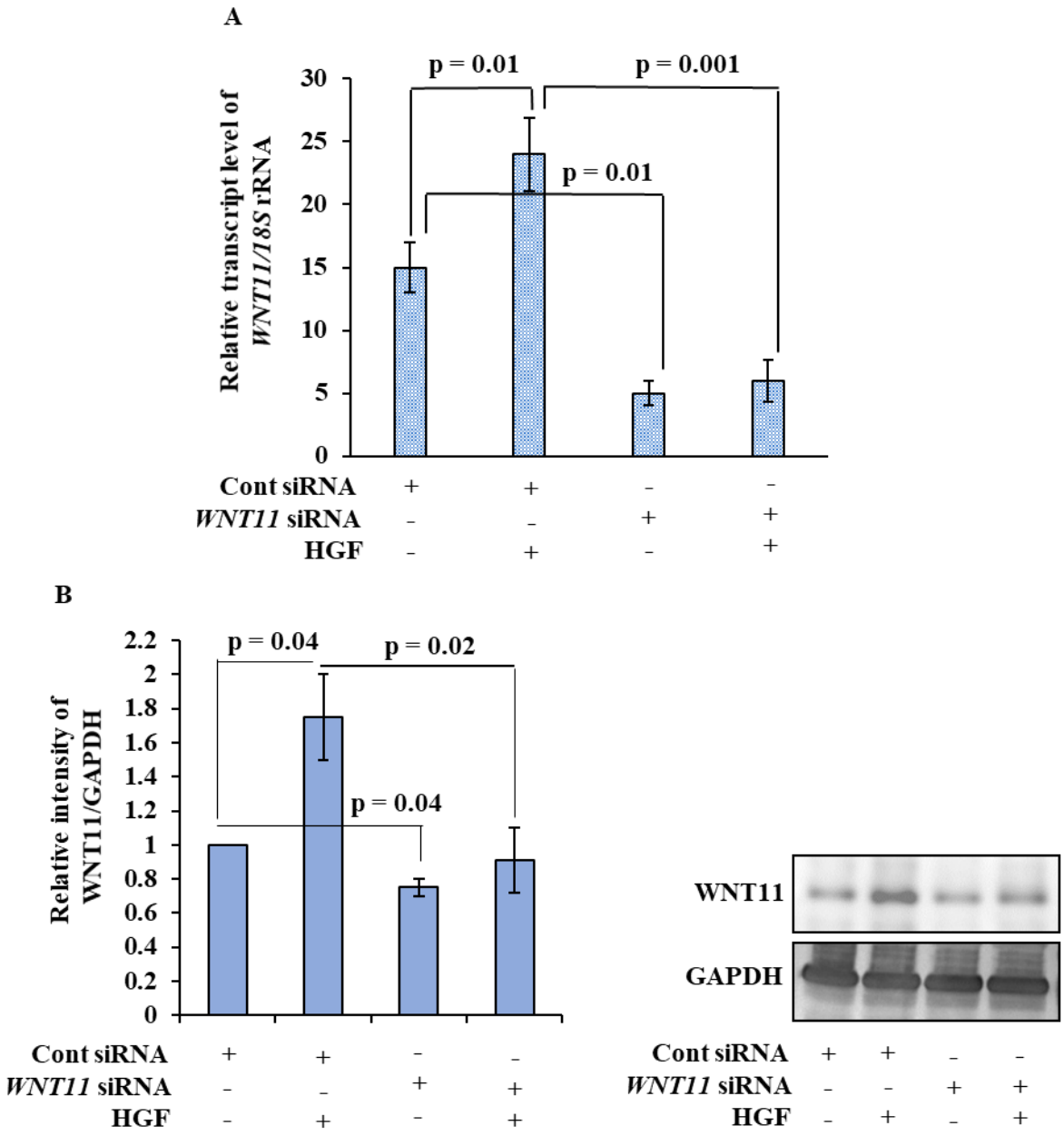

Fig. 4.10 Confirmation of *WNT11* silencing by RT-qPCR and Western blotting in HTR-8/SVneo cells after HGF treatment: HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in 6-well culture plate and grown under humidified atmosphere with 5% CO_2 at 37ºC. Next day, cells were transfected with control and *WNT11* siRNA respectively. After 48 h of transfection, cells were treated with and without HGF for 24 h as described in *Materials and Methods*. Silencing of WNT11 was confirmed by RT-qPCR and Western blotting. **Panel A** represents the expression profile of *WNT11* at the transcript levels in control and *WNT11* silenced cells after treatment with and without HGF (50 ng/mL) for 24 h. Each bar represents relative expression after normalization with *18S* rRNA and values are expressed as mean ± *s.e.m* of three independent experiments. **Panel B** shows the densitometric profile of WNT11 at protein level by Western blotting in control and *WNT11* silenced cells respectively, after treatment with and without HGF for 24 h. Ear bar represents relative intensity of WNT11 with respect to untreated control siRNA silenced cells after normalization with GAPDH. Values shown are mean ± *s.e.m* of three independent experiments. Representative Western blot profiles of WNT11 and GAPDH are appended on the right hand side of **Panel B**.

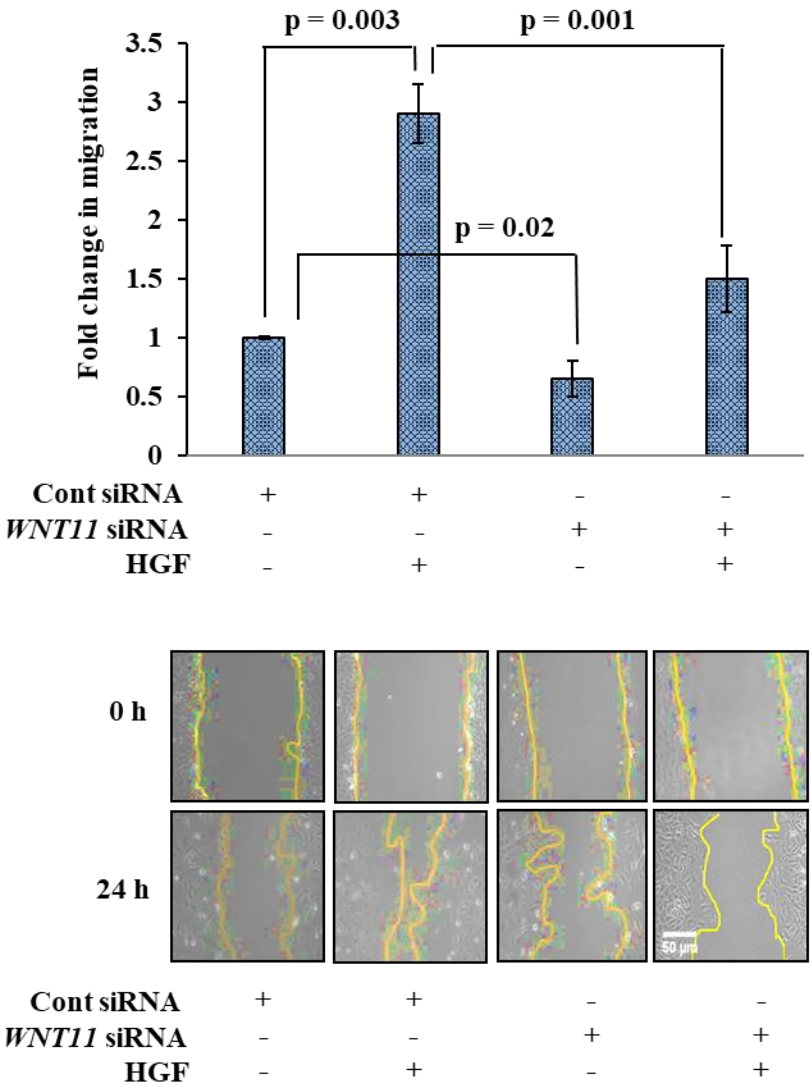

Fig. 4.11 Effect of WNT11 silencing on the HGF-mediated migration of HTR-8/SVneo cells: HTR-8/SVneo cells were transfected with *WNT11* and control siRNA and subsequently used to study their migration by scratch wound healing migration assay as described in *Materials and Methods*. Each bar graph represents the fold change in migration of cells transfected with control and *WNT11* siRNA respectively subsequent to treatment with and without HGF (50 ng/mL) for 24 h, as compared to untreated control siRNA transfected cells. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative images are appended below. Pictures were taken at 0 and 24 h. Scale bar represents 50 µm.

expression of ITGA2 was higher amongst the integrin studied, it would be interesting to further study its effect on HTR-8/SVneo cells migration. In this direction**,** HTR-8/SVneo cells were transfected with *ITGA2* and control siRNA and further treated in the presence and absence of HGF for 24 h. Silencing of ITGA2 was confirmed at transcript and protein level by RT-qPCR and Western blotting, respectively, as described in *Materials and Methods.* Significant decrease ($p = 0.02$) in the expression of *ITGA2* at transcript level was observed in HGF treated *ITGA2* siRNA transfected cells as compared to HGF treated control siRNA-transfected cells. In addition, significant decrease ($p = 0.03$) in the basal level of *ITGA2* transcript was also observed in ITGA2 siRNA transfected cells as compared to control siRNA transfected cells without HGF treatment (Fig. 4.12A). Similarly, reduced expression of ITGA2 at protein level was also observed in HGF-treated *ITGA2* silenced cells ($p = 0.02$) as compared to control siRNA transfected cells treated with HGF (Fig. 4.12B). Further, significant decrease in the basal levels of ITGA2 was also observed in *ITGA2* siRNA transfected cells as compared to control siRNA transfected cells in the absence of treatment with HGF. After confirmation of silencing, *ITGA2* silenced cells and control siRNA transfected cells were used in scratch wound migration assay. Interestingly, significant decrease (~40%; $p = 0.002$) in migration was observed in *ITGA2* silenced cells as compared to control siRNA transfected cells after treatment with the HGF (50 ng/mL) for 24 h. Moreover, the decrease in basal migration (without HGF treatment) was also observed in HTR-8/SVneo cells transfected with *ITGA2* siRNA, which was statistically significant ($p = 0.01$) as compared to control counterpart (Fig. 4.13). These results suggest that ITGA2 may be involved in HGF-mediated trophoblastic cells migration.

ITGAV silencing impaired the HGF-mediated increase in HTR-8/SVneo cells migration

Apart from ITGA2, significant increase in expression of ITGAV was also observed in HGF treated and untreated cells, as mentioned previously. Previous studies have shown a decrease in ITGAV expression in cytotrophoblast cells of preeclamptic placenta

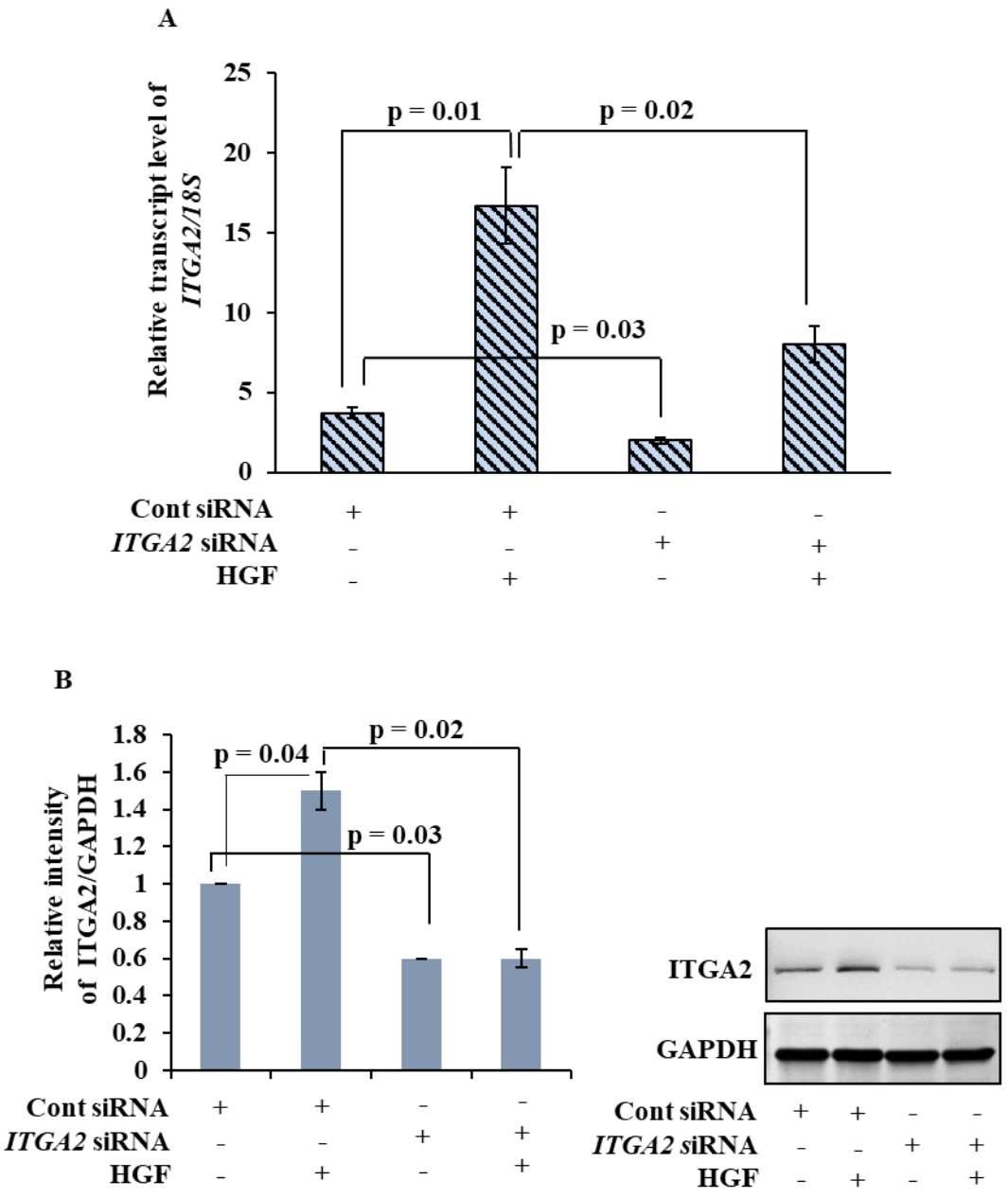

Fig. 4.12 Confirmation of *ITGA2* silencing by RT-qPCR and Western blotting in HTR-8/SVneo cells after HGF treatment: HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in 6-well culture plate and grown under humidified atmosphere with 5% CO_2 at 37°C. Next day, cells were transfected with control and *ITGA2* siRNA respectively. After 48 h of transfection, cells were treated with and without HGF for 24 h as described in *Materials and Methods*. Silencing of ITGA2 was confirmed by RT-qPCR and Western blotting. **Panel A** represents the expression profile of ITGA2 at the transcript levels in control and *ITGA2* silenced cells after treatment with and without HGF (50 ng/mL) for 24 h. Each bar represents relative expression with respect to untreated control at 0 h after normalization with *18S* rRNA and values are expressed as mean ± *s.e.m* of three independent experiments. **Panel B** shows the densitometric profile of ITGA2 at protein level by Western blotting in control and *ITGA2* silenced cells respectively, after treatment with and without HGF for 24 h. Ear bar represent relative intensity of ITGA2 with respect to untreated control siRNA silenced cells after normalised with GAPDH. Values shown are mean ± *s.e.m* of three independent experiments. Representative Western blots profiles of ITGA2 and GAPDH are appended on the right hand side of the **Panel B**.

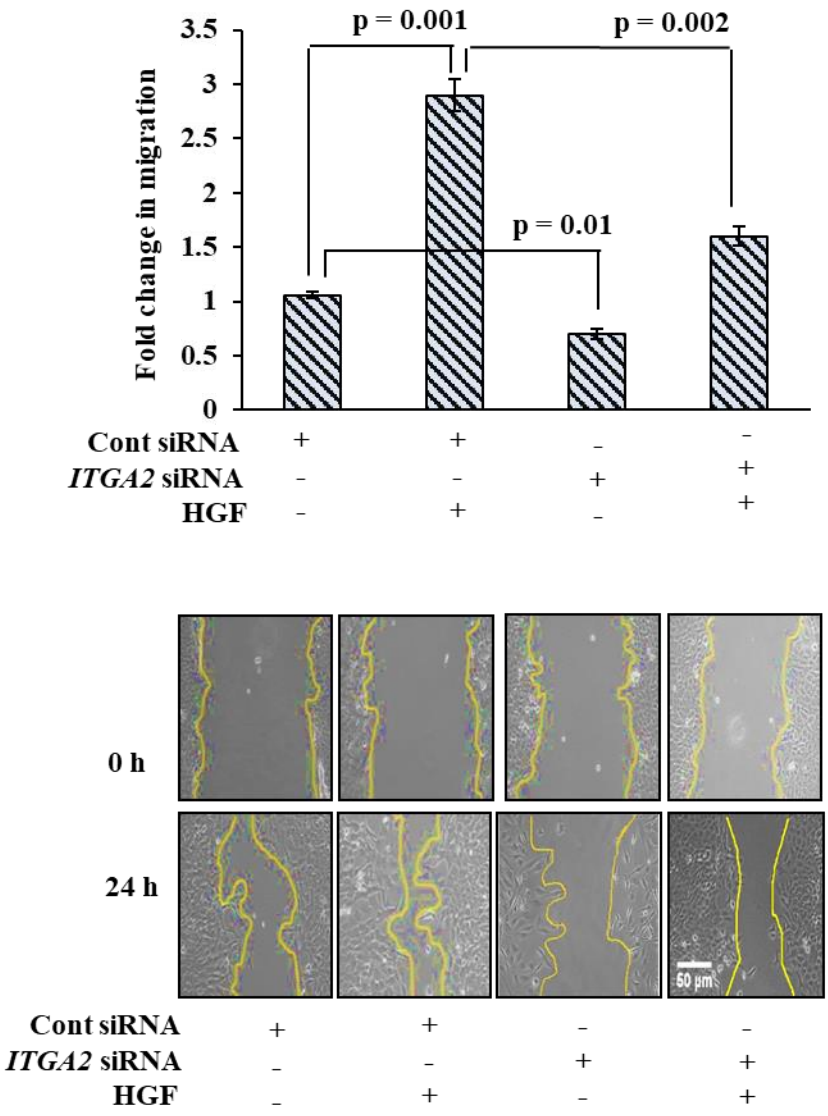

Fig. 4.13 Effect of *ITGA2* silencing on migration of the HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells were transfected with *ITGA2* and control siRNA and subsequently used to study their migration by scratch wound healing migration assay as described in *Materials and Methods*. The bar graph shows the fold change in migration of cells transfected with control and *ITGA2* siRNA respectively subsequent to treatment with and without HGF (50 ng/mL) for 24 h. The results are expressed as mean ± *s.e.m* of fold change in migration, as compared to control siRNA transfected cells without HGF treatment, observed in three independent experiments. Representative images are appended below. Pictures were taken at 0 and 24 h. Scale bar represents 50 µm.

(Vatansever *et al*., 2003). To link, its role in trophoblastic cells migration, ITGAV was knockdown in HTR-8/SVneo cells by using siRNA mediated gene silencing approach. The confirmation of ITGAV knock down in transfected cells was checked by RT-qPCR and Western blotting. Analysis of RT-qPCR results showed significant decrease (HGF treated, $p = 0.02$; untreated, $p = 0.04$) in *ITGAV* transcript level in *ITGAV* siRNA transfected cells as compared to control siRNA transfected cells treated in presence and absence of HGF after 24 h respectively (Fig. 4.14A). Similar results were also observed at protein level in *ITGAV* silenced cells (HGF treated, $p = 0.04$; untreated $p = 0.02$) as compared to control siRNA transfected cells with and without HGF treatment respectively (Fig. 4.14B). Surprisingly, silencing of ITGAV in HTR-8/SVneo cells led to loss in their adherence ability. Due to this, 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 as described in *Materials and Methods.* A significant decrease (~ 48%, $p = 0.003$) in the migration of *ITGAV* silenced HTR-8/SVneo cells treated with HGF for 24 h was observed, as compared to control siRNA transfected cells treated with HGF for the same time duration. In addition, basal migration (without HGF treatment) was also reduced by ~38% in *ITGAV* silenced cells as compared to control siRNA transfected cells (Fig. 4.14C). These results suggest that ITGAV integrin is important for adherence and migration of HTR-8/SVneo cells.

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

HGF is also known as pleotropic cytokine, it activates several signal transduction pathways on binding to C-met receptor. The C-met receptor undergo phosphorylation resulting in the recruitment of Src homology 2 domain, which further activates downstream signaling pathways like RAS, PI3-kinase, PKA and MAPK (Organ and Tsao, 2011). To find the downstream signaling pathways activated by HGF treatment in trophoblastic cells, HTR-8/SVneo cells were treated with optimized concentration of HGF (50 ng/mL) for 10, 30 and 60 min and subsequently cell lysates were prepared to

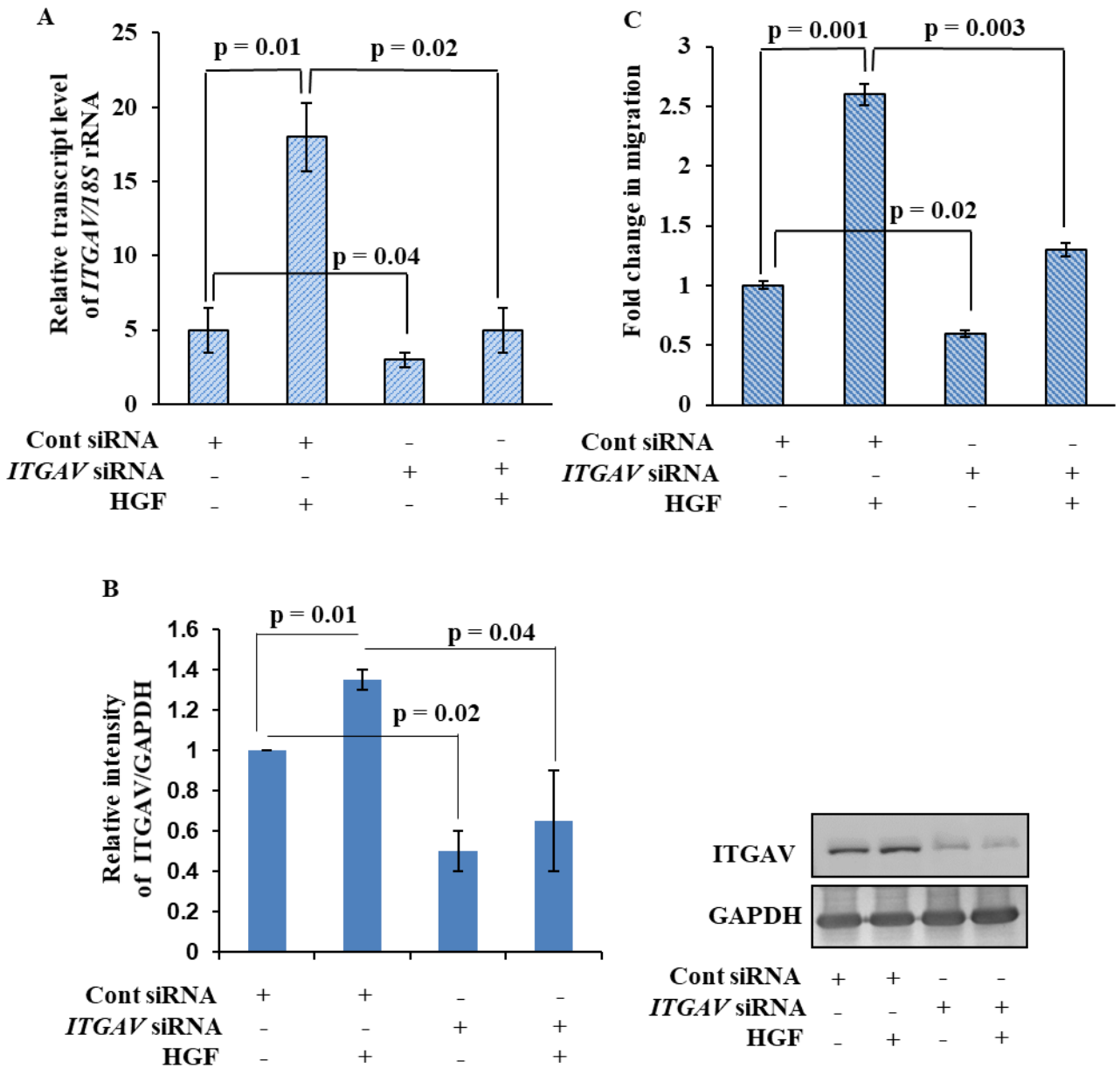

Fig. 4.14 Effect of *ITGAV* silencing by siRNA on the migration of HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in 6-well culture plate under humidified atmosphere with 5% CO_2 at 37ºC. Next day, cells were transfected with control and *ITGAV* siRNA respectively. After 48 h of transfection, cells were used to study migration in transwell assay in presence or absence of HGF (50 ng/mL) as described in *Materials and Methods*. Silencing of *ITGAV* was confirmed at transcript as well as at protein level by RT-qPCR and Western blotting. **Panel A** represents the expression profile *ITGAV* at the transcript levels in control and *ITGAV* silenced cells after treatment with and without HGF (50 ng/mL) for 24 h. Each bar represents relative expression after normalization with *18S* rRNA and values are expressed as mean ± *s.e.m* of three independent experiments. **Panel B** shows the densitometric profile of ITGAV at protein level by Western blotting in control and *ITGAV* silenced cells respectively after treatment with and without HGF for 24 h. Each bar represent relative intensity of ITGAV with respect to untreated control siRNA silenced cells after normalised with GAPDH. Values shown are mean ± *s.e.m* of three independent experiments. Representative Western blot profiles of ITGAV and GAPDH are appended on the right hand side of **Panel B**. **Panel C** represents the fold change in migration of HTR-8/Svneo cells transfected with control or *ITGAV* siRNA respectively, subsequent to treatment with and without HGF (50 ng/mL) for 24 h. The results are expressed as mean ± *s.e.m* of fold change in migration, as compared to control siRNA transfected cells without HGF treatment, observed in three independent experiments.

assessed the activation of MAPK and PKA signaling by Western blots as described in *Materials and Methods*. Activation status of signaling pathways was assess from the phosphorylation of protein involved or their quantitative changes. Treatment of HTR-8/SVneo cells with HGF led to an increase in the pERK1/2 and pPKA levels (Fig. 4.15). Time kinetics analyze revealed significant increase in phosphorylation of ERK1 (~2.8 fold) and ERK2 (~2.2 fold) at 10 min followed by gradual decrease till 60 min after treatment with HGF, as compared to 0 min control after normalized with total ERK at their respective time points (Fig. 4.15A). Similarly, in case of PKA signaling, phosphorylation of PKA was significantly increased by ~1.6 fold at 10 and 60 min after HGF treatment as compared to 0 min (Fig. 4.15B). These results suggest that treatment of HTR-8/SVneo cells with HGF led to activation of both MAPK and PKA signaling pathways.

MAPK/PKA signaling pathways are involved in HGF induced expression of WNT ligands and integrins

To investigate, whether the activation of MAPK and PKA signaling pathways by HGF has any role in increased expression of WNT ligands and integrins, HTR-8/SVneo cells were pre-treated with pharmacological inhibitor, U0126 to inhibit MAPK signaling and H89 to inhibit PKA signaling pathways as per manufacturer's instructions. Subsequently, cells were treated in the presence and absence of HGF for 24 h. After 24 h, cells were harvested in cell lysis buffer and lysates were further processed for Western blotting to assess the levels of WNT4, WNT11, ITGA2 & ITGAV as described in *Materials and Methods*. Inhibition of MAPK signaling by pre-treatment with U0126 led to significant decrease in the expression of WNT11 ($p = 0.003$) in HTR-8/SVneo cells subsequent to treatment with HGF as compared to cells that were not pre-treated with U0126 but treated with HGF (Fig. 4.16B). However, inhibition of MAPK signaling did not result in any significant change in the expression of WNT4 under similar experimental conditions (Fig. 4.16A). On the other hand, in case of integrins, significant decrease in the expression of ITGA2 ($p = 0.04$) was observed in HTR-8/SVneo cells pre-treated with

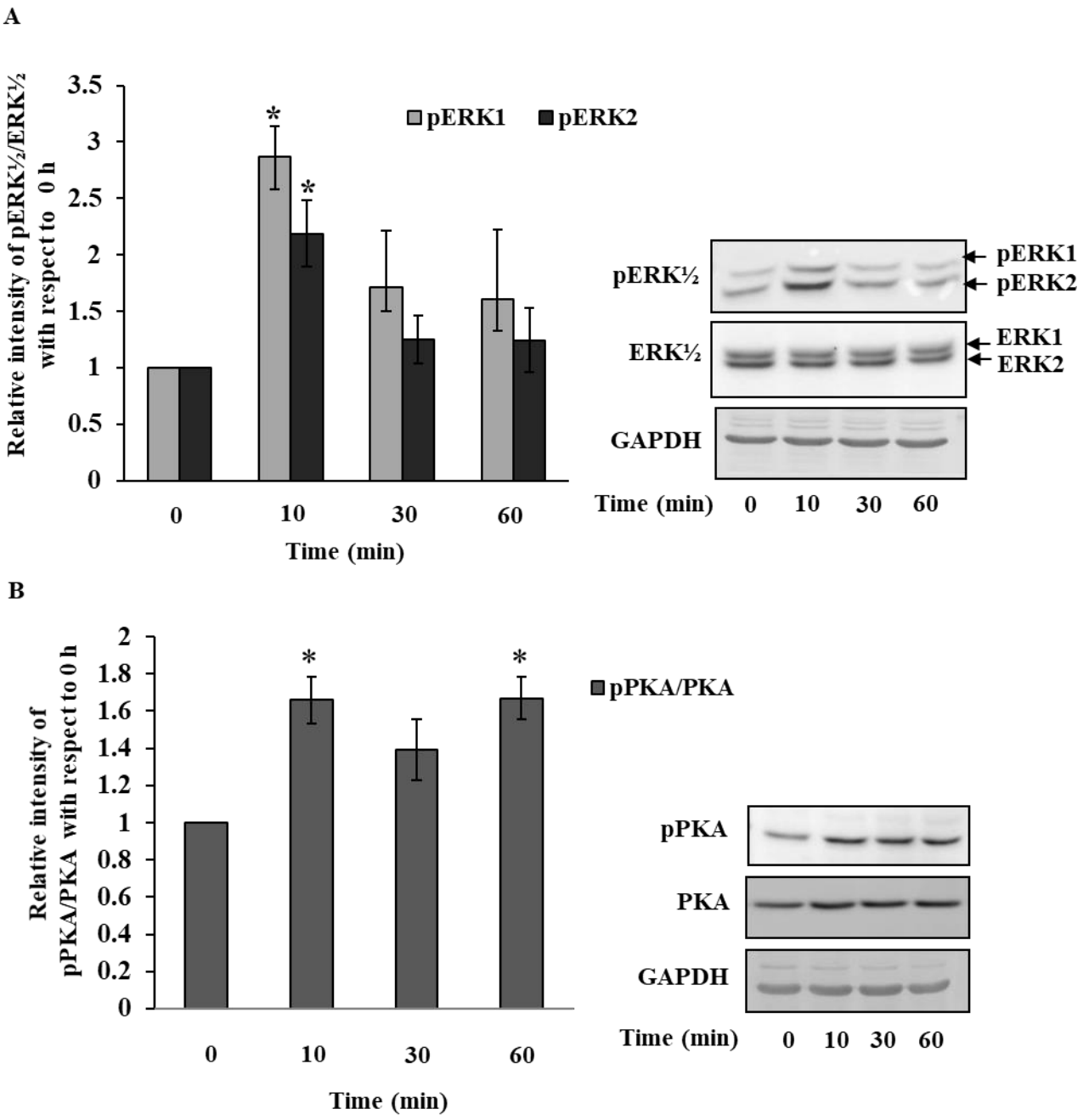

Fig. 4.15 Activation of MAPK & PKA signaling pathways by HGF in HTR-8/SVneo cells. HTR-8/SVneo cells were treated with HGF (50 ng/mL) for varying time periods (10, 30 and 60 min) followed by Western blot analysis to determine the activation of ERK½ and PKA as described in *Materials and Methods*. **Panel A** represents the densitometric plot showing the relative increase in phosphorylated ERK½ (pERK½) in HGF treated cells with respect to untreated control as compared to total ERK½. **Panel 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. 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 of the respective panels. * denotes $p \leq 0.05$ with respect to untreated control.

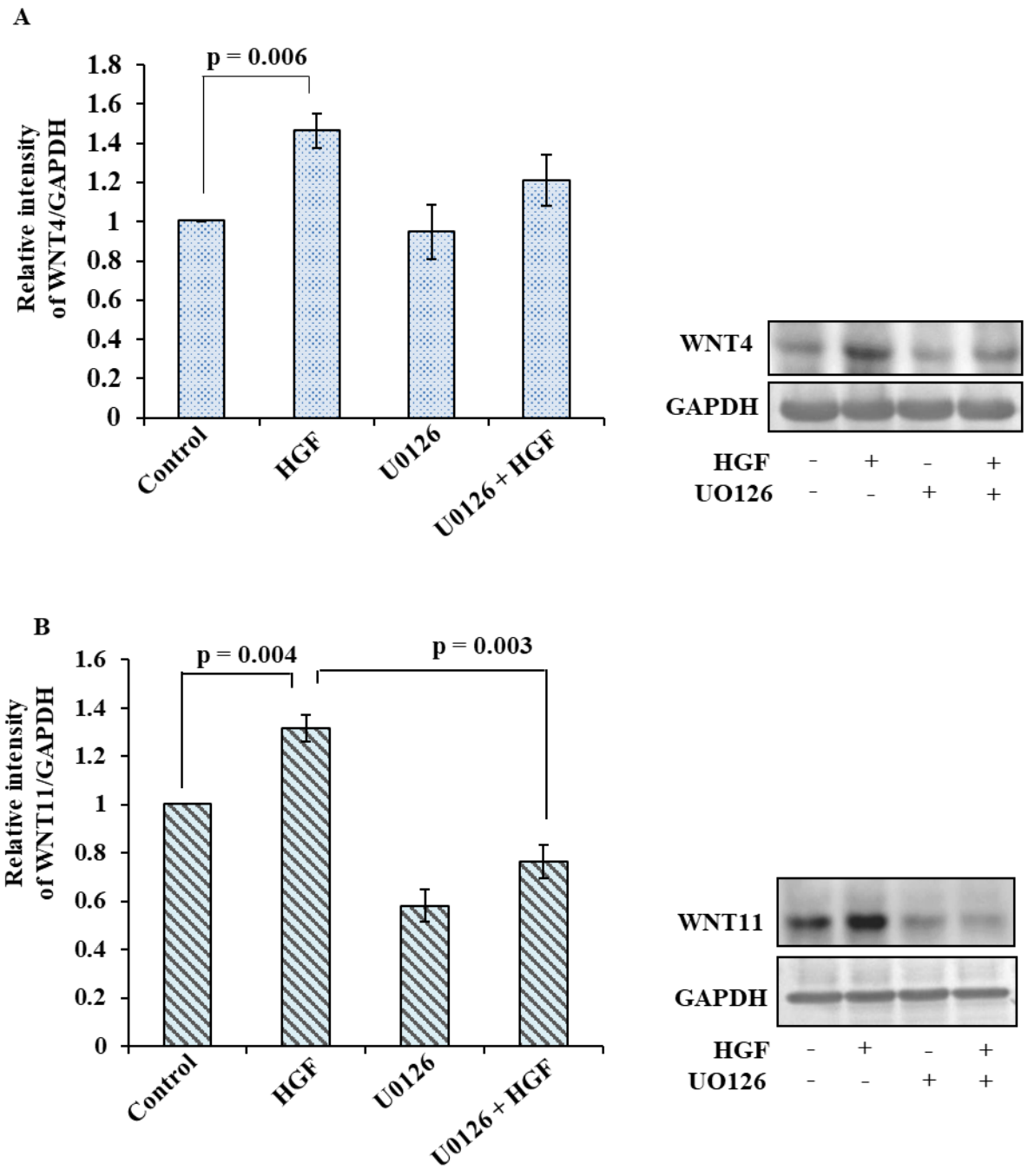

Fig. 4.16 Effect of inhibition of MAPK signaling on HGF induced expression of WNT4 & WNT11 in HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate and grown under humidified atmosphere of 5% CO_2 at 37ºC and allowed to grow as monolayer. The monolayer of cells were pre-treated with and without MAPK inhibitor U0126 (10 µM) for 2 h and were subsequently treated with and without HGF (50 ng/mL) for 24 h. After incubation for 24 h, cell lysates were prepared to asses the expression of WNT4 and WNT11 by Western blots as described in *Materials & Methods*. **Panels A and B** represent the densitometric profile of WNT4 and WNT11 in HGF treated, U0126 pre-treated, and U0126 pre-treated cells subsequently treated with HGF as compared to untreated cells after normalization with GAPDH. Data are shown as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside each panels respectively.

U0126 and subsequently treated with HGF, as compared to control cells treated with HGF (Fig. 4.17A). In contrast, no significant changes in expression of ITGAV were observed in cells treated with HGF after MAPK inhibition as depicted in Western blot profiles (Fig. 4.17B).

Under similar experimental set up, the effect of inhibition of PKA signaling by H89 on the expression of WNT4, WNT11, ITGA2 and ITGAV was also investigated by Western blots. HTR-8/SVneo cells pre-treated with H89 and subsequently treated with HGF for 24 h showed ~40% decrease in expression of WNT4 and WNT11 (WNT4, $p = 0.04$; WNT11, $p = 0.007$), as compared to cells that were not pre-treated with H89, but later treated with HGF (Fig. 4.18A, B). Likewise, in case of integrin, PKA inhibition led to significant reduction in the expression of ITGA2 and ITGAV (ITGA2, $p = 0.002$; ITGAV, $p = 0.007$) in cells pre-treated with H89 followed by treatment with HGF as compared to cells that were not pre-treated with H89 but subsequently treted with HGF (Fig. 4.19A, B). To summarize the above results, it can be stated that MAPK and PKA signaling pathways may be playing an important role in the HGF-mediated expression of WNT ligands and integrins in HTR-8/SVneo cells.

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

In general, cellular processes are regulated by the interdependence or cross-talk between the various signaling pathways or the effector proteins inside the cell. Although, HGF/c-Met and integrin signaling and HGF/c-Met and WNT signaling are known to be involved in migration, invasion and differentiation (Chan *et al*., 2006), very little is known about the cross-talk between WNT and integrin signaling. To delineate the cross-talk between these two signaling proteins, the expression of WNT4 and WNT11 was checked by RT-qPCR in *ITGA2* and *ITGAV* silenced cells and vice-versa as described in *Materials and Methods*. The RT-qPCR analysis of *ITGA2* and *ITGAV* transcripts in *WNT4* silenced cells revealed significant decrease (*ITGA2*, $p = 0.03$; *ITGAV*, $p = 0.01$) in their expression as

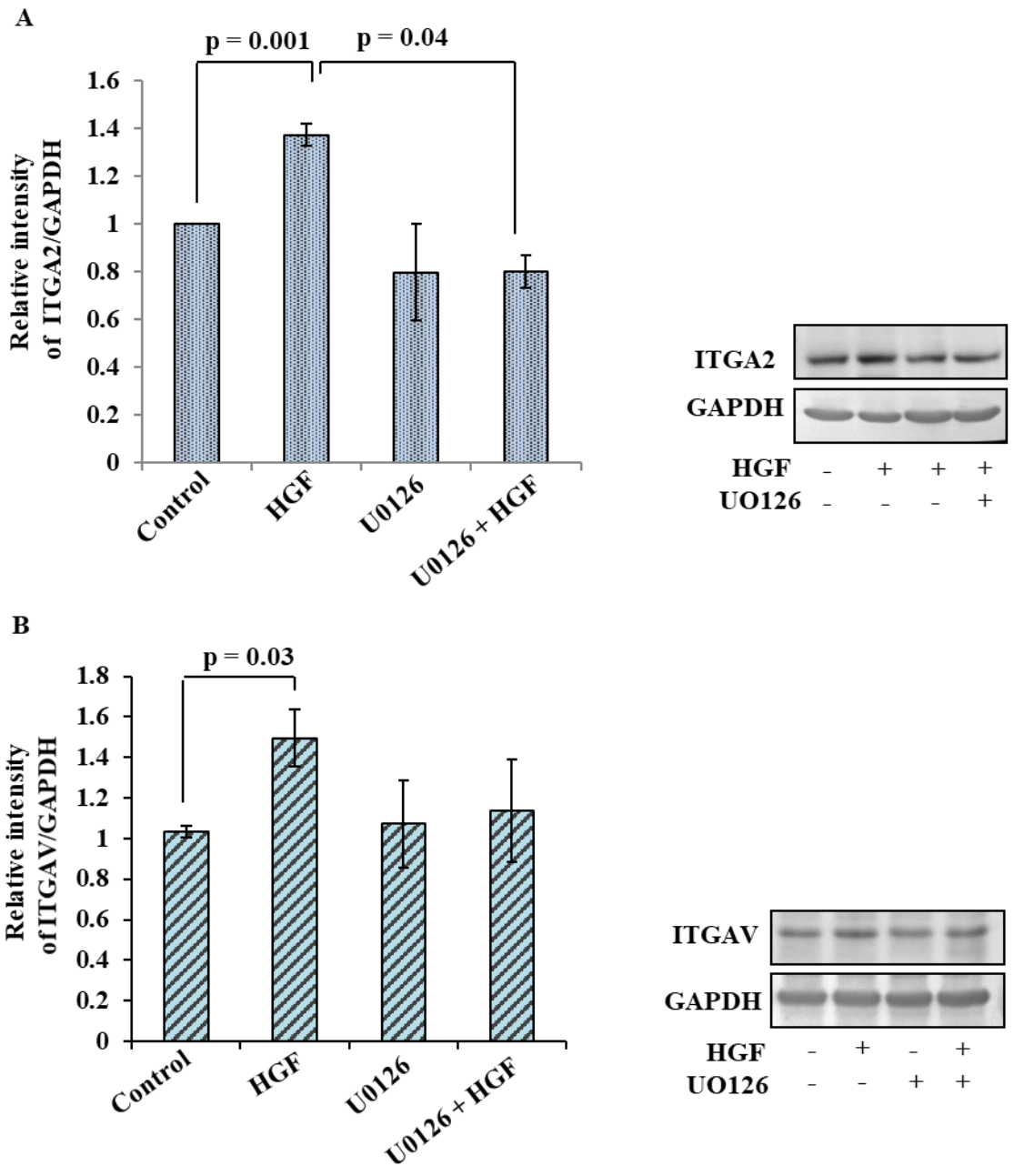

Fig. 4.17 Effect of inhibition of MAPK signaling on HGF induced expression of ITGA2 & ITGAV in HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate under humidified atmosphere of 5% CO_2 at 37°C and allowed to grow as monolayer. The monolayer of cells were pre-treated with and without MAPK inhibitor U0126 (10 µM) for 2 h and were subsequently treated with and without HGF (50 ng/mL) for 24 h. After incubation for 24 h, cell lysates were prepared to asses the expression of ITGA2 and ITGAV by Western blots as described in *Materials & Methods*. **Panels A and B** represent the densitometric profiles of ITGA2 and ITGAV in HGF treated, U0126 pre-treated and U0126 pre-treated cells subsequently treated with HGF as compared to untreated cells after normalization with GAPDH. Data are shown as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside each panel respectively.

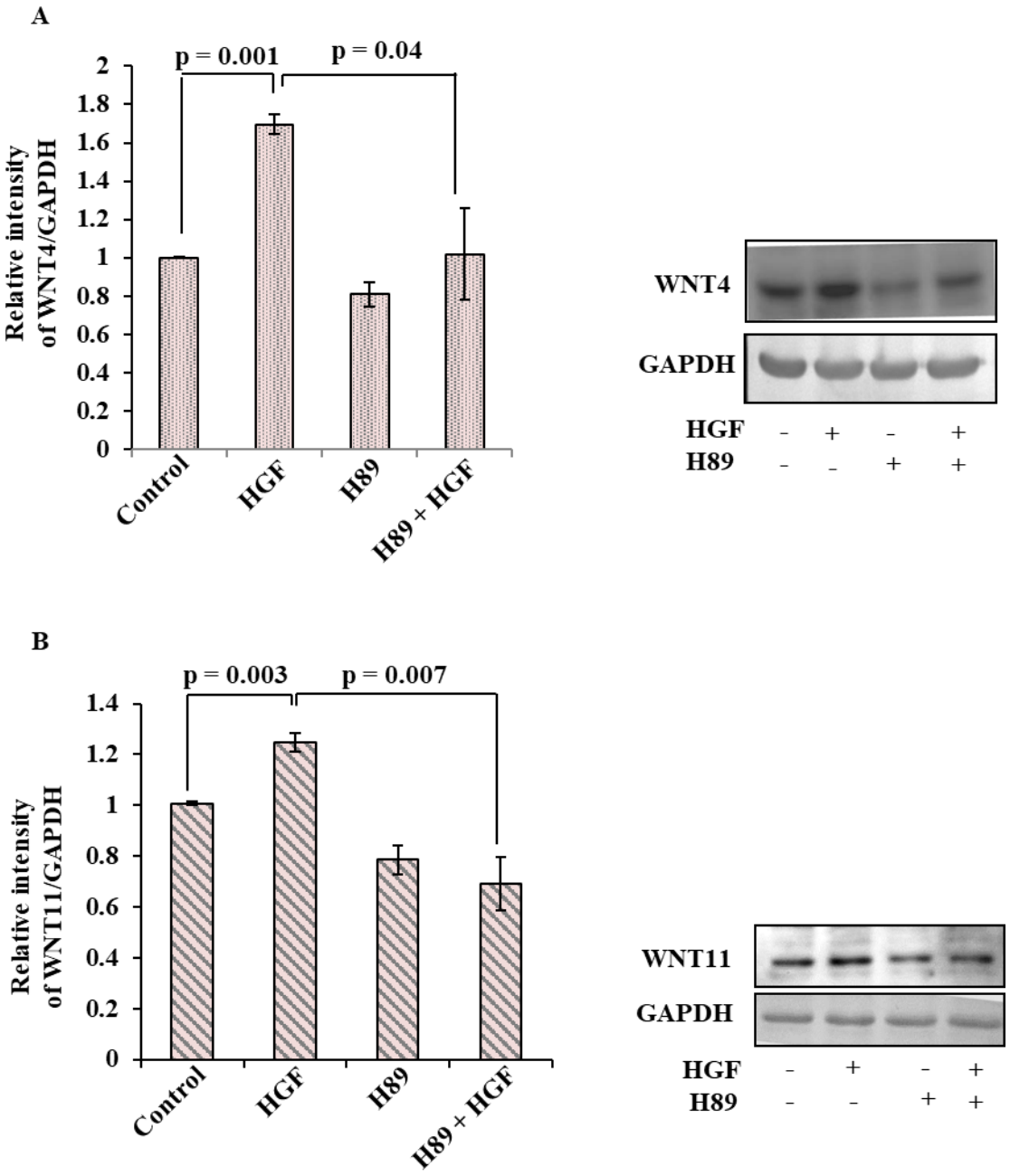

Fig. 4.18 Effect of inhibiting PKA signaling on HGF induced expression of WNT4 & WNT11 in HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate under humidified atmosphere of 5% CO_2 at 37ºC and allowed to grow as monolayer. The monolayer of cells were pre-treated with and without PKA inhibitor H89 dihydrochloride (10 µM) for 2 h and were subsequently treated with HGF (50 ng/mL) for 24 h. After incubation for 24 h, cell lysates were prepared to asses the expression of WNT4 and WNT11 by Western blots as described in *Materials & Methods*. **Panels A and B** represent the densitometric profiles of WNT4 and WNT11 in HGF treated, H89 pre-treated, and H89 pre-treated cells subsequently treated with HGF as compared to untreated cells after normalization with GAPDH. Data is shown as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside each panel respectively.

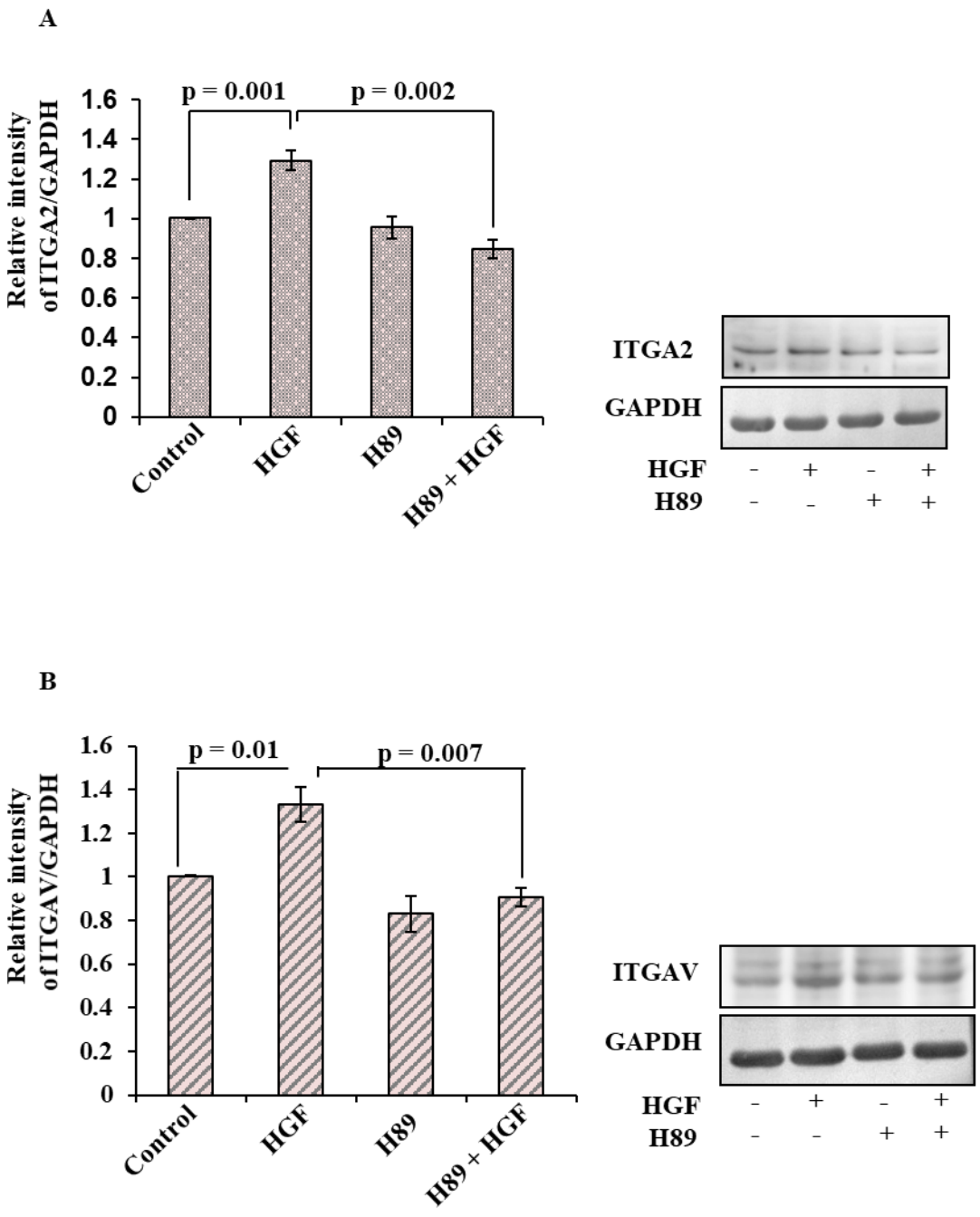

Fig. 4.19 Effect of inhibiting PKA signaling on HGF induced expression of ITGA2 & ITGAV in HTR-8/SVneo cells: HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6-well culture plate under humidified atmosphere of 5% CO_2 at 37ºC and allowed to grow as monolayer. The monolayer of cells were pre-treated with and without PKA inhibitor H89 dihydrochloride (10 µM) for 2 h and cells were subsequently treated with and without HGF (50 ng/mL) for 24 h. After incubation for 24 h, cell lysates were prepared to asses the expression of ITGA2 and ITGAV by Western blots as described in *Materials & Methods*. **Panels A and B** represent the densitrometric profiles of ITGA2 and ITGAV in HGF treated, H89 pre-treated and H89 pre-treated cells subsequently treated with HGF as compared to untreated cells after normalization with GAPDH. Data is shown as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside each panel respectively.

compared to control siRNA transfected cells after treatment with HGF for 24 h (Fig. 4.20A, B). However, in case of *WNT11* silenced cells significant decrease in only *ITGAV* ($p = 0.01$) transcript was observed as compared to control siRNA transfected cells treated with HGF, while no significant differences in the expression of *ITGA2* were observed on *WNT11* knockdown under the similar experimental conditions (Fig. 4.21A, B).

Similarly, expression of *WNT4* and *WNT11* was also analyzed in *ITGA2* and *ITGAV* silenced HTR-8/SVneo cells. A significant decrease in the level of both *WNT4* and *WNT11* (*WNT4*, $p = 0.01$; *WNT11*, $p = 0.04$) transcripts was observed in *ITGA2* silenced cells as compared to control siRNA transfected cells treated with HGF for 24 h (Fig. 4.22A, B). Likewise, in case of *ITGAV* silenced cells, significant reduction in the transcript level of *WNT4* and *WNT11* (*WNT4*, $p = 0.001$; *WNT11*, $p = 0.04$) was also observed as compared to control siRNA transfected cells after HGF treatment (Fig. 4.23A, B). These observations suggest that expression of WNT ligands and integrins are interdependent or regulate each other's expressions.

Role of β-catenin in HGF-mediated increase in the migration of HTR-8/SVneo cells

β-catenin mediated signaling has been known to play an important role during embryonic development, tissue homeostasis and carcinogenesis. In human pregnancy, abnormal expression of β-catenin has been reported in the EVTs of the placenta obtained from hydatidiforms moles (type of pregnancy disorder) as compared to normal cells, indicating dysregulation of its expression can led to abnormal trophoblast development (Zhang *et al*., 2017). Signaling pathways like HGF/c-Met, WNT and integrins are known to stabilize the β-catenin level inside the cytoplasm, which lead to its translocation in the nucleus and acts as transcription factor for the target effector proteins of the these signaling pathways. To examine the role of β-catenin in HGF-mediated migration of HTR-8/SVneo cells, the expression of β-catenin was checked at transcript as well as at protein level, by RT-qPCR and Western blotting, respectively. Significant increase in the transcript levels of *β-catenin* was observed in HGF treated HTR-8/SVneo cells as

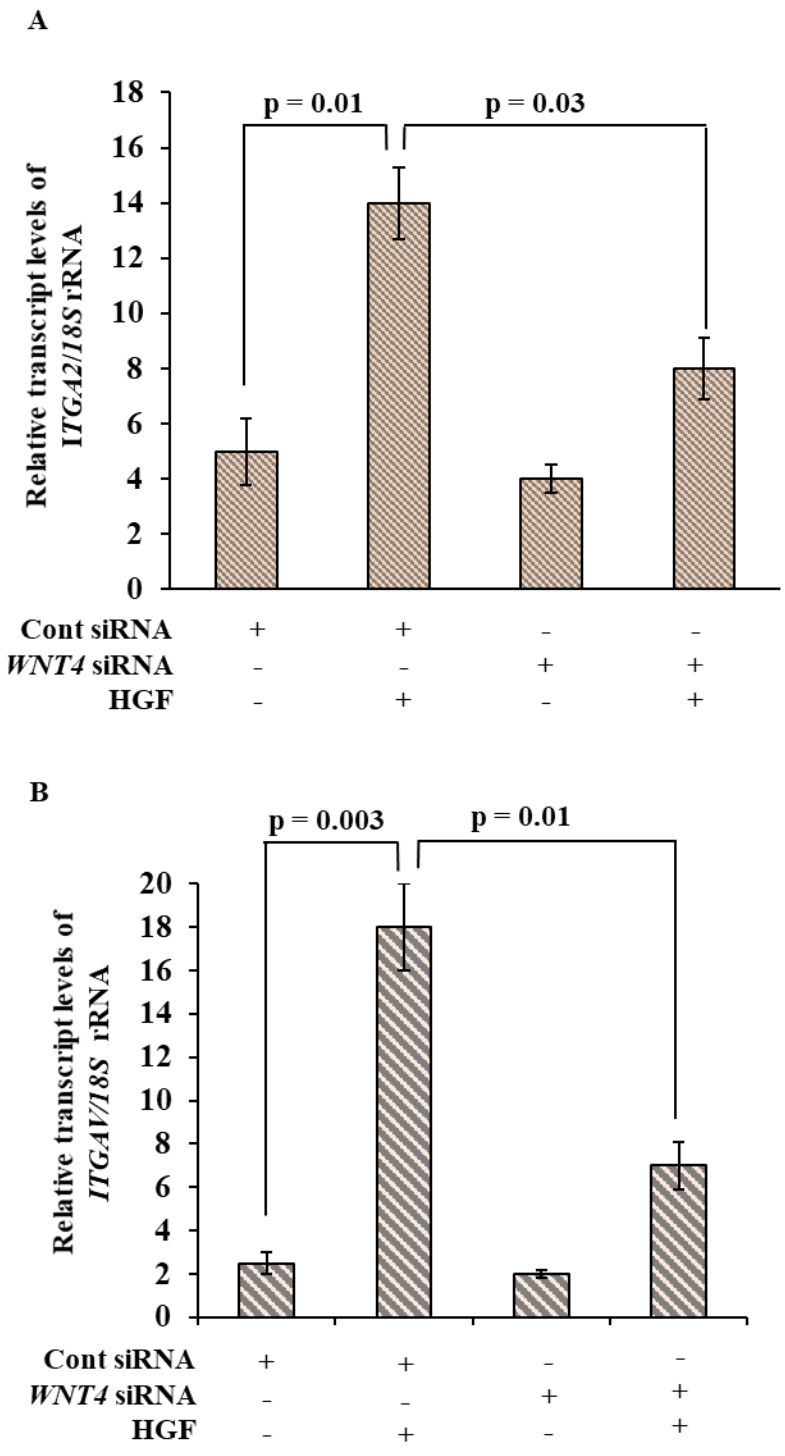

Fig. 4.20 Effect of *WNT4* silencing on the expression of *ITGA2* and *ITGAV*: *WNT4* silenced HTR-8/SVneo cells were used in wound healing assay and subsequently treated in the presence and absence of HGF (50 ng/mL) for 24 h. Total RNA was isolated from the cells post wound healing assay and *ITGA2* and *ITGAV* transcript levels were determined by RT-qPCR as described in *Materials and Methods*. **Panel A** compares *ITGA2* transcript levels between control siRNA transfected and *WNT4* silenced cells with/without HGF treatment. **Panel B** compares *ITGAV* transcript levels between control siRNA transfected and *WNT4* silenced cells after treatment in presence and absence of HGF for 24 h. In both the panels each bar represents relative expression after normalization with *18S* rRNA used as internal control. The values are expressed as mean ± *s.e.m* of three independent experiments.

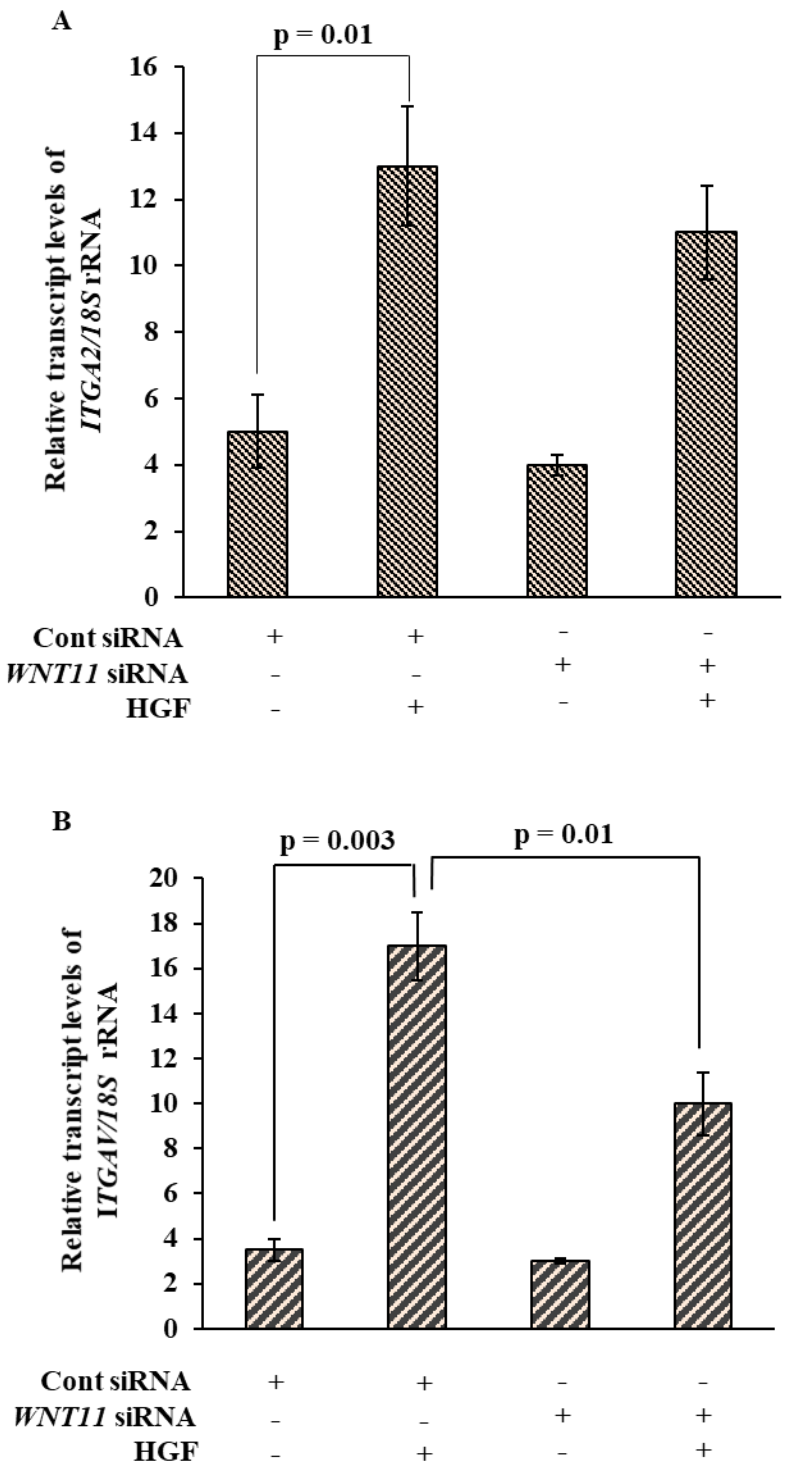

Fig. 4.21 Effect of *WNT11* silencing on the expression of *ITGA2* and *ITGAV*: *WNT11* silenced HTR-8/SVneo cells were used in wound healing assay and subsequently treated with and without HGF (50 ng/mL) for 24 h. Total RNA was isolated from the cells post wound healing assay to determine the *ITGA2* and *ITGAV* transcript levels by RT-qPCR as described in *Materials and Methods*. **Panels A and B** compare *ITGA2* and *ITGAV* transcript levels respectively, between control siRNA transfected and *WNT11* silenced cells with and without HGF treatment. Each bar represents relative expression after normalization with *18S* rRNA used as an internal control. Values are expressed as mean ± *s.e.m* of three independent experiments.

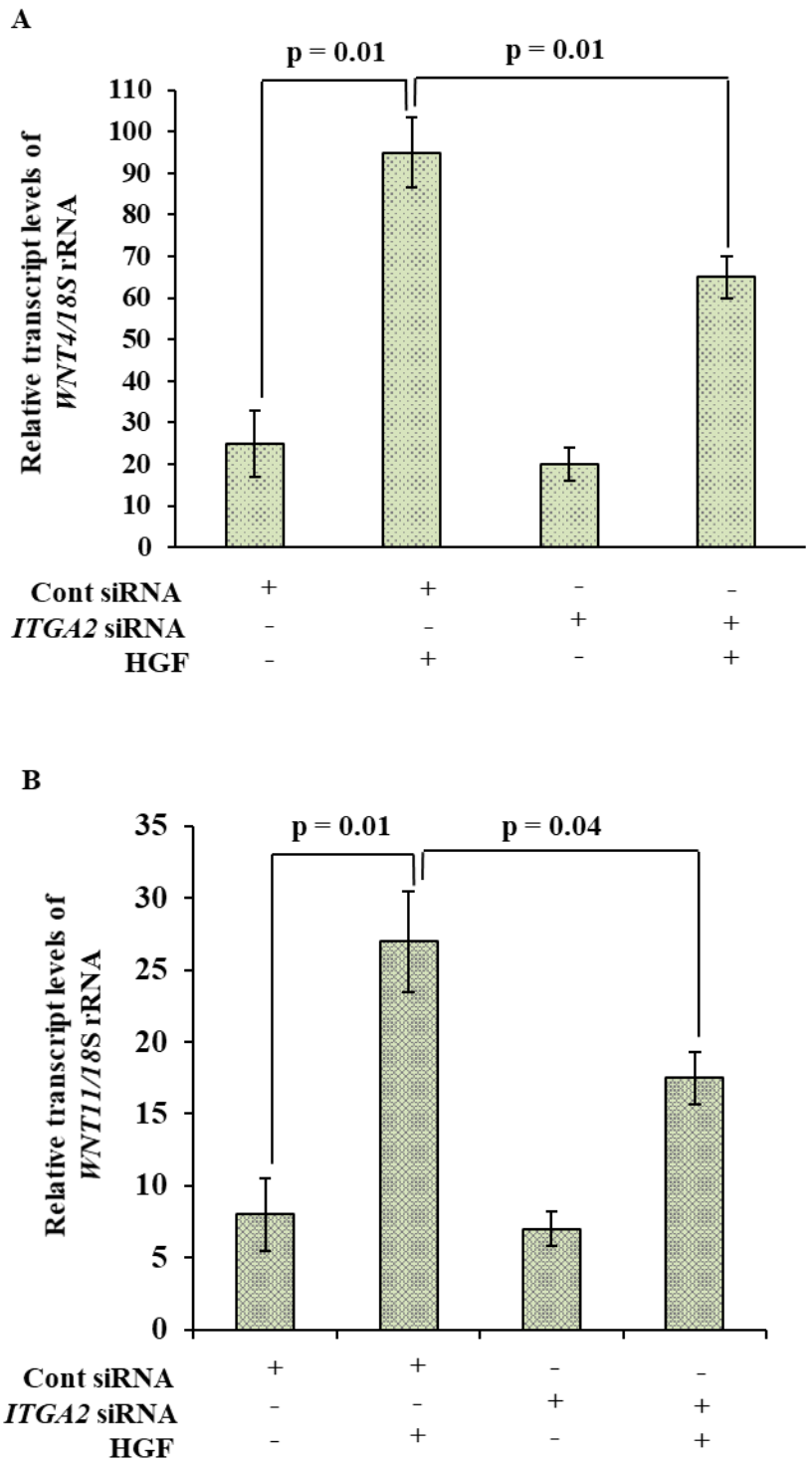

Fig. 4.22 Effect of *ITGA2* silencing on the expression of *WNT4* and *WNT11*: *ITGA2* silenced HTR-8/SVneo cells were used in wound healing assay and subsequently treated in presence and absence of HGF (50 ng/mL) for 24 h. Total RNA was isolated from the cells after 24 h post wound healing assay to determine the *WNT4* and *WNT11* transcript levels by RT-qPCR as described in *Materials and Methods*. **Panels A and B** compare *WNT4* and *WNT11* transcript levels between control siRNA transfected and *ITGA2* silenced cells with/without HGF treatment. Each bar represents relative expression after normalization with *18S* rRNA used as internal control. Values are expressed as mean ± *s.e.m* of three independent experiments.

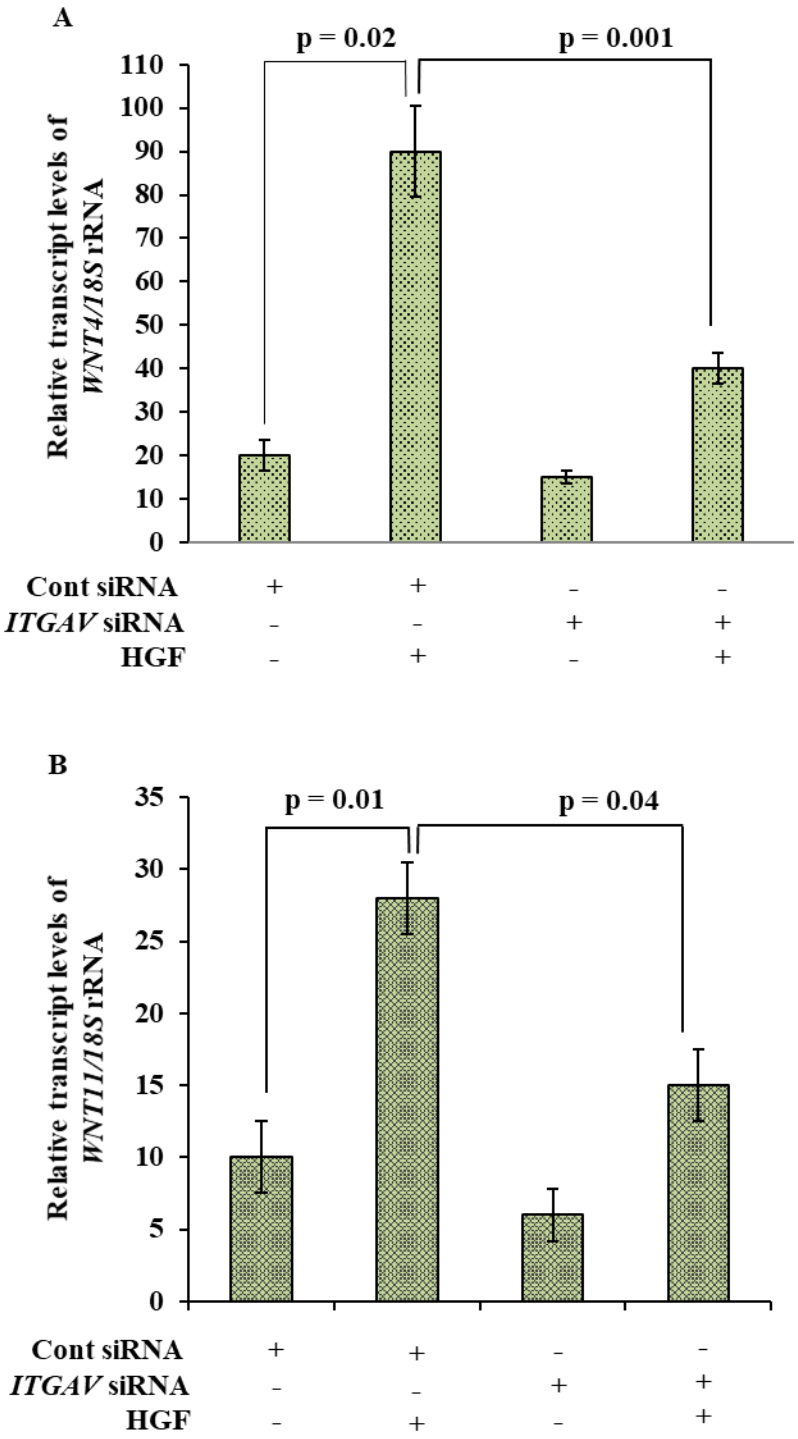

Fig. 4.23 Effect of *ITGAV* silencing on the expression of *WNT4* and *WNT11*. *ITGAV* silenced HTR-8/SVneo cells were used in wound healing assay and subsequently treated with and without HGF (50 ng/mL) for 24 h. Subsequently, total RNA was isolated and *WNT4* and *WNT11* transcript levels were determined by RT-qPCR as described in *Materials and Methods*. **Panel A** represents the transcript levels of *WNT4* in control siRNA and *ITGAV* transfected cells with/without HGF (50 ng/mL) treatment. **Panel B** represents the transcript levels of *WNT11* in control siRNA and *ITGAV* transfected cells after treatment with and without HGF (50 ng/mL). In both the panels, each bar represents relative expression after normalization with *18S* rRNA. The values are expressed as mean ± *s.e.m* of three independent experiments.

compared to untreated control after 24 h (Fig. 4.24A). Since β-catenin has been known to be present in both cytoplasm and nucleus of the cell, its expression in cytoplasmic as well as nuclear fractions was investigated by Western blotting. The Western blots analysis showed significant increase in β-catenin levels in the cytoplasmic (~1.7 fold) as well as in the nuclear (~2.0 fold) fractions of the cells treated with HGF as compared to untreated cells (Fig. 4.24B). To associate stabilization of β-catenin with trophoblast migration, in HTR-8/SVneo cells, β-catenin was knockdown by siRNA mediated gene silencing approach and subsequently used in scratch wound migration assay in the presence and absence of HGF as described in *Materials and Methods*. A significant decrease ($p = 0.03$) in the expression of β-catenin was observed in HTR-8/SVneo cells transfected with *β-catenin* siRNA as compared to cells transfected with control siRNA after treatment with HGF for 24 h. In addition, significant decrease ($p = 0.001$) at the basal level was also observed in cells transfected with *β-catenin* siRNA as compared to control siRNA transfected cells without HGF treatment (Fig. 4.25A). After confirmation of silencing, *β-catenin* knockdown cells were further used in scratch wound migration assay. A significant reduction in migration was observed in *β-catenin* knockdown cells as compared to control siRNA transfected cells treated in the presence of HGF for 24 h, whereas no significant change in basal migration was observed (Fig. 4.25B).

Since β-catenin has been suggested to be downstream connecting link for both WNT ligands and integrin signaling, it is hypothesized that β-catenin might be a common target for WNT ligands and integrins by which they regulate HGF-mediated HTR-8/SVneo cell migration. In this direction, the expression of β-catenin in HTR-8/SVneo cells silenced for *WNT4/WNT11* and *ITGA2/ITGAV* was further studied. Interestingly, 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 *ITGA2* and *ITGAV* (ITGA2, $p = 0.001$; ITGAV, $p = 0.02$) silenced cells as compared to control siRNA transfected cells after treatment with HGF for 24 h. However, no significant changes in the expression of β-catenin were observed in *WNT4/WNT11/ITGA2/ITGAV* knockdown cells in absence of HGF treatment

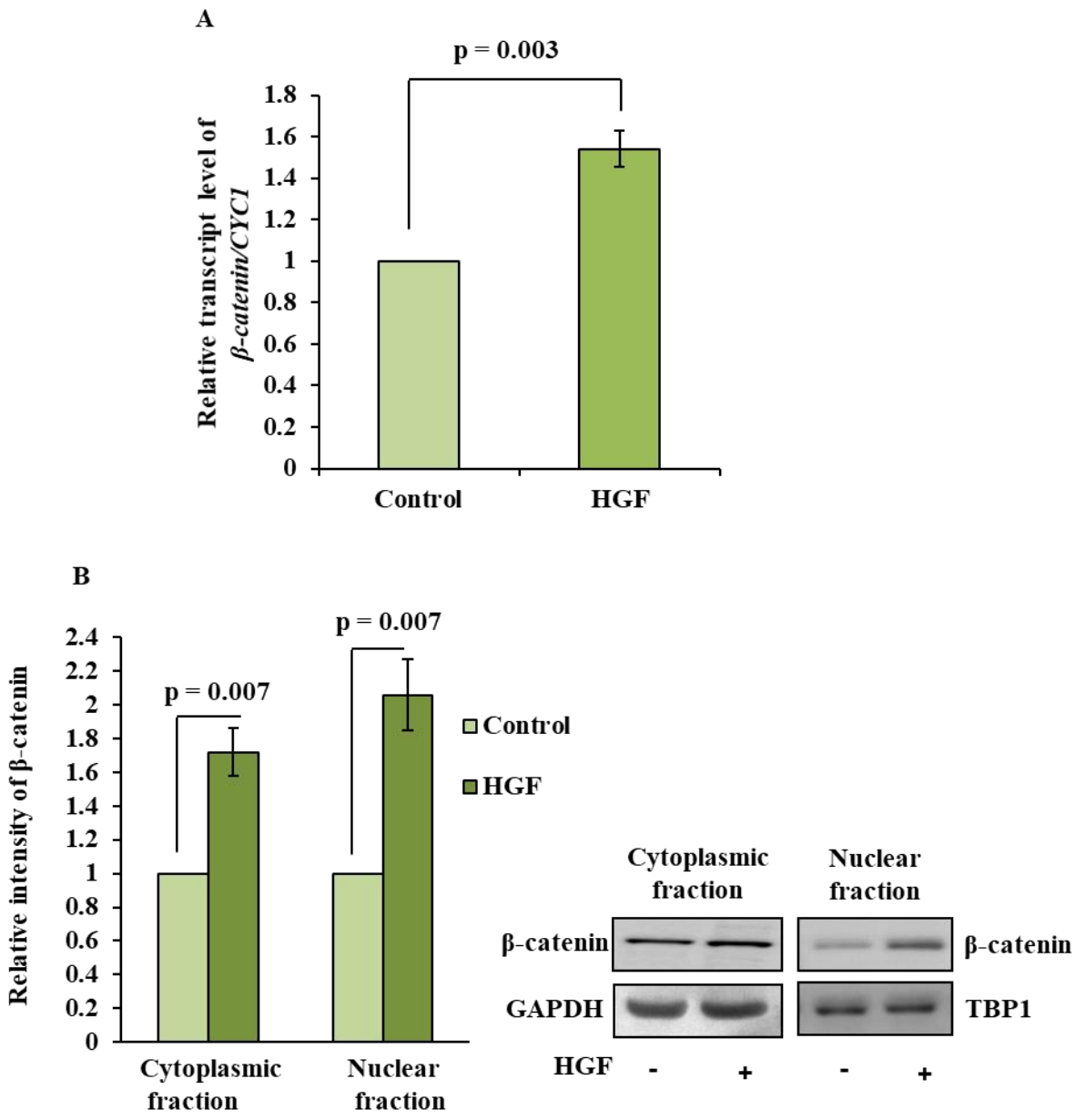

Fig. 4.24 Expression of β-catenin in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells were treated with or without HGF (50 ng/mL) for 24 h. The transcript level of *β-catenin* was measured by RT-qPCR; protein level was measured in both cytoplasmic and nuclear fractions by Western blotting as described in *Materials and Methods.* **Panel A** shows transcript profile of *β-catenin*, the bars represent relative expression after normalization with cytochrome C1 (*CYC1*) used as loading control. Values are expressed as mean ± *s.e.m* of three independent experiments. **Panel B** shows protein level of β-catenin in cytoplasmic and nuclear fractions respectively after treatment with and without HGF (50 ng/mL). Each bar represents relative expression after normalization with GAPDH/TBP1. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative blots are appended alongside. GAPDH/TBP1 were used as cytoplasmic/nuclear internal loading controls respectively.

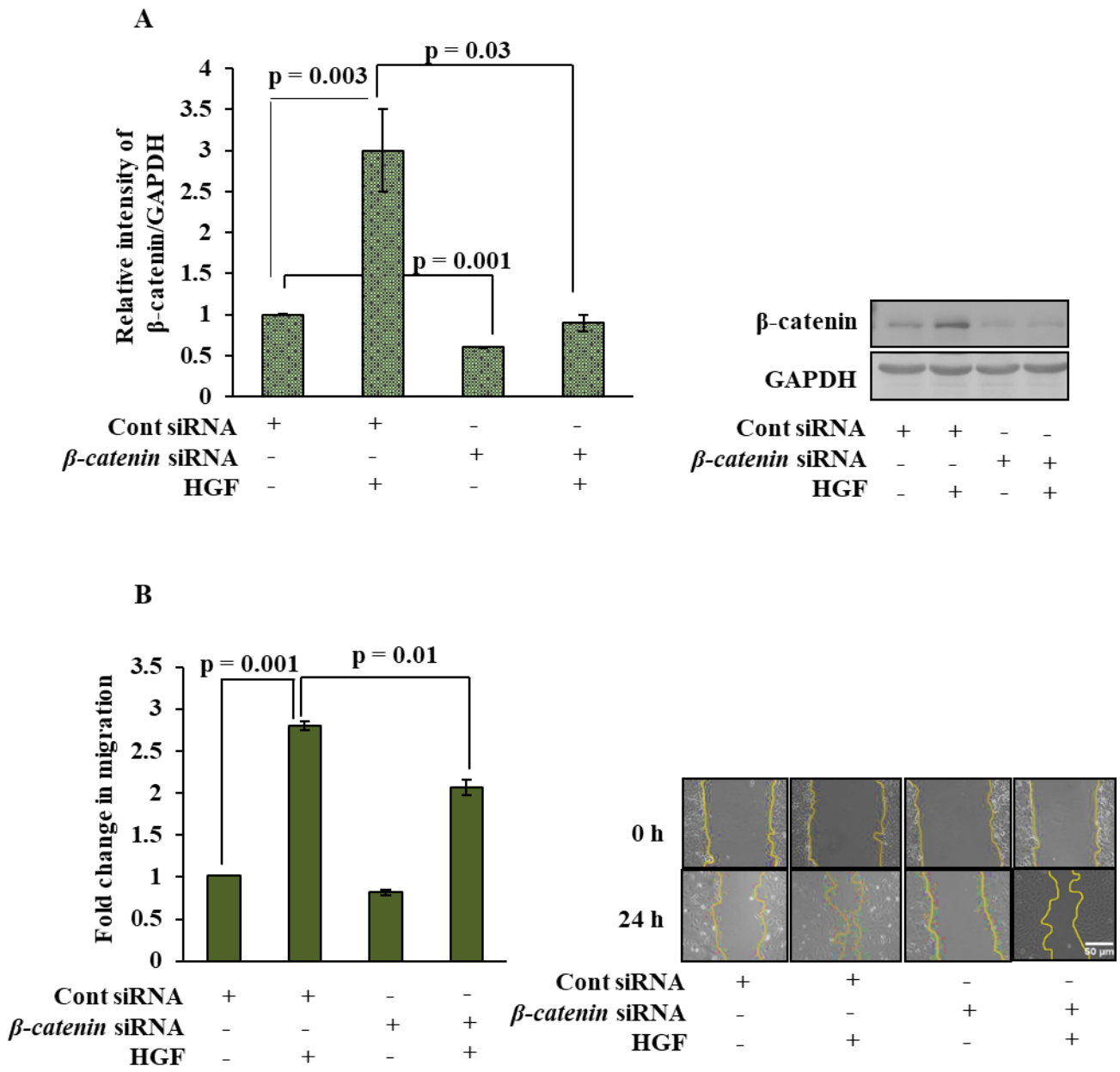

Fig. 4.25 Effect of silencing of β-catenin on the migration of HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells (0.15 x 10^6/well) were seeded in 6-well culture plate under humidified conditions with 5% CO_2 at 37°C. Next day, cells were transfected with *β-catenin* and control siRNA. After 48 h of transfection, cells were used for wound healing assay in the presence and absence of HGF (50 ng/mL) as described in *Materials and Methods*. Silencing of β-catenin was confirmed by Western blotting. **Panel A** shows densitometric analysis of β-catenin in control siRNA and *β-catenin* siRNA transfected cells in presence and absence of HGF. Each bar represents the relative expression of β-catenin after normalization with GAPDH with respect to untreated control siRNA transfected cells. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative blots for β-catenin in control and *β-catenin* siRNA transfected cells treated with and without HGF are appended alongside. **Panel B** shows the fold change in migration of cells transfected with control or *β-catenin* siRNA respectively, subsequent to treatment with and without HGF (50 ng/mL) after 24 h. The results are expressed as mean ± *s.e.m* of fold change in the migration with respect to control siRNA transfected cells without HGF treatment (50 ng/mL), observed in three independent experiments. Representative images of cells at 0 h and 24 h are appended alongside. Scale bar is 50 µm..

as compared to control siRNA transfected cells without HGF treatment (Fig. 4.26). These observations suggest that β-catenin might be common denominator for WNT and integrin pathways through which they regulate the migration process in HTR-8/SVneo cells upon treatment with HGF.

Discussion

In human pregnancy, cytokines and growth factors secreted by diverse cell types at fetal-maternal interface plays an important role during implantation by regulating trophoblast cell migration and invasion. HGF is known to be secreted by STB, EVTs, endothelial and mesenchymal cells, while its receptor is mainly expressed on trophoblast cells (Wolf *et al*., 1991). Deciphering the role of HGF in trophoblast migration and its associated signaling pathways and effector molecules might help in better understanding of the reasons of reported low level of HGF in pregnant women suffering from preeclampsia and IUGR (Somerest *et al*., 1998a, 1998b). In this study, HTR-8/SVneo cell line is used as model system to study trophoblast migration. The dose dependent increase in migration observed on treatment with HGF as presented in the present thesis is in agreement with the previous studies (Cartwright *et al*., 2002; Liu *et al*., 2012). The possible reason for increase in migration might be due to fact that HGF also acts as scatter factor (Grant *et al*., 1993). Since, maximum migration of HTR-8/SVneo cells was observed at 50 ng/mL of HGF, thus, this concentration was chosen for further experiments. The concentration of HGF used in the present study may be in physiological range, as HGF levels in plasma of pregnant women at 15-20th week of gestation was approximately 670 ng/mL (Kim *et al*., 2012), whereas in amniotic fluid its level was ~45-50 ng/mL in pregnant women at 20-29th week of gestation as reported by previous clinical studies (Horibe *et al*., 1995).

In the first trimester placenta, the transcripts of fourteen WNT ligands namely *WNT1, WNT2, WNT2B, WNT3, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT9B, WNT10A, WNT10B* and *WNT11* were found to be expressed. In the same study, mRNA

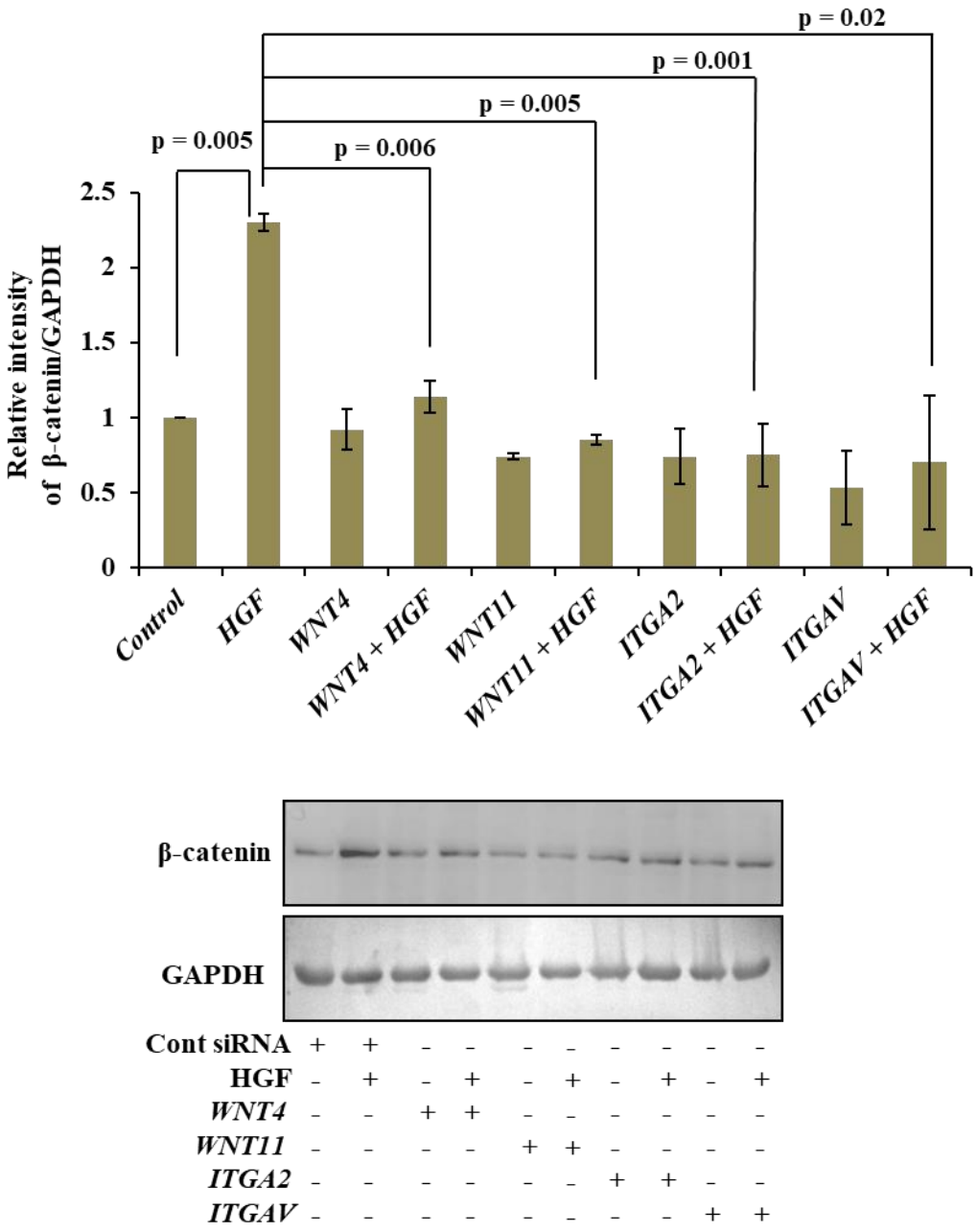

Fig. 4.26 Effect of silencing of *WNT4/WNT11/ITGA2/ITGAV* on the expression of β-catenin in HTR-8/SVneo cells treated with HGF: HTR-8/SVneo cells (0.15 x 10^6/well) were seeded in 6-well culture plate and grown under humidified conditions of 5% CO_2 at 37°C. Next day, cells were transfected with *WNT4*, *WNT11, ITGA2, ITGAV* and control siRNA as described in *Materials and Methods*. After 48 h of transfection, cells were further incubated in the presence and absence of HGF (50 ng/mL) for 24 h. Subsequently, cell lysates were prepared and processed for Western blotting to check the expression of β-catenin. The bar graph shows the densitometric analysis of β-catenin in control siRNA and *WNT4/WNT11/ITGA2/ITGAV* silenced cells in presence and absence of HGF. Each bar represents the relative expression of β-catenin after normalization with GAPDH with respect to untreated control siRNA transfected cells. Values are expressed as mean ± *s.e.m* of three independent experiments performed under above experimental conditions. Representative blots of β-catenin from one of the experiments is appended below.

expression of the *WNT2*, *WNT7A*, *WNT10B* was moderate, whereas the expression of *WNT2B*, *WNT3*, *WNT4*, *WNT5A*, *WNT5B*, *WNT7B*, *WNT9B* and *WNT11* was highly upregulated in HTR-8/SVneo cells (Sonderegger *et al*., 2007). In this context, only those WNT ligands which are highly up regulated in the previous study, were chosen to study their role in HTR-8/SVneo cell migration. Amongst these WNT ligands, maximum fold change in expression was observed for WNT4 and WNT11 both at transcript as well as protein levels. However, lower fold increase at protein level as compared to transcript level was observed in both WNT4 and WNT11, which suggest the role of regulatory mechanism at post-transcription/post translational level. It could be due to many reasons, for example in case of Xenopus *WNT8* (Xwnt8), the presence of nucleotide regulatory element negatively regulates adenylation and thereby represses translation (Tian *et al*., 1999). Besides this, distinct expression pattern of different isoforms of WNT ligands has been reported in case of Xwnt8 and *WNT2B* in different human cancer cell lines (Cui *et al*., 1995; Katoh *et al*., 2000). Moreover, it might be possible that these alternately generated isoforms might undergo translation in different polysomes, thus resulting in variable protein expressions. Since WNT ligands are secreted glycoproteins, the post translation modifications like glycosylation and acylation also regulate their stability and expression (Tanaka *et al*., 2002; Nusse, 2003). Until now, association of individual WNT ligands, in context of human trophoblast migration and invasion has not been established except WNT3A (Sonderegger *et al*., 2010). To link the HGF mediated increase in WNT4 and WNT11 ligands with HTR-8/SVneo cells migration, siRNA mediated gene silencing approach was used using commercially available siRNA against *WNT4* and *WNT11*, which are well documented in previous studies carried out in human epithelial cells BEAS-2B and human hepatocellular cancer cell lines (Toyama *et al*., 2010; Durham *et al*., 2013). Interestingly, silencing of *WNT4* and *WNT11* with their respective siRNA led to significant decrease in HTR-8/SVneo cells migration by ~30% and ~40%, respectively, as compared to control siRNA transfected cells on treatment with HGF. In addition, basal migration of HTR-8/SVneo cells was also decreased significantly in absence of HGF after WNT4 and WNT11 knockdown (Figs. 4.9, 4.11). Till now, there is

no information pertaining to the role of WNT4 and WNT11 in trophoblast migration. However, WNT4-β-catenin pathway is known to be involved in BMP2 induced human endometrial stromal cell differentiation (HESCs) (Li *et al*., 2013). Besides this, WNT4 also plays an important role during mammalian gonad development, as *WNT4*$^{-/-}$ knockout XX gonads in mice showed decrease in endothelial cell migration and coelomic formation (Jeays Ward *et al*., 2003). Moreover, WNT4-mediated signaling was also required for the initiation and elongation of mullerian duct in ontogenesis of female reproductive tract in mice. Further in same studies, it was shown that overexpression of WNT4 in mouse embryonic fibroblast cells have higher migration rate as compared to control cells, thereby confirmed its role in cell migration (Prunskaite-Hyyryläinen *et al*., 2016). On the other hand, WNT11 has been reported to regulate intestinal epithelial cells (IEC6) migration via activation of non-canonical signaling pathways which involved activation of protein kinase C and Ca^{2+}/Calmodulin-dependent protein kinase I (Ouko *et al*., 2004). Similarly, it also promotes migration of cancer cell lines (derived from breast and prostate tissues) under the stimulation of transcription complex ERRα/β-catenin in an autocrine manner (Dwyer *et al*., 2010). In contrast, it inhibits the migration of human hepatocellular cancer cell lines via activation of RhoA and Rho kinase, which inhibit Rac1 activity resulting in suppression of migration and motility (Toyama *et al*., 2010). These studies suggest that function of WNT11 is dependent on cell types and external environment.

The process of integrin switching is the integral part of EVT cells differentiation, migration and invasion in response to alteration in ECM network. Failure in integrin switching can give rise to pregnancy complications as mentioned in previous studies where immunohistochemistry data showed that invading cytotrophoblast cells of preeclamptic placenta were unable to up-regulate the expression of integrin αVβ3 (Zhou *et al.*, 1997b). Similarly, studies done in the differentiating invasive cytotrophoblasts have shown up-regulation of integrin α5β1 & α1β1 and down-regulation of α6β1 (Damsky *et al*., 1992, 1994). In addition, the iEVTs isolated from preeclamptic placenta

failed to down-regulate ITGB4 integrin subunit and unable to up-regulate ITGA1 integrin subunit, as compared to cells from normal pregnancy placenta (Zhou *et al*., 1993). In case of trophoblast migration, switching of integrins has been under the effect of various cytokines likes TGF-β, IGF2 and IGFBP1 (Irving and Lala, 1995). In addition, HGF treatment of epithelial, endothelial and colon cancer cells also led to activation of integrins, while integrin clustering activates c-met receptor of HGF suggesting a cross-talk between the two signaling pathways (Chan *et al*., 2006). In this context, the expression of various integrins reported to be expressed in HTR-8/SVneo cells were studied in the presence and absence of HGF (Fig. 4.4). Amongst all the integrins studied, significant increase in the expression of ITGA2 and ITGAV both at transcript and protein levels was observed in cell treated with and without HGF. Higher fold change in expression at transcript level as compared to protein level was observed in ITGA2/ITGAV on treatment with HGF. Similar observations with respect to integrins have been reported previously in breast cancer cell line T47-D and in human trabecular meshwork (HTM) cells (Chan *et al*., 2006; Lindberg *et al*., 2010). This can be either due to better stability and increased half-life of mRNA transcripts (Sachs 1993) or due to regulatory control elements present on the promoter sites at 5' UTR regions, which act as translational control for the integrin transcripts (Birkenmeier *et al*., 1991). To establish the significance of these two integrin proteins, knockdown experiments were carried out using siRNA and cell migration was assessed in the respective knock down cells. The siRNA employed against ITGAV in the present study has been previously used in human kidney epithelial cell line TCL-598 (Schiller *et al*., 2009). Silencing of *ITGA2* and *ITGAV* by siRNA led to significant decrease in cell migration at basal level as well as after HGF treatment in HTR-8/SVneo cells (Figs. 4.13, 4.14). Clinical studies have shown a decrease in immunostaining of ITGAV in cytotrophoblast cells of preeclamptic placenta (Vatansever *et al*., 2003). On the other hand, no significant changes were observed in the expression profiles of *ITGB1*, *ITGB3*, *ITGB4* and *ITGB5* (Fig. 4.4B). Similar findings with respect to *ITGB1* were observed in first trimester trophoblast cells treated with estradiol and IL-1α (Das and Basak, 2003). The expression of β chain is ubiquitous and

essential to the cells as knockout studies reveal embryo lethality (Lei *et al*., 2008). No significant changes in the β-chain expression that were observed in the present studies may be due to fact that the cytoplasmic tail of β integrin is more homologous as compared to diverse alpha chain. Most of the downstream signaling proteins (FAK, talin, kindling, Src, paxillin etc) bind to the cytoplasmic tail of β chain (Harburger and Calderwood, 2009). Thus it can only be hypothesize that the type of ligands signal from ECM is sensed by diverse α chain while its effect on the cell is transduced by the conserved β chain of integrins. Since extracellular matrix ligands bind to integrin receptor through I domain of α chain in association with β chain to activate downstream signalling (Liddington and Ginsberg, 2002), to find which combination of α and β chains is occurring in the present study, immunofloresence studies were carried out using antibodies against α2β1 and αV (β1, β3 & β5) (Figs. 4.6, 4.7). Treatment of HTR-8/SVneo cells with HGF led to increased expression of α2β1 and αVβ5 (Fig. 4.6). Increase in the expression of α2β1 was also reported in human melanoma and pancreatic cancer cell line cells during their migration on ECM substrate (Etoh *et al*., 1993; Grzesiak and Bouvet, 2006). Previous studies have reported that HGF treatment promotes the αVβ3 clustering via activating focal adhesion components in HTU-5 and HTU-34 cell lines (Trusolino *et al*., 1998). However, in the present study, increased expression of αVβ5 without any significant changes in the expression of αVβ1 and αVβ3 has been observed after treatment with HGF (Fig. 4.7). This suggest that different regulatory mechanisms are activated in trophoblast cells and cancer lines after HGF treatment resulting in different integrin profile.

Understanding the molecular basis of trophoblast migration, require deeper understanding of different signaling pathways. HGF is known to regulate trophoblast migration through up regulation of inducible nitric oxide (iNOS) via MAPK signaling pathway and also by activation of cAMP induced PKA signaling pathway through Rap1 (Cartwright *et al*., 2002; Chen *et al*., 2013). Besides this, HGF also activates NF-kB and JNK signaling during migration of glioma and brain's endothelial cells (Rush *et al*., 2007; Esencay *et*

al., 2010). In the present study, an increase in phosphorylation level of ERK½ and PKA in HTR-8/SVneo cells after treatment with HGF has been observed (Figs. 4.15, 4.27). Previous studies have also reported activation of MAPK and PKA with respect to WNT signaling (Yun *et al*., 2005; Gallegos *et al*., 2012). As HGF treatment led to up regulation of WNT ligands and integrins and it also activates MAPK and PKA signaling, there must be a link between these proteins. To prove this assumption, expression of WNT ligands and integrins are checked in the presence and absence of pharmacological inhibitors of respective signaling pathways. A decrease in HGF induced WNT4/WNT11 expression in HTR-8/SVneo cells pre-treated with inhibitor of PKA signaling has been observed, suggesting its role in regulation of WNT ligands expression. On the other hand, HTR-8/SVneo cells pre-treated with U0126 and subsequently treated with HGF showed decrease in the expression of only WNT11 ligand and does not affect WNT4 expression (Fig. 4.16B, A). The MAPK inhibition studies done in mesenchymal progenitor cells also showed decrease in WNT7A expression on subsequent treatment with TGF-β (Tuli *et al*., 2003). Similarily, a significant decrease in ITGA2 expression in U0126 treated cells and reduced expression of both of ITGA2 & ITGAV in H89 treated cells on subsequent treatment with HGF has been observed (Figs. 4.17A, 4.19). Interestingly, MAPK signaling is also known to regulate ITGA2 expression in Mardin Darby Canine kidney (MDCK) cells treated with HGF (Liang and Chen 2001).

In general, almost every cellular process is regulated by interdependence or cross-communication of different signaling pathways. To find the same between WNT ligands and integrins in HGF-mediated HTR-8/SVneo cells 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 RT-qPCR analysis showed that in *WNT4* silenced cells, expression of *ITGA2* transcript was significantly decreased (~35%) whereas silencing of *WNT11* has no significant effect on its expression (Fig. 4.20A, 4.21A). On the other hand, *ITGAV* expression was significantly compromised in both *WNT4* and *WNT11* silenced cells (Fig. 4.20B, 4.21B). These observations indirectly link HGF to

integrin expression through WNT ligands during HTR-8/SVneo cells migration. Simultaneously, the expression of *WNT4* and *WNT11* in cells silenced for *ITGA2* and *ITGAV* has also been studied. The expression of *WNT4* and *WNT11* were significantly reduced in cells silenced for both *ITGA2* and *ITGAV* (Figs. 4.22, 4.23). These observations give rise to a new paradigm in WNT and integrin signaling pathways, 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 (Liu *et al*., 2009).

To regulate different cellular processes, transcription dependency on β-catenin is increasingly being seen as output of numerous pathways acting in synergy or independently. For example, stiffness in ECM leads to increased expression of β-catenin by the activation of integrin/focal adhesion kinase (FAK) pathway. The β-catenin then accumulates inside nucleus and binds to WNT1 promoter leading to its increased expression. This integrin-activated β-catenin/WNT pathway further regulates mesenchymal stem cell differentiation and primary chondrocytes phenotype maintenance (Du *et al*., 2016). The other connecting link between WNT and integrin signaling has been suggested to occur through integrin ligation and FAK activation, in synergy with WNT signaling through a Grb2-rac-jnk-c-jun pathway (Crampton *et al*., 2009). On the other hand, it has been reported that WNT3A regulates β1 integrin *via* integrin linked kinase (ILK) protein during the migration and adhesion of vascular smooth muscles cells (Wu *et al*., 2014). The above studies have suggested the role of various interlinking proteins like β-catenin, FAK and ILK between WNT and integrin signaling pathways. Besides this, HGF is also known to stabilizes the β-catenin, through c-MET via GSK mediated phosphorylation of LRP receptor in renal epithelial cells (Koraishy *et al*., 2014). In the prsent study, increase in expression and nuclear localization of β-catenin in HGF treated HTR-8/SVneo cells has also been observed (Fig. 4.24B). In addition, siRNA mediated silencing of β-catenin showed decrease (~30%) in HTR-8/SVneo cells migration as compared to control siRNA transfected cells after treatment with HGF (Fig.

4.25B). The partial decrease in the HGF mediated migration of HTR-8/SVneo cells subsequent to silencing of β-catenin by siRNA as observed in the present study may be due to the fact that HGF can also activate other downstream transcription factors like ZEB1, snail and slug as observed in various cancer cell lines (Ogunwobi and Liu, 2011; Han *et al*., 2016), which may be involved in HGF-mediated increase in migration of these cells. Moreover, 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 (Fig. 4.26). Taking the clue from above mentioned studies, we can assume or hypothesized that β-catenin may be a common downstream target for WNT ligands and integrins pathways (Fig. 4.27). It is also possible that interdependence of these pathways may involve other downstream denominators or may act independently during HGF-mediated HTR-8/SVneo cells migration.

How HGF mediates increase in migration of HTR-8/SVneo cells by activating MAPK & PKA signaling pathways and their effect on WNT ligands and integrins expressions which in turn regulates expression of β-catenin has been summarized in schematic form in Fig. 4.27.

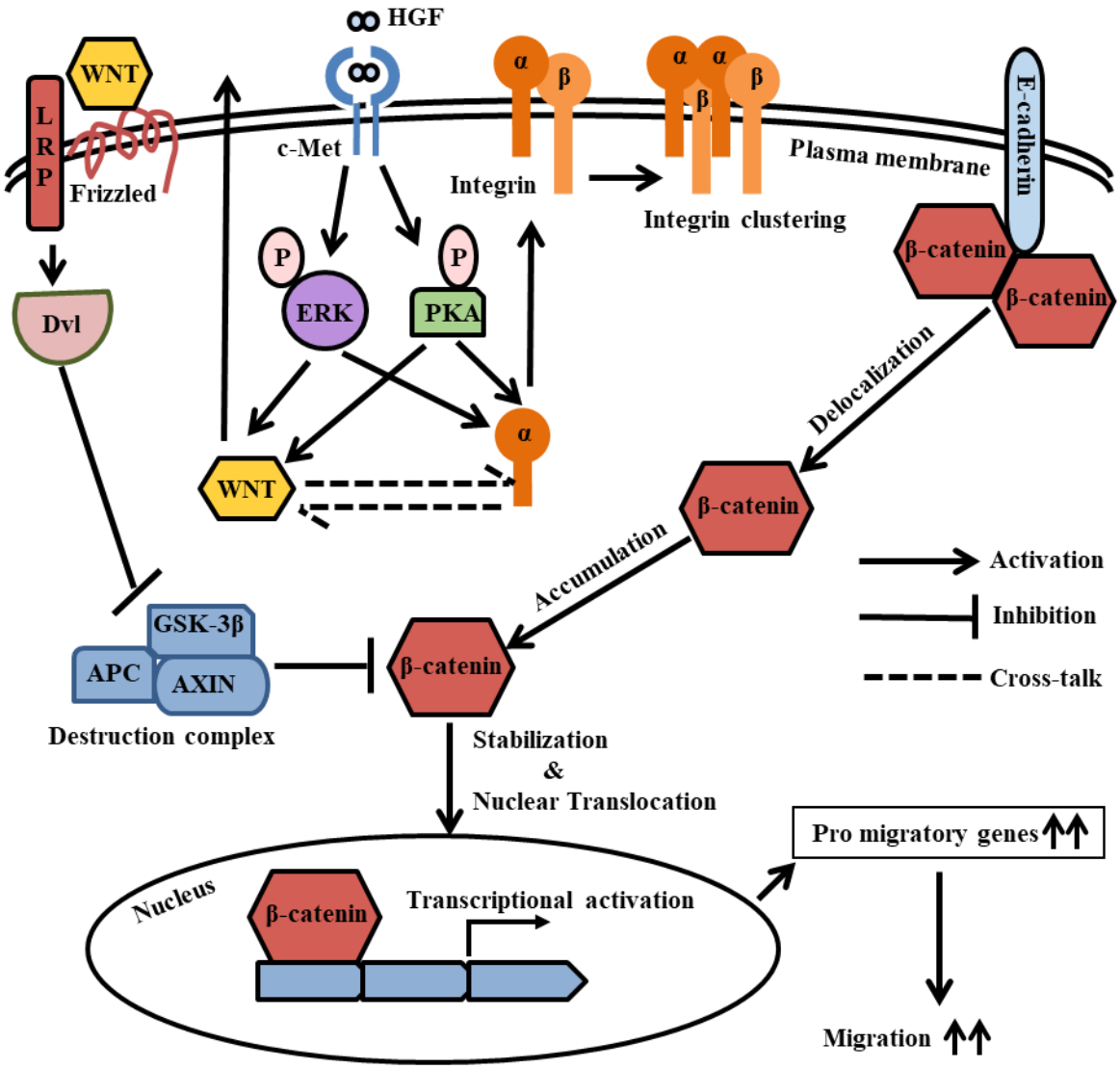

Fig. 4.27 The mechanistic model for HGF-mediated HTR-8/SVneo cells migration: HGF regulates WNT and integrin expression via activation of ERK/PKA signaling pathways after binding to its c-Met receptor. Cross-communication between WNT ligands and integrin is also shown. Integrin translocate to plasma membrane and undergo integrin clustering leading to delocalisation of E-cadherin-β-catenin complex and thereby lead to 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 prevent recruitment of destruction complex (GSK-3β/Axin/APC), hence protect β-catenin from degradation. The active form of β-catenin translocate 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 cells migration

Chapter – II

Effect of Hypoxia and HGF on migration and invasion of trophoblast cells: Role of signaling pathways and effector proteins

Background

In human pregnancy, development of placenta is initiated under a low oxygen environment, before the establishment of maternal circulation to embryo (Soares *et al*., 2017). This low oxygen or hypoxic condition is essential for the early development of both embryo and placenta, as they are still immature to protect themselves from oxidative stress (Leese, 2002). In addition, hypoxia is also required to keep the embryonic cells in pluripotent state (Forristal *et al*., 2010) and it also act as inducer for proliferation, differentiation and migration/invasion of cytotrophoblast cells (Zhou *et al*., 1998; Tuuli *et al*., 2011). Thus, it is imperative to investigate the regulatory mechanisms associated with trophoblast migration/invasion under hypoxic conditions.

Hypoxia promotes the migration and invasion of cancer cells through activation of various signaling pathways along with secretion of various cytokines, MMPs, and uPA, which directly or indirectly influence ECM remodelling (Petrova *et al*., 2018). In human pregnancy, the expression of some MMPs like MMP1, MMP2 and MMP3 primarily occur in trophoblast cells, while the production of MMP9 is up-regulated by external factors like growth factors, cytokines, oxygen and pathological conditions (Staun-Ram *et al*., 2004; Weiss *et al*., 2007). Further, it is the ratio of MMPs/TIMPs which regulate the integrity of ECM and thus control migration and invasion of the cells.

In response to hypoxia, the expression of oxygen sensitive transcription factors such as HIFs are also up regulated in placental tissues (Semenza and Wang, 1992). It is well known that expression of HIF-1α is primarily regulated by changes in oxygen concentration; however, in pregnancy different cytokines and hormones also regulate HIF-1α expression under different physiological conditions (Pringle *et al*., 2010). HIF-1α is known to be involved in trophoblastic cells migration and invasion by regulating expression of certain genes like uPAR (Meade *et al*., 2007) and TGF-β3 (Nishi *et al*., 2004). However, we still do not know how HIF-1α is regulated under hypoxia during

trophoblast migration in presence of HGF. Although, the importance of HGF and its associated signaling pathways under normoxic conditions has been widely discussed in previous chapter, however, its role in trophoblastic cell migration/invasion under hypoxic conditions has not been fully elucidated.

Thus an attempt has been made in this chapter, to decipher the role of HGF and its associated signaling pathways in trophoblastic cells migration/invasion under normoxic and hypoxic conditions. The expression of various MMPs and TIMPs responsible for trophoblast migration/invasion was also studied. The significance of MAPK and PI3K signaling pathways during HGF-mediated migration/invasion of HTR-8/SVneo cells under normoxic/hypoxic conditions has also been addressed using respective pathway inhibitors. Moreover, silencing of HIF-1α and its effect on HGF-mediated HTR-8/SVneo cell migration under hypoxia was also investigated.

Results

HGF-mediated increased in trophoblastic cells migration and invasion under hypoxic conditions involve up-regulation of MMPs

In early pregnancy, low oxygen is an extrinsic factor which regulates trophoblastic cells functions including proliferation, migration and invasion. To investigate the effect of hypoxia on trophoblastic cells migration and invasion, HTR-8/SVneo cells were used in Scratch wound migration assay and Matrigel invasion assay as described in *Materials and Methods*. Significant increase in migration (~40%, $p = 0.01$) and invasion (~ 2.3 fold, $p = 0.001$) was observed in HTR-8/SVneo cells incubated under hypoxic (2% O_2) conditions as compared to cells under normoxia (20% O_2) after 24 h (Figs. 4.28, 4.29). As HGF is known to facilitate tumor cell growth, migration and invasion under low oxygen (Lee *et al*., 2014), its effect on trophoblastic cell migration under hypoxic conditions was also investigated. HTR-8/SVneo cells were treated with optimized concentration of HGF (50 ng/mL) under hypoxia for 24 h. Interestingly, HGF treatment

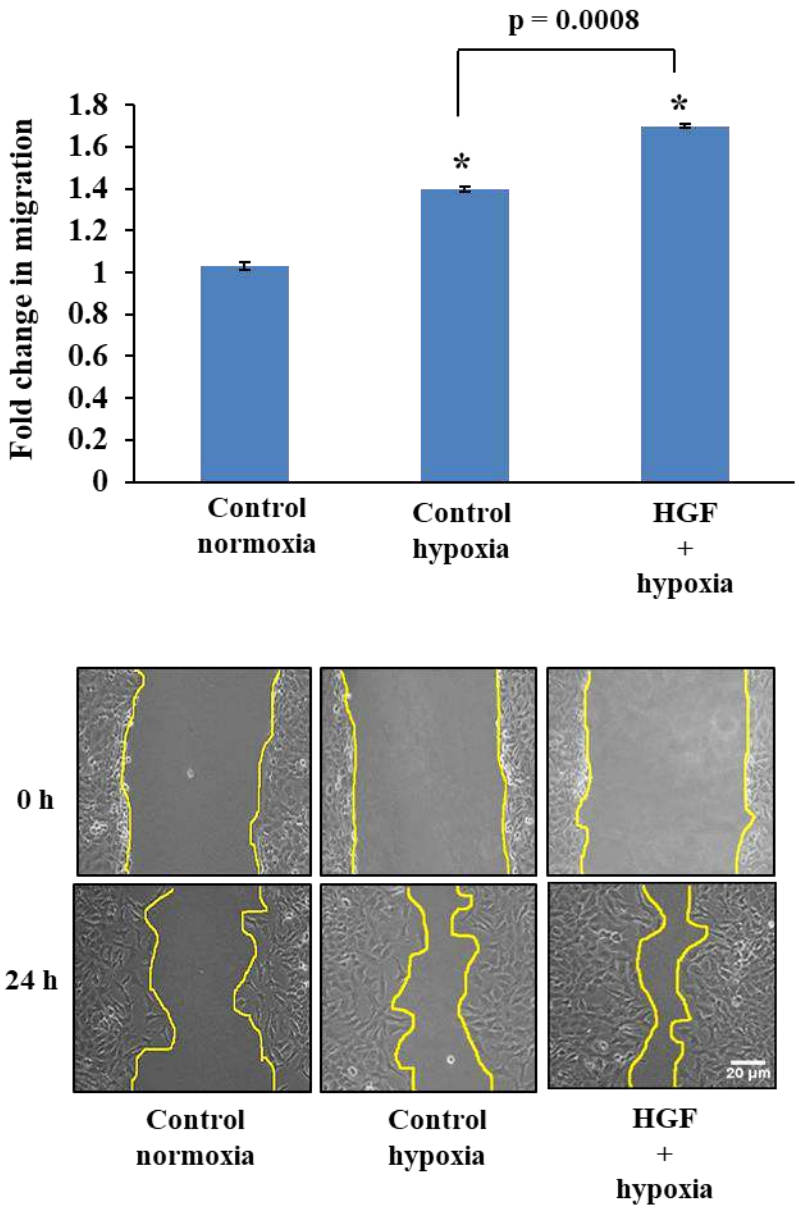

Fig. 4.28 Effect of HGF treatment on HTR-8/SVneo cells migration under hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were grown in 6-well culture plate under normoxic (20% O_2) conditions at 37°C with 5% CO_2 in a humidified chamber to form a monolayer. Cells were pre-treated with mitomycin-C (5 µm) for 2 h to inhibit cell proliferation. Monolayer of cells were scratched in horizontal as well as vertical directions. Detached cells were removed by washing with plain medium and subsequently treated with and without HGF (50 ng/mL) for 24 h under hypoxic (2% O_2) conditions. Another set of cells after scratching, were incubated for 24 h under normoxic conditions without any treatment with HGF. The fold change in migration was calculated based on the area of wound closure after 24 h. The results are shown as mean ± *s.e.m* of three independent experiments. Representative images at 0 and 24 h with and without HGF (50 ng/mL) treatment under normoxic and hypoxic conditions are appended below. Scale bar represents 20 µm. * denotes $p \leq 0.05$ as compared to untreated cells under normoxia, which was considered as statistically significant.

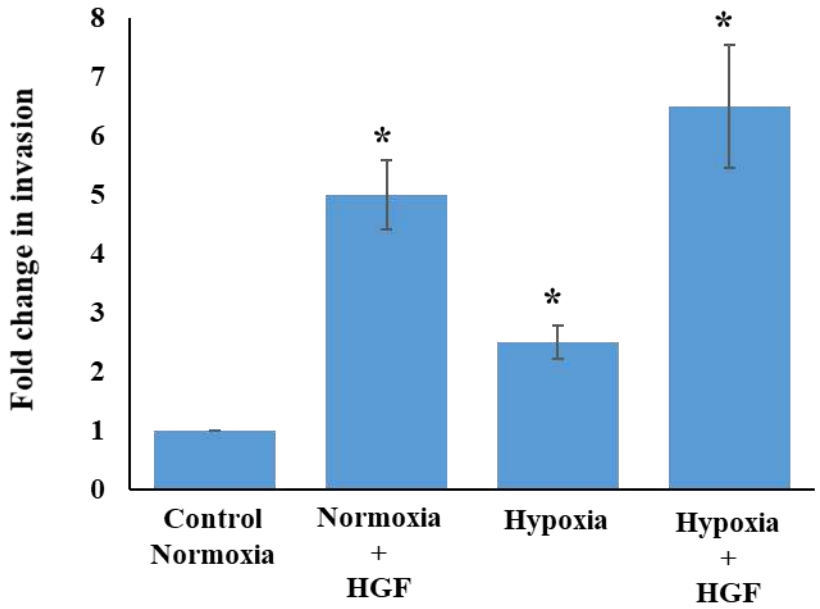

Fig. 4.29 Effect of HGF treatment on HTR-8/SVneo cells invasion under normoxic and hypoxic conditions: Cell invasion was measured by Matrigel invasion assay. HTR-8/SVneo cells (0.1 x 10^6/well) were seeded in upper chamber of transwell either in presence or absence of HGF (50 ng/mL) for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions at 37ºC with 5% CO_2 as described in *Materials and Methods*. The bar graph represents relative fold change in invasion of HTR-8/SVneo cells in HGF treated cells under normoxic conditions and untreated & HGF treated cells under hypoxic conditions as compared to the untreated control cells cultured under normoxia. Values are expressed as mean ± *s.e.m* of three independent experiments. * denotes $p \leq 0.05$ as compared to untreated cells under normoxia, which was considered statistically significant.

led to a significant increase in the migration ($p = 0.0008$) and invasion (~3 folds, $p = 0.002$) of HTR-8/SVneo cells, when compared to untreated cells under hypoxic conditions (Fig. 4.28, 4.29). These results suggest that in synergy with hypoxia, HGF promotes trophoblastic cells migration and invasion. In addition, we also observed ~5-fold increase in the invasion of HGF treated cells, when compared to untreated cells under normoxic conditions (Fig. 4.29).

Secretion of MMPs, which break down ECM increases during invasion and migration of EVTs (Cockle *et al*., 2007, Zhu *et al*. 2012). To determine, which class of MMPs are involved during trophoblastic cells migration and invasion under normoxic and hypoxic conditions, the expression of MMP1, MMP2, MMP3 and MMP9 was checked at transcript as well as protein level by RT-qPCR and Western blotting as described in *Materials & Methods.* A significant increase in the transcript levels of *MMP1*, *MMP2* and *MMP9* was observed under hypoxic conditions as compared to untreated cells under normoxia after 24 h (Table 4.1). However, at protein level, significant ($p = 0.01$) increase in only MMP1 was observed in cells incubated under hypoxia *versus* normoxia (Fig. 4.30A). On the other hand, in case of HGF treated HTR-8/SVneo cells under hypoxic conditions, a significant increase in the transcript level of *MMP1* and *MMP9* was observed as compared to untreated cells under hypoxia (Table 4.1). Similar observation was also made at protein level as depicted from Western blot profiles (Figs. 4.30A, 4.31B). Under normoxic conditions, RT-qPCR analysis revealed up-regulation in the expression of *MMP2*, *MMP3* and *MMP9* at transcript levels in HGF treated cells as compared to untreated cells (Table 4.1) while at protein level, significant (MMP2, $p = 0.02$; MMP3, $p = 0.03$) increase in only MMP2 and MMP3 was observed in Western blot profiles of HTR-8/SVneo cells treated with HGF as compared to untreated cells (Figs. 4.30B, 4.31A). Since MMPs are secretory proteins, their enzymatic activity by substrate Zymography was also checked as described in *Materials & Methods*. Since expression of MMP2 and MMP3 was higher in HGF treated cells under normoxic conditions, we check their enzymatic activity in culture supernatant in similar conditions. Significant ($p =$

Table 4.1: Relative transcript levels of *MMPs*, *TIMPs* and *MMPs*/*TIMP1* ratio in HTR-8/SVneo cells with and without HGF treatment under normoxic/hypoxic conditions by RT-qPCR

MMPs* & *TIMPs	**Relative ΔCt of the transcript levels with respect to untreated cells under normoxia (Mean ± *s.e.m*)**						
	Normoxia	**Normoxia + HGF**	**Hypoxia**	**Hypoxia + HGF**	**Normoxia *vs* Normoxia + HGF (p-value)**	**Hypoxia *vs* Hypoxia + HGF (p-value)**	**Normoxia *vs* Hypoxia (p-value)**
MMP1	1.0 ± 0.01	1.27 ± 0.11	2.50 ± 0.17[c]	6.83 ± 0.2[b]	0.06	0.001[b]	0.001[c]
MMP2	1.0 ± 0.01	2.87 ± 0.12[a]	2.50 ± 0.21[c]	3.03 ± 0.12	0.001[a]	0.11	0.002[c]
MMP3	1.0 ± 0.01	6.73 ± 0.46[a]	1.43 ± 0.18	2.16 ± 0.14	0.002[a]	0.08	0.07
MMP9	1.0 ± 0.01	1.34 ± 0.04[a]	1.96 ± 0.08[c]	4.60 ± 0.26[b]	0.001[a]	0.0006[b]	0.003[c]
TIMP1	1.0 ± 0.01	0.69 ± 0.04[d]	2.12 ± 0.19	1.03 ± 0.08[e]	0.002[d]	0.006[e]	0.004
TIMP2	1.0 ± 0.01	1.51 ± 0.17	0.8 ± 0.08	1.54 ± 0.49	0.03	0.21	0.08
TIMP3	1.0 ± 0.01	1.98 ± 0.44	2.65 ± 0.47	0.44 ± 0.15[e]	0.09	0.01[e]	0.02
TI*MP4*	1.0 ± 0.01	1.45 ± 0.20	0.89 ± 0.34	0.70 ± 0.24	0.08	0.67	0.77
MMP1*/*TIMP1	1.0 ± 0.01	1.85 ± 0.15[f]	1.19 ± 0.10	6.71 ± 0.65[g]	0.004[f]	0.001[g]	0.13
MMP2*/*TIMP1	1.0 ± 0.01	4.18 ± 0.26[f]	1.20 ± 0.20	2.95 ± 0.14[g]	0.0002[f]	0.002[g]	0.32
MMP3*/*TIMP1	1.0 ± 0.01	9.87 ± 1.03[f]	0.68 ± 0.10	2.10 ± 0.07[g]	0.001[f]	0.004[g]	0.04
MMP9*/*TIMP1	1.0 ± 0.01	1.97 ± 0.19[f]	0.93 ± 0.08	4.54 ± 0.59[g]	0.007[f]	0.003[g]	0.47

[a]Significant increase in MMPs on treatment with HGF under normoxia; [b]Significant increase in MMPs on treatment with HGF under hypoxia; [c]Significant increase in MMPs in hypoxia as compared to normoxia; [d]Significant decrease in TIMPs on treatment with HGF under normoxia; [e]Significant decrease in TIMPs on treatment with HGF under hypoxia; [f]Significant increase in MMPs/TIMP1 ratio under normoxia after HGF treatment; [g]Significant increase in MMPs/TIMP1 ratio under hypoxia after HGF treatment

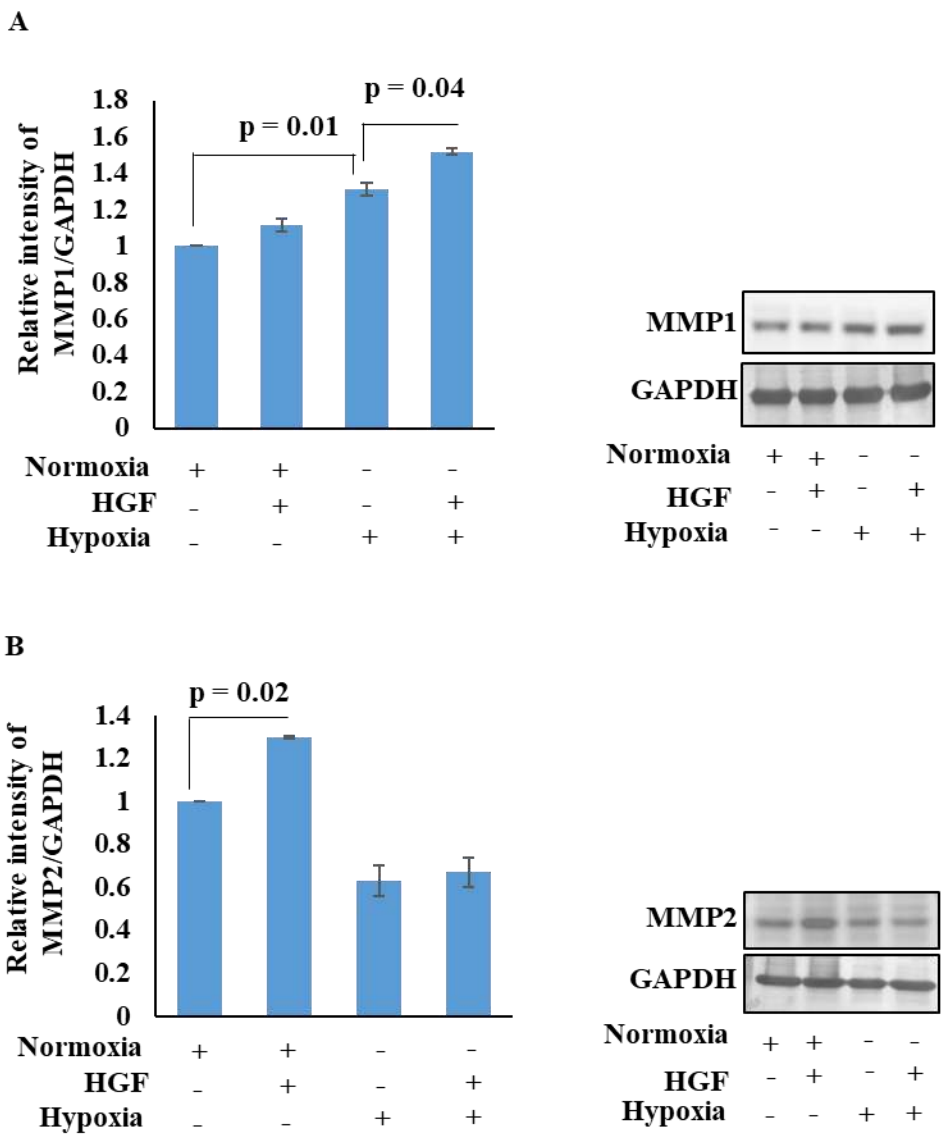

Fig. 4.30 Expression profile of MMP1 and MMP2 in HTR-8/SVneo cells treated with HGF under normoxic and hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were grown overnight in 6-well culture plate at 37ºC with 5% CO_2 in normoxic (20% O_2) conditions. Next day, cells were serum starved for 6 h, followed by treatment with and without HGF (50 ng/mL) for 24 h in normoxic as well as hypoxic (2% O_2) conditions. Subsequently, cell lysates were prepared to determine expression of MMP1 and MMP2 at protein level by Western blotting as described in *Materials and Methods*. **Panels A and B** show densitometric analysis of MMP1 and MMP2 proteins in cell lysate. GAPDH was used as a loading control. Each graph shows relative intensity of MMP1 and MMP2 protein respectively as compared to untreated cells under normoxic conditions. Values are expressed as mean ± *s.e.m* of band intensity of three independent experiments. Representative blots are appended alongside in each panel..

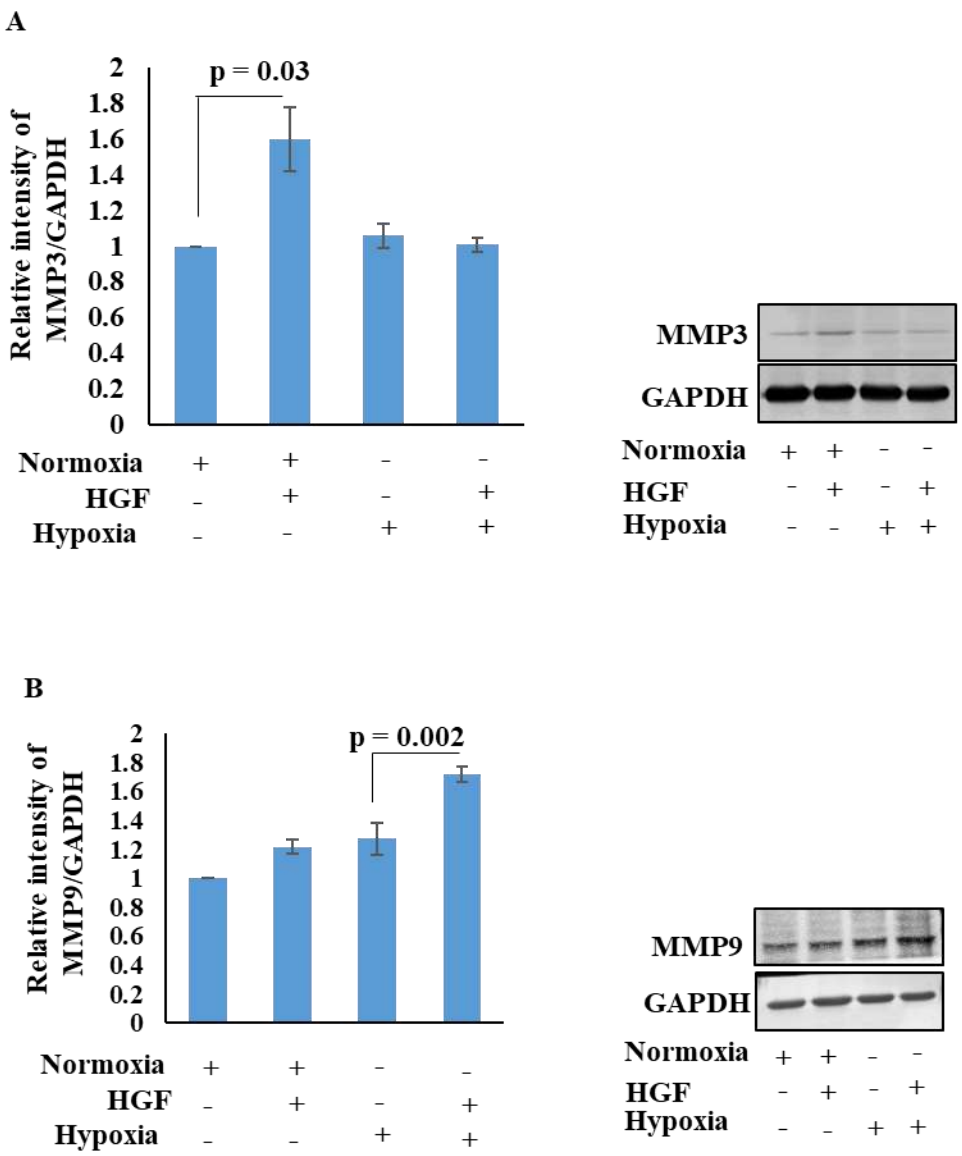

Fig. 4.31 Expression profile of MMP3 and MMP9 in HTR-8/SVneo cells treated with HGF under normoxic and hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were grown overnight in 6-well culture plate at 37ºC with 5% CO_2 in normoxic (20% O_2) conditions. Next day, cells were serum starved for 6 h, followed by treatment with and without HGF (50 ng/mL) for 24 h in normoxic (20% O_2) as well as hypoxic (2% O_2) conditions. Subsequently, cell lysates were prepared to determine expression of MMP3 and MMP9 at protein level by Western blotting as described in *Materials and Methods*. **Panels A and B** show densitometric analysis of the MMP3 and MMP9 proteins in cell lysate. Each bar graph represents the relative intensity of MMP3 and MMP9 respectively, as compared to untreated cells under normoxic conditions. GAPDH was used as a loading control. Values are expressed as mean ± *s.e.m* of band intensity of three independent experiments. Representative blots are appended alongside in each panel.

0.001) increase in enzymatic activity of MMP2 was observed by gelatin zymography in HGF treated cells as compared to untreated control under normoxia (Fig. 4.32A). However, casein zymography for MMP3 did not reveal any enzymatic activity in culture supernatant harvested from HGF treated and control cells incubated under normoxic conditions. Similarly, enzymatic activity of MMP1 and MMP9, which were up-regulated at protein level under hypoxic conditions were also quantified. Gelatin zymogram for MMP9 revealed significant ($p = 0.01$) increase in MMP9 enzymatic activity in the culture supernatant of HGF treated cells as compared to untreated control incubated under hypoxic conditions for 24 h (Fig. 4.32B). However, under similar experimental conditions MMP1 in the culture supernatant harvested from untreated and HGF treated cells did not show any enzymatic activity in collagen Zymography.

TIMPs regulate the activities of MMPs, by inhibiting their activity (Visse and Nagase 2003). During placentation, the ratios of MMPs versus TIMPs regulate the integrity of ECM and thus control migration and invasion of the trophoblast cells (Luo *et al*. 2011). To find, which class of TIMPs are involved during trophoblastic cells migration/invasion, the expression of *TIMP1, TIMP2, TIMP3* and *TIMP4* were investigated at transcript level by RT-qPCR (Table 4.1). HGF treatment of HTR-8/SVneo cells led to a significant decrease in *TIMP1* transcript level under normoxic conditions, whereas under hypoxic conditions, both *TIMP1* and *TIMP3* were down-regulated in HGF treated HTR-8/SVneo cells as compared to the untreated controls under similar experimental conditions (Table 4.1). On the other hand, in case of *TIMP2* and *TIMP4*, no significant decrease was observed at transcript level However, Western blot analyses revealed a significant ($p = 0.04$) decrease in only TIMP1 in HTR-8/SVneo cells treated with HGF as compared to untreated cells incubated under hypoxic conditions (Fig. 4.33A). No significant change in protein expression of TIMP3 was observed between untreated and HGF treated HTR-8/SVneo cells incubated under normoxic as well as hypoxic conditions (Fig. 4.33B). Since only TIMP1 was decreased in HGF treated cells as compared to their respective untreated counterparts, we calculated the ratio between MMPs *versus* TIMP1 at mRNA

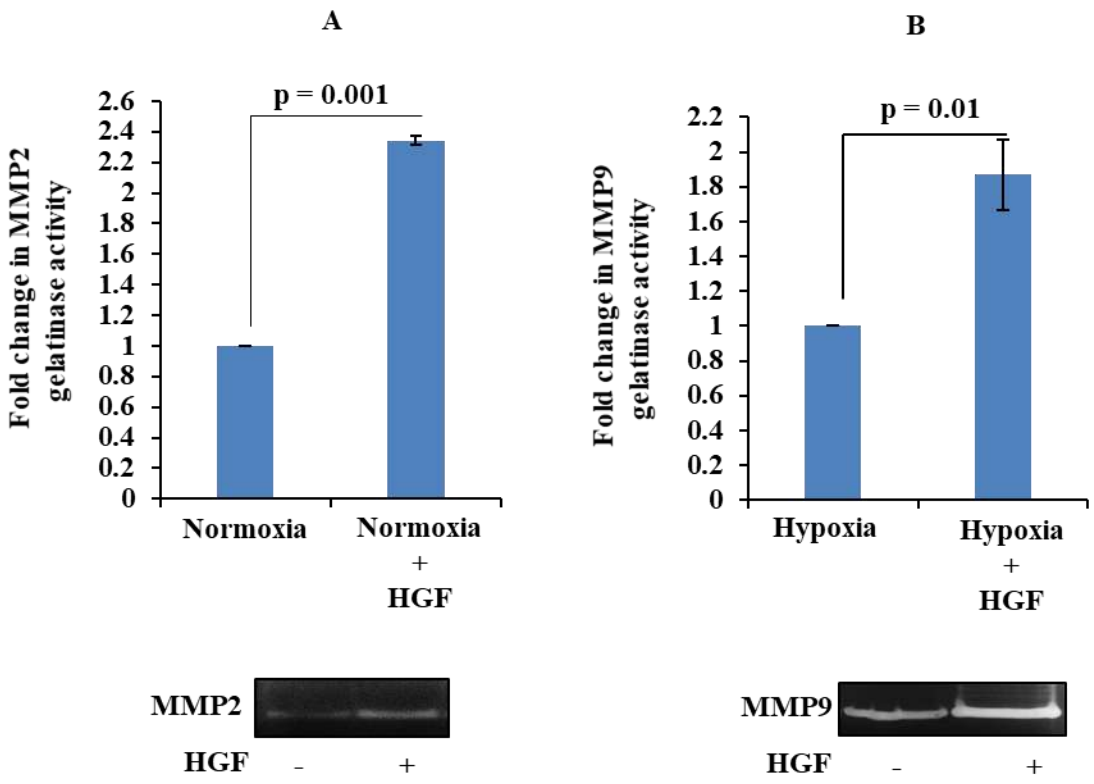

Fig 4.32 Expression profile of secreted MMPs by Zymography: HTR-8/SVneo cells (0.2 x 10^6/well) were grown overnight in 6-well culture plate under normoxia (20% O_2) at 37ºC with 5% CO_2 in a humidified incubator. Next day, cells were serum starved for 24 h, followed by treatment with HGF (50 ng/mL) for 24 h either under normoxic (20% O_2) or hypoxic (2% O_2) conditions. Culture supernatant were harvested and concentrated. Concentrated supernatant was analysed by gelatin Zymography as described in *Materials and Methods*. **Panel A** shows the densitometric analysis of MMP2 under normoxic conditions and **Panel B** shows densitometric analysis of MMP9 under hypoxic conditions. The values are expressed as mean ± *s.e.m* of three independent experiments. Representative zymogram are appended below.

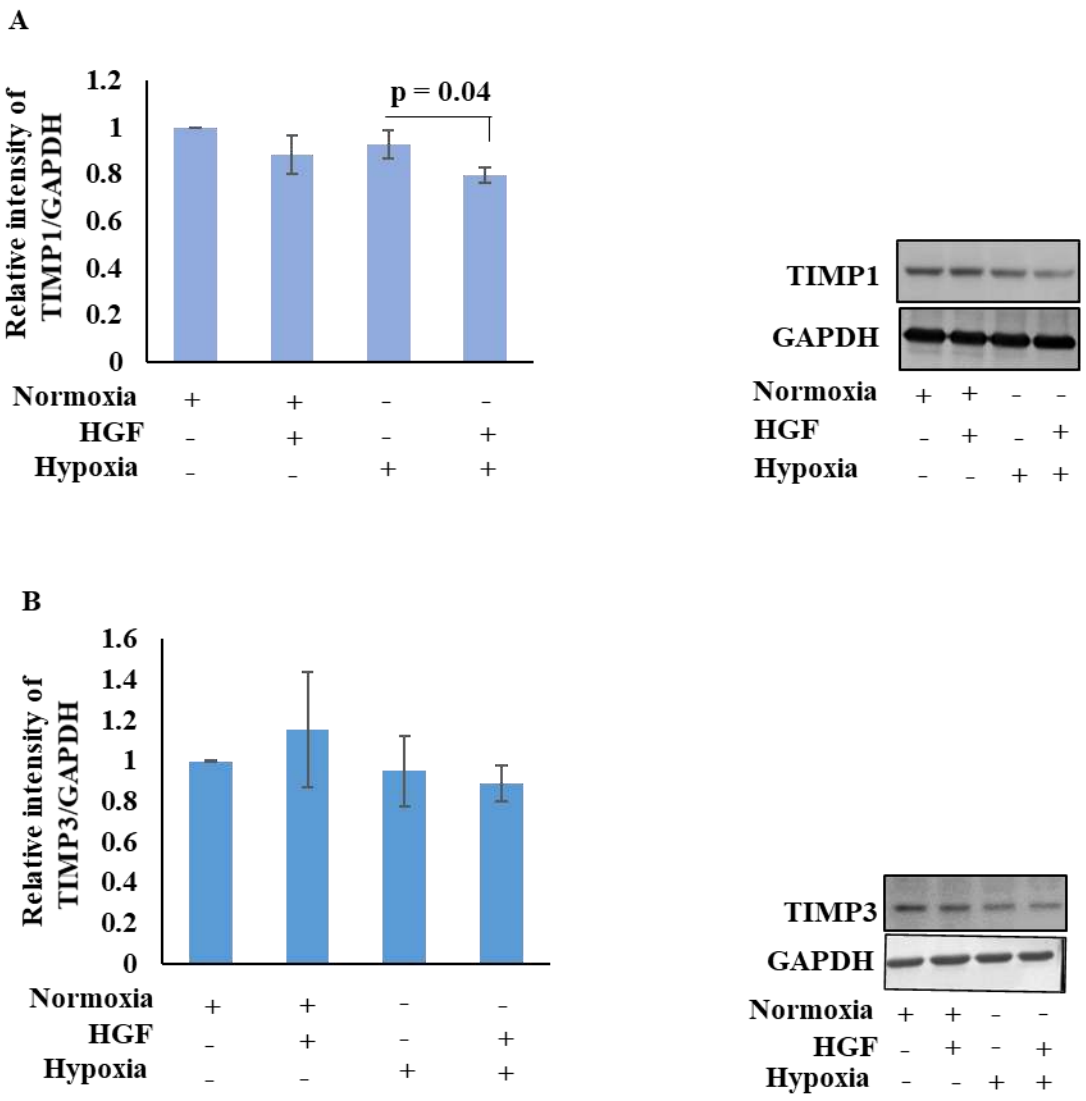

Fig. 4.33 Expression profile of TIMP1 and TIMP3 in HTR-8/SVneo cells treated with HGF under normoxic and hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were grown overnight in 6-well culture plate at 37ºC with 5% CO_2 in normoxic (20% O_2) conditions. Next day, cells were serum starved for 6 h, followed by treatment with HGF (50 ng/mL) for 24 h in normoxic as well as hypoxic (2% O_2) conditions. Subsequently, cell lysates were prepared to determine expression of TIMP1 and TIMP3 at protein level by Western blotting as described in *Materials and Methods*. **Panels A and B** show densitometric analysis and representative Western blots of TIMP1 and TIMP3 proteins in cell lysate. GAPDH was used as a loading control. Values are expressed as mean ± *s.e.m* of band intensity of three independent experiments..

as well as at protein level. Interestingly, analysis of MMPs *versus* TIMP1 ratios revealed a significant increase at the transcript levels in *MMP1*/*TIMP1*, *MMP2*/*TIMP1*, *MMP3*/*TIMP1* and *MMP9*/*TIMP1* ratio both in normoxic and hypoxic conditions subsequent to treatment with HGF as compared to respective untreated controls (Table 4.1). Moreover, at protein level significant increase in MMP2/TIMP1 ($p = 0.01$) and MMP3/TIMP1 ($p = 0.003$) ratio was observed in HGF treated cells under normoxia as compared to untreated control cells (Fig. 4.34A), while significant increase in MMP1/TIMP1 ($p = 0.0001$) and MMP9/TIMP1 ($p = 0.01$) ratio was observed in HGF treated HTR-8/SVneo cells as compared to untreated cells under hypoxic conditions (Fig. 4.34B).

MAPK and PI3K signaling pathways are involved in HGF-mediated HTR-8/SVneo cell migration/invasion

Under normal physiological conditions, HGF promotes trophoblast cells migration via activation MAPK and PI3K signaling pathways (Cartwright *et al*. 2002; Liu *et al*. 2012). However, under hypoxic condition, the importance of HGF and associated signaling pathways in trophoblast migration has not been fully elucidated. To determine, which signaling pathways were activated during HGF-mediated trophoblastic cells migration/invasion under hypoxic conditions, HTR-8/SVneo cells were serum starved in normoxic conditions and subsequently treated with and without HGF for 10, 30 and 60 min under hypoxic conditions. After each time point, cells were lysed and processed for Western blotting to determine ERK½ and Akt phosphorylation levels as described in *Materials and Methods*. Significant increase in pERK2 at 10 min and both pERK1 and pERK2 at 30 and 60 min were observed in cells exposed to hypoxia without HGF treatment (Fig. 4.35A). On the other hand, HTR-8/SVneo cells treated with HGF under hypoxic conditions, showed significant increase in pERK1 and pERK2 at all the time points as compared to untreated control. Time kinetics analysis for pERK½ revealed a maximal increase at 10 min (pERK1 ~4.8 fold; pERK2 ~3.0 fold) after HGF treatment

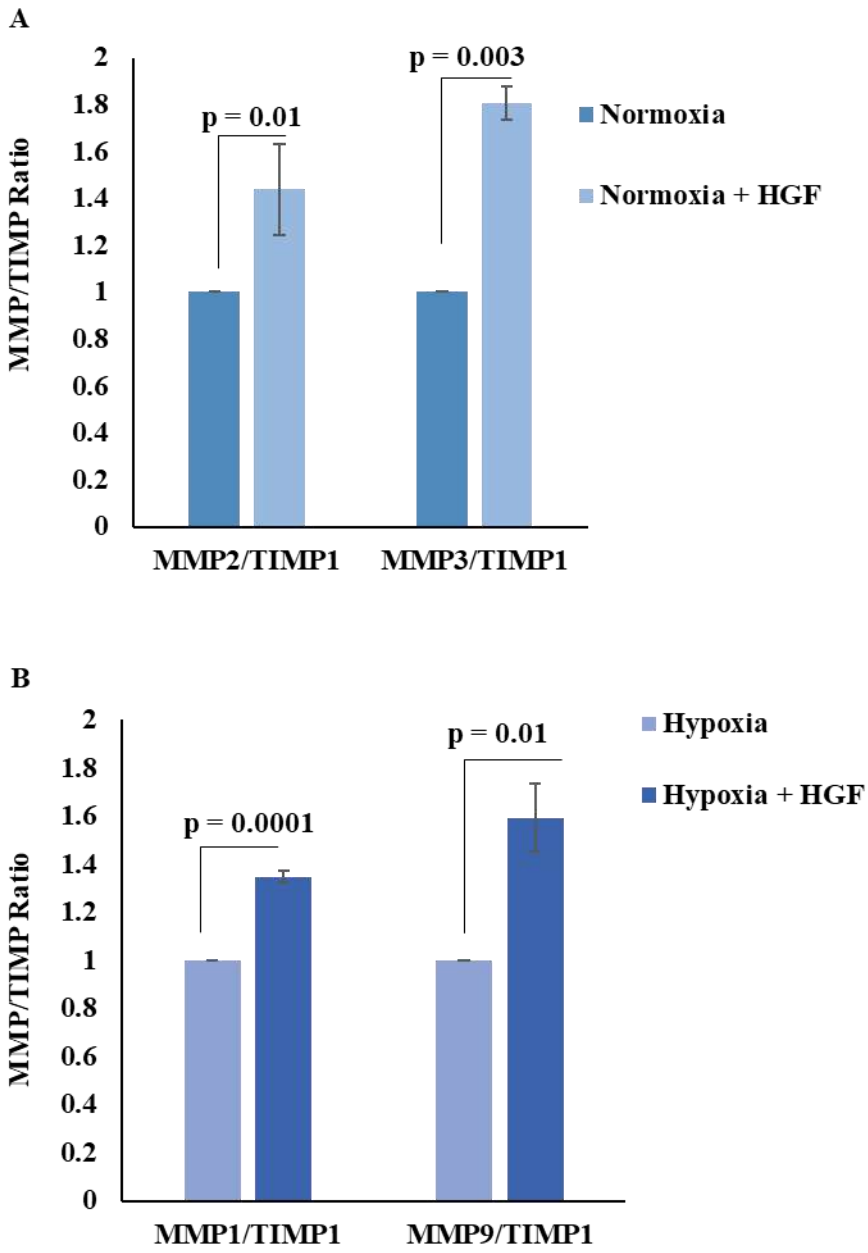

Fig. 4.34 MMP/TIMP ratio in HTR-8/SVneo cells treated with HGF: MMP/TIMP ratio was calculated from the densitometric analysis of Western blots of MMPs and TIMPs in untreated and subsequently treated with HGF cells under normoxic and hypoxic conditions. **Panel A** shows the bar graph of MMP2/TIMP1 and MMP3/TIMP1 ratio under normoxic conditions in untreated and HGF treated cells, whereas **Panel B** shows MMP1/TIMP1 and MMP9/TIMP1 ratio under hypoxic conditions. Values are expressed as ratio of mean ± *s.e.m* of three independent experiments. Value of HGF untreated cells were taken as 1.

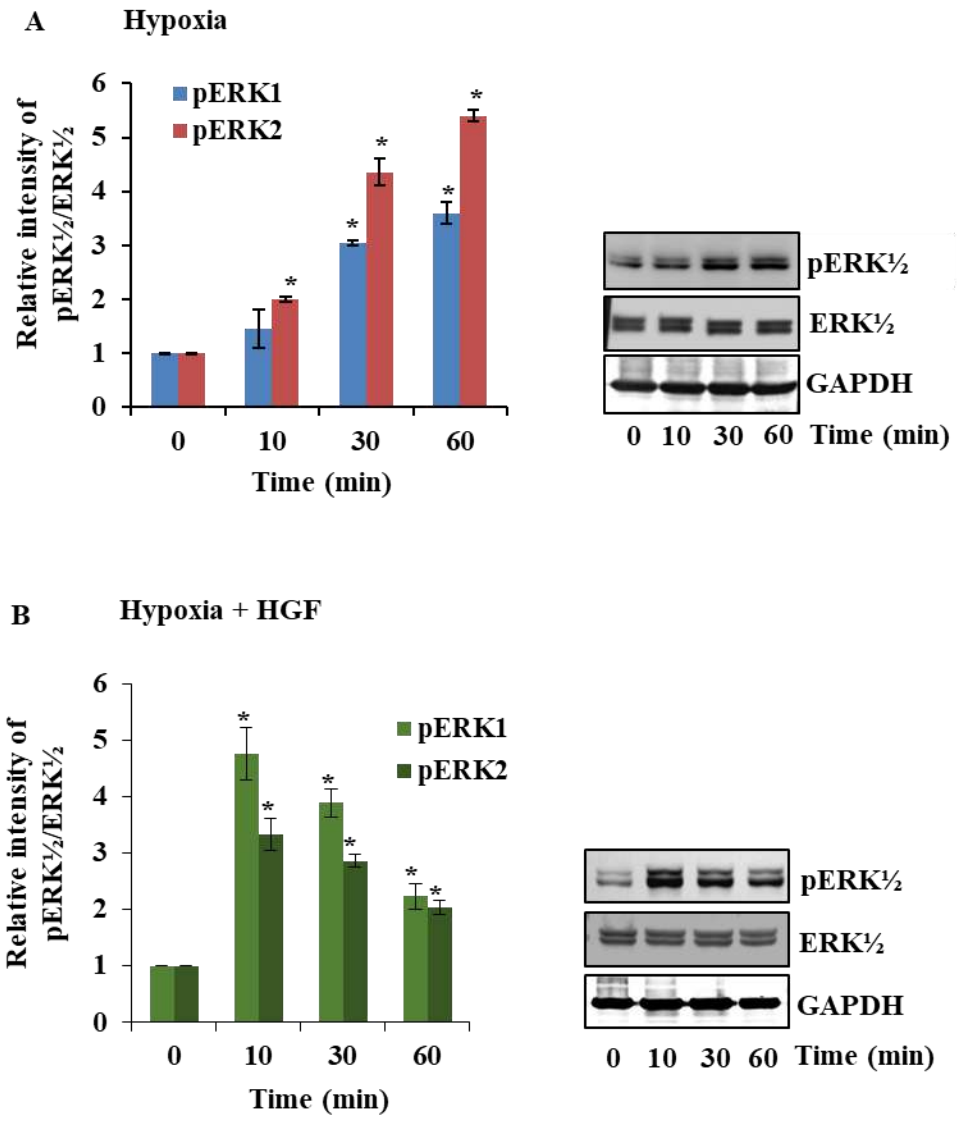

Fig. 4.35 Activation of MAPK signaling pathways in untreated and HGF treated HTR-8/SVneo cells under hypoxic conditions. HTR-8/SVneo cells (0.2 x 10^6/well) were seeded in 6 well culture plate under humidified conditions with 5% CO_2 at 37°C. Next day, cells were serum starved for 6 h in same culture conditions and incubated in the presence and absence of HGF (50 ng/mL) for varying time periods (10, 30 and 60 min) under hypoxic (2% O_2) conditions followed by Western blot analysis to determine activation of ERK½ as described in *Materials and Methods*. **Panel A** represents the densitometric plot showing the relative increase in phosphorylated ERK½ (pERK½) in untreated cells under hypoxia, with respect to control (0 min) as compared to total ERK½. **Panel B** represents the densitometric plot showing the relative increase in phosphorylated ERK½ (pERK½) in HGF treated cells with respect to control as compared to total ERK½. GAPDH was simultaneously developed as loading control for each experimental set. Representative blots of p-ERK½, ERK½ and GAPDH are appended alongside in panels **A** & **B.** The data is expressed as fold change with respect to control cells at 0 h and values are shown as mean ± *s.e.m* of at least three experiments.. * denotes $p \leq 0.05$ with respect to untreated control, which was considered statistically significant.

followed by gradual decrease till 60 min (Fig. 4.35B). The activation of MAPK signaling in presence of HGF under normoxic conditions has already been described in Chapter I of *Results and Discussion*.

In case of PI3K signaling pathways, under hypoxia, no significant changes in the phosphorylation level of Akt (Thr308) were observed (Fig. 4.36A). On the other hand, under similar conditions, HGF treatment of HTR-8/SVneo cells led to a rapid increase in level of pAkt (Thr308). Time kinetics studies revealed a sharp increase in pAkt (Thr308) expression level (~25 fold) within 10 min of HGF treatment as compared to untreated cells at 0 min. However, pAkt (Thr308) levels decreases from 10 min onwards, but still significantly higher as compared to untreated control (Fig. 4.36B). Treatment of HTR-8/SVneo cells with HGF under normoxia also led to a significant increase in pAkt (Thr308) at 10 min (Fig. 4.36C).

After confirmation of activation of MAPK and PI3K signaling in HGF treated HTR-8/SVneo cells, the next experiment was to determine, whether ERK½ and PI3K signaling have any role in HGF-mediated HTR-8/SVneo cells migration/invasion under hypoxic conditions. To prove this, cells were pre-treated with pharmacological inhibitors for MAPK (U0126) and PI3K (LY294002) signaling as per manufacturer's instructions and further used in Scratch wound migration assay in the presence and absence of HGF under hypoxic conditions as described in *Materials and Methods*. Significant ($p = 0.002$) decrease in migration was observed in HTR-8/SVneo cells, which were pre-treated with U0126 but subsequently treated with HGF for 24 h as compared to cells treated with HGF, but without U0126 pre-treatment (Fig. 4.37). Moreover, inhibition of PI3K signaling pathways by LY294002 also led to a significant ($p = 0.004$) decrease in the migration of HTR-8/SVneo cells treated with HGF as compared to cells that were not pre-treated with LY294002 but subsequently treated with HGF (Fig. 4.37). Besides migration, effect of pharmacological inhibitors of MAPK and PI3K was also studied during invasion of HTR-8/SVneo cells under normoxic and hypoxic conditions by

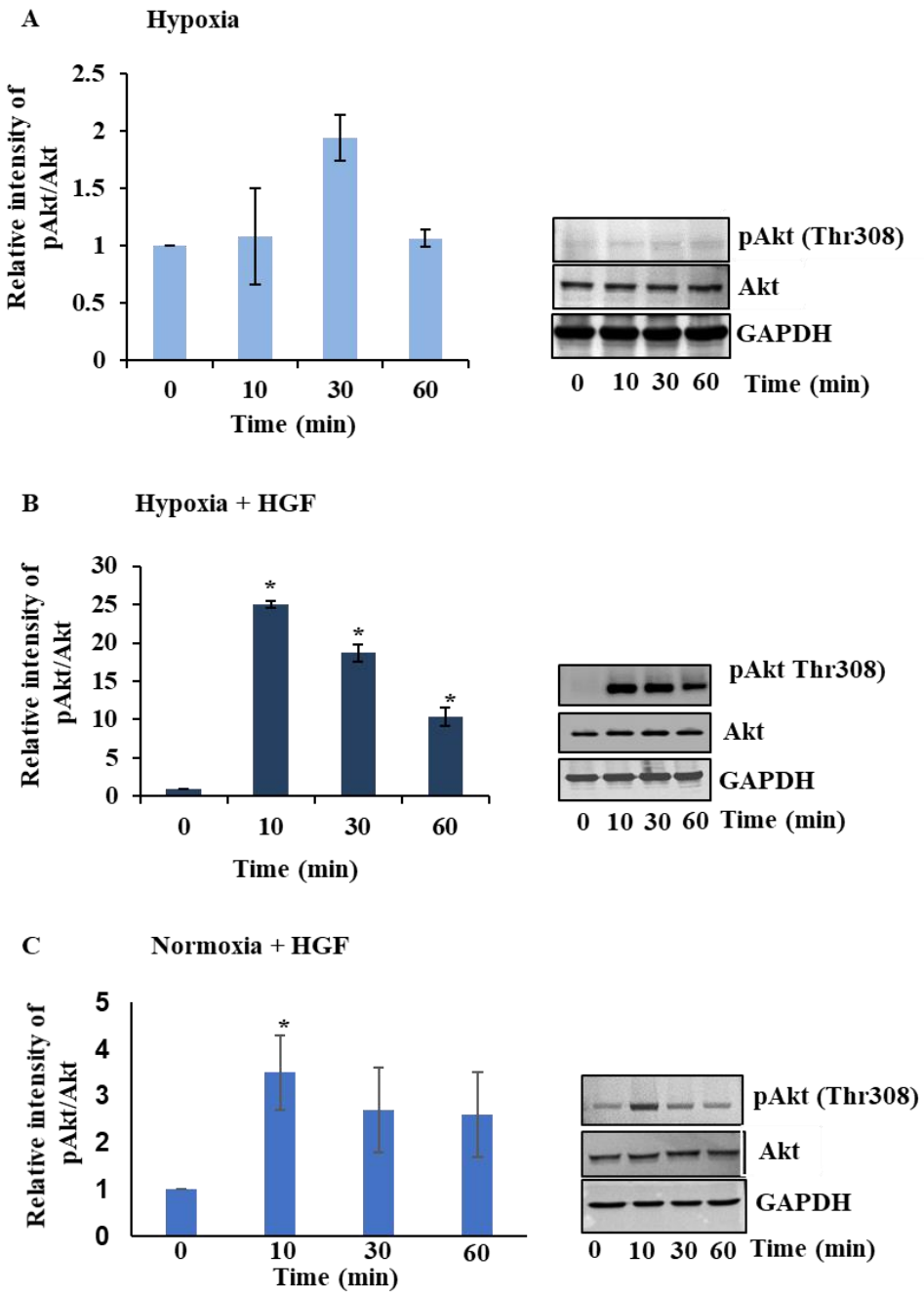

Fig. 4.36 Activation of PI3K pathway in HGF treated HTR-8/SVneo cells under normoxic and hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were cultured in 6-well plate for 24 h under normoxic conditions. Subsequently, cells were serum starved for 6 h, followed by treatment with and without HGF (50 ng/mL) for varying time periods (10, 30 and 60 min) under hypoxic (2% O_2) and normoxic (20% O_2) conditions. After treatment with HGF, cell lysate was prepared and processed to determine the activation of pAkt by Western blotting as described in *Materials and Methods*. **Panels A and B** represent the densitometric plot showing the relative increase in pAkt (Thr 308) in untreated and HGF treated cells, respectively, kept under hypoxic conditions with respect to 0 min as compared to total Akt. **Panel C** represents the densitometric plot showing the relative increase in pAkt (Thr308) in HGF treated cells with respect to untreated control at 0 min as compared to total Akt under normoxic conditions. GAPDH was included as loading control for each set of experiments. Representative blots of pAkt, Akt and GAPDH are shown alongside in **panels A, B** and **C.** The data is expressed as fold change with respect to cells at 0 min; values are shown as mean ± *s.e.m* of three independent experiments. * denotes $p \leq 0.05$ which was considered statistically significant as compared to untreated cells at 0 min.

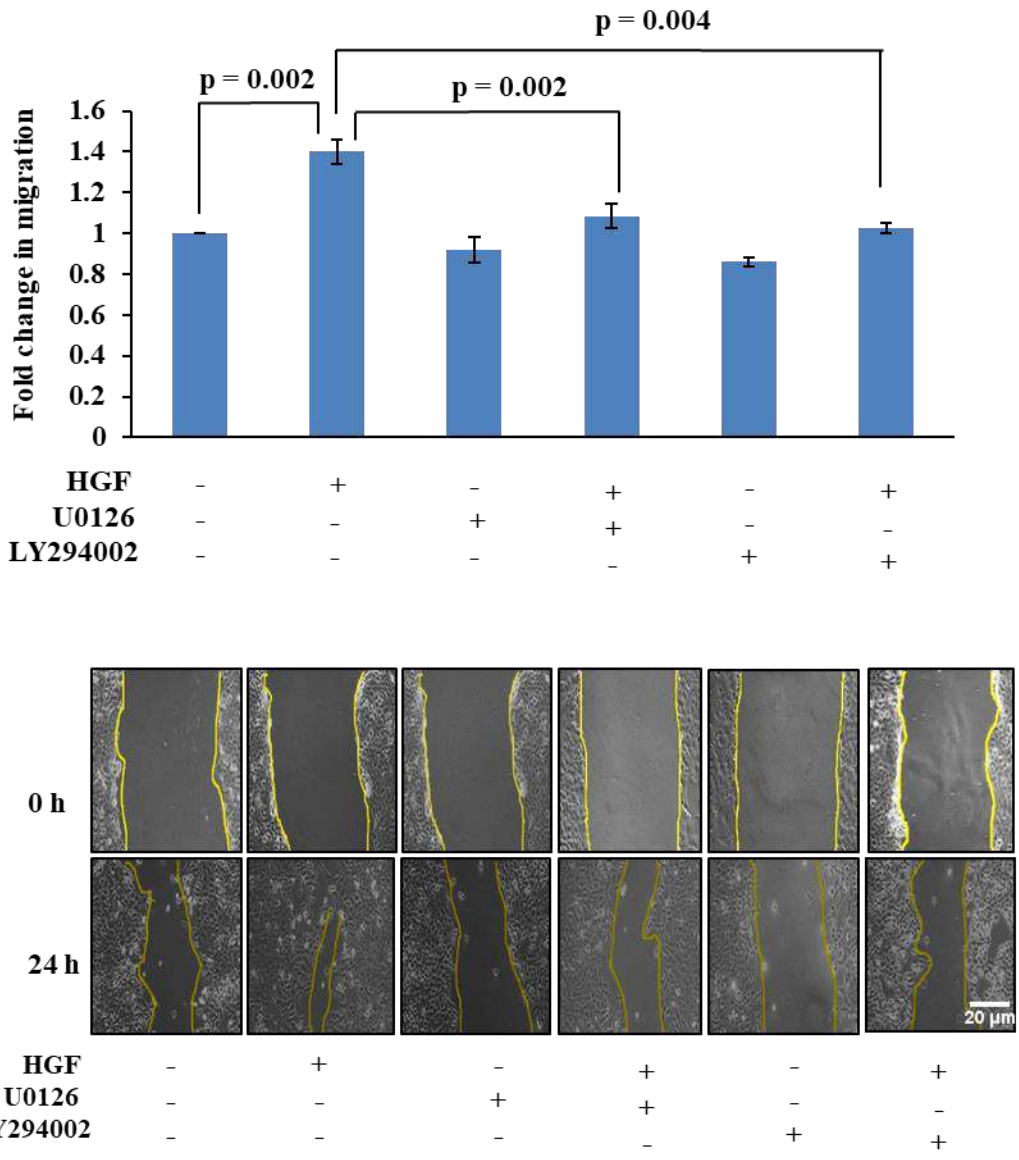

Fig. 4.37 Effect of inhibition of MAPK and PI3K signaling pathways on HGF-mediated HTR-8/SVneo cells migration under hypoxic conditions: HTR-8/SVneo cells (0.2 x 10^6/well) were cultured in 6-well culture plate at 37°C in 5% CO_2 under normoxic (2% O_2) conditions to form a monolayer. After formation of monolayer, cells were serum starved for 6 h followed by treatment with MAPK inhibitor U0126 (10 µm) or PI3K inhibitor LY294002 (10 µM), and subsequently used in wound healing migration assay under hypoxic (2% O_2) conditions in the presence or absence of HGF with appropriate controls as described in *Materials and Methods*. The bar graph show fold change in migration under different experimental conditions as compared to untreated control. Data is shown as mean ± *s.e.m* of three independent experiments. Representative images at 0 h and 24 h are also shown in the panel. Scale bar represents 20 µm.

Matrigel invasion assay. Inhibition of both MAPK and PI3K signaling led to a significant decrease ($p = 0.01$) in invasion of HTR-8/SVneo cells treated with HGF as compared to cells, which were not pre-treated with MAPK and PI3K inhibitors but subsequently treated with HGF under normoxic conditions (Fig. 4.38A). Similarly, significant decrease (MAPK, $p = 0.01$; PI3K, $p = 0.03$) in invasion of HTR-8/SVneo cells pre-treated with U0126 and LY294002 followed by treatment with HGF was also observed under hypoxia as compared to cells not pre-treated with U0126 and LY294002 but treated with HGF for 24 h (Fig. 4.38B). These findings suggest that both MAPK and PI3K signaling pathways have a role in HGF-mediated HTR-8/SVneo cells migration/invasion both under normoxic and hypoxic conditions.

HGF-mediated increase in HTR-8/SVneo cell migration under hypoxia involve up-regulation of HIF-1α

Under hypoxic conditions, HIF-1α protein stabilizes inside the cytoplasm and translocate towards nucleus, where it acts as a transcription factor and activates genes responsible for cell migration and invasion. Although, the expression of HIF-1α is constitutively maintained throughout pregnancy (Pringle *et al*., 2010), abnormal expression has been reported in preeclamptic placenta (Caniggia and Winter, 2002). To determine, whether HGF has any regulatory influence on HIF-1α, HTR-8/SVneo cells were serum starved and subsequently incubated under hypoxia for 24 h in the presence and absence of HGF as described in *Materials and Methods*. RT-qPCR analysis revealed a significant ($p = 0.003$) increase in transcript level of *HIF-1α* in HGF treated HTR-8/SVneo cells as compared to untreated control (Fig. 4.39A). In addition, significant increase ($p = 0.02$) in expression of HIF-1α at protein level was also observed in nuclear fraction of HGF treated cells (Fig. 4.39B). To further confirm the nuclear translocation of HIF-1α protein, immunofluorescence studies were carried out in the presence and absence of HGF under hypoxia as described in *Materials and Methods*. HTR-8/SVneo cells treated with HGF

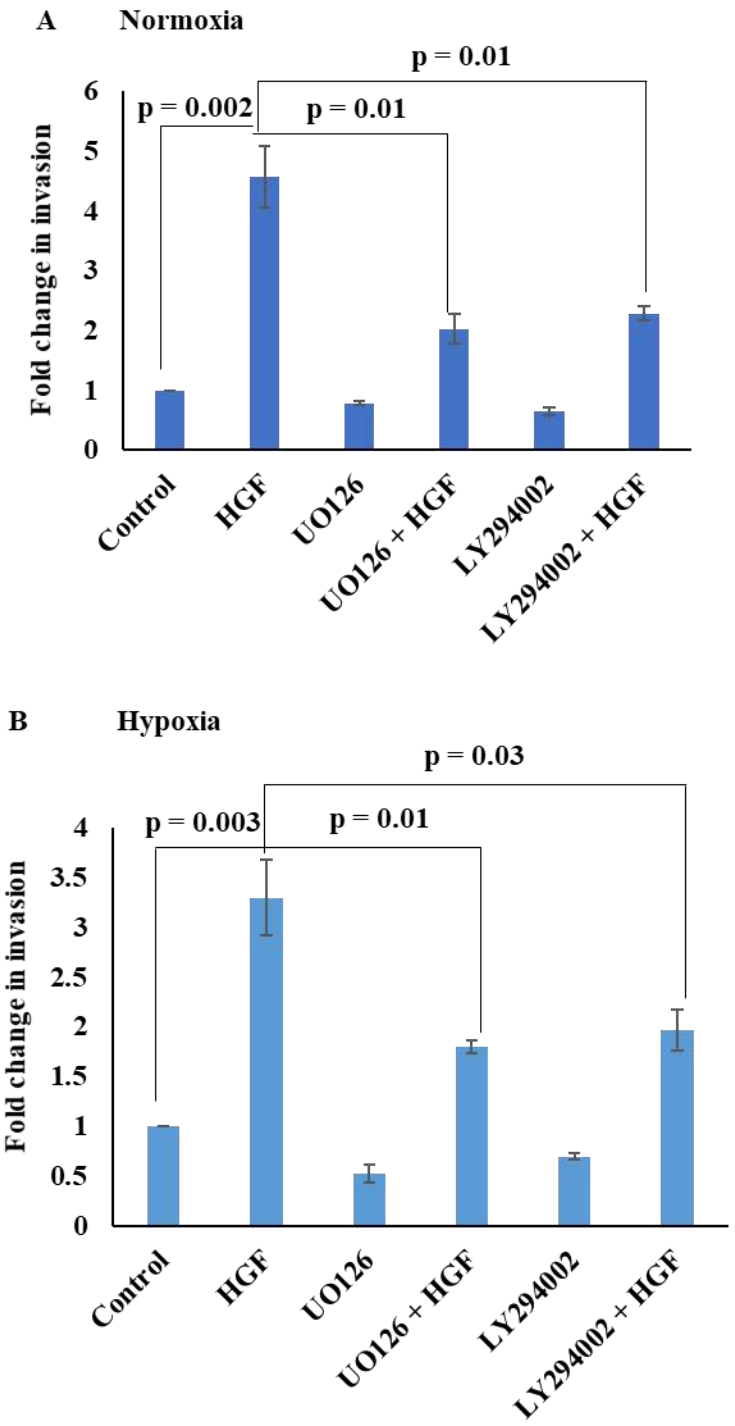

Fig. 4.38 Effect of inhibition of MAPK and PI3K signaling pathways on HTR-8/SVneo cells invasion after treatment with HGF under normoxic and hypoxic conditions: Following U0126 and LY294002 pre-treatment of HTR-8/SVneo cells, invasion assay was performed either in presence or absence of HGF (50 ng/mL) under normoxic (20% O_2) and hypoxic (2% O_2) conditions as described in *Materials and Methods*. **Panel A** shows relative fold change in invasion of varying treatment groups (HGF treated, U0126 pre-treated and U0126 pre-treated cells subsequently treated with HGF, LY294002 pre-treated and LY294002 pre-treated cells subsequently treated with HGF) as compared to the untreated HTR-8/SVneo cells under normoxic conditions. **Panel B** shows relative fold change in invasion of varying treatment groups (HGF treated, U0126 pre-treated and U0126 pre-treated cells subsequently treated with HGF, LY294002 pre-treated and LY294002 pre-treated cellssubsequently treated with HGF) as compared to the untreated HTR-8/SVneo cells under hypoxic conditions. Values are expressed as mean ± *s.e.m* of three independent experiments performed in duplicates.

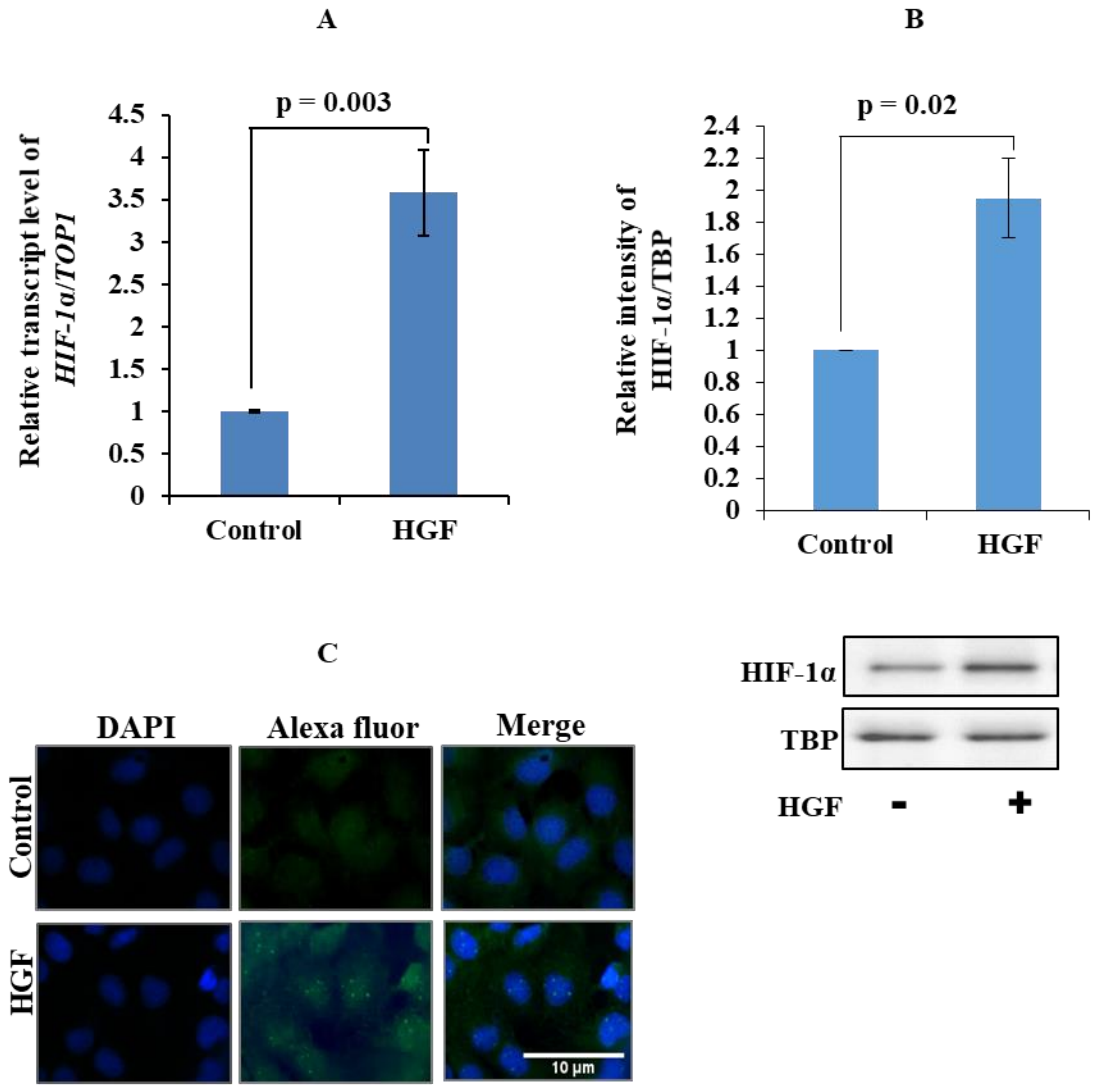

Fig. 4.39 HIF-1α expression and localization in HTR-8/SVneo cells treated with HGF under hypoxia: HTR-8/SVneo cells (0.2 x 10^6/well) were cultured in 6-well plate for overnight at 37°C in 5% CO_2 at 37°C under normoxic (20% O_2) conditions followed by serum starvation for 6 h. Subsequently, cells were treated in the presence and absence of HGF (50 ng/mL) for 24 h under hypoxic (2% O_2) conditions. After treatment, cells were processed to prepare cell lysate and transcript levels of *HIF-1α* was determined by RT-qPCR. In addition, cells were processed to prepare nuclear fraction to study HIF-1α expression profile by Western blotting. Further, in another set of experiment, HIF-1α localization in nucleus was detected by immunofloresence as described in *Materials and Methods*. **Panel A** shows transcript profile of *HIF-1α*, the bars represent relative intensity after normalization with *TOP1* as an internal loading control, and data is expressed as mean ± *s.e.m* of three different experiments performed in duplicates. **Panel B** shows protein profile of HIF-1α, the bar represents relative expression after normalize with TBP as an internal loading control and data expressed as mean ± *s.e.m* of three different experiment. Representative blots are appended below the graph. **Panel C** HTR-8/SVneo cells were cultured on coverslip and treated with HGF (50 ng/mL) for 24 h in hypoxic conditions followed by fixation in chilled methanol. The HIF-1α (green) was detected inside nucleus stained with DAPI as described in *Materials and Methods*. Scale bar represent 10 µm.

for 24 h showed higher HIF-1α protein localization (green dots) inside the nucleus (blue) as compared to cells without HGF treatment (Fig. 4.39C).

After confirmation of HGF role in stabilization of HIF-1α protein, its role in HGF induced migration of HTR-8/SVneo cells under hypoxic conditions were investigated. HIF-1α was knockdown by siRNA mediated gene silencing approach as described in *Material and Methods*. Silencing of HIF-1α was confirmed at protein level by Western blotting (Fig. 4.40A). Significant decrease in HIF-1α expression was observed in both untreated and HGF treated cells transfected with *HIF-1α* siRNA as compared to cells transfected with control siRNA treated with and without HGF treatment respectively. After confirmation of silencing, *HIF-1α* silenced HTR-8/SVneo cells were used in scratch wound migration assay to assess their migration potential. Significant ($p = 0.002$) reduction in migration was observed in HGF treated *HIF-1α* knockdown cells as compared to control siRNA transfected cells incubated with HGF for 24 h under hypoxic conditions (Fig. 4.40B). On the other hand, no significant change was observed in basal migration of HTR-8/SVneo cells transfected with *HIF-1α* siRNA as compared to control siRNA transfected cells incubated for 24 h without HGF treatment under hypoxic conditions (Fig. 4.40B).

HGF-mediated activation of MAPK and PI3K signaling pathways regulate HIF-1α expression under hypoxic conditions

From the above results, it is clear that both MAPK/PI3K signaling and HIF-1α transcription factor regulate the HGF-mediated migration of HTR-8/SVneo cells. To determine, whether the activation of MAPK and PI3K signaling pathways by HGF are also involved in up-regulation of HIF-1α expression, HTR-8/SVneo cells were pre-treated with MAPK inhibitor (U0126) and PI3K inhibitor (LY294002) as described in *Materials and Methods*. The efficacy of these inhibitors was confirmed by Western blotting. Interestingly, Western blots profile revealed significant ($p = 0.03$) decrease in

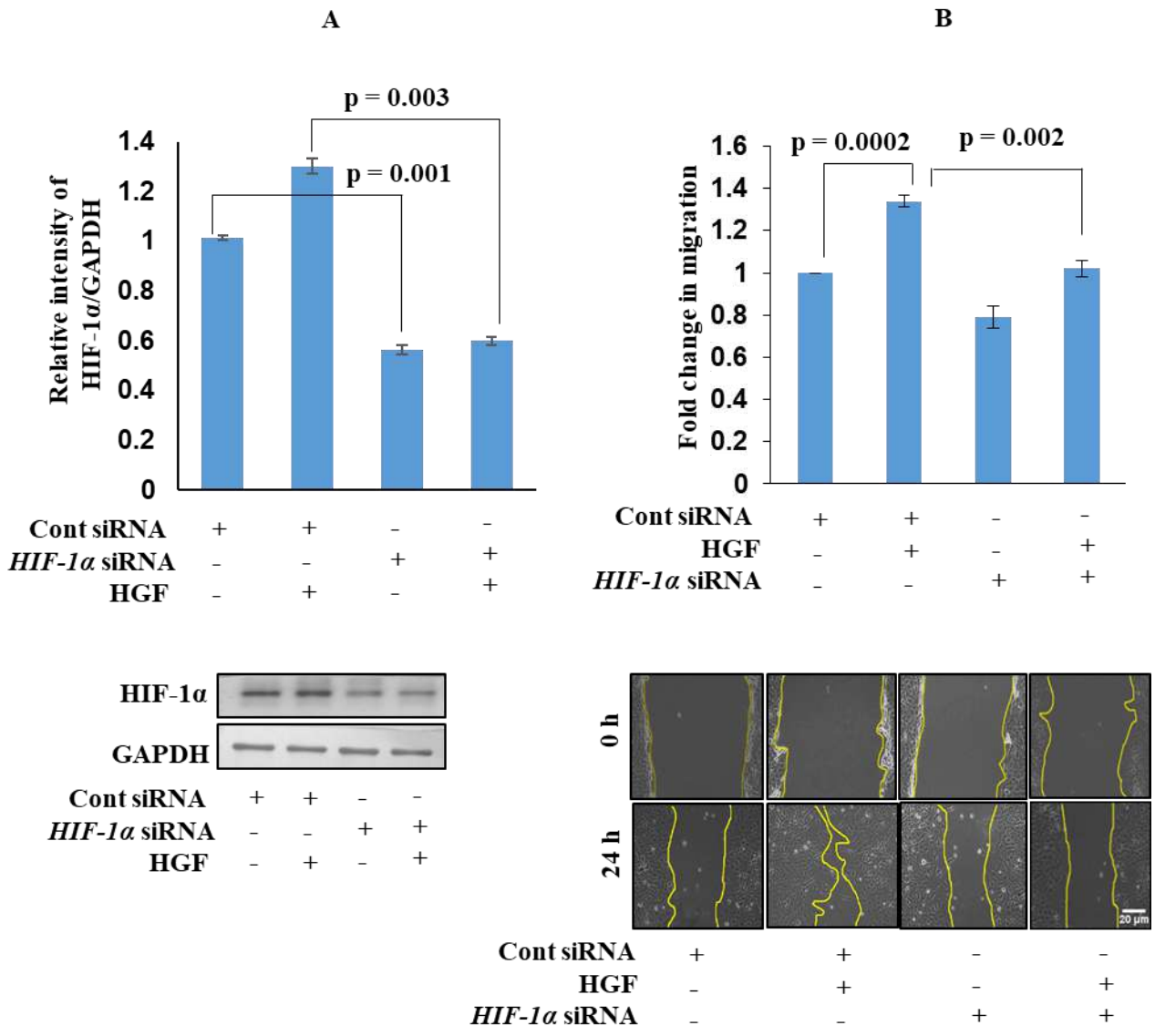

Fig. 4.40 Effect of *HIF-1α* silencing on HGF-mediated HTR-8/SVneo cells migration under hypoxic conditions: HTR-8/SVneo cells were transfected with control and *HIF-1α* siRNA. After 48 h of transfection, cells were used to study migration by scratch wound assay as described in *Materials and Methods*. Silencing of HIF-1α expression was confirmed by Western Blotting. **Panel A** shows densitometric analysis of relative intensity of HIF-1α expression in control siRNA and *HIF-1α* siRNA transfected cells after subsequent treatment with and without HGF. Values are expressed as mean ± *s.e.m* of three independent experiments. Representative blots are appended below. **Panel B** shows fold change in migration of cells transfected with *HIF-1α* siRNA as compared to control siRNA, subsequent to treatment with and without HGF (50 ng/mL) for 24 h. Representative images are appended below. Values are expressed as mean ± *s.e.m* of three independent experiments. Scale bar represent 20 µm.

HIF-1α expression in the nuclear fraction of cells pre-treated with U0126 and later treated with HGF as compared to HGF treated control cells without pre-treatment with MAPK inhibitor U0126 (Fig. 4.41). Similarly, a significant ($p = 0.04$) decrease in HIF-1α expression in cells pre-treated with PI3K inhibitor and subsequently treated with HGF (50 ng/mL) as compared to cells not pre-treated with LY294002 but treated with HGF for 24 h was also observed (Fig. 4.41). On the other hand, no significant changes were observed in basal expression of HIF-1α in HTR-8/SVneo cells pre-treated with either U0126 or LY294002 as compared to untreated counterparts under similar experimental conditions (Fig. 4.41). These results suggest that the expression and stabilization of HIF-1α is regulated by MAPK and PI3K signaling pathways during HGF-mediated migration of HTR-8/SVneo cells under hypoxic conditions.

Discussion

During early phase of pregnancy, physiological hypoxia plays a significant role in different embryonic processes, including placentation, angiogenesis and haematopoiesis (Dunwoodie 2009). The low O_2 environment at this stage of pregnancy is maintained by EVTs, which plug the lumen of spiral arteries to prevent maternal blood flow in the developing placenta, (Genbacev *et al*., 1997). However, non-physiological hypoxia at utero-placental interface is associated with pregnancy complications like preeclampsia (due to shallow invasion of EVTs) and IUGR (van Patot *et al*., 2012). Since, low levels of HGF have been reported in preeclamptic and IUGR patients (Wolf *et al*., 1991); decoding the role of HGF in trophoblast migration and associated signaling pathways under hypoxic conditions might reveal the molecular aspects of the above pregnancy complications. In the present study, HTR-8/SVneo cells exposed to 2% oxygen (hypoxic conditions) showed significant increase in migration/invasion as compared to cells incubated in presence of 20% oxygen (normoxic conditions) (Figs. 4.28, 4.29), which is in agreement with the previous studies done in trophoblastic cells (Wang *et al*., 2015; Zhu *et al.,* 2017). However, these studies employed different cell line as model i.e JEG3

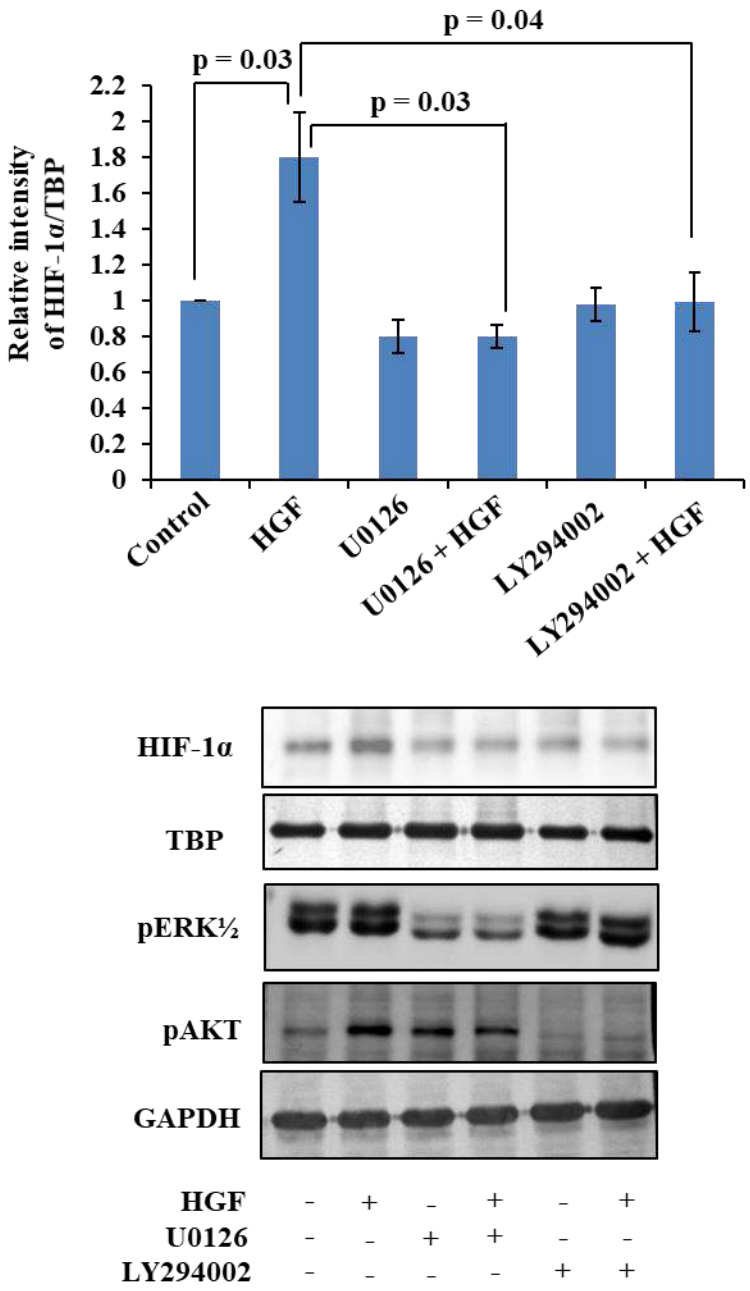

Fig. 4.41 Effect of inhibition of MAPK and PI3K signaling on HIF-1α expression in HGF-treated HTR-8/SVneo cells under hypoxia: HTR-8/SVneo cells (0.2 x 10^6/well) were cultured in 6-well plate for overnight at 37°C in 5% CO_2 under normoxic (20% O_2) condition followed by serum starvation for 6 h. Cells were pre-treated with U0126 (10 µm) and LY294002 (10 µm) for 2 h. Subsequently, cells were treated with/without HGF for 24 h under hypoxic (2% O_2) conditions. The expression of HIF-1α was checked by Western blotting as described in *Materials and Methods*. The bar graph showed the densitometric profiles of HIF-1α in untreated, U0126 and LY294002 pre-treated and further treated in the presence or absence of HGF. Data is shown as mean ± *s.e.m* of three independent experiments. Representative blots are appended below. Inhibition of MAPK and PI3K signaling was confirmed by down regulation of pERK½ and pAkt in the cells treated with U0126 and LY294002 respectively. TBP was used as nuclear loading control for normalization of HIF-1α expression, while GAPDH was used for normalisation of pERK½ and Akt expression.

and different migration assay system (Transwell assay). The increase in invasion as reported by other groups under hypoxic conditions was suggested to have occurred through up-regulation and activation of urokinase receptor expression and MMPs (Graham *et al*., 1998; Lash *et al*., 2007). In addition, in presence of HGF, significant increase in HTR-8/SVneo cells migration and invasion under both normoxic as well as hypoxic conditions was also observed (Figs. 4.28, 4.29). The increase in migration after HGF treatment under normoxic conditions has already been mentioned in previous chapter. It might be possible that increase in migration observed in HGF treated HTR-8/SVneo cells in present study, may involve activation of c-Met protein as reported by previous studies done in JEG3 cell line and glioblastoma cell lines under similar conditions (Hayashi *et al.,* 2005; Eckerich *et al*., 2007). Similarly, under normoxic conditions, c-met dependent HGF-mediated increase in invasion has been reported in trophoblastic cell line ED27 and pancreatic cell line COLO-357 (Kauma *et al*., 1999; Matsushita *et al*., 2007). Further, these studies suggest that higher expression of c-Met protein on the cell membrane might increase the sensitivity of these cells towards HGF, resulting in higher amplification of HGF signaling and subsequently increase in cell migration/invasion. Thus, in other words, we can summarize that hypoxia in synergy with HGF led to increase in HTR-8/SVneo cells migration/invasion.

MMPs play a crucial role during migration and invasion of different type of cells. The activity of these proteases is controlled by TIMP, which regulate the proteolytic cleavage of active domain of MMPs in the ECM. In general, trophoblastic cells express wide variety of MMPs, from MMP1 to MMP28 except MMP20 and MMP25 (Anacker *et al*., 2011). However, in the present study the role of four MMPs that is MMP1, MMP2, MMP3 and MMP9, which are widely known to be associated with migration/invasion of EVT cells has been studied. Significant increase in the expression of MMP1 both at transcript as well as proteins levels in HTR-/SVneo cells treated with HGF under hypoxic conditions was observed (Table 4.1, Fig. 4.30A). MMP1 also known as interstial collagenase degrades interstitial collagens type I, II and III and is abundantly expressed in

EVTs closer to decidua (Huppertz *et al*., 1998). Besides this, MMP1 also plays an important role in cytotrophoblast invasion, as lower levels of this protein has been reported in placental complications like preeclampsia and fetal growth restriction (FGR) (Lian *et al*., 2010; Deng *et al*., 2015). In present study, in absence of HGF, significant increase in MMP1 expression was also observed under hypoxia as compared to cells incubated under normoxia, which suggest that HTR-8/SVneo cells switch to MMP1, during migration and invasion under hypoxic conditions. Increase in expression of MMP9 both at mRNA and protein levels was also observed (Table 4.1, Fig. 4.31B) in HGF treated HTR-8/SVneo cells under hypoxic conditions. However, under normoxia, increase in expression of MMP2 and MMP3 was observed in HGF treated HTR-8/SVneo cells as compared to untreated cells (Figs. 4.30B, 4.31A). These results are in agreement with previous study, where increase in MMP2 expression under normoxic condition was observed, while no change in MMP9 expression was seen in HTR-8/SVneo cells after HGF treatment (Liu *et al*., 2012). Moreover, these results, further concur with the previous reports, where it has been shown that hypoxia favours secretion of MMP9 over MMP2 during trophoblast invasion as revealed in first trimester trophoblast cell lines (Onogi *et al*., 2011; Kobara *et al*., 2013). MMP3 also known as stremolysin-1 is known to be widely expressed in placental and trophoblast cultures (Maquoi *et al*., 1997). However, decrease in expression of MMP3 was observed in EVT cells located near spiral arteries of the placenta of preeclamptic women (Reister *et al.*, 2006), which suggest that MMP3 plays an important role in EVT migration and invasion. Besides HGF treatment, IL-1β also increases MMP3 expression levels and regulate trophoblast cells motility through up regulation of IGF/IFGBP network as reported by others (Coppock *et al*., 2004; Husslein *et al.*, 2009).

Apart from MMPs we also studied the expression of different TIMPs, however significant decrease in only TIMP1 was observed in HGF treated HTR-8/SVneo cells under hypoxic conditions (Fig. 4.33A). Imbalances in the MMP/TIMP ratios have been implicated in pregnancy disorders (Fortunato *et al*., 1999) and can be act as biomarker for

pregnancy' complications, as it is used in determining the severity of other diseases (Ferrer-Aguero *et al*., 2009). Previous study has reported higher level of MMP9:TIMP1 and MMP9:TIMP2 ratios in women with *preterm* labor as compared to *term* (Sundrani *et al*., 2017). In the present study, a significant increase in the ratio of MMP2:TIMP1 and MMP3:TIMP1 in HGF treated cells under normoxia and MMP9:TIMP1 and MMP1:TIMP1 in HGF treated cells under hypoxia was observed (Fig. 4.34A, 4.34B).

Previous studies have established the role of HGF in trophoblast migration through activation of MAPK and PI3K signaling pathways under normoxic condition (Cartwright *et al*., 2002). The role of MAPK signaling in HTR-8/SVneo cells treated with HGF under normoxic conditions was also reported in the previous chapter of the thesis. However, it is not clear which signaling pathways are involved in HGF-mediated increase in HTR-8/SVneo cells migration/invasion under hypoxic conditions. Here in this study, phosphorylation of both ERK½ and Akt proteins has been observed in response to HGF treatment under hypoxic conditions (Figs. 4.35B, 4.36B). In addition, gradual increase in pERK½ levels in untreated cells under hypoxia was also observed (Fig. 4.35A), which is in agreement with the previous findings reported in human microvascular endothelial cells-1 (HMEC-1) (Minet *et al*., 2000). A previous study has reported a significant increase in the levels of pAkt and p38, but no change in pERK½ levels in human ovarian carcinomas cells Hey-A8 (Xu *et al*., 2004). However, in the present study no significant increase in the expression of pAkt (Thr308) was observed in HTR/SVneo cells under hypoxia alone (Fig. 4.36A). This suggests that hypoxia mediated cell signaling pathways behave differently in cancer and trophoblastic cell lines. The activation of ERK1 phosphorylation was observed to be significantly higher than ERK2 in HGF treated HTR-8/SVneo cells, while ERK2 and not ERK1 was up regulated in HGF induced motility of non-small cell lung carcinoma (NSCLC) (Radtke *et al*. 2013). Thus it may be possible that phosphorylation of ERK1 may be more relevant in regulating HGF-mediated HTR-8/SVneo cells migration, while ERK2 control HGF-mediated increase in migration of cancer cells. The activation of both the ERK½ and PI3K signaling is important in HGF-

mediated migration/invasion of HTR-8/SVneo cells, as inhibition of MAPK by U0126 and PI3K pathway by LY294002 led to concomitant decrease in cell migration/invasion as compared to HTR-8/SVneo cells treated with HGF without pre-treatment of pharmacological inhibitor (Figs. 4.37, 4.38A, 4.38B).

It is well known that placenta responds to hypoxia through stabilization and increase in expression of oxygen sensitive transcription factors such as HIFs (Semenza and Wang 1992). The expression of HIF-1α increases during early gestation (5-8th weeks) in low oxygen concentration and gradually drops around 10-12th weeks of pregnancy with an increase in the placental oxygen level (Caniggia *et al*. 2000). Although HIF-1α is regulated by hypoxia in general, others factors like cytokines and hormones also regulate its expression under normoxic and hypoxic conditions. In the present study, a significant increase in expression of HIF-1α at protein level in the nucleus of HGF treated HTR-8/SVneo cells incubated under hypoxic conditions was observed in Western blot and immunofluorescence (Fig. 4.39). This is in agreement with previous studies, where increase in expression of HIF-1α in nuclear extract of HepG2 hepatoma cells treated with HGF under normoxia as well as in hypoxia has been reported (Tacchini *et al*., 2001, 2004). Further, upon silencing, 30% decrease in migration of HIF-1α knockdown cells treated with HGF was observed in contrast to HGF-treated control siRNA transfected cells under hypoxic conditions (Fig. 4.40B). In addition, in the present study it was further confirmed that the MAPK and PI3K signaling regulate the HGF-mediated increase in expression of HIF-1α in HTR-8/SVneo cells under hypoxic conditions. Significant decrease in expression of HIF-1α expression was observed in cells pretreated with U0126 and LY294002 and subsequently treated with HGF as compared to control cells treated with HGF only (Fig. 4.41). In contrast, previous study in HepG2 cells showed that the blockade of only PI3K pathway inhibited HGF-mediated HIF-1α expression, while MAPK inhibition has no effect on HIF-1α expression after HGF treatment (Tacchini *et al*., 2001). These observations suggest HGF-mediated regulation of HIF-1α expression varies differently in different cell types.

How HGF regulates the expression of different MMPs and TIMP under both normoxic and hypoxic conditions, along with activation of MAPK and PI3K signaling pathways, which in turn regulate expression of HIF-1α under hypoxic conditions, has been summarized in the form of schematic diagram in Fig. 4.42.

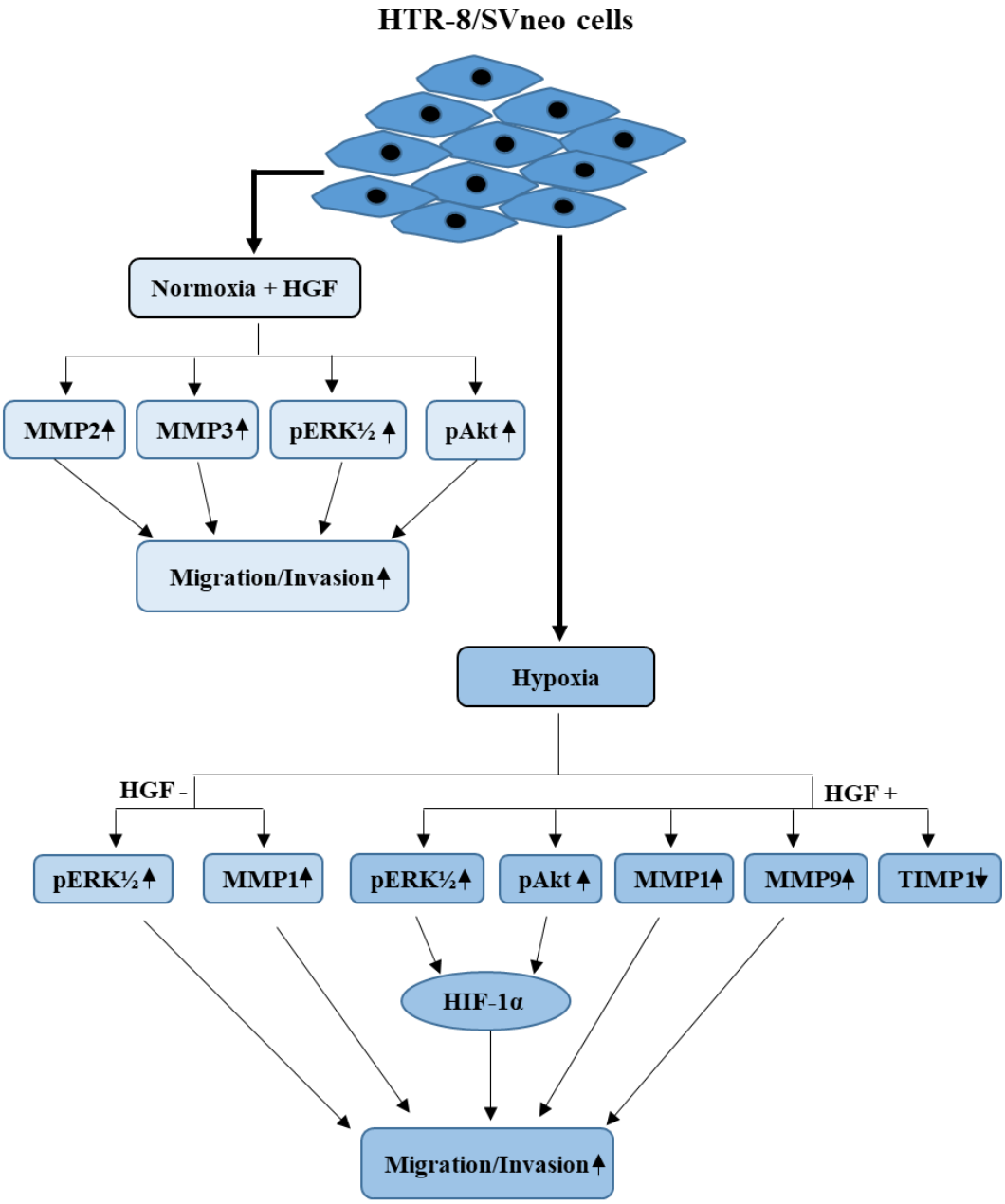

Fig. 4.42 HGF-mediated migration/invasion of HTR-8/SVneo cells under normoxic and hypoxic conditions: Schematic representation of the role of MMPs and MAPK/PI3K signaling pathways in HTR-8/SVneo cells migration/invasion following treatment with HGF under normoxic and hypoxic conditions. Treatment of HTR-8/SVneo with HGF under normoxia led to activation of ERK½ and Akt concomitant with increased expression of MMP2 and MMP3 which increase cell migration/invasion. Under hypoxia alone, increase in expression of MMP1 and activation of pERK½ were observed, which may be responsible for increase in migration/invasion at basal level. Treatment of HTR-8/SVneo cells with HGF under hypoxia led to an increase in expression of MMP1 & MMP9, downregulation of TIMP1 and activation of both ERK½ and Akt signaling pathways which may be responsible for further increase in migration/invasion as compared to cells without treatment with HGF under hypoxia. Activation of ERK½ & Akt under hypoxia after treatment with HGF additionally recruit HIF-1α in the nucleus that facilitate HTR-8/SVneo cells migration under hypoxia.

Summary

During embryo implantation, the inadequate migration and invasion of trophoblastic cells is one of the reasons for pregnancy related complications like preeclampsia and IUGR. Understanding the fundamentals of migration process, through delineating the signaling pathways and effector proteins might reveal the crucial insights for better understanding of molecular basis of these pregnancy's complications. In an attempt to understand the migration and invasion of trophoblastic cells, HTR-8/SVneo cell line was used as experimental model system. This cell line has been employed by several investigators to study migration and invasion under different experimental conditions. The trophoblast's cells migration process is precisely controlled by maternal factors and factors released and/or expressed by trophoblastic cells themselves such as integrins, E-cadherin, proteases, cytokines, and growth factors. In present thesis, the emphasis was given on HGF-mediated migration and invasion of trophoblastic cells under normoxic and hypoxic conditions. HGF also known as pleotropic cytokine is secreted by STB, EVT, endothelial and mesenchymal cells (Wolf *et al*., 1991). The importance of HGF during pregnancy was first suggested in clinical studies, where its level was found to be downregulated in IUGR placentae. HGF is known to stimulate migration and invasion of different trophoblastic cell lines by activation of MAPK and PI3K signaling pathways and upregulation of transcription factors (*hlx* & *hlx1*) and effectors proteins (MMP2, MMP9, iNOS; Cartwright *et al*., 2002; Rajaraman *et al*., 2010; Liu *et al*., 2012). In the current thesis, treatment of HTR-8/SVneo cells with HGF for 24 h led to a dose dependent increase in their migration in a scratch wound migration assay; and HGF at a concentration of 50 ng/mL showed maximum migration of these cells. Further, treatment of HTR-8/SVneo cells with optimized concentration of HGF (50 ng/mL) also led to activation of MAPK & PKA signaling pathways as observed by increase in the expression of pERK½ and pPKA. These results are in agreement with the previous studies.

During pregnancy, the expression of fourteen WNT ligands has been reported in first trimester placenta (Sonderegger *et al*., 2007); however, functional role of individual

WNT ligands has not been explored yet. In the present study, an effort was made to investigate the role of different WNT ligands in HTR-8/SVneo cell migration. Out of seven WNT ligands, the expression of WNT4 and WNT11 were found to be up regulated in both untreated as well as HGF treated HTR-8/SVneo cells. Further, silencing of WNT4 and WNT11 using siRNA led to a significant decrease in basal as well as HGF-mediated HTR-8/SVneo cell migration as compared to control siRNA transfected cells. Since integrin switching is also an important phenomenon, during trophoblast cell migration the expression of various integrin α and β chains was studied in presence and absence of HGF. The expression of ITGA2 and ITGAV was found to be higher as compared to α chain of other integrins studied. Silencing of both ITGA2 and ITGAV by siRNA, led to a significant decrease in basal as well as HGF-mediated migration of HTR-8/SVneo cells. On the other hand, no significant changes were observed amongst β chains of integrin on treatment with and without HGF, suggesting that β chain might have some different function during HGF-mediated migration of HTR-8/SVneo cells. Further, to find which combination of integrin α and β chain get activated in HGF mediated migration of HTR-8/SVneo cells, indirect immunofluorescence studies were done on the cells treated with and without HGF. The expression of integrin α2β1 and αVβ5 was found to be up regulated in HGF treated cells as compared to untreated control cells. Most of the previous studies have focused on activation of downstream signaling pathways upon activation of WNT ligands. However, in present thesis, the regulation of WNT ligands expression has been explored. Since, HGF led to an increased expression of WNT4 & WNT11 and activation of MAPK & PKA signaling, it was hypothesized that HGF might regulate the expression of WNT4 and WNT11 ligands through MAPK and PKA signaling pathways. To confirm the same, the expression of WNT4 and WNT11 was checked in cells pre-treated with pharmacological inhibitors U0126 and H89 of MAPK and PKA signaling respectively. Significant decrease in expression of only WNT11 was observed after HGF treatment in HTR-8/SVneo cells pre-treated with U0126 as compared to cells not pre-treated with U0126 but subsequently treated with HGF. Moreover, PKA inhibition by H89 led to a significant decrease in expression of both

WNT4 and WNT11 after treatment with HGF for 24 h, as compared to cells not pre-treated with H89, but later treated with HGF. Similarly, regulation of integrin expression during HGF-mediated migration of HTR-/SVneo cells was also assessed by pathways inhibition studies. MAPK pathway inhibition led to a significant decrease in expression of only ITGA2 in HGF treated HTR-8/SVneo cells as compared to cells treated with HGF, but not pre-treated with U0126. While on pre-treatment with H89, HTR-8/SVneo cells showed significant decrease in expression of both ITGA2 and ITGAV in cells treated with HGF as compared to cells not pretreated with H89 but subsequently treated with HGF. These results for the first time highlighted the importance of MAPK and PKA signaling pathways in the expression of WNT ligands and integrins.

Taking a cue from existing literature, an indirect cross-communication between WNT ligands and integrins expression in HGF-mediated trophoblastic cell migration was hypothesized. To establish the same, the expression of *WNT4* and *WNT11* transcripts were quantified by RT-qPCR in *ITGA2* and *ITGAV* silenced HTR-8/SVneo cells and *vice-versa*. It was observed that expression of *ITGA2* transcript was significantly decreased in HGF-treated-*WNT4* silenced cells, while no significant change in expression of *ITGA2* transcript was observed in HGF-treated *WNT11* silenced cells as compared to HGF-treated control siRNA transfected cells respectively. On the other hand, *ITGAV* expression was significantly compromised in both *WNT4* and *WNT11* silenced cells after treatment with HGF as compared to control siRNA transfected cells on HGF treatment. Similarly, on silencing of *ITGA2* and *ITGAV* by siRNA in presence of HGF, expression of both *WNT4* and *WNT11* transcripts were significantly reduced in HGF treated HTR-8/SVneo cells as compared control siRNA transfected cells subsequently treated with HGF. These observations give rise to a new paradigm in WNT and integrin signaling pathway as results shown here showed their interdependence. Previous studies have suggested the role of various interlinking proteins like β-catenin, FAK and ILK between WNT and integrin signaling pathways (Crampton *et al*., 2009; Wu *et al*., 2014; Du *et al*., 2016). HGF treatment of HTR-8/SVneo cell led to increase in expression of β-catenin

both in cytoplasm as well as in nucleus as revealed by Western blotting. Further, knockdown of β-catenin by siRNA also led to decrease in HGF-mediated migration of HTR-8/SVneo cells as compared to HGF treated control siRNA transfected cells. In present thesis, an effort was made to investigate the common denominator for both WNT and integrin pathways. It was found that on silencing of *WNT4/WNT11/ITGA2/*ITGAV by respective siRNAs, expression of β-catenin was significantly down regulated as compared to control siRNA transfected cells after HGF treatment. Although it is known that β-catenin is a downstream target for WNT signaling and also indirectly linked to integrin signaling, but, present findings further established that β-catenin can act as a connecting link between WNT and integrin signaling, as observed in present study in HGF-mediated migration of trophoblastic cells (Fig. 4.27).

Besides cytokines, growth factor and signaling pathways, oxygen tension also plays an important role during trophoblastic cell migration and invasion. During first trimester of pregnancy, low partial pressure of O_2 of 18-40 mmHg was maintained by EVT cells as they plug lumen of spiral arteries. Failure in invasion and migration of EVTs into spiral arteries, can lead to pregnancy complications like preeclampsia and IUGR (Poston and Raijmakers, 2004). Keeping this in view, HTR-8/SVneo cells migration and invasion was studied in presence and absence of HGF under hypoxic conditions. HTR-8/SVneo cells incubated under hypoxic (2% O_2) conditions showed increase in the migration in scratch wound migration assay and the invasion in matrigel invasion assay as compared to the cells incubated under normoxic (20% O_2) conditions. To rule out the possibility of proliferation under hypoxia, HTR-8/SVneo were pre-treated with Mitomycin-C. The results observed in present study are in agreement with the previous studies done in trophoblastic cells. The increase in migration and invasion observed under hypoxia was further enhanced after treatment with HGF as compared to untreated control cells. As mentioned earlier, the role of MMPs has been reported in previous studies during migration and invasion of trophoblastic cells under various physiological conditions. In present study, differential expression of MMPs was observed in HTR-8/SVneo cells

incubated in presence and absence of HGF under normoxic and hypoxic conditions. Western blot analysis revealed up regulation of MMP1 under hypoxia as compared to cells under normoxia without HGF treatment. However, in presence of HGF, expression of MMP2 and MMP3 under normoxia and MMP1 and MMP9 under hypoxia was up-regulated under normoxic and hypoxic conditions respectively as compared to cells incubated in absence of HGF under similar conditions. As TIMPs are known to be the regulator of MMPs, significant decrease in expression of only TIMP1 was observed under hypoxia alone. However, on calculating the MMP/TIMP ratio, significant increase in ratio of MMP2:TIMP1 & MMP3:TIMP1 under normoxia and MMP1:TIMP1 & MMP9:TIMP1 ratio under hypoxia was observed in HGF treated HTR-8/SVneo cells as compared to untreated cells under respective similar conditions. Thus it can be concluded that it is the MMP:TIMP ratio which determine the fate of migration and invasion during HGF-mediated migration of trophoblastic cells. Role of cell signaling pathways in presence of HGF was known in previous studies and also established by present thesis in first chapter; however, the role of signaling pathways under hypoxia and in presence of HGF was not established. To investigate the same, HTR-8/SVneo cells incubated under hypoxia, showed increased in phosphorylation of both ERK1 and ERK2 at various time points as compared to cells at 0 min. However, no significant change in expression of pAkt was found under similar culture conditions and time points, which suggested that MAPK is the main signaling pathway activated during hypoxia in HTR-8/SVneo cells. Further, in HGF treated HTR-8/SVneo cells, Western blot profiles revealed increase in phosphorylation of ERK½ at 10 min in both under normoxic as well as hypoxic conditions. Similarly, significant increase in pAkt levels was also observed both under normoxic and hypoxic conditions after 10 min of HGF treatment; however, increase in the fold change of pAkt was higher in cells incubated under hypoxic conditions as compared to normoxia in presence of HGF. To further correlate the activation of MAPK and PI3K signaling pathways by HGF with respect to trophoblastic cell migration and invasion, pathways inhibition studies were done using pharmacological inhibitors U0126 and LY294002. Inhibition of MAPK by U0126 led to a significant decrease in invasion

of HTR-8/SVneo cells treated with HGF as compared to cells not pre-treated with U0126, but subsequently treated with HGF under normoxia. Similarly, under hypoxic conditions, on pre-treatment with U0126, significant decrease was observed in HGF-mediated HTR-8/SVneo cells migration and invasion as compared to cells which were not pre-treated with U0126, but subsequently treated with HGF. In case of PI3K pathway inhibition by LY294002 under normoxia, a significant decrease was observed in invasion of HTR-8/SVneo cells treated with HGF as compared to cells not pre-treated with LY294002, but later treated with HGF. Furthermore, under hypoxia, blockade of PI3K signaling pathways significantly inhibited the HGF-mediated migration and invasion of HTR-8/SVneo cells as compared to control cells which were not pre-treated with LY294002, but subsequently treated with HGF. These observations highlighted the significance of MAPK and PI3K signaling pathways during HGF-mediated migration and invasion of trophoblastic cells.

The placenta responds to hypoxia through stabilization and increase in expression of HIFs. During pregnancy different cytokines and hormones also regulate the HIF-1α expression under different physiological conditions. To ascertain whether HIF-1α has a role in HGF-mediated migration of trophoblastic cells under hypoxia, HTR-8/SVneo cells were treated with HGF. Western blots and immunofluorescence data revealed an increase in HIF-1α expression inside the nucleus in HTR-8/SVneo cells treated with HGF as compared to untreated control under hypoxia. Further, HIF-1α silencing by siRNA led to reduction in HGF-mediated migration of HTR-8/SVneo cells under hypoxic conditions. In addition, in present study it was further confirmed that the MAPK and PI3K signaling regulate the HGF-mediated increase in expression of HIF-1α in HTR-8/SVneo cells under hypoxic conditions. From the observations, it can be concluded that treatment of HTR-8/SVneo cells with HGF under normoxia favours MMP2 and MMP3 expression and activation of MAPK and PI3K pathways, while in hypoxia increased expression of MMP1 & MMP9, downregulation of TIMP1, activation of both ERK½ &

Akt signaling pathways and transcription factor HIF-1α may be responsible for increase in migration/invasion of HTR-8/SVneo cells treated with HGF (Fig. 4.42).

To sum-up, in the present thesis, the role of WNT ligands and integrins during HGF-mediated migration of HTR-8/SVneo cells has been demonstrated. In addition, significance of MAPK and PKA signaling pathways in regulation of WNT ligands and integrin expression has also been verified. Further, the results presented herein also suggest cross-communication between WNT ligands and integrin through active participation of β-catenin. Besides this, role of MMPs during HGF-mediated migration and invasion of HTR-8/SVneo cells under normoxic and hypoxic conditions has been documented. In addition, the relevance of MAPK and PI3K pathways along with their regulatory control on HIF-1α expression has also been demonstrated in HGF-mediated HTR-8/SVneo migration under hypoxia.

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