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

Abstract

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Preeclampsia is a leading cause of maternal mortality and responsible for over 60,000 pregnant women deaths/year around the globe and also known to complicate 5% of all pregnancies. However, the percentage of preeclampsia is seven fold higher in developing countries like India as compared to other developed nations. According to revised definition of preeclampsia released by *International Society for the study of Hypertension in Pregnancy* (ISSHP), it is defined as hypertension developing after 20 weeks of gestation with one or more of the following: proteinuria, maternal organ dysfunction (including renal, hepatic, hematological, or neurological complications), or fetal growth restriction (FGR). Although, the symptoms appear in second trimester, the defective cellular events or processes occur during first trimester of pregnancy, are suggested to be the cause of preeclampsia. During the first trimester of pregnancy, interstial extravillous trophoblast (iEVT) and endovascular extravillous trophoblast (enEVT) arise from the extravillous trophoblast (EVT) lineage, migrate and invade the endometrium layer and remodel the uterine spiral arteries. Inadequate migration and invasion of iEVT and enEVT leads to defective placenta, which cause pregnancy complication like FGR, which later develop into preeclampsia and intrauterine growth restriction (IUGR). Thus, it is important to study the molecular mechanisms associated with trophoblast cells migration and invasion. Various cytokines, growth factors and chemokines, present at maternal-fetal interface, regulates the process of trophoblast cells migration and invasion. Among them, the expression of hepatocyte growth factor (HGF) is reported to be decreased in IUGR placenta. In addition to cytokines, differential expression of effector proteins like integrins and matrix metalloproteniases (MMPs) and oxygen tension also influence the migration and invasion of EVTs. Taking a cue from previous clinical studies, HGF was selected in present thesis to study the migration and invasion of trophoblastic cells. The objective of present thesis was to decipher the molecular signaling pathways and effector proteins in HGF-mediated trophoblastic cells migration under normoxic and hypoxic conditions, to better understand the pregnancy complications that may occur due to low HGF level. HTR-8/SVneo cell line, which is similar to first trimester EVTs, was used as an experimental model system. This cell line has been employed by several investigators to study migration and invasion under different experimental conditions. In the present study, treatment of HTR-8/SVneo cells with varying concentration of 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. In addition, treatment of HTR-8/SVneo cells with optimized concentration of HGF (50 ng/mL) also led to activation of mitogen activated protein kinase (MAPK) and protein kinase A (PKA) signaling pathways as observed by increase in the expression of pERK½ and pPKA by Western blotting.

The expression of fourteen WNT ligands has been reported in first trimester placenta. The transcript profile of seven WNT ligands was studied by quantitative reverse transcription polymerase chain reaction (RT-qPCR) during trophoblastic cell migration; however, the expression of WNT4 and WNT11, at transcript as well as protein level was found to be up-regulated at 24 h in both untreated as well as HGF treated HTR-8/SVneo cells. Further, siRNA mediated silencing of WNT4 and WNT11, led to a significant decrease in basal as well as HGF-mediated HTR-8/SVneo cell migration as compared to control siRNA transfected cells. In addition, transcript and protein levels of various integrin α and β chains was also 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 using 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, indirect immunofluorescence revealed increase in expression of integrin α2β1 and αVβ5 in HGF treated cells as compared to untreated control cells. Inhibition of MAPK signaling with pharmacological inhibitors U0126, led to decrease in expression of WNT11 in HGF treated HTR-8/SVneo cells as compared to cells not pre-treated with U0126 but subsequently treated with HGF. Moreover, PKA inhibition by H89 also 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. MAPK signaling pathway inhibition also led to a significant decrease in expression of 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.

To study the cross-communication between WNT ligands and integrins, the expression of *WNT4* and *WNT11* transcripts were quantified by RT-qPCR in *ITGA2* and *ITGAV* silenced HTR-8/SVneo cells and *vice-versa*. The expression of *ITGA2* transcript was significantly decreased in HGF-treated-*WNT4* 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, 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. HGF treatment of HTR-8/SVneo cells also 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. To investigate the common denominator for both WNT and integrin pathways, silencing of *WNT4/WNT11/ITGA2/*ITGAV was carried out by respective siRNAs. The expression of β-catenin was significantly down regulated in *WNT4/WNT11/ITGA2/*ITGAV silenced cells as compared to control siRNA transfected cells after HGF treatment. In conclusion, the present study highlighted for the first time, the importance of MAPK and PKA signaling pathways in the expression of WNT ligands and integrins. In addition, cross-communication between WNT ligands and integrin shown here give rise to a new paradigm in WNT and integrin signaling pathways. Moreover, the connecting link between WNT and integrins signaling, in the form of β-catenin has also been established during HGF-mediated migration of trophoblastic cells.

In order to study the role of hypoxia, 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. The increase in migration and

invasion observed under hypoxia was further enhanced after treatment with HGF as compared to untreated control cells. In present study, differential expression of MMPs were 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 were up-regulated under normoxic and hypoxic conditions respectively, as compared to cells incubated in absence of HGF under similar experimental conditions. Significant decrease in expression of TIMP1 was observed under hypoxia alone. In addition, significant increase in ratio of MMP2:TIMP1 & MMP3:TIMP1 under normoxia and MMP1:TIMP1 & MMP9:TIMP1 under hypoxia was observed in HGF treated HTR-8/SVneo cells as compared to untreated cells under respective experimental conditions. To decipher the signaling pathways, HTR-8/SVneo cells incubated under hypoxia showed increase 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 led to a significant decrease 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.

Further, to study the role of hypoxia inducible factor-1α (HIF-1α) in HGF-mediated migration of trophoblastic cells, HTR-8/SVneo cells were treated with HGF under hypoxia. 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 cells under hypoxia. Further, HIF-1α silencing using siRNA led to a significant decrease 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. In conclusion, 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 their migration/invasion.