IDENTIFICATION OF NOVEL NOTCH TARGET GENES THAT ARE MEDIATORS OF NOTCH IN INDUCING EPITHELIAL TO MESENCHYMAL TRANSITION AND MIGRATION/INVASION

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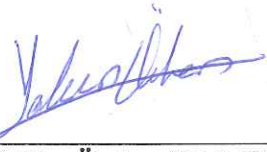İzmir Institute of Technology

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ABSTRACT

IDENTIFICATION OF NOVEL NOTCH TARGET GENES THAT ARE MEDIATORS OF NOTCH IN INDUCING EPITHELIAL TO MESENCHYMAL TRANSITION AND MIGRATION/INVASION

Notch signaling has first been described in murine mammary gland by the proviral integration of mouse mammary tumor virus (MMTV) into the Notch4 locus (*Int3* locus) which resulted expression of constitutively active form of Notch4 and transformation of mammary epithelial cells. Notch1 is highly expressed in breast cancer and constitutively active form of Notch1 induces neoplasm. In breast cancer, overexpression of active Notch1 receptor (NICD) promotes epithelial-mesenchymal transition (EMT) via Snail induction which demonstrates the role of Notch signaling in induction of metastasis through EMT. However, the downstream mediators of Notch in EMT, migration and invasion processes are still elusive. In this study, we hypothesized that Notch signaling induces EMT and migration via regulating one or more of the seven candidate genes that are SEMA6D, SEMA3C, CXCR7, CXCL14, CCL20, HMGA2 and CYR61 which were shown to be differentially regulated by Notch signaling in breast cells in microarray data. The candidate genes are involved in EMT and migration in different cell types and tissues.

We showed that Notch1 activation in normal breast epithelial cell line MCF10A significantly increased both mRNA and protein expressions of SEMA6D and CYR61 while it significantly reduced SEMA3C and HMGA2 mRNA levels. Notch inhibition led to significant reduction in mRNA expression of CYR61, CCL20 and HMGA2 and protein expression of CYR61 only, while the rest of candidate genes were affected slightly in breast cancer cell line, MDA-MB-231. We chose SEMA6D for further investigation because there is no data indicating the role of SEMA6D in breast cancer in the literature. SEMA6D could be mediator of Notch signaling to induce EMT because it partially rescues negative effect of Notch inhibition on EMT markers. Notch independent effect of SEMA6D suggested that SEMA6D may be involved in inhibiting EMT whereas, it induced migration and cell viability in MDA-MB-231 cell line. Further analysis is required to reveal the role of SEMA6D in EMT and migration.

ÖZET

NOTCH YOLAĞININ EPİTELYAL-MEZENKİMAL GEÇİŞTE VE MİGRASYON/İNVAZYON TETİKLEMEDE ARACI OLARAK KULLANILDIĞI YENİ HEDEF GENLERİNİN TANIMLANMASI

Notch yolağı'nın meme kanserindeki rolü ilk olarak MMTV (Mouse mammary tumor virus) virüsünün Notch4 lokusuna (*Int3* lokus) entegre olup yolağın sürekli olarak aktif olmasına ve buna bağlı olarak meme tümörü oluşumuna yol açmasıyla ortaya çıkmıştır. Meme kanseri hasta örneklerinde aktif Notch1 ifadesinin fazla olduğu ve Notch1'in sürekli aktivasyonunun neoplazm oluşumuna yol açtığı gözlenmiştir. Meme kanserinde, Notch1 almacının aşırı ekspresyonunun epitelyal-mezenkimal geçişi (EMG), Snai1 üzerinden tetiklediği gösterilmiştir. Fakat Notch yolağının EMG, migrasyon ve invazyonu gerçekleştirmek için hangi alt mekanizmaları kullandığı henüz tam olarak anlaşılmamıştır. Bu çalışmada, Notch'a bağlı EMG, migrasyon ve invazyona yol açan yeni Notch hedef genlerinin tanımlanması amaçlanmıştır. Mikrodizi analizlerinden faydalanılarak, Notch ile ilgisi bilinmeyen fakat, metastazla ilgili çalışmalarda yer alan yedi gen aday olarak belirlenmiştir. Normal meme hücrelerinde, Notch aktivasyonuna bağlı olarak aday genlerin ifadelerinin değiştiği gösterilmiştir. Bu genler, SEMA3C, SEMA6D, CXCL14, CXCR7, CCL20, CYR61 ve HMGA dır.

MCF10A normal meme epitel hücre hattında, Notch1 aktivasyonu SEMA6D ve CYR61 mRNA ve protein ifadelerinde anlamlı artışa sebep olurken, HMGA2 ve SEMA3C genlerinin ifadesinde anlamlı azalışa sebep olmuştur. MDA-MB-231 meme kanseri hücre hattında, Notch yolağının inhibasyonu CYR61, CCL20 ve HMGA2 mRNA ifadelerinde ve sadece CYR61 protein ifadesinde anlmalı azalışa sebep olurken, diğer aday genlerinin ifadesinde çok az değişme sebep olmuştur. Bu geneler arasından SEMA6D daha ileri ki araştırmalar için seçilmiştir çünkü literatürde SEMA6D'nin meme kanserindeki rolünü gösteren bir çalışma yoktur. SEMA6D'nin Notch inhibasyonunun EMG belirteç molekülleri üzerindeki negatif etkisini kısmen kurtardığı görülmüştür ve Notch yolağının, EMG ve migrasyonu tetiklemek için SEMA6D yi aracı olarak kullanıyor olabileceği gösterilmiştir. SEMA6D'nin Notch sinyalinden bağımsız etkisi araştırılmıştır ve SEMA6D'nin MDA-MB-231 meme kanseri hücrelerinin yaşama kapasitesini anlamlı artırdığı ve migrasyonu tetiklediği gözlemlenmiştir.

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CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Breast cancer is the most commonly diagnosed cancer type and the second leading cause of cancer related deaths after lung and bronchus cancer in women world-wide in spite of improvements in early detection and treatment strategies. Treatment of breast cancer can vary depending on tumor characteristics such as size of tumor and extent of spread. Treatment usually includes mastectomy (surgical removal of the breast), surgical removal of the tumor and surrounding tissue, radiotherapy, chemotherapy, hormonal therapy and targeted therapy. Women having breast cancer that is positive for hormone receptors benefit from hormonal therapy and also breast cancer patients who overexpress HER2 (growth promoting protein) can be treated with targeted therapy (American Cancer Society, 2016). 5-year survival rates for localized and regional (spread to regional lymph nodes) cases are 61% and 31% respectively while it decreases to 6% for breast cancer that has metastasized or metastatic breast cancer (National Cancer Institute, 2009). Therefore, the main cause of breast cancer related deaths is not the primary tumor itself, but its metastases at distant sites. It is important to identify prognostic markers in order to predict the risk of metastasis development accurately in patients because only approximately half of 80% breast cancer patients who receive adjuvant chemotherapy show recurrence and die of metastatic breast cancer. Thus, many patients will be over-treated and suffer the diverse side-effects of chemotherapy unnecessarily (Weigelt et al. 2005).

1.2. Tumor Metastasis

Metastases is the main cause of cancer mortality in cancer patients due to resistance of spreading tumor cells to existing therapies. The development of metastases from primary tumors is a multi-step process known as the invasion metastatic cascade

which includes dissemination of cancer cells to distant organs followed by adaptation to foreign microenvironment and colonization. Accumulation of genetic and epigenetic changes within tumor cells drive the metastatic cascade. Invasion-metastatic cascade includes (1) local invasion of tumor cells into extracellular matrix (ECM) and stromal cell layer, (2) transendothelial migration of tumor cell into blood vessels called intravasation, (3) survival in the blood stream, (4) arrest at distant organ, (5) extravasation, (6) survival in the foreign microenvironment to form micro-metastases and (7) subsequent proliferation and colonization (Fidler 2003) (Figure 1.1). In addition to interaction within cancer cells, communication between cancer cells and non-neoplastic stromal cells also play crucial role in metastatic cascade.
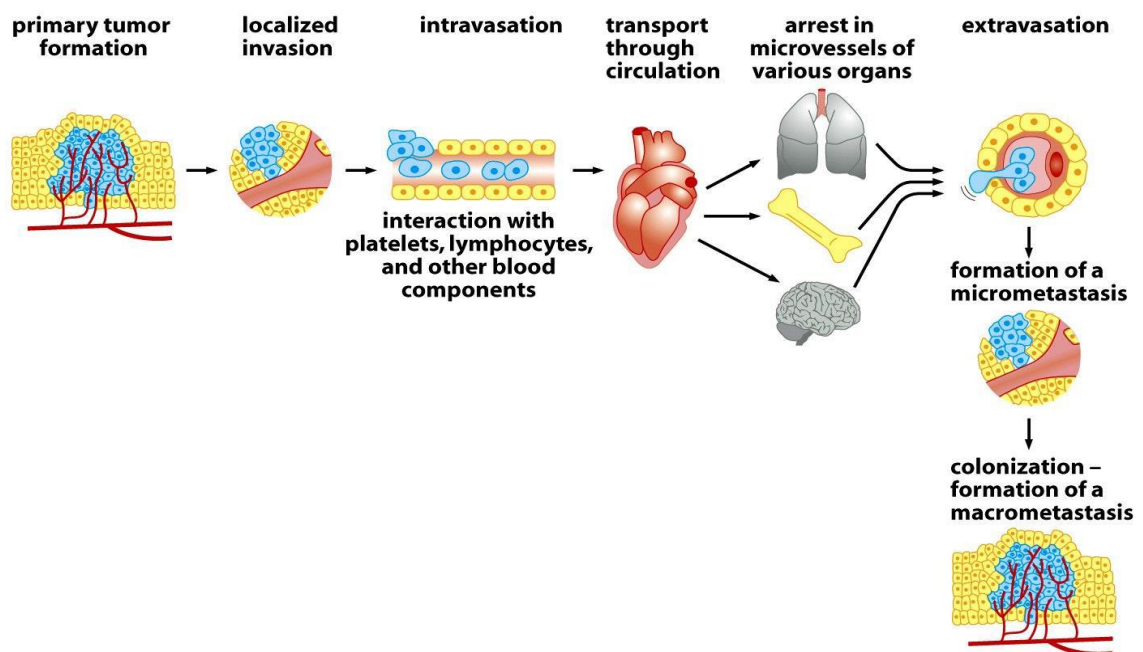
Figure 14-4 The Biology of Cancer (© Garland Science 2007)

Figure 1.1. The main steps of Invasion-metastatic cascade (The Biology of Cancer,2007)

1.2.1 Local Invasion

Cancer cells invade surrounding tumor-associated stroma and other adjacent tissues (Friedl & Wolf 2003). At first, cancer cells must degrade the basement membrane (BM) in order to invade nearby tissues. The basement membrane is a

specialized extracellular matrix that separate epithelial and stromal components of epithelial tissues. In addition to structural role of the BM, it plays role in signaling events which leads to change in proliferation, survival, cell polarity and invasiveness (Bissell & Hines 2011). It has been indicated that tissue architecture of normal epithelial cells act as a barrier in order to protect tissue against cancer cell invasion. For instance, myoepithelial cells serve as a barrier to invasion in the mammary gland (Hu et al. 2008). Most type of carcinomas invade moving by collectively (epithelial sheets or detached clusters) or as single cells (mesenchymal or amoeboid cell types). Single cell invasion is incompatible with the epithelial tissue organization so tumor cells alter their phenotypic and morphological properties by undergoing epithelial -mesenchymal transition (EMT) which occurs during embryonic morphogenesis and cancer progression (Thiery et al. 2009).

1.2.2. Epithelial- Mesenchymal Transition (EMT)

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells lose their polarized epithelial character and gain mesenchymal phenotype including elevated migratory capacity, invasiveness, resistance to apoptosis and production of high level of extracellular matrix components (Kalluri & Neilson 2003). There are a number of different molecular processes that drive EMT. Activation of transcription factors (TFs) including Slug, Snail, Twist, ZEB1 and ZEB2 which repress expression of epithelial markers and induce expression of mesenchymal markers organize acquisition of mesenchymal phenotype. Many of these transcription factors directly repress the expression of E-cadherin which is an epithelial marker. These involved TFs are used as biomarkers to show cells have passed through EMT (Thiery et al. 2009; Kalluri & Weinberg 2009) (Figure 1.2). miRNAs are also important regulators of EMT program. They either promote epithelial or mesenchymal phenotype depending on the type of miRNA by post-transcriptional regulation (Thiery et al. 2009).
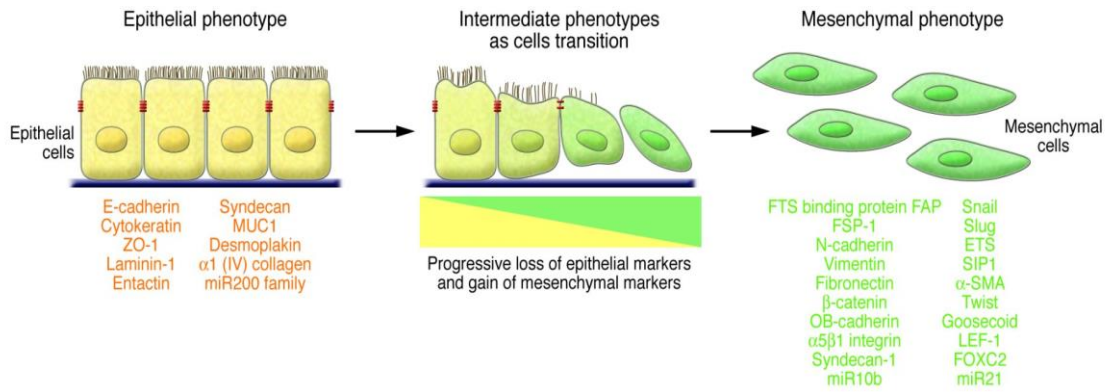
Figure 1.2. EMT- List of commonly used Epithelial and Mesenchymal markers (Kalluri & Weinberg 2009)

Extracellular matrix degrading enzymes are key players in EMT process. Cancer cells must degrade BM to invade so they activate several matrix metalloproteinases (MMPs) which are important regulators of tumor microenvironment and lead to BM loss (Kessenbrock et al. 2010).

When the cancer cells enter the stroma, they encounter fibroblasts, endothelial cells, adipocytes and bone marrow-derived cells including macrophages, other immune cells and mesenchymal stem cells (Joyce & Pollard 2009). These cells found in stroma can affect the aggressiveness of carcinoma cells by different type of signaling. For example, in breast cancer, IL-6 secreted by stroma cells induce invasion of MCF7 and MDA-MB-231 cells through Notch activation (Studebaker et al. 2008).

EMT is divided into three discrete biological subtypes depending on the biological context. Type 1 EMTs are associated with embryogenesis, implantation and organ development. It can form mesenchymal stem cells which ultimately undergo mesenchymal to epithelial transition. Type 2 EMTs are related to fibrosis, tissue regeneration and wound healing. In addition, they are also associated with inflammation in contrast to type1 EMTs. Cancer cells undergo type 3 EMT which make them invasive and migratory (Zeisberg & Neilson 2009).

1.2.3. Intravasation

Intravasation is defined as the entrance of cancer cells into blood vessels or lymphatic system. Although, dissemination via the blood system has been considered as the major route, there is emerging evidence that lymphatic spread also play important role in dissemination of cancer cells. Cancer cells must have ability to cross pericyte

and endothelial cell barrier which is facilitated by molecular changes (Gupta & Massagu 2006). Tumor cells promote the generation of new blood vessels generally through vascular endothelial growth factors (VEGFs) which is called angiogenesis. The neovasculature formed by tumor cells is more prone to leakiness compared to normal blood vessels so neovasculature leads to intravasation (Carmeliet & Jain 2011).

1.2.4. Survival in the Circulation

Cancer cells that are intravasated into circulation are called circulating tumor cells (CTCs). CTCs must survive in spite of stresses in the circulation system in order to reach the target organ site. They seem to lack integrin-dependent anchorage to ECM components that is essential for survival. Normally, epithelial cells undergo anoikis in the absence of anchorage. In addition, they must overcome the shear stress and cells of the innate immune system in the circulation (Guo & Giancotti 2004; Joyce & Pollard 2009).

1.2.5. Arrest at Distant Organ Site

According to seed and soil hypothesis, metastasis is based on the cross-talk between cancer cells (seed) and specific organ microenvironments (soil). Therefore, metastasis of cancer cells to distant organ sites is limited (Fidler 2003). Variety of factors such as chemokines and metadherin play role in CTCs arrest and molecular events drive organ-specific arrest of CTCs are still elusive.

1.2.6. Extravasation

Extravasation is the process, in which CTCs exit from the capillary entering into the target organ site. Latter form of extravasation is similar to earlier step of intravasation. For extravasation of tumor cells, physical barrier must be disrupted. This disruption is accomplished by the factors that are secreted by primary tumor cells. These secreted factors induce vascular hyper-permeability and disrupt distant microenvironment which enable extravasation. Factors such as angiopoietin-like-4,

EREG, COX-2, MMP-1 and MMP-2 perturb pulmonary vascular endothelial cell-cell junctions in order to promote extravasation of breast cancer cells into the lungs (Nguyen et al. 2009; Gupta et al. 2007; Padua et al. 2008).

1.2.7. Metastatic Colonization

Metastatic colonization is the growth of disseminated tumor cells in the distant organ microenvironment. There are genes that are fundamental for organ specific metastatic colonization and their expression is specific for the cancer cells that metastasize those organs. Disseminated tumor cells need to make foreign microenvironment more hospitable niche in order to escape dormancy and initiate proliferation (Nguyen et al. 2009).

1.3. Notch Signaling Pathway

Notch signaling is one of the signaling pathways that play role in breast cancer and inhibitors of Notch signaling have been used in clinical trials in order to improve outcome of breast cancer (Guo et al. 2010). In addition to its role in breast cancer growth, recently the role of Notch signaling in breast cancer metastasis has been investigated.

Notch genes encode transmembrane receptors that were discovered in flies by partial loss of function which results in notches at the wing margins of flies in 1917 (Morgan 1917). Notch signaling is an evolutionary conserved mechanism from invertebrates to mammals. Notch signaling plays role in cell fate decisions and developmental processes in a broad spectrum through local cell-cell interactions by regulating differentiation, proliferation and survival (Guo et al. 2010).

1.4. Structure of Notch Receptors and Ligands

Drosophila has a single Notch receptor and two ligands named Delta (D1) and Serrate (Ser) while mammals such as mice and humans have four Notch receptors (NOTCH1-4) (Weinmaster et al. 1992; del Amo et al. 1993; Lardelli & Urban 1993;

Lardelli et al. 1994) and five ligands which are Delta-like-1, -3, -4 (homologous of Delta) and Jagged1 and Jagged2 (homologous of Serrate) (Artavanis-tsakonas et al. 1999; Miele 2006).

Notch receptors are members of single-pass type 1 transmembrane protein. Notch protein family is synthesized as single precursor protein then it undergoes Sl cleavage by furin-like convertase in the golgi network and the two fragments are linked non-covalently at the cell surface to form heterodimeric receptor. Mature Notch protein family consists of an extracellular subunit (NEC), a transmembrane subunit (NTM) and a cytoplasmic subunit (Fiúza & Arias 2007). NEC contains 29-34 tandem epidermal growth factor (EGF)-like repeats which mediate ligand binding. NTM consists of a conserved negative regulatory region with three cysteine-rich Notch Lin12 repeats which prevents ligand independent signaling (Ranganathan et al. 2011). Cytoplasmic subunit of Notch protein is composed of RAM domain that has role in DNA binding with six ankyrin repeats (ANK), two nuclear localization signals (NLS), followed by a transactivation domain (TAD) and Pro-Glu-Ser-Thr (PEST) residue that regulates receptor half-life (Kopan & Ilagan 2009; Tien et al. 2009). Although, Notch receptors share many similarities in structure, they have differences in the extracellular and intracellular parts. The number of EGF-like repeats are different in the extracellular part. Notch 1 and Notch2 receptors have 36 EGF repeats whereas Notch3 has 34 and Notch4 has 29 EGF-like repeats. In the intracellular part, Notch1 contains strong TAD and Notch2 a weak TAD but Notch3 and Notch4 don't have TAD (Figure 1.3) (Radtke & Raj 2003).

JAG1, JAG2 and Dll-1 are type 1 cell surface proteins and they are composed of the Delta/Serrate/LAG-2 (DSL), Delta and OSM-11-like proteins (DOS) and EGF repeats, whereas Dll-3 and Dll-4 ligands don't contain DOS domains. DOS and DLL domains are important for binding to Notch receptor. However, some membrane bound and secreted non-canonical ligands which lack DSL and DOS domains can activate Notch signaling both *in vivo* and *in vitro* (D'Souza et al. 2008). In addition, they also differ in the number of EGF-like repeats and space between them in addition to presence of cysteine-rich domain (CR) found in Ser, JAG1 and JAG2 (Figure 1.3) (Radtke & Raj 2003).
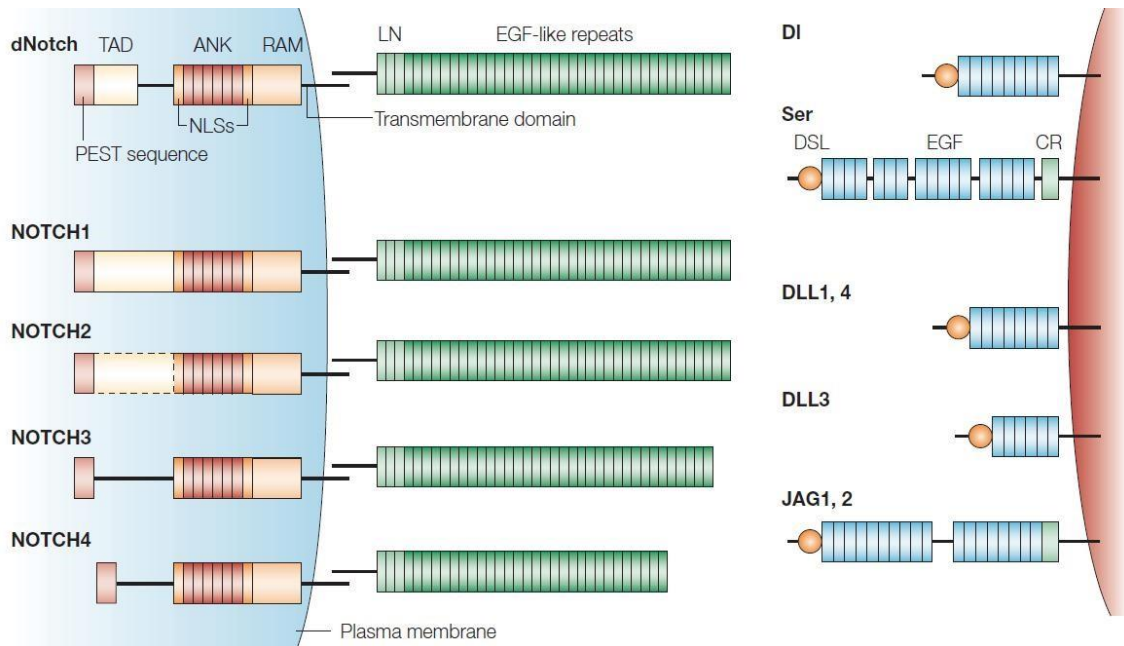
Figure 1.3. Notch Receptors and Notch Ligands in Drosophila and Human (Radtke & Raj 2003)

1.5. Notch Processing and Signaling

Notch signaling is initiated by physical interaction of Notch ligand with mature Notch receptor between two adjacent cells through EGF-like repeats which results in dissociation of extracellular subunit of Notch (NEC) from transmembrane subunit (NTM). The dissociation occurs after two sequential cleavages of NTM at S2 and S3 sites. NTM is cleaved by Tumor necrosis factor-α-converting enzyme (TACE)/ADAM at Site 2 resulting in dissociation of NEC from heterodimer and creates membrane bound Notch extracellular truncation which is cleaved by Presenilin-1 dependent γ-secretase complex at Site 3 (Mumm et al. 2000). S3 cleavage results in the release of Notch intracellular domain (NICD) which translocate to the nucleus where it interacts with DNA binding transcription factor CSL (RBP-Jk, CBF-1) in order to turn on the expression of its target genes. Activated NICD-CSL complex displaces co-repressors complex including CIR (CBF-1 Interacting Corepressor), SMRT/N-CoR, SHARP and HDAC-1 (Histone Deacetylase-1) and recruits co-activators such as SKIP, MAML-1 (Mastermind-like Protein-1) and ternary complex is formed. The ternary complex recruit transcription factors called p300, PCAF and GCN5 which have intrinsic histone acetyl transferase activity. The CSL-dependent transduction is called as canonical Notch

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pathway while CSL-independent transduction is called non-canonical Notch pathway (Figure 1.4) (Fiúza & Arias 2007; Borggrefe & Oswald 2009).
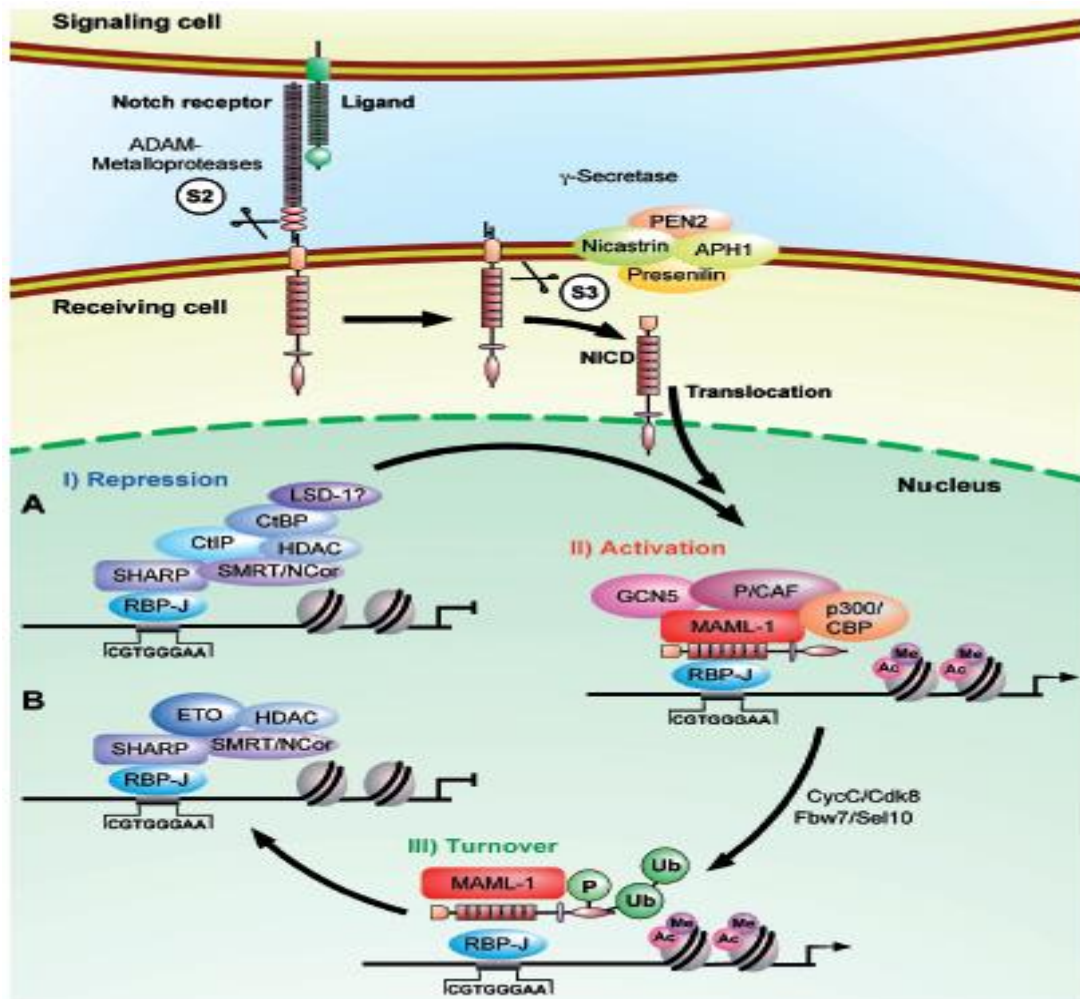
Figure 1.4. Molecular Steps Involved in Canonical Notch Signaling (Borggrefe & Oswald 2009)

1.6. Target Genes of Notch Signaling

The best characterized target genes of canonical Notch signaling are Hairy and Enhancer-of-split- related transcription suppressor of the Hes (Hes1-7) and Hey families (Hey1, Hey2, HeyL, HesL/HelT, Dec1/BHLHB2, Dec2/BHLHB3). Hey and Hes proteins have helix-loop-helix domain that is important for formation of homo- or heterodimers to repress their target genes. (Iso et al. 2003). Each member of Hey family can be induced by Notch signaling whereas, only Hes1, Hes5 and Hes7 genes are

induced by Notch signaling but Hes2, Hes3 and Hes6 seem to be Notch independent. Hes4 needs to be investigated (Fischer et al. 2004). The expression profile of Hey and Hes proteins are different depending on cell type and type of cancer. For example, Hes1, Hey1 and Hey2 are expressed in endothelial cells while Hes1 and Hes3 are expressed in undifferentiated embryonic stem cells (Katoh 2007). Other known Notch targets include genes that play role in the control of cell cycle and cell survival such as *cyclin D1*, NF-κB, *Deltex,* p21WAF1/Cip1 and *c-myc*. In addition, Notch1, Notch3, bcl2, HoxA5, 9 and 10 other Notch target genes that have been reported (Borggrefe & Oswald 2009; Weng et al. 2006).

1.7. Functions of Notch Signaling

Notch signaling plays a key role in diverse cellular processes including hematopoiesis, neurogenesis, vascular development, skin differentiation, myogenesis and immune response through regulating cell proliferation, apoptosis, differentiation and cell fate determination (Wu & Griffin 2004).

Notch signaling is firstly studied in *Drosophila* and it has been shown that Notch signaling restricts neural differentiation by inhibiting pro-neural genes (Parks et al. 1997). In mammals, Notch signaling triggers terminal differentiation in human skin and it also stimulates differentiation of keratinocytes in mouse (Borggrefe & Oswald 2009).

Notch signaling has divergent context-dependent role that it is crucial a determinant of proliferation versus growth arrest, survival versus death and differentiation versus stemness (South et al. 2012).

1.8. Role of Notch Signaling in Cancer

Notch signaling is dysregulated in several human disease including cancer and neurodegenerative disorders. Notch signaling can act as either oncogene or tumor suppressive in a context-dependent manner (South et al. 2012).

The first link between Notch signaling and malignancy was identified in human T cell neoplasia (Ellisen et al. 1991). In humans, dysregulated expression of wild type Notch receptors, ligands and targets is detected in many solid tumors including cervical, head and neck, renal, endometrial cancers, hepatocellular carcinoma (Gao et al. 2008),

mesotheliomas, malignant melanomas and hematological malignancies including Hodgkin lymphomas, anaplastic large-cell non Hodgkin lymphomas and subset of acute myeloid leukemia (Nickoloff et al. 2003).

The oncogenic role of Notch signaling is first documented in the T-cell leukaemias. Notch1, Notch2 and some Notch ligands are overexpressed in human colon cancers (Zagouras et al. 1995). Notch1 inhibits apoptosis (p53-dependent) in cervical cancer via activating Akt pathway (Pathway et al. 2003).

Notch has also tumor suppressor role in addition to its oncogenic activity during tumorigenesis. It has been shown that Notch acts as tumor suppressor in skin and inactive form of Notch1 leads to basal cell carcinoma (BSC) and squamous cell carcinoma (SCC) (Radtke & Raj 2003).

1.9. Notch Signaling in Breast Cancer

The oncogenic role of Notch signaling in breast cancer was first seen in the murine mammary gland by the proviral integration of mouse mammary tumor virus (MMTV) into the *Notch4* locus (*Int3* locus). Insertion of the provirus into the *Notch4* locus leads to over-expression of Notch4-ICD and transforms mammary epithelial cells (Gallahan & Callahan 1987).

Notch1 also shows oncogenic activity in the mammary gland like Notch4. Notch1 locus is an another target for MMTV provirus integrational activity. Insertion of the provirus in the Notch1 locus results in over-expression of 3' truncated Notch1 transcripts and protein products that transforms HC11 mouse mammary (Diévart et al. 1999). In addition, Notch1 was indicated as downstream mediator of oncogenic Ras which is mutated in early breast cancer and leads to breast carcinogenesis (Weijzen et al. 2002). Breast cancer patients co-expressing Notch1 and JAG1 showed poorer survival rate compared to patients with low expression of these genes (Reedijk et al. 2005).

In vivo studies showed that Notch3-ICD upregulation in mammary glands results in the formation of mammary tumor which means Notch3 has transforming potential (Hu et al. 2006).

Although Notch signaling plays role as an oncogene, recent studies show that Notch signaling can also act as tumor suppressor in breast tissue. Breast cancer patients

with Notch2 expression showed better survival rate and constitutively active Notch2-ICD increased apoptosis in MDA-MB-231 human adenocarcinoma cell line (O'Neill et al. 2007). Human primary epithelial cells expressing Notch1-ICD showed decrease in proliferation (Yalcin-Ozuysal et al. 2010; Mazzone et al. 2010). The dose of Notch activation is crucial in the phenotypic responses because dosage determines the balance between growth-stimulative and growth-suppressive effect (Mazzone et al. 2010).

1.10. Notch Signaling in- EMT, Invasion and Metastases

Recently, the role of Notch pathway in metastasis has been studied in addition to its role in primary tumor growth. Several research revealed that deregulated Notch signaling play role in tumor metastasis in different tumor types (Hu et al. 2012). Jagged1 is more expressed in metastatic prostate cancer compared with benign prostatic tissue or localized cancer in prostate cancer patient's samples so high Jagged1 expression is related to recurrence of prostate cancer (Santagata et al. 2004). Activation of Notch1 enhances tumor metastasis in melanoma in animal models, gastric cancer in patient samples and pancreatic cancer (Hu et al. 2012).

In zebrafish embryos and endothelial cell line *in vitro*, overexpression of Notch1-ICD promotes mesenchymal transformation and EMT via Snail induction. (Timmerman et al. 2004). Slug was identified as a direct Notch target gene in primary breast cancer cells (Leong et al. 2007) and Slug, like Snail, can induce EMT through E-cadherin repression in primary breast cancer (Martin et al. 2005). In addition, expression of Jagged1 promotes breast cancer invasiveness and metastasis through induction of Slug in MDA-MB-468 breast cancer cell line (Chen et al. 2010). Lastly, inhibition of Notch pathway in breast cancer patients prevented development of brain metastasis (Witzel et al. 2016).

Several studies indicated that Notch signaling promotes tumor metastasis through EMT, migration and invasion. However, the downstream mediators of Notch in these processes are still elusive. In order to understand the mediators of Notch in induction of EMT, migration and invasion, seven candidate genes (HMGA2, CXCL14, CYR61, CXCR7, CCL20, SEMA3C and SEMA6D) were selected. Selected candidate genes were differentially regulated by ectopically expressed Notch1 in normal human breast epithelial cell line (Mazzone et al. 2010), human and mouse primary breast

epithelial cells (Yalcin-Ozuysal et al. 2010). The candidate genes are associated with tumor metastasis according to the literature however, their connection with Notch signaling and their role in Notch induced EMT, migration and invasion are not known.

1.11. Candidate Genes in Breast Cancer

HMGA2 belongs to a high mobility group A non-histone protein family that change chromatin structure by binding the minor groove of AT-rich DNA sequences. HMGA proteins regulate expression of many genes either positively or negatively by interacting transcription machinery but they don't have transcriptional activity. In addition to regulation of gene regulation, HMGA proteins are involved in the differentiation, cell cycle, senescence and viral integration. During embryogenesis, they are highly expressed but their expression level is quite low in adult tissue (Fusco & Fedele 2007). Several studies showed that HMGA protein family has oncogenic activity. HMGA2 induces neoplastic transformation and promote metastasis (Sgarra et al. 2004). Elevated expression of HMGA2 is associated with poor prognosis in breast cancer patient (Langelotz et al. 2003). HMGA2 directly binds to the promoter of SNAI1 and leads to EMT (Thuault et al. 2008).

CCL20, CXCR7 and CXCL14 candidate genes belong to chemokine family that control cell migration. Chemokines are family of small cytokines (8-14kDa). They are divided into four classes according to location of the cysteine residues. Four classes include CXC, CC, C and CX3C chemokines (Baggiolini et al. 1997; Luster 1998). The chemokine receptors are G-protein coupled receptors and receptor-chemokine interaction mediates communication between cancer cells and tumor microenvironment. They can act as chemo-attractant for cell migration to distant sites. While some chemokines promote tumor progression, others increase anti-tumor immunity (Seema et al. 2011)

CCL20 enhanced the migration and invasiveness of MDA-MB-231 breast cancer cell line *in vitro* (KIM et al. 2009). Expression level of CCL20 is more in metastatic breast cancer cells compared to non-metastatic breast cancer cells (Roberti et al. 2012).

CXCL14 increased cell proliferation and migration capacity in breast cancer (Pelicano et al. 2009; Allinen et al. 2004). Little is known about novel chemokine, CXCL14, it needs further investigation.

CXCR7 is a G-protein coupled receptor for CXCL12 and CXCL11 chemokines. CXCR4 is also receptor for CXCL12 chemokine so CXCR4 and CXCR7 can form both homodimers and heterodimers which affects the CXCR4 signaling both positively and negatively. CXCR7 is highly expressed in many cancer type including breast cancer and tumor vasculature (Miao et al. 2007). CXCR7 reduced invasion capacity of breast cancer cells through suppression of CXCR4 signaling but it promoted primary tumor growth through angiogenesis (Hernandez et al. 2011).

CYR61 (CCN1) belongs to CCN protein family. CYR61 is a secreted, cysteine-rich and heparin binding protein and it is associated with cell surface and extracellular matrix. CYR61 plays role in cell adhesion, migration, proliferation, apoptosis and angiogenesis (Leask & Abraham 2006; Babic et al. 1998). It has been shown that CYR61 is a tumor promoting factor and regulator of breast cancer progression and it induces invasiveness *in vitro* and induce tumorigenesis *in vivo* (Tsai et al. 2002). Treatment of breast cancer cells with anti-CYR61 antibodies inhibits cell proliferation, migration and invasion through downregulating AKT and ERK phosphorylation in vitro. Anti-CYR61 antibodies suppress primary tumor growth and lymph node metastasis *in vivo* breast cancer mouse models (Lin et al. 2012). CYR61 is also associated with Laryngeal squamous cell carcinoma invasion and lymph node metastasis by inducing EMT program (Liu et al. 2015). Lastly, CYR61 induces cholangiocyte proliferation through CCN1//αvβ5/NF-κB/NOTCH-JAG1 axis (Kim et al. 2015).

There are two candidate genes, SEMA6D and SEMA3C that belong to Semaphorin (SEMA) family. Semaphorins are classified into eight classes (classes 1-7 and V-viral), classes 1 and 2 are expressed in invertebrates while classes 3-7 are found in vertebrates and class V is encoded by viruses. Class 2, 3 and V Semaphorins are secreted as a soluble molecule, while rest of members exist in membrane bound form. Primary receptors for Sempahorins are Plexin family but Class 3 semaphorins except SEMA3E bind to neuropilin receptor family. Binding of class 3 semaphorins to neuropilins leads to direct activation of PlexinA1-4 receptors. Membrane bound semaphorins bind to Plexin receptor family (Figure 1.5) (Harburg & Hinck 2010; Casazza et al. 2007).
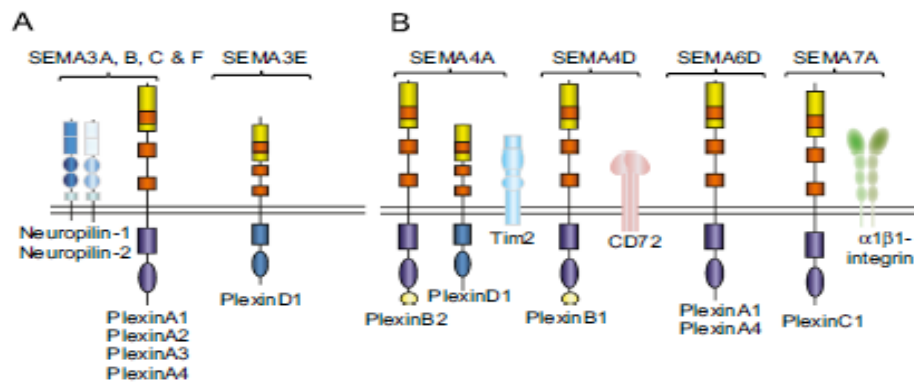
Figure 1.5. Schematic representation of the interaction between Semaphorins and their receptors. (A): Secreted semaphorins, (B): transmembrane semaphorins. (Eissler & Rolny 2013)

Semaphorins play role in cell adhesion, migration and invasion, organogenesis, immunoreaction, carcinogenesis and axon guidance (Morris et al. 2006). A number of semaphorins are upregulated in tumors (Harburg & Hinck 2010).

SEMA3C is a secreted semaphorin. It has been shown that Twist (EMT inducer) induced expression of SEMA3C during embryonic development (Lee & Yutzey 2011). In contrast to other class 3 semaphorins, SEMA3C promotes cell migration and ADAMTS1 regulate its activity so SEMA3C and ADAMTS1 act coordinately leading to an increase migration capacity of breast cancer cells (Esselens et al. 2010).

SEMA6D is a membrane bound semaphorin that can act both through cell-cell contacts and distantly through dispersion of its cleaved ectodomain. PlexinA1 and PlexinA4 are receptor for SEMA6D (Eissler & Rolny 2013). It has been shown that SEMA6D induces migration of the myocardial cells during heart development (Toyofuku et al. 2004). SEMA6D and its receptor PlexinA1 are overexpressed in gastric carcinoma (Zhao et al. 2006). SEMA6D may have also pro-proliferative activity by regulating NF-κB signaling pathway (Catalano et al. 2009). Tie2 expressing monocytes have induced expression of SEMA6D which suggest that it may activate angiogenesis by PlexinA1/VEGFR2 signaling (Pucci et al. 2009; Catalano et al. 2009). Recently, SEMA6D has been indicated as an oncogene in human osteosarcoma (Moriarity et al. 2015). Lastly, SEMA6D expression is associated with patient survival of triple negative breast cancer patients. Breast cancer patients expressing high level of SEMA6D showed decreased expression level for E-cadherin, while they are expressing increased level of SNAI2, ZEB1 and ZEB2 (approximately 2 fold) compared to patients expressing low level of SEMA6D (Chen et al. 2015).

CHAPTER 2

AIM OF THE RESEARCH PROJECT

Breast cancer is the second leading cause of cancer related deaths world-wide among women in spite of the improved treatment strategies. The main cause of death is not the primary tumor itself, but its metastasis at distant sites. Therefore, understanding the mechanism behind metastasis is crucial for developing better treatments. It has been shown that Notch signaling causes formation of mammary gland tumor. In addition to its role in primary tumor formation, Notch signaling plays role in important mechanisms of metastasis process which are epithelial-mesenchymal transition (EMT), migration and invasion. However, molecular mechanisms behind those effects of Notch signaling need to be understood. Therefore, we hypothesized that Notch signaling induces EMT, migration and invasion via one or more of the seven candidate genes that are SEMA6D, SEMA3C, CXCR7, CXCL14, CCL20, CYR61 and HMGA2. In this study, we aim to investigate whether Notch signaling induces EMT, migration and invasion via regulation of those genes.

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Line and Cell Culture

MDA MB 231 human breast cancer cell line, MCF10A human normal breast epithelial cell line, 293T human embryonic kidney cell line and NIH/3T3 mouse embryonic fibroblast cell line were used during this study. 293T cell line was utilized for virus production, while NIH/3T3 cell line for virus titration experiments. Since MCF10A cell line has no Notch activity, Notch pathway was activated in MCF10A while inhibition of Notch signaling was performed in MDA MB 231 cell line which has high Notch activity.

MCF10A cells were cultured in high glucose DMEM-F12 (Gibco, Cat# 31-330-038) supplemented with 5% Donor Horse Serum (BI, Cat# 04-004-1B), 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B), 20ng/mL EGF (Sigma, Cat#E9644), 10μg/mL Insulin (Sigma, Cat#I1882), 100 ng/mL Cholera Toxin (Sigma, Cat# C8052), 0.5 μg/mL Hydrocortisone (Sigma, Cat# H0888).

MDA MB 231 and 293T cells were maintained in high glucose DMEM (Gibco, Cat# 41966-029) supplemented with 10% Fetal Bovine Serum (FBS) (BI, Cat# 04-007-1A), and 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B).

NIH/3T3 cells were grown in high glucose DMEM (Gibco, Cat# 41966-029) supplemented with 10% New Born Calf Serum (NBCS) (BI, Cat# 04-102-1A), and 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B).

All cell lines were maintained at $37^{o}C$ under 5% CO_2.

MCF10A and MDA MB 231 cell lines were purchased from the ATTC while 293T and NIH/3T3 cell lines were provided by Prof. Cathrin Brisken Lab (EPFL, ISREC).

3.2. Virus Production and Titration

3.2.1. Retro and Lenti-virus Production

Retro and Lenti-viral systems were utilized for exogenous gene expression and RNA interference experiments. Both retro and lenti viruses were produced by using 293T human embryonic kidney cell line due to its high transfection efficiency. For retro-virus production, pcl10A vector containing retro-virus packaging genes was used while psPAX2 packaging and pMD2.G envelope vectors were used for lent-virus production.

Notch signaling was activated by MSCV-NICD retrovirus which express Notch1 intracellular domain. Empty vector MSCV was used as control. In order to produce retrovirus, 239T cells were seeded at 4×10^6 cells/10cm plates at 24 hours prior to transfection. 2 μg retroviral vector (MSCV or MSCV-NICD), 2 μg packaging vector (pcl10A) and 12 μl transfection reagent, Fugene HD (Promega, Cat#E2311) were mixed in 1:3 ratios in 500 μl serum-free medium and incubated for 30 minutes at room temperature. After incubation, the mixture was added to the plated 293T cells drop by drop for both control and gene of interest vectors. The medium was changed 24 hours after transfection. The produced viruses were released into the medium so at 48 and 72 hours, medium containing viruses were harvested, aliquoted and stored at -80 ^{o}C.

Notch inhibition, SEMA6D overexpression and SEMA6D silencing were performed by lentiviral system. For Notch inhibition and SEMA6D knock down, lentiviruses containing shRNA against mRNA of gene of interest were produced by using 293T cell line. Notch inhibition via CSL silencing was performed by shRNA against CSL (shCSL vector) and shGFP vector was used as a control. For SEMA6D overexpression, lentiviruses containing SEMA6D cDNA were produced in 239T cell line too. pLX304-SEMA6D vector and as a control pLX304-LacZ vector were used. In order to produce lentivirus, 239T cells were seeded at 4×10^6 cells/10cm plates at 24 hours prior to transfection. At an optimized ratio (1:3), 1.5 μg psPAX2, 0.5 μg pMDG.2, 2 μg interested lentiviral vector and 12 μl transfection reagent, Fugene HD (Promega, Cat#E2311), were mixed in 500 μl serum-free medium and incubated for 30 minutes at room temperature. Then the mixture was added to the plated 293T cells. 24

hours after transfection, medium was changed. At 48 hours and 72 hours, viruses were harvested, aliquoted and stored at -80 $^{\circ}$C.

3.2.2. Virus Titration

Virus titration is required to quantify and determine efficiency of produced viruses. NIH/3T3 mouse embryonic fibroblast cell line was used. NIH/3T3 cells were seeded at 150.000 cells/well into 6-well plate one day before. NIH/3T3 cells were infected with 10^{-3}, 10^{-4}, 10^{-5} diluted viruses including 8 μg/ml polybrene. After 24 hours, medium was changed and at third day after infection, infected NIH/3T3 cells were transferred to 10 cm plates and selection done by 400μg/mL Geneticin (Gibco, Cat#10131-019) or 2μg/mL puromycin (Sigma Aldrich, Cat#P8833) depending on the resistant gene of viral vector. Antibiotics killed uninfected cells and infected cells grow and formed colonies. Selection was ended when all non-transduced cells died. After selection was ended, cells were washed with 1X PBS twice and stained with the 0.5% crystal violet dye for 10 minutes. Then excess dye was removed by washing with 1X PBS for three times. The number of colonies were counted which demonstrated efficiency of viruses. According to colony number, amount of virus that was used for infection was determined for both control and gene modulating viruses.

3.3.   Infection of Cell Lines by Viruses

Notch activation experiments were performed by using MCF10A cell line since MCF10A has no Notch activity. MCF10A cells were seeded at $2,5x10^5$ cell/well into 6-well plate one day before infection. Cells were infected with MSCV control and Notch1 intracellular domain expressing MSCV-NICD retro-viruses. Notch inhibition experiments were performed on MDA MB 231 cell line due to its high Notch activity. MDA MB 231 cells were cultured at $3,5x10^5$ cell/well in to 6-well plate 24 hours prior to infection. Cells were infected with shGFP control and shCSL lenti-viruses.

SEMA6D was overexpressed in MDA MB 231 cell line because it has low SEMA6D expression level while SEMA6D knockdown was performed in MCF10A cell line. $3,5x10^5$ MDA MB 231 cell/well in 6-well plate were infected with pLX304-LacZ

control and pLX304-SEMA6D lenti-viruses 24 hours after seeding. $2,5x10^5$ MCF10A cell/well in 6-well plate were infected with shNS control and shSEMA6D lenti-viruses 24 hours after seeding. For EMT, migration and invasion analysis, $35x10^4$ MDA MB 231 cell/well in 6 well plate were double infected with pLX304-SEMA6D, shCSL and their control viruses (pLX304-LacZ & shGFP). $2,5x10^5$ MCF10A cell/well in 6-well plate were also double infected with MSCV-NICD, shSEMA6D and their control viruses (MSCV & shNS).

The same procedure was followed for all infection experiments. In all infections, polybrene (Sigma Aldrich, Cat# 107689) was used at 8 µg/mL final concentration in order to increase efficiency of infection. 24 hours after infection, medium was changed and at 72 hours infected cells were flash frozen by taking plates on to liquid nitrogen after washing by cold 1X PBS twice. After flash freeze, plates were stored at 80 ^{o}C for RNA and protein isolation.

3.4. mRNA Isolation and Semi-Quantitative Real Time RT-PCR (qRT-PCR)

Total mRNA was isolated by Pure-link RNA Mini Kit (Ambion, Cat# 12183018A) according to manufacturer's instructions. The concentration was determined and complementary DNA (cDNA) was synthesized from 1 µg RNA by using reverse transcriptase and random hexamer primers (Thermo Scientific, Ca#K1622). mRNA expression levels of genes of interest were determined by semi-quantitative real time RT-PCR (qRT-PCR) by using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Cat# K0252) and specific forward and reverse primers for cDNAs. Maxima SYBR Green Master Mix has SYBR Green I, Taq DNA polymerase, dNTPs and buffer which is mixed with forward, reverse primers and 1/8 diluted cDNA. The qRT-PCR experiments were performed in triplicate on BioRad IQ5 detection system.

The threshold was set within the logarithmic phase of target gene amplification plot and C_t values at which amplification detected were calculated. Relative mRNA expressions were calculated according to delta-delta Ct method. Non template controls were included in each experiment. TATA box binding protein (TBP) was used as a

reference for a relative quantification of expression of target genes. Two tailed student's t-test was used for statistical calculations.

Table 3.1. Sequences of forward and reverse primers used for qRT-PCR.

TBP-Forward Primer	5'-TAGAAGGCCTTGTGCTCACC-3'
TBP-Reverse Primer	5'-TCTGCTCTGACTTTAGCACCTG-3'
HEY2-Forward Primer	5'-AGGGAGGAGGCGGGC-3'
HEY2-Reverse Primer	5'-CCCTCTCCTTTTCTTTCTTGCC-3'
HEY1-Forward Primer	5'-GGCTGGTACCCAGTGCTTT-3'
HEY1- Reverse Primer	5'-GATAACGCGCAACTTCTGCC-3'
SEMA6D-Forward Primer	5'-TTTCCCAGTTGAGGGCAGTC-3'
SEMA6D-Reverse Primer	5'-AGGGCGTCCTCTAAAAACCG-3'
SEMA3C-Forward Primer	5'-ACCAAGAGGAATGCGGTCAG-3'
SEMA3C-Reverse Primer	5'-TGTTGACAAGGCTACGCAGT-3'
CYR61-Forward Primer	5'-AAGGGGCTGGAATGCAACTT-3'
CYR61-Reverse Primer	5'-CTGCCCGGTAACTTTGACCA-3'
HMGA2-Forward Primer	5'-GCCCTCTCCTAAGAGACCCA-3'
HMGA2-Reverse Primer	5'-CTGCCTCTTGGCCGTTTTTC-3'
CXCR7-Forward Primer	5'-TGTGGGTTACAAAGCTGCCA-3'
CXCR7-Reverse Primer	5'-GAGGCGGGCAATCAAATGAC-3'
CXCL14-Forward Primer	5'-AAGGGACCCAAGATCCGCTA-3'
CXCL14-Reverse Primer	5'-GACACGCTCTTGGTGGTGAT-3'
CCL20-Forward Primer	5'-GTCTGTGTGCGCAAATCCAA-3'
CCL20-Reverse Primer	5'-GACAAGTCCAGTGAGGCACA-3'
E-CADHERIN-Forward Primer	5'-CAGCACGTACACAGCCCTAA-3'
E-CADHERIN-Reverse Primer	5'-GGTATGGGGGCGTTGTCATT-3'
ZO-1-Forward Primer	5'-ATGGAGGAAACAGCTATATGGGA-3'
ZO-1-Reverse Primer	5'-CCAAATCCAAATCCAGGAGCC-3'
SNAI1-Forward Primer	5'-CTAGGCCCTGGCTGCTACAA-3'
SNAI1-Reverse Primer	5'-TGTGGAGCAGGGACATTCG-3'
SNAI2-Forward Primer	5'-CTCCTCATCTTTGGGGCGAG-3'
SNAI2-Reverse Primer	5'-TTCAATGGCATGGGGGTCTG-3'
ZEB1-Forward Primer	5'-CCCAGGTGTAAGCGCAGAAA-3'
ZEB1-Reverse Primer	5'-GTCTGGTCTGTTGGCAGGTC-3'
ZEB2-Forward Primer	5'-ATAAGGGAGGGTGGAGTGGAA-3'
ZEB2-Reverse Primer	5'-GTTAATTGCGGTCTGGATCGTG-3'
VIMENTIN-Forward Primer	5'-GCTAACCAACGACAAAGCCC-3'
VIMENTIN-Reverse Primer	5'-CGTTCAAGGTCAAGACGTGC-3'

3.5. Protein Isolation and Western Blot

Protein expression levels of gene of interest were detected by western blot method.

3.5.1. Protein Isolation

Total protein was isolated by using RIPA lysis buffer (containing 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 50 mM Tris-HCl (pH 7.5), 1% DOC and 5 mM EDTA) which was mixed with 1X protease inhibitor, 1 mM DTT, phosphatase inhibitors, 1 mM sodium orthovanadate (Na_3VO_4) and 50 mM NaF. Cells were scraped with lysis buffer and lysed cells were collected into eppendorf tubes and homogenized by insulin 26G syringe. The cell lysates were incubated on ice for 20 minutes then they were centrifuged at 14000 rpm at 4 ^{o}C for 20 minutes. Supernatant containing total protein was transferred into fresh eppendorfs and stored at -80 ^{o}C.

3.5.2. Quantification of Proteins

Concentration of isolated proteins were determined by Bradford assay in order to load equal amount of protein to SDS- polyacrylamide gel. In order to generate standard curve, BSA calibrator samples were used at different concentrations (0.2, 0.5, 1, 2, 4, 8 mg/ml). 10 µl standards and protein samples were dissolved in 800 µl water in 1 cm cuvette then 200 µl 5X Bradford dye solution was added. Each sample was prepared as duplicates. Spectrophotometric measurement was performed at 595 nm. Concentration of isolated proteins were calculated according to equation generated by standard curve.

3.5.3. Western Blot Procedure

Total protein (20µg-100µg), 5µl 5X loading dye and water up to 25 µl were mixed and boiled at 90 ^{o}C for 10 minutes. Samples and 5µl protein marker (NEB, Cat# P7712S) were resolved by electrophoresis on 5% stacking and 10-15 % resolving SDS-

polyacrylamide gel. Then proteins were transferred to PVDF membrane at 250 mA for 2-3 hours on ice. Membranes were blocked with either 5% milk powder or 5% BSA 1X TBS-Tween20 (TBS-T) for 2-4 hours depending on the optimized conditions for specific antibodies. After blocking, membranes were immunoblotted with primary antibody according to optimized protocol for 2 hours at room temperature or overnight at 4^oC. After primary antibody incubation, membranes were washed with 1X TBS-T for 10 minutes, 3 times and then membranes were incubated with secondary antibodies at recommended concentration in 1X TBS-T for 2 hours at room temperature. After that, membranes were washed with 1X TBS-T for 10 minutes, 3 times again and the target proteins were detected with ECL system (Millipore, Cat# WBLUR100) by using Biorad VersaDoc Imaging System (4000 MP).

Primary antibodies against the following proteins were used: rabbit anti-Hey2 (Abcam, Cat# AB184246, 1/500 in 5% milk powder), rabbit anti-SEMA6D (Abcam, Cat # AB191169, 1/250 in 5% milk powder), rabbit anti-CCN1 (Abcam, Cat# AB127988, 1/400 in 5% milk powder), rabbit anti-CXCR7 (Abcam, Cat# AB 38089, 1/500 in 5% milk powder), rabbit anti-CXCL14 (Abcam, Cat# AB 46010, 1/1000, in 5% BSA), rat anti-SEMA3C (Abcam, Cat# AB 135167, 1/500 in 5% milk powder) and rabbit anti-β-actin (Abcam, Cat# AB 75186, 1/1000 in 5% milk powder). Secondary antibodies were goat polyclonal anti-rabbit (Dako, Cat# P0448, 1:2000 in TBS-T) and mouse monoclonal anti-rat (Abcam, Cat# AB106718, 1/1000 in TBS-T). Intensity of all proteins were normalized to β-actin and each data was normalized to the control group. Quantification of western blot images were performed by gel images tool of ImageJ. Two tailed Student's t-test was used for statistical analysis.

3.6. Cell Migration

In order to see effects of modification on migration capacity of MDA MB 231 and MCF10 cell lines, scratch wound healing assay was performed. Cells were seeded at 7.5 X 10^5 cells/well in 6-well plate and grown to confluency for 48 hours. MCF10A cell were seeded at 5 X 10^5 cells/well in 6-well plate and grown to confluency for 24 hours. Confluent monolayer was scratched with a 10μl micropipette tip and washed with 1X PBS twice in order to remove floating cells. Then fresh L15 medium (supplemented with 10% FBS for MDA MB 231 and 5% Donor Horse Serum, 1%

Penicillin/Streptomycin, 20ng/mL EGF, 10µg/mL Insulin, 100 ng/mL Cholera Toxin, 0.5 µg/mL Hydrocortisone for MCF10A cell line.) was added. Images were taken for 48 hours at 1 hour intervals with Zeiss microscopy from 3 different positions per conditions.

3.7. MTT Assay

MTT assay was performed in order to see the effect of modification on cell viability. Stable MDA-MB-231 cells (SEMA6D& Control) were seeded at $1,5 \times 10^4$ cells/well in 12 well plate. At day 2, 4, 7 and 9 MTT assay was performed. Stable MDA-MB-231 cells were incubated with 10% MTT in DMEM for 4 hours at 37°C, 5% CO_2. After incubation, DMSO was added to harvest dye and transferred to 96-well plate for spectrometric measurements. OD measurements were done at 570 and 650 nm with Thermo multiskan spectrum.

CHAPTER 4

RESULTS

4.1. Notch1 Activation Regulates mRNA Expression of Candidate Genes in MCF10A

To determine whether Notch signaling regulates expression of candidate genes (HMGA2, CCL20, CXCL14, CXCR7, CYR61, SEMA3C and SEMA6D), we activated Notch signaling by introducing Notch1 Intra-Cellular Domain (N1-ICD) in MCF10A human breast epithelial cell line which doesn't show Notch activity. 72 hours after infection by retro-virus containing N1-ICD, mRNA levels of the candidate genes were detected by semi-quantitative Real Time RT-PCR.

Activation of Notch1 signaling in MCF10A cells was confirmed by checking the mRNA expression levels of known target genes of Notch signaling such as Hey1 and Hey2. Expression levels of Hey1 and Hey2 were increased approximately 8 fold and 300 fold respectively which confirms activation of Notch signaling in MCF10A cells. Notch1 activation upregulated mRNA expressions of CXCL14, SEMA6D, CCL20, CYR61 and CXCR7. Increase in SEMA6D and CYR61 was significant, whereas CXCL14, CCL20 and CXCR7 couldn't reach significant levels. Notch1 activation downregulated mRNA expressions of HMGA2 and SEMA3C significantly (Figure 4.1). Our results suggest that activation of Notch1 differentially regulates mRNA expression levels of CXCL14, SEMA6D, CCL20, CYR61, CXCR7, HMGA2 and SEMA3C genes.
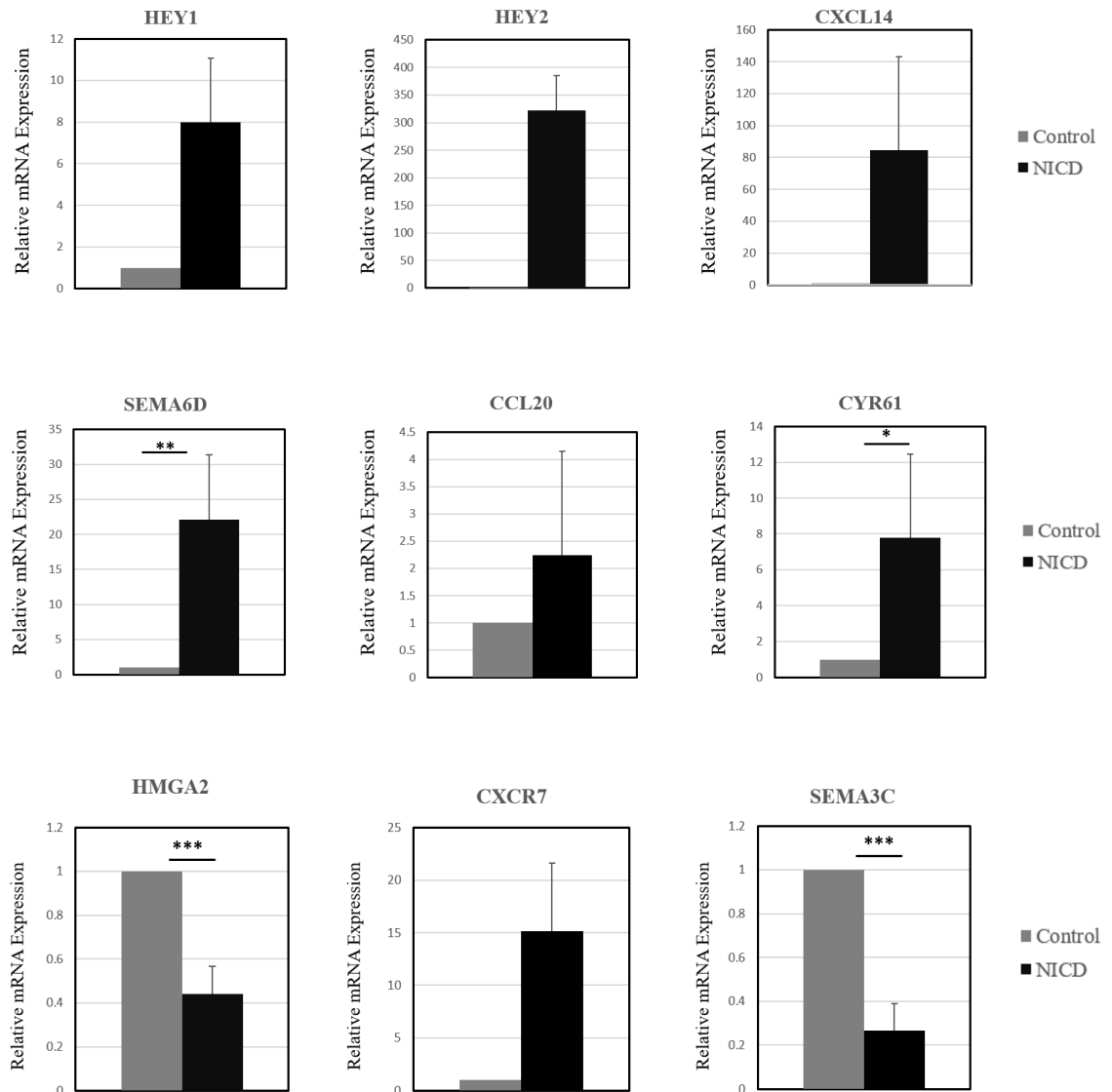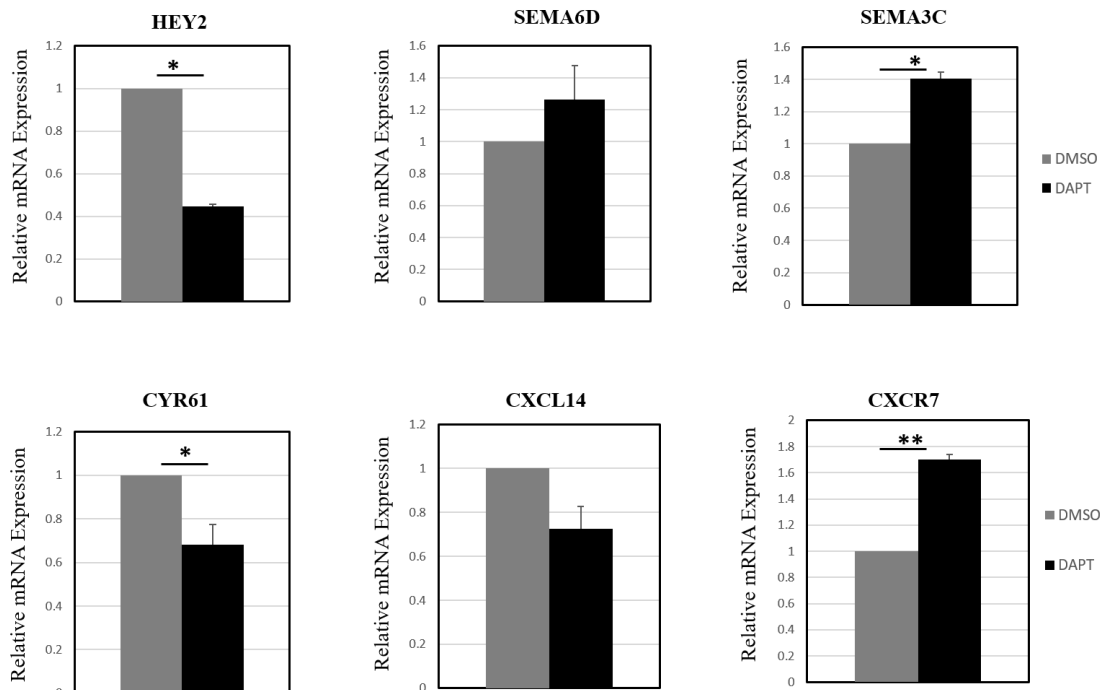
Figure 4.1. Real Time RT-PCR results of Notch1 activation in MCF10A. 72 hours after infection, mRNA levels of Notch targets Hey2 and Hey1; candidate genes CXCL14, SEMA6D, CCL20, CYR61, HMGA2, CXCR7 and SEMA3C were detected. TBP was used for normalization. Three independent experiments were conducted. (*$p < 0.05$, **$p < 0.02$, ***< 0.003)

4.2. Notch Inhibition Regulates mRNA Expression of Candidate Genes in MDA-MB-231

In order to determine whether Notch signaling regulates expression of candidate genes in breast cancer cells, Notch signaling was inhibited by two different methods in human breast cancer cell line MDA-MB-231 which shows high Notch activity.

4.2.1. Notch Inhibition by γ-secretase Inhibitor

γ-secretase inhibitors are one way of inhibiting Notch signaling. γ-secretase is responsible for release of NICD after activation of Notch receptor by Notch ligands. DAPT is a γ-secretase inhibitor that is frequently used to inhibit both canonical and non-canonical Notch pathway. MDA-MB-231 cells were treated with 90 µM DAPT for 24 hours to inhibit Notch pathway which is determined according to optimization with different concentrations and time points. After 24 hours DAPT treatment, mRNA levels of Notch target gene Hey2 and candidate genes were detected by semi-quantitative Real Time RT-PCR. Expression of Hey2 significantly reduced by DAPT, which indicates inhibition of Notch signaling was accomplished. Notch inhibition evoked significant upregulation in SEMA3C and CXCR7 whereas, increase in SEMA6D and HMGA2 couldn't reach significant level. mRNA expression levels of CXCL14, CCL20 and CYR61 were decreased by Notch inhibition. CYR61 expression was significantly decreased approximately 40% while CXCL14 and CCL20 was decreased nearly 30% and 10% respectively but they didn't reach significant level (Figure 4.2).
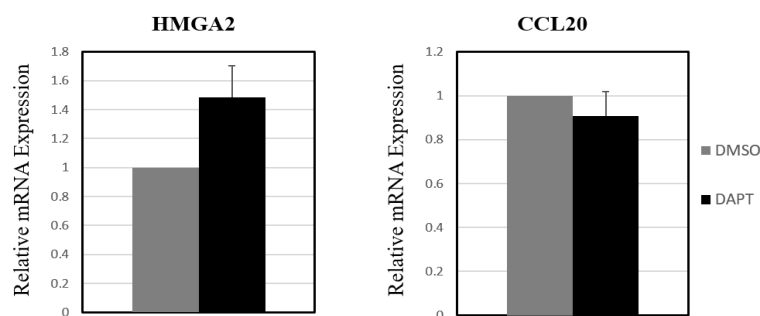
Figure 4.2. Real Time RT-PCR results of Notch inhibition by DAPT in MDA-MB-231. 24 hours after DAPT treatment, mRNA levels of Notch target Hey2; candidate genes CXCL14, SEMA6D, CCL20, CYR61, HMGA2, CXCR7 and SEMA3C were detected. TBP was used for normalization. Three independent experiments were conducted. (*p< 0.05, **p<0.02, ***<0.003)

4.2.2. Notch Inhibition by CSL Transcription Factor Silencing

CSL is the key transcription factor involved in canonical Notch pathway. It interacts with NICD in order to turn on expression of target genes of Notch. Therefore, silencing of CSL results in inhibition of Notch signaling.

In order to inhibit Notch signaling, lenti-viruses including shRNA sequence against CSL mRNA (shCSL) or shRNA sequence against GFP mRNA (control) were introduced to MDA-MB-231 cells. 72 hours after infection, mRNA levels of Hey2 and candidate genes were measured by semi-quantitative Real Time RT-PCR.

Hey2 expression was decreased approximately 80% which is an indication of successful inhibition of Notch signaling. Notch inhibition via CSL silencing resulted in significant reduction in mRNA expression levels of CYR61, HMGA2 and CCL20. It also decreased CXCL14 and SEMA6D mRNA expression however, these are not significant. Notch inhibition slightly affected expression of SEMA3C and CXCR7 genes (Figure 4.3).
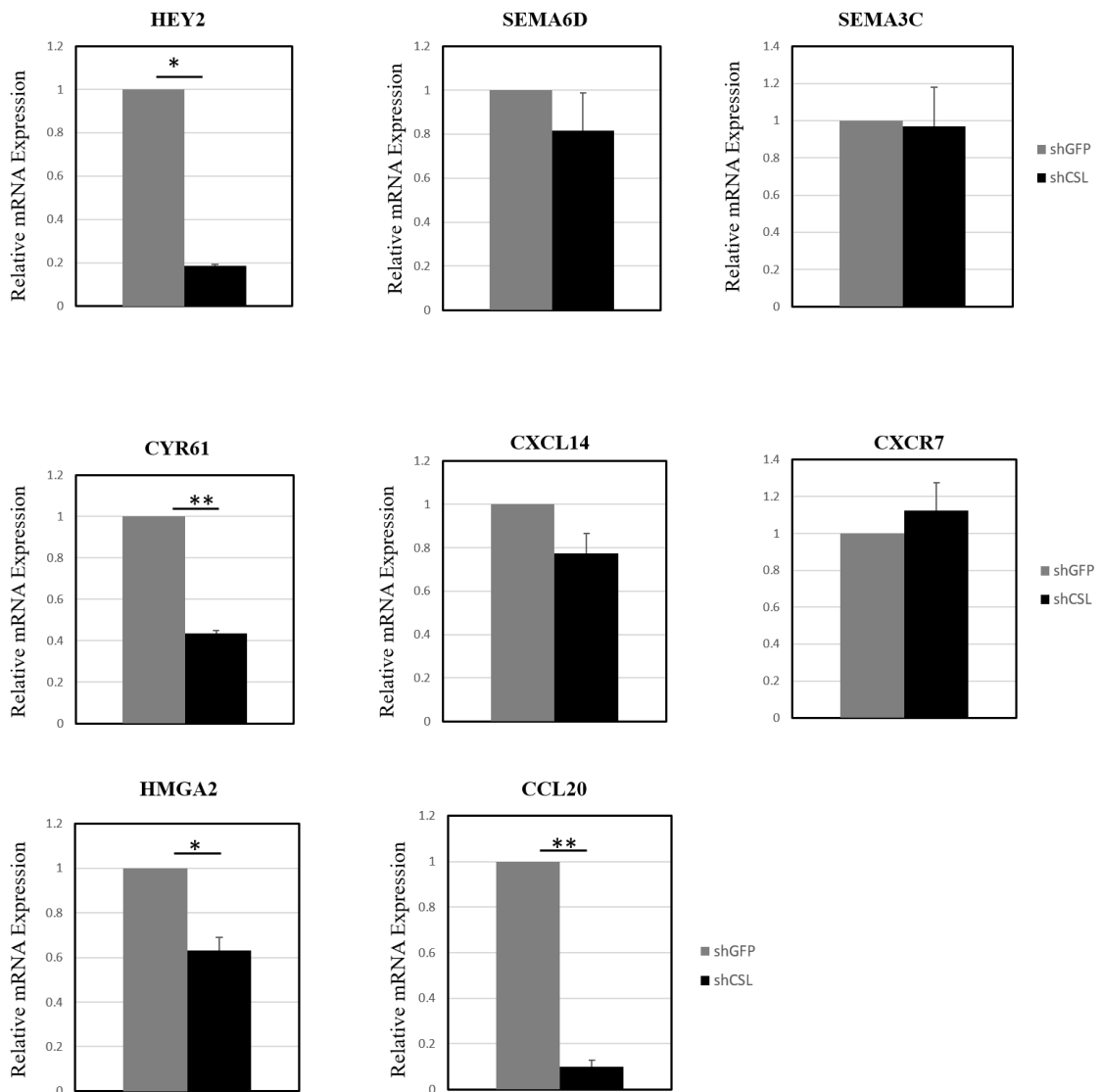
Figure 4.3. Real Time RT-PCR results of Notch inhibition by CSL inhibition in MDA-MB-231. 72 hours after infection, mRNA levels of Notch target Hey2; candidate genes CXCL14, SEMA6D, CCL20, CYR61, HMGA2, CXCR7 and SEMA3C were measured. TBP was used for normalization. Three independent experiments were conducted. (*p< 0.05, **p<0.02, ***<0.003)

Depending on mRNA expression results, Notch inhibition differentially regulates expression of candidate genes. Although, both DAPT and shCSL treatment lead to Notch inhibition, they showed different pattern in regulation of SEMA6D, SEMA3C, HMGA2 and CXCR7 genes. However, they lead to similar expression pattern for CYR61, CCL20 and CXCL14.

When Notch inhibition and Notch1 induction results are considered, upregulation of CXCL14, CYR61, CCL20 and SEMAD under active Notch1 in MCF10A and downregulation of CXCL14, CYR61, CCL20 and SEMA6D via CSL

silencing in MDA-MB-231 show that SEMA6D, CYR61, CXCL14 and CCL20 are regulated transcriptionally by canonical Notch pathway in human breast epithelial cells.

4.3. Notch1 Activation Regulates Protein Expressions of Candidate Genes in MCF10A

Real Time RT-PCR results of Notch1 activation in MCF10A cells showed that SEMA6D, SEMA3C, CYR61, CXCL14 and CXCR7 genes were highly regulated through Notch1 activation. Therefore, these genes were selected for protein expression analysis in order to confirm Real Time RT-PCR results. Notch signaling was activated by introducing Notch1 Intra-Cellular Domain (N1-ICD) in MCF10A human breast epithelial cell line which doesn't show Notch activity. 72 hours after infection by retro-virus containing N1-ICD, protein expression of those genes were checked by Western Blot. Hey2 protein level was increased nearly 3.5 fold that confirms activation of Notch signaling in MCF10A cells. Notch1 activation led to significant increase in SEMA6D (approximately 1.5 fold) and CYR61 (approximately 25 fold) protein levels which are consistent with mRNA expression results in Notch1 activated MCF10A cells. Notch1 activation also evoked nearly 1.5 fold increase in CXCR7 protein level but it didn't reach to significant value. Protein level of SEMA3C was slightly affected by Notch1 induction. CXCL14 antibody didn't work so we couldn't detect protein expression of CXCL14.
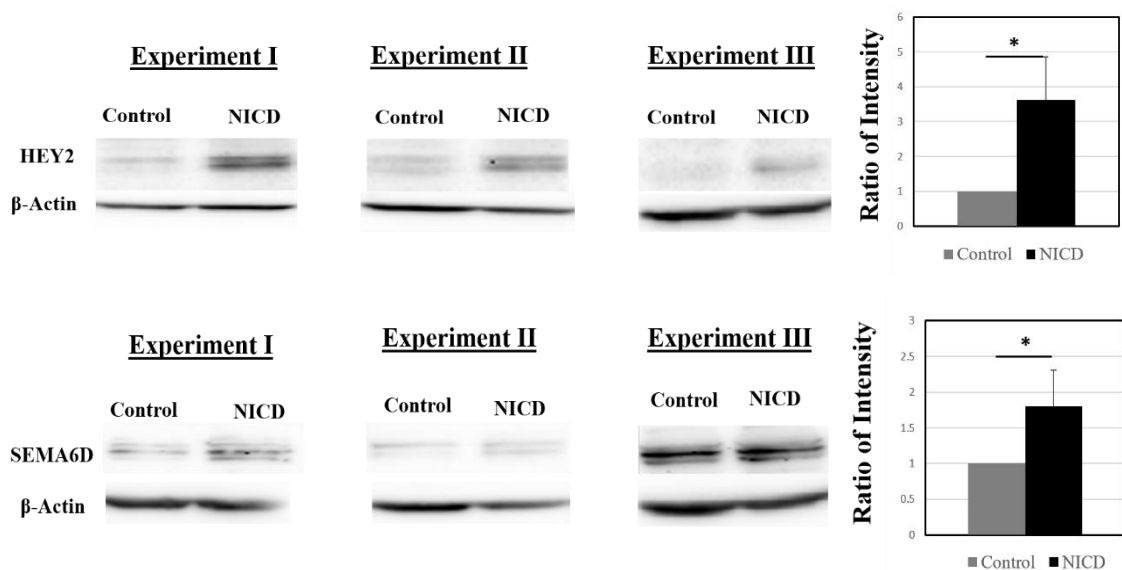
Figure 4.4. Western Blot results of Notch1 activation in MCF10A. 72 hours after infection, protein level of Notch target gene Hey2; candidate genes SEMA6D, CYR61, SEMA3C, and CXCR7 were detected. β-Actin was used as loading control. Three independent experiments were conducted. (*p< 0.05, **p<0.02)

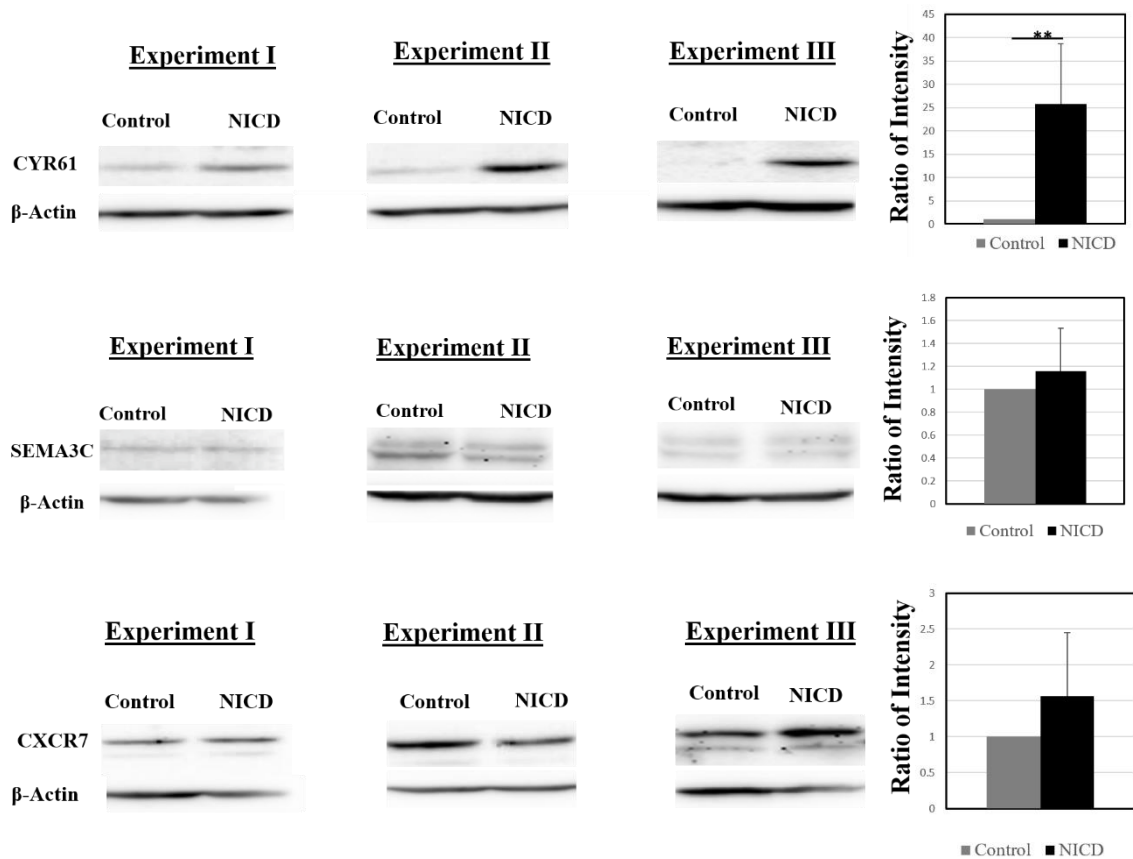## 4.4. Notch Inhibition Regulates Protein Expressions of Candidate Genes in MDA-MB-231

We chose CSL silencing method rather than DAPT to inhibit Notch signaling for protein expression analysis because DAPT also inhibits cleavage of other γ-secretase target proteins. So we wanted to see the effect of specific Notch inhibition. Lenti-viruses including shRNA sequence against CSL mRNA (shCSL) or shRNA sequence against GFP mRNA (control) were administered to MDA-MB-231 cells. 72 hours after infection, protein expression levels of Hey2, SEMA6D, CYR61 and CXCR7 were detected. Hey2 protein expression was reduced approximately 50% by CSL silencing. Notch inhibition slightly affected protein expression of SEMA6D while it decreased CYR61 expression 70% but it is not significant which are consistent with mRNA

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expression results. CXCR7 protein expression level was increased by Notch inhibition which is inconsistent with Real Time RT-PCR results of Notch inhibition in MDA-MB-231cells (Figure 4.5).
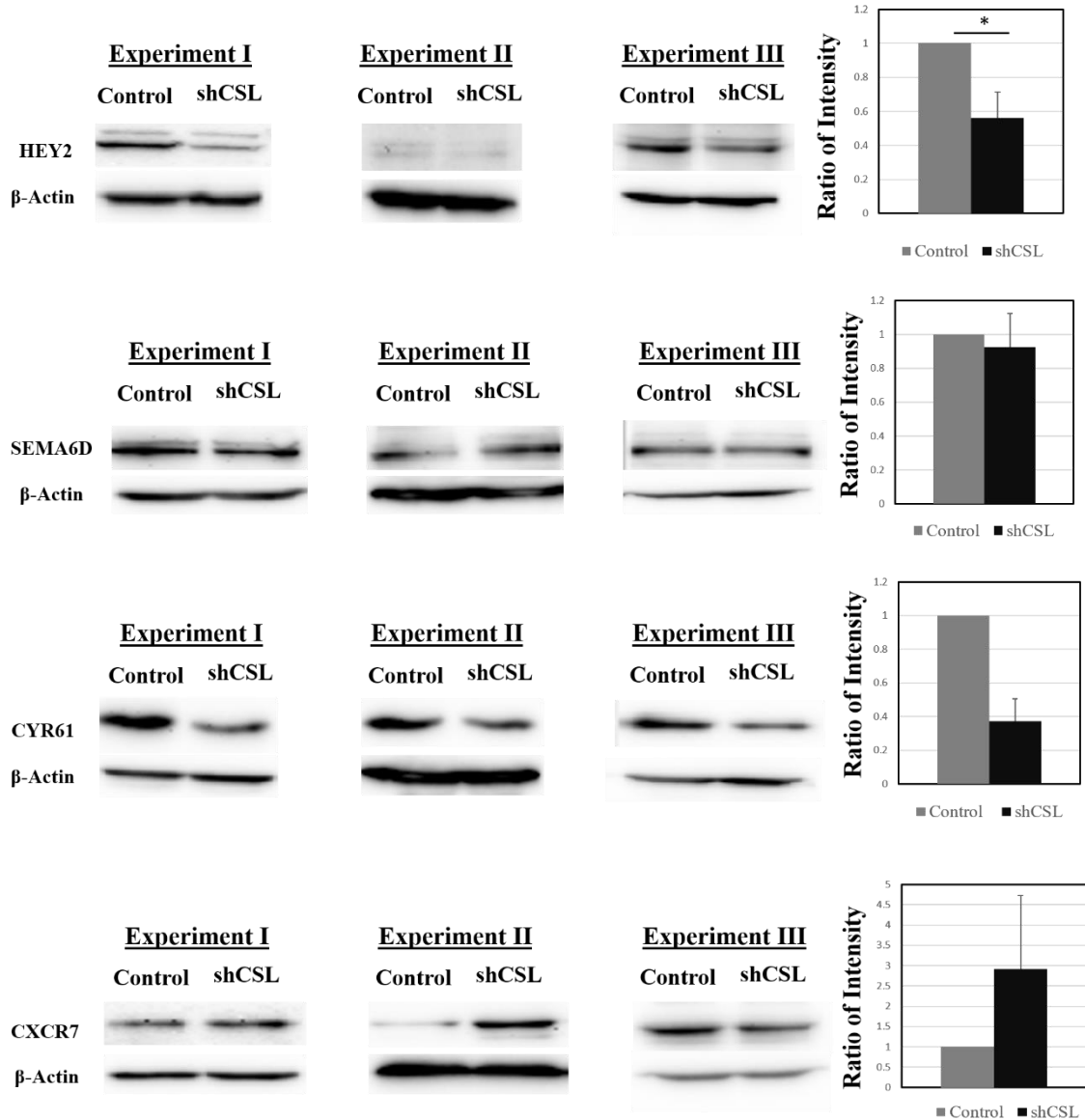
Figure 4.1. Western Blot results of Notch inhibition via CSL silencing in MDA-MB-231. 72 hours after infection protein level of Notch target gene Hey2; candidate genes SEMA6D, CYR61 and CXCR7 were detected. β-Actin was used as loading control. Three independent experiments were performed. (*p< 0.05)

We chose SEMA6D for EMT analysis and migration experiments, because it was significantly upregulated by Notch activation by approximately 20 fold and 2 fold in mRNA and protein level respectively in normal breast epithelial cell line MCF10A.

Although, we didn't detect any significant change in SEMA6D expression in CSL inhibition, it is a promising gene because it is involved in embryonic development (Toyofuku et al. 2004) and has oncogenic properties in osteosarcoma (Moriarity et al. 2015) and in asbestos induced mesothelioma (Catalano et al. 2009). There is no data indicating role of SEMA6D in breast cancer.

4.5. SEMA6D Under Notch Signaling Slightly Affects EMT Markers in MDA-MB-231

Activation of Notch1 induces EMT via SNAI2 induction (Timmerman et al. 2004) and metastasis in melanoma, pancreatic cancer and gastric cancer (Hu et al. 2012). Therefore, we investigated whether SEMA6D plays role under Notch signaling during Notch induced EMT in breast cancer. For this purpose, four conditions that are Control-Control (I), Control-SEMA6D (II), shCSL-Control (III) and shCSL-SEMA6D (IV) MDA-MB-231 cells were generated by double infection. In both shCSL-control (III) and shCSL-SEMA6D (IV), Notch was successfully inhibited as shown by reduction in Notch target gene Hey2 mRNA level. SEMA6D overexpression is also achieved in both Control-SEMA6D and shCSL-SEMA6D conditions. Overexpression of SEMA6D in shCSL-SEMA6D group led to increase in ZO-1, ZEB1, ZEB2 and SNAI2 mRNA, which were reduced upon Notch inhibition in shCSL-control group. Overexpression of SEMA6D in shCSL-SEMA6D group resulted in slight decrease in e-Cadherin and Vimentin mRNA compared to shCSL-Control group. SNAI1 mRNA level largely remained similar in group III and IV (Figure 4.6). SEMA6D overexpression partially rescues the negative effect of Notch inhibition on EMT markers such as ZO-1, ZEB1, ZEB2 and SNAI2. However, for the second and third experiments, when cells were treated with shCSL virus, expression of HEY2 increased so inhibition of Notch signaling cannot be achieved (Figure 4.7). In this case, e-cadherin expression is reduced, while SNAI1 was induced as it would be expected if Notch signaling would have been activated.

HEY2

SEMA6D

Control- Control
Control-SEMA6D
shCSL-Control
shCSL-SEMA6D

e-Cadherin

ZO-1

Control- Control
Control-SEMA6D
shCSL-Control
shCSL-SEMA6D

ZEB1

ZEB2

Control- Control
Control-SEMA6D
shCSL-Control
shCSL-SEMA6D

SNAI1

SNAI2

Control- Control
Control-SEMA6D
shCSL-Control
shCSL-SEMA6D

34
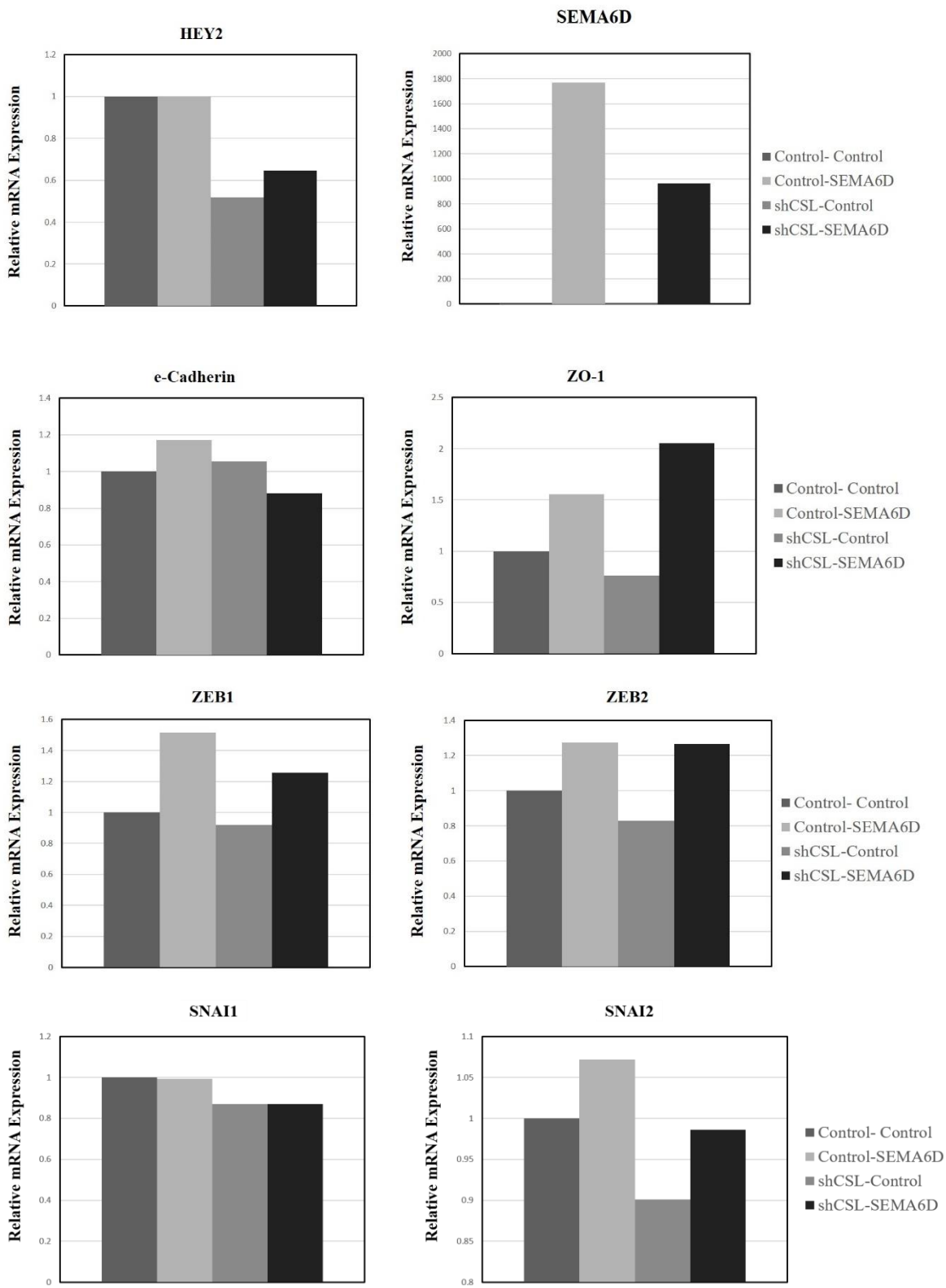
Vimentin

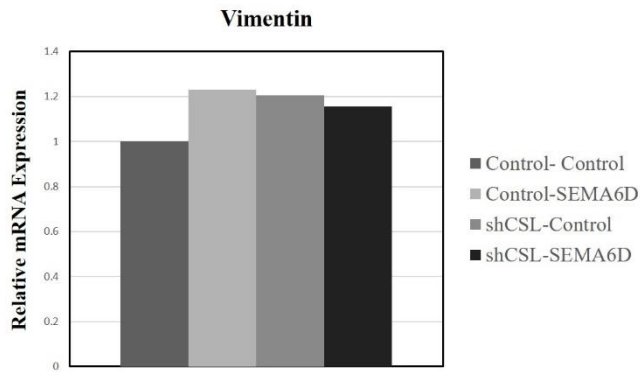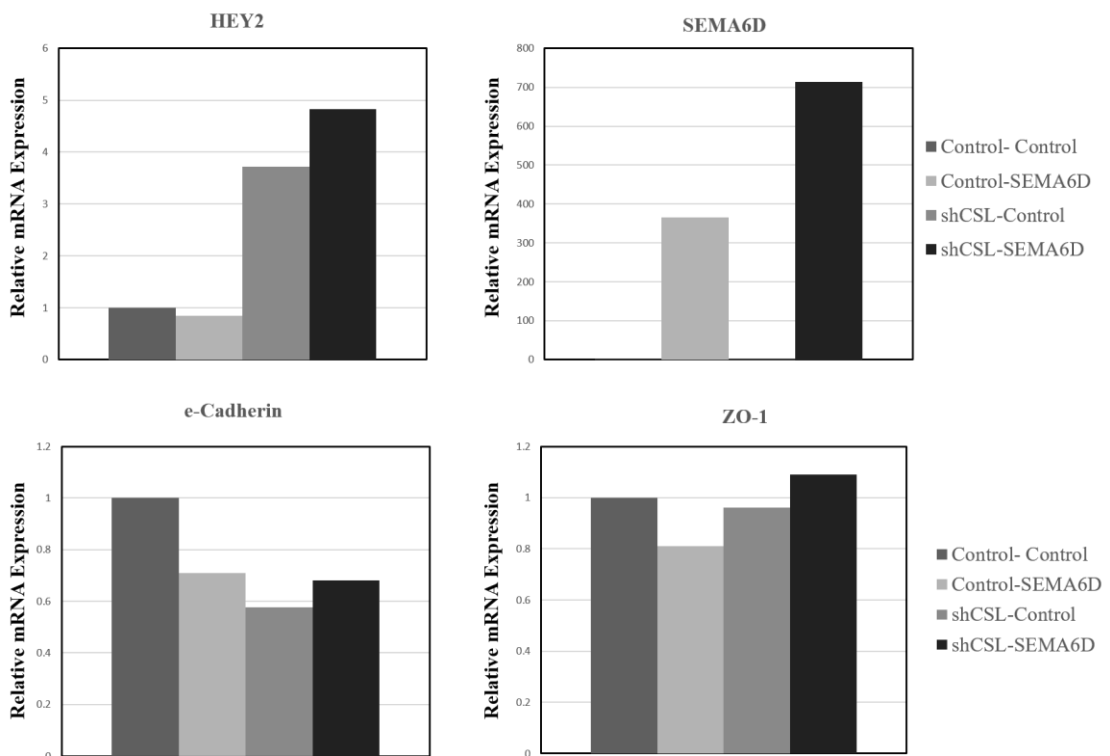Figure 4.6. Relative mRNA expression levels of Notch target gene Hey2 and SEMA6D and of EMT markers including ZO-1, e-Cadherin, ZEB1, ZEB2, SNAI1, SNAI2 and Vimentin in MDA-MB-231 cells infected with (I) two control viruses, (II) control and SEMA6D cDNA viruses, (III) CSL shRNA and control viruses and (IV) CSL shRNA and SEMA6D cDNA viruses. mRNA levels were detected 72 hours after infection. TBP was used for normalization.
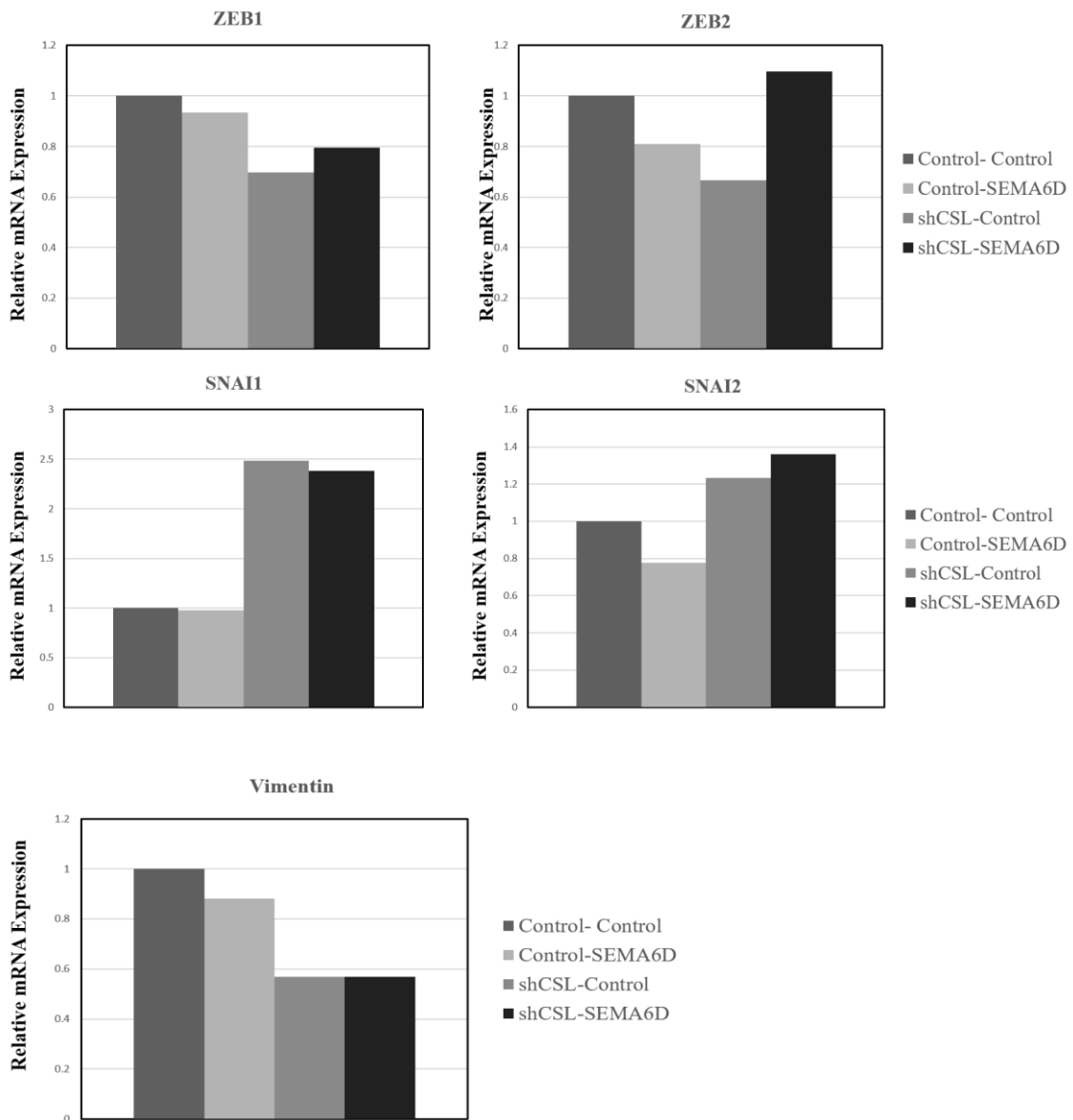
Figure 4.7: Relative mRNA expression levels of Notch target gene Hey2 and SEMA6D and of EMT markers in MDA-MB-231 cells infected with (I) two control viruses, (II) control and SEMA6D cDNA viruses, (III) CSL shRNA and control viruses and (IV) CSL shRNA and SEMA6D cDNA viruses. mRNA levels were detected 72 hours after infection. TBP was used for normalization.

4.6. SEMA6D Rescues Anti-Proliferative Effect of Notch inhibition in MDA-MB-231

Notch signaling can act both as an oncogene and tumor suppressive depending on the cellular context and the dosage (Mazzone et al. 2010; South, Cho, and Aster 2012). Notch1 is activated in breast cancer cell lines which induces the proliferation

(Weijzen et al. 2002). We investigated the proliferative effect of SEMA6D under Notch signaling in MDA-MB-231 cells. We generated stable MDA-MB-231 cell lines which are (I) control-control, (II) control-SEMA6D, (III) shCSL-control, (IV) shCSL-SEMA6D. 350.000 cell/well were seeded to 6 well/plate for each condition. After double infection, cells were selected with 4µg/ml blasticidin and 2µg/ml puromycin antibiotics in order to select cells which are infected with two viruses. Control-SEMA6D group has higher cell density compared to control-control group. Cell density was reduced by Notch inhibition via CSL silencing compared to control-control group and cells formed clusters. However, cell density of shCSL-SEMA6D is higher compared to shCSL-SEMA6D group (Figure 4.8). This data suggest that SEMA6D rescued anti-proliferative effect of Notch inhibition in MDA-MB-231 cells.
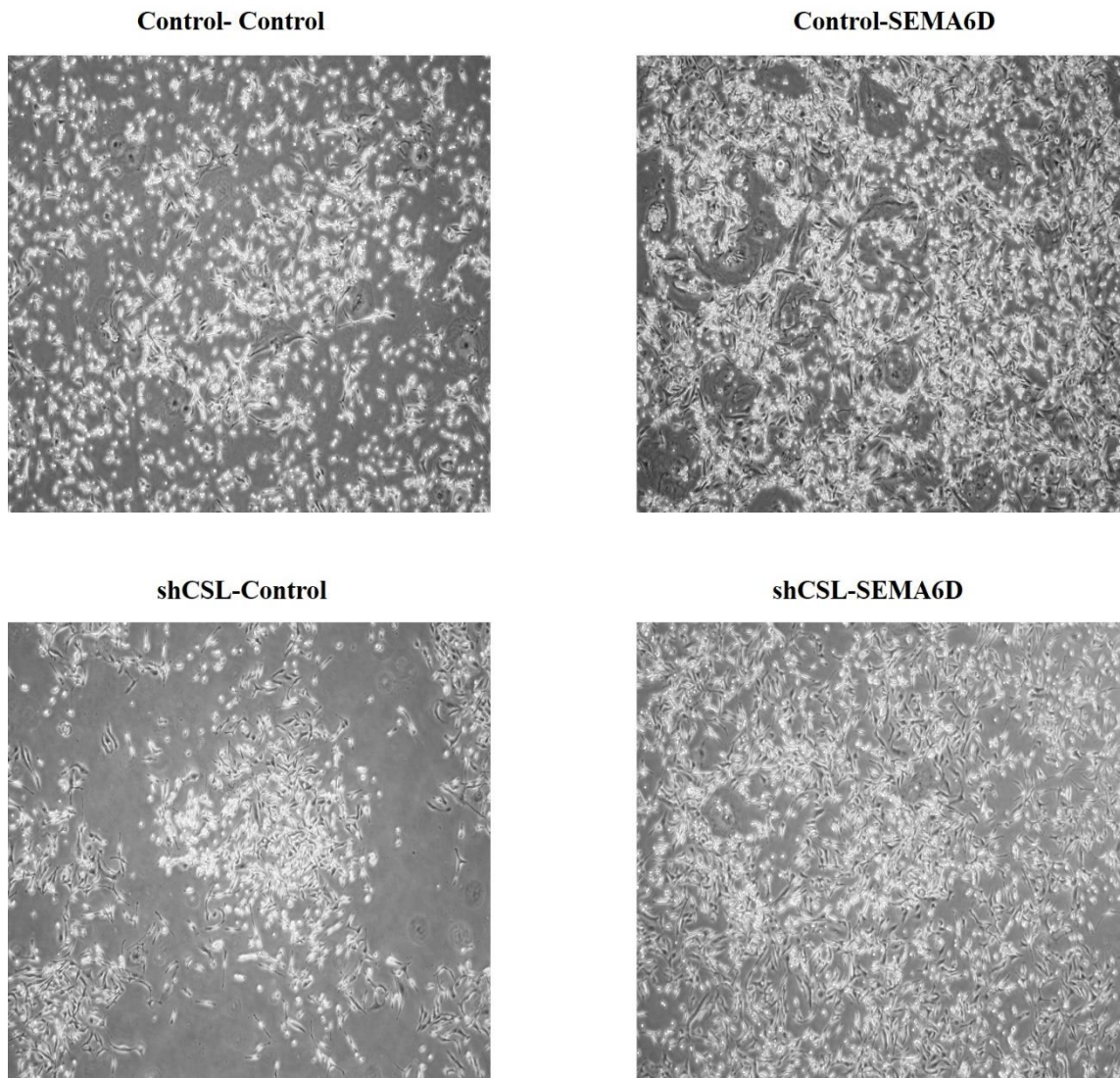
Figure 4.8. Photomicrographs of MDA-MB-231 cells infected with (I) two control viruses, (II) control and SEMA6D cDNA viruses, (III) CSL shRNA and control viruses and (IV) CSL shRNA and SEMA6D cDNA viruses. Photomicrographs indicate cell densities of the four different groups.

4.7. SEMA6D Overexpression Slightly Affects EMT Markers in MDA-MB-231

In order to see the effect of SEMA6D overexpression on EMT markers, Lenti-viruses having SEMA6D cDNA or LacZ (control) were introduced to MDA-MB-231 cells. We chose MDA-MB-231 cells because it has lower SEMA6D expression compared to MCF10A and it is hard to induce EMT in MCF10A due to its strong epithelial character. When SEMA6D is overexpressed in MCF10A, it might tend to

protect its epithelial character by increasing expression of cell junction proteins. EMT analysis was performed for both transient (72 hours after infection) and stable MDA-MB-231 cells by checking the mRNA expression levels of ZO-1, e-Cadherin which are epithelial markers and Vimentin, ZEB1, ZEB2, SNAI1 and SNAI2 that are mesenchymal markers. We generated stable MDA-MB-231 overexpressing SEMA6D and its control (LacZ) by selecting with 4 μg/ml blasticidin antibiotic to eliminate the cells which weren't infected. Overexpression of SEMA6D was confirmed with approximately 550 folds increase in SEMA6D compared to control in stable MDA-MB-231 cells. Overexpression of SEMA6D slightly increased expression of epithelial markers such as ZO-1 and e-Cadherin and mesenchymal markers, ZEB1, ZEB2, Vimentin and SNAI1, whereas it led to slight reduction in SNAI2 mesenchymal markers. SNAI2 expression was decreased nearly 35% by SEMA6D overexpression (Figure 4.9).
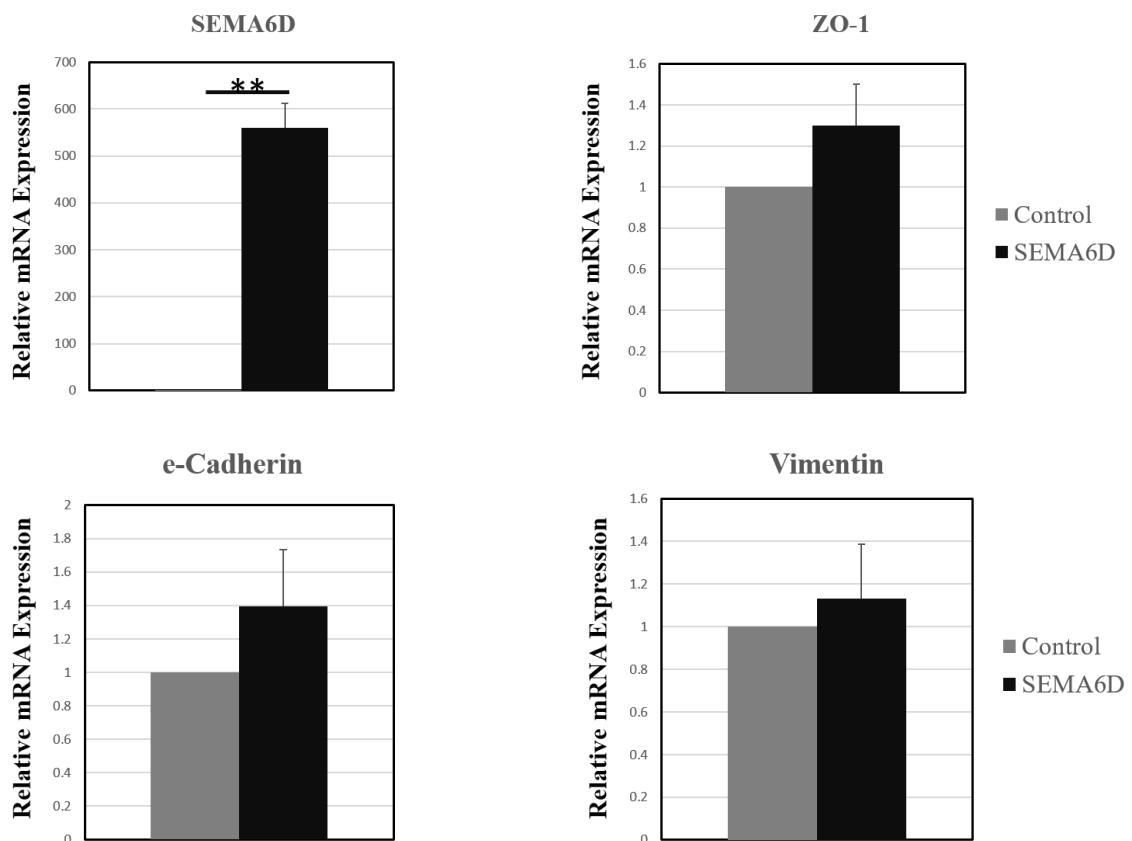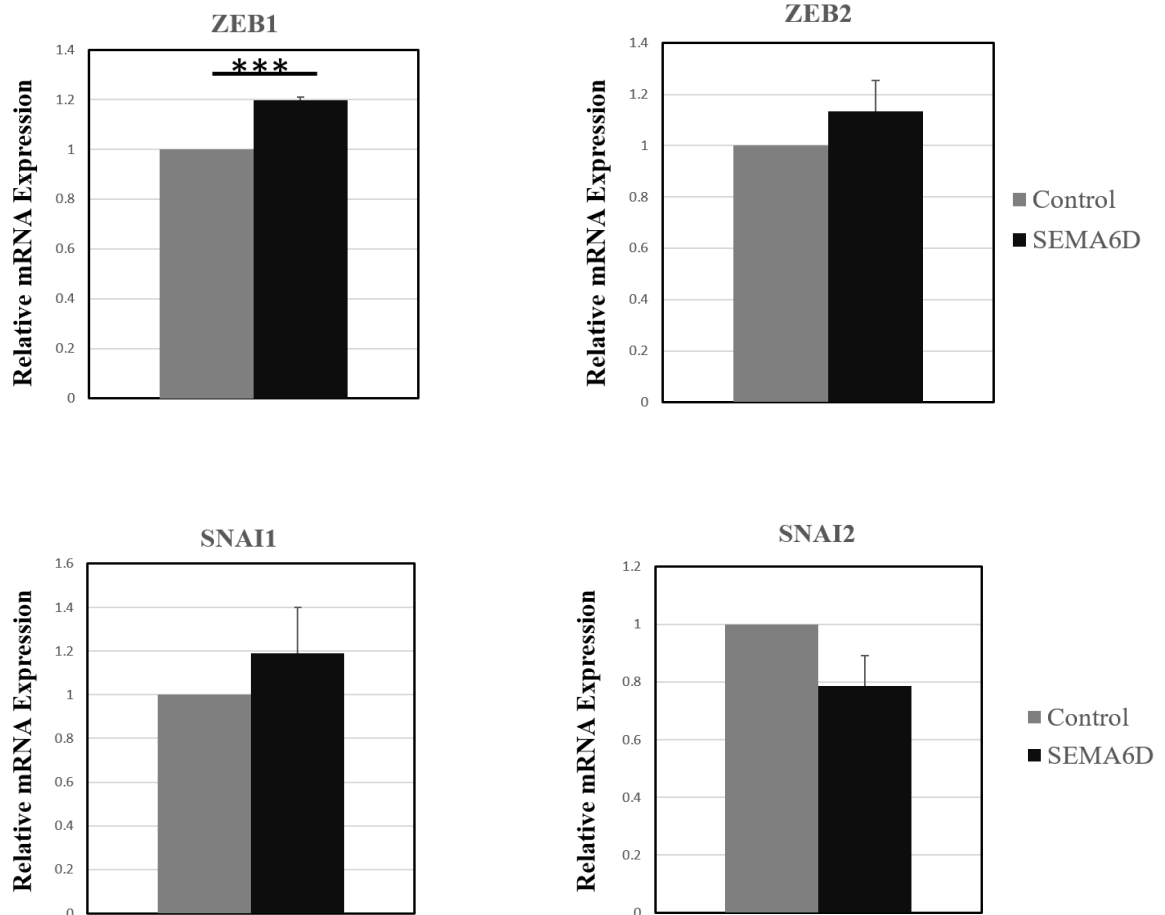
Figure 4.9. Real Time RT-PCR results of stable MDA-MB-231 cells overexpressing SEMA6D. After selection of stable cells by 4 µg/ml blasticidin, mRNA levels of SEMA6D and EMT markers that are ZO-1, e-Cadherin, Vimentin, ZEB1, ZEB2, SNAI1 and SNAI2 were detected. TBP was used for normalization. One experiment was performed.

mRNA levels of SEMA6D and EMT markers were checked 72 hours after infection. SEMA6D overexpression was confirmed by more than 400 folds increase in expression of SEMA6D. SEMA6D overexpression resulted in slight change in ZO-1, Vimentin, ZEB1, ZEB2 and SNAI1 EMT markers. While expression of e-Cadherin and SNAI2 was reduced by the overexpression of SEMA6D genes (Figure 4.10)
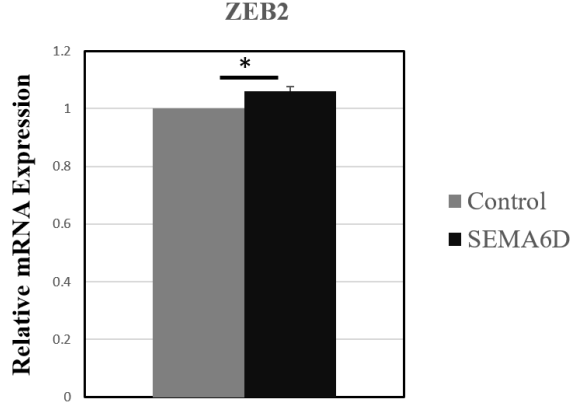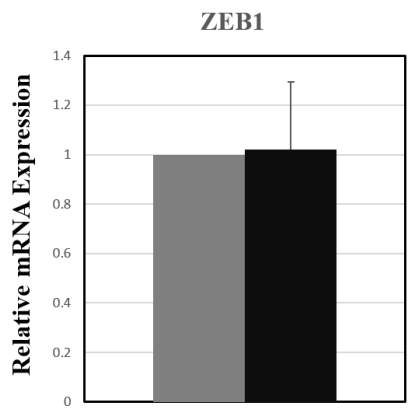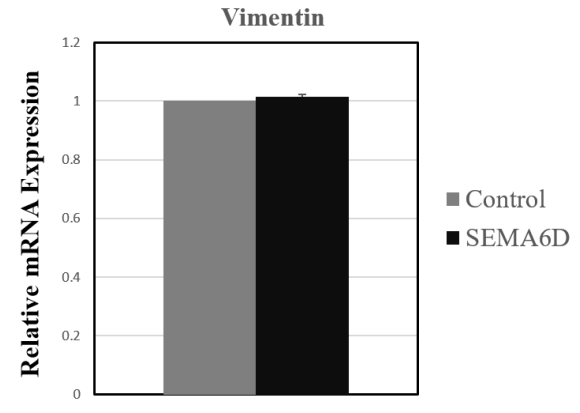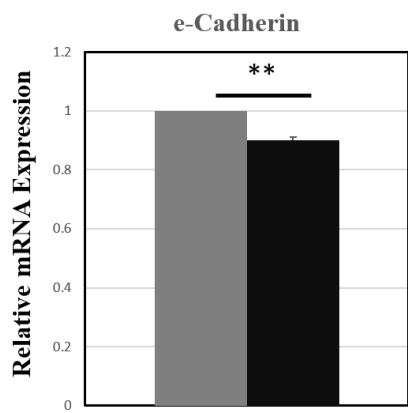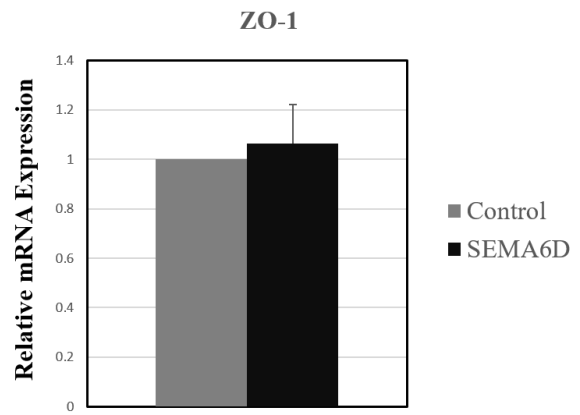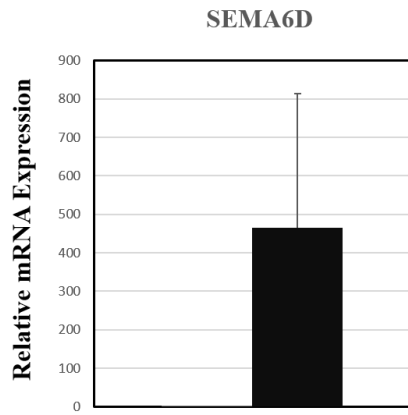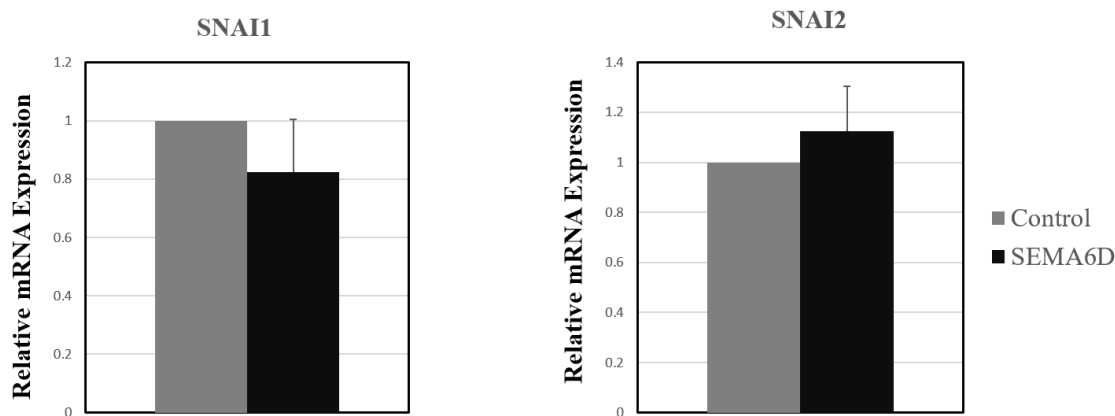
Figure 4.10. Real Time RT-PCR results of SEMA6D overexpression in MDA-MB-231 cells. After 72 hours after infection, mRNA levels of SEMA6D and EMT markers that are ZO-1, e-Cadherin, Vimentin, ZEB1, ZEB2, SNAI1 and SNAI2 were detected. TBP was used for normalization. One experiment was performed.

4.8. SEMA6D Overexpression Induces Cell Viability in MDA-MB-231 cells

In order to investigate the Notch independent effect of SEMA6D on cell viability, we performed MTT assay for SEMA6D overexpressing stable MDA-MB-231 cell line. Cell viability was increased by the SEMA6D overexpression compared to control in day 2 and 4. At day 9, cell viability of MDA-MB-231 cells overexpressing SEMA6D was reduced slightly compared to control (Figure 4.11). It might be caused by the confluency. MDA-MB-231 SEMA6D overexpressing cells reached confluency faster because it has increased level of cell viability compared to control.
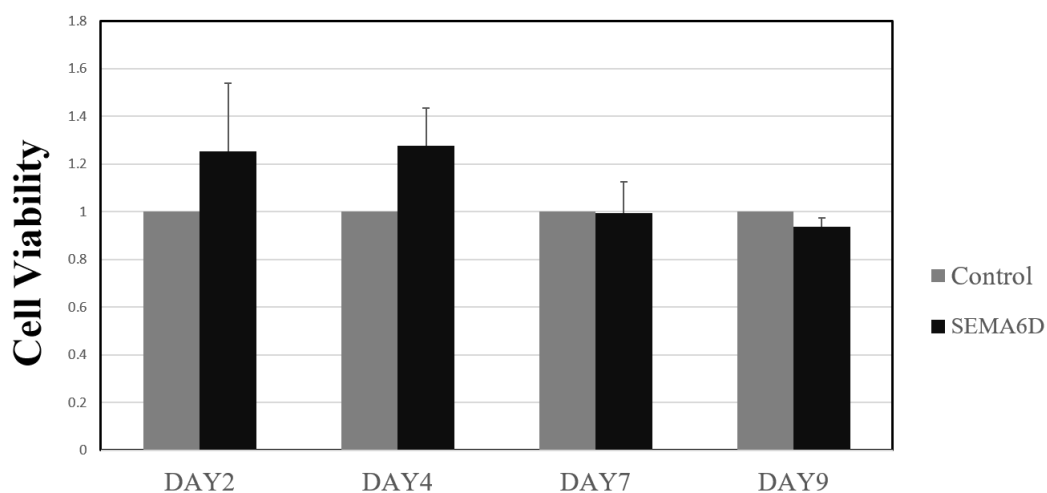
Figure 4.11. MTT assay results indicating cell viability of stable MDA-MB-231 cell overexpressing SEMA6D and the control.

4.9. SEMA6D Overexpression Induces Migration in MDA-MB-231 cells

SEMA6D induces migration of myocardial cells during heart development (Toyofuku et al. 2004). We hypothesized that SEMA6D may induce migration of breast cancer cells, MDA-MB-231. Stable MDA-MB-231cells were used for scratch wound healing assay. MDA-MB-231 cells overexpressing SEMA6D closed the gap by 60% while control group closed by 40% at 12 hours. At 24 hours, while control group closed the gap about 70%, cells overexpressing SEMA6D closed the 90% of the gap (Figure 4.12). This data suggest that SEMA6D induces migration of MDA-MB-231 cells independent of Notch signaling.
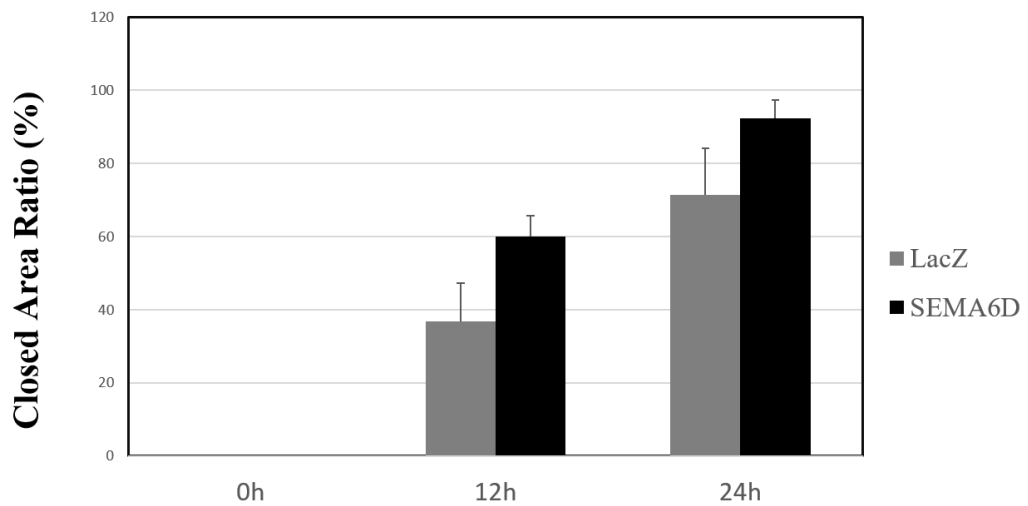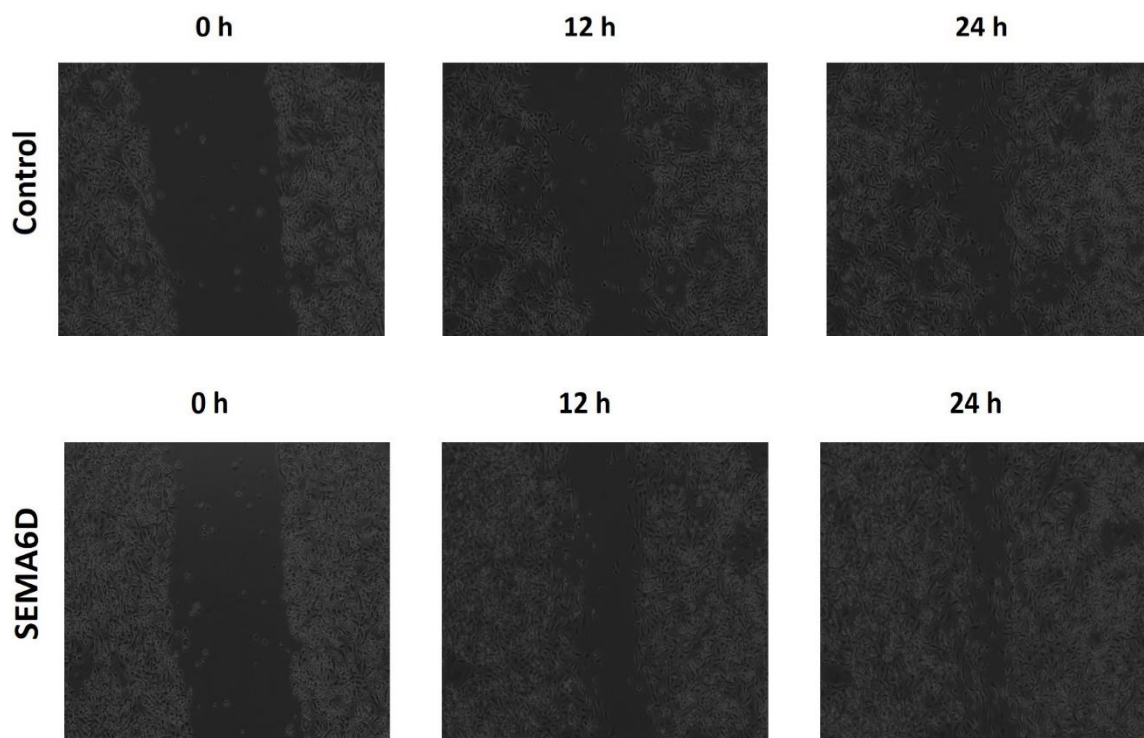
Figure 4.12. Photomicrographs of scratch wound healing assay of stable MDA-MB-231 cells overexpressing SEMA6D and Control group. The graph shows the quantification of the closed area by MDA-MB-231 cells.

CHAPTER 5

DISCUSSION

Notch signaling has first been described in murine mammary gland by the proviral integration of mouse mammary tumor virus (MMTV) into the Notch4 locus (*Int3* locus) which resulted in transformation of mammary epithelial cells (Jhappan et al. 1992). Notch1 is highly expressed in breast cancer and constitutively active form of Notch1 induces neoplasm (Kiaris et al. 2004; Reedijk et al. 2005). In breast cancer xenograft models, overexpression of active Notch1 receptor-ICD promotes epithelial-mesenchymal transition (EMT) via Snail induction which demonstrates the role of Notch signaling in induction of metastasis through EMT (Leong et al. 2007). Inhibition of Notch pathway in breast cancer patients prevented development of brain metastasis (Witzel et al. 2016). However, the downstream mediators of Notch in EMT and migration processes are still elusive. We hypothesized that Notch signaling induces EMT and migration via regulating one or more of the seven candidate genes that are SEMA6D, SEMA3C, CXCR7, CXCL14, CYR61, HMGA2 and CCL20 which were shown to be differentially regulated by Notch signaling in normal breast epithelial cells (Mazzone et al. 2010; Yalcin-Ozuysal et al. 2010).

Activation of Notch1 in MCF10A cells leads to differential regulation of candidate genes in both mRNA level and protein level which confirms previous findings (Mazzone et al. 2010; Yalcin-Ozuysal et al. 2010). While mRNA expression levels of CXCL14, SEMA6D, CCL20, CYR61 and CXCR7 were increased, HMGA2 and SEMA3C mRNA expression levels were reduced by Notch1 activation. Protein expression levels of SEMA6D, CYR61 and CXCR7 were consistent with mRNA levels whereas, SEMA3C didn't show consistency. The regulation of the candidate genes was also supported by Notch inhibition in MDA-MB-231 breast cancer cells. However, Notch inhibition by DAPT and CSL silencing showed different regulation patterns for mRNA expressions of candidate genes. It may be caused by the Notch independent effect of DAPT which also inhibits cleavage of other γ-secretase target proteins such as E-cadherin, Src, CD44, ErbB4 (Lammich et al. 2002). DAPT inhibits all Notch signaling whereas, CSL silencing leads to inhibition of canonical Notch pathway.

Therefore, we preferred CSL silencing for protein expression analysis in order to examine the effect of specific Notch inhibition on candidate genes. Inhibition of Notch signaling via CSL silencing resulted in reduction mRNA levels of SEMA6D, CYR61, CXCL14, HMGA2 and CCL20 while mRNA levels of CXCR7 and SEMA3C remained similar. Protein expressions of SEMA6D and CYR61 are consistent with the mRNA levels. Therefore, our data suggest that CYR61 are tightly regulated by Notch signaling in both MCF10A and MDA-MB-231 cells. SEMA6D was significantly upregulated by Notch activation by approximately 20 fold and 2 fold in mRNA and protein level respectively in normal breast epithelial cell line MCF10A. Although, we didn't detect any significant change in SEMA6D expression in CSL inhibition, SEMA6D was selected for EMT, migration and cell viability analysis because it is a promising gene as it is involved in embryonic development (Toyofuku et al. 2004) and has oncogenic properties in osteosarcoma (Moriarity et al. 2015) and in mesothelioma cells (Catalano et al. 2009). There is no data indicating role of SEMA6D in breast cancer.

Activation of Notch1 induces EMT via SNAI2 induction (Timmerman et al. 2004) and metastasis in melanoma, pancreatic cancer and gastric cancer (Hu et al. 2012). Therefore, we asked whether SEMA6D plays role under Notch signaling during Notch induced EMT in breast cancer. SEMA6D overexpression partially rescues the negative effect of Notch inhibition on EMT markers such as ZO-1, ZEB1, ZEB2 and SNAI2. Experiment was repeated for 3 times, but Notch inhibition cannot be accomplished in the second and third experiments. Expression of Notch target gene, Hey2, increased with shCSL virus treatment. Cell may have mechanism to compensate inhibition of Notch signaling. Notch inhibition with shCSL experiments should be repeated or alternative inhibition approaches should be tested in order to elucidate Notch dependent effect of SEMA6D on EMT markers.

Stable MDA-MB-231 cells overexpressing SEMA6D slightly reduced mRNA levels of ZEB1 and Vimentin that are mesenchymal markers. The rest of EMT markers including ZEB2, SNAI1, SNAI2, e-Cadherin and ZO-1 weren't affected by SEMA6D overexpression. Transient infection of MDA-MB-231 cells with lenti-virus containing SEMA6D cDNA led to slight reduction in mRNA levels of ZEB1 and SNAI1 whereas the rest remained similar. SEMA6D may inhibit EMT because it reduced expression of mesenchymal markers, however, protein levels of EMT markers also need to be examined.

SEMA6D has been indicated as an oncogene in human osteosarcoma (Moriarity et al. 2015) and asbestos induced mesothelioma (Catalano et al. 2009). Therefore, we investigated the effect of SEMA6D on cell viability of MDA-MB-231 cells. According to MTT results, SEMA6D overexpression led to increase in cell viability of MDA-MB-231 cells. However, MTT shows the cell viability, in order to show whether SEMA6D plays role as an oncogene or tumor suppressive in breast cancer, proliferation assays such as propidium iodide staining or BrdU assay should be performed. In addition, soft agar colony formation assay can be also performed to see whether SEMA6D causes transformation of normal breast epithelial cells.

SEMA6D induces migration of myocardial cells during heart development (Toyofuku et al. 2004). We assessed the effect of SEMA6D on migration of MDA-MB-231 cells by scratch wound healing assay. Stable MDA-MB-231 cells overexpressing SEMA6D closed the gap faster compared to control. So, SEMA6D may induce migration. However, cells weren't treated with any drugs in order to stop proliferation. The closure can be caused by proliferation rather than migration. Because MTT results showed that SEMA6D increases cell viability, and mesenchymal markers are also partially downregulated by SEMA6D. In order to see the migratory effect of SEMA6D on MDA-MB-231 cells, cells should be treated with drugs such as Mitomycin C or cultured with less serum to minimize the proliferation.

CHAPTER 6

CONCLUSION

Recently, the role of Notch signaling in metastasis has been investigated besides its role in primary tumor growth. Several studies indicated that deregulated Notch signaling has been observed in metastatic tumors in different tumor types (Hu et al. 2012). In this study, we tried to reveal the downstream mediators of Notch signaling in Notch induced EMT, migration and invasion. First, we assessed regulation of the candidate genes, SEMA6D, SEMA3C, CXCL14, CCL20, CXCR7, CYR61 and HMGA2 by Notch signaling in MCF10A and MDA-MB-231 cell lines both in mRNA and protein level. We showed that expression of CXCL14, SEMA6D, CCL20, CYR61 and CXCR7 were increased whereas, expression of SEMA3C and HMGA2 were reduced upon Notch1 activation in normal breast epithelial cell line, MCF10A. Expression of CYR61, CCL20 and HMGA2 were reduced upon Notch inhibition in breast cancer cell line, MDA-MB-231 while the rest of candidate genes were affected slightly. Depending on RT-qPCR and western blot results, CYR61 genes were tightly regulated by Notch signaling in MCF10A and MDA-MB-231 cell lines. SEMA6D was significantly upregulated by Notch activation in mRNA and protein level in MCF10A. Although, we didn't detect any significant change in SEMA6D expression upon Notch inhibition, SEMA6D was selected for further analysis because SEMA6D is more novel and promising gene compared to CYR61.

The role of SEMA6D in Notch induced EMT was investigated. SEMA6D overexpression partially rescues the negative effect of Notch inhibition on EMT markers such as ZO-1, ZEB1, ZEB2 and SNAI2. Therefore, SEMA6D could be mediator of Notch signaling to induce EMT.

Notch independent effects of SEMA6D were investigated. SEMA6D overexpression slightly regulated mRNA levels of EMT markers. It led to partial decrease in expression of mesenchymal markers including ZEB1, SNAI and Vimentin. So, SEMA6D could be involved in inhibiting EMT.

SEMA6D also affects migration capacity of MDA-MB-231 cells. Overexpression of SEMA6D induced migration of MDA-MB-231. SEMA6D might

induce migration via EMT or not. We have to reveal proliferation effect for final conclusion.

Finally, SEMA6D overexpression induced cell viability of MDA-MB-231 cells. Therefore, SEMA6D may have oncogenic role in breast cancer but it needs further investigation.

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