CYR61 - NOTCH INTERACTION DURING EPITHELIAL-TO-MESENCHYMAL TRANSITION, MIGRATION AND INVASION IN BREAST CANCER CELLS

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ABSTRACT

CYR61 – NOTCH INTERACTION DURING EPITHELIAL TO MESENCHYMAL TRANSITION, MIGRATION AND INVASION IN BREAST CANCER CELLS

Notch signaling is one of the major pathways involved in development and tumorigenesis. Activated Notch is correlated with increased migration, invasion and epithelial-to-mesenchymal-transition (EMT) in breast cancer. However, mechanism of Notch-mediated cancer progression is poorly understood. CYR61 is a secreted protein and its upregulation is also related to increased breast tumorigenesis and EMT. Microarray analyses revealed that CYR61 was differentially expressed in response to Notch activation in breast epithelial cells. We hypothesized that CYR61 is a downstream mediator of Notch during EMT, migration and invasion.

To test whether Notch requires CYR61 during EMT, migration and invasion, two complementary approaches were followed: (i) CYR61 expression was silenced by using shRNA in MCF10A epithelial breast cell line in the presence of Notch activation, (ii) CYR61 was over-expressed in MDA-MB-231 cancer breast cell line in the absence of Notch activity. Then, expression of EMT markers was analyzed in mRNA and protein levels via RT-qPCR and immuno-blotting, respectively. Migration and invasion ability of cells were investigated by wound healing assay and a lab-on-a-chip-system, respectively. Here, it was shown that CYR61 inhibition decreased Notch-induced EMT, migration and invasion of MCF10A and CYR61 overexpression in the absence of Notch activity partially rescued Notch-mediated invasion in MDA-MB-231 cell lines. Our findings suggest that CYR61 may act in downstream of Notch and is regulated by Notch. When we consider importance of CYR61 in Notch-induced EMT and cancer progression, targeting CYR61 may hold promise to develop novel strategies for treatment of breast cancer in early stages.

ÖZET

MEME KANSERİ HÜCRELERİNDE MEZENKİMAL GEÇİŞ (EMT), MİGRASYON VE İNVAZYON SIRASINDA CYR61-NOTCH ETKİLEŞİMİ

Notch sinyal yolağı gelişim ve tümör oluşumunda yer alan ana yolaklardan biridir. Notch aktivasyonu meme kanserinde migrasyon, invazyon ve mezenkimal geçiş (EMT) ile ilişkilidir. Fakat Notch aracılığıyla kanser gelişiminin mekanizması tam olarak anlaşılamamıştır. CYR61 salgılanan bir proteindir ve fazla ifadelenmesinin meme tümörleşmesi ile ilgili olduğu bilinmektedir. Microarray analizi CYR61 geninin Notch aktivasyonuyla farklı ifadelendiği açığa çıkarmıştır. Bu tez çalışmasında, CYR61'in EMT, migrasyon ve invazyon sırasında Notch alt aracısı olduğu hipotez edilmiştir.

Notch yolağının EMT, migrasyon ve invazyon sırasında CYR61 e ihtiyacı olup olmadığını anlamak için iki tamamlayıcı yaklaşım takip edildi: (i) MCF10A epitelyum meme hücre hattında Notch varlığında CYR61 ifadelenmesi shRNA kullanılarak susturuldu, (ii) MDA-MB-231 meme kanseri hücre hattında Notch eksikliğinde CYR61 fazla ifadelendi. Sonrasında, EMT belirteçlerinin ifadelenmesi mRNA ve protein seviyesinde sırasıyla RT-qPCR ve immün-blotlama yöntemleriyle analiz edilmiştir. Hücrelerin migrasyon ve invazyon yetenekleri sırasıyla yara iyileşme analizi ve lab-on-a-chip sistemi ile araştırıldı. Burada, CYR61'in MCF10A hücre hattında Notch tarafından indüklenen EMT, migrasyon ve invazyonda önemli olduğu ve Notch aktivitesi olmadığında CYR61 in fazla ifadelenmesinin Notch tarafından indüklenen invazyonu MDA-MB-231 kanser hücrelerinde kısmi olarak kurtardığı gösterilmiştir. Bu bulgular CYR61 in Notch yolağının alt mekanizmasında rol alabileceğini ve Notch tarafından kontrol edilebileceğini önermektedir. CYR61'in Notch tarafından indüklenen EMT ve kanser gelişimdeki önemi düşünüldüğünde, CYR61'in hedef alınması, meme kanserini erken aşamalarda tedavi etmek için yeni tedavi stratejileri geliştirmede ümit vaat etmektedir.

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CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Cancer is a disease which is resulted from uncontrolled cell growth and finally form malignant tumor. Depending on where initial tumor grows, they are named specifically such as breast cancer which is one of the common cancer types and second leading cause of cancer related death among women[1][4]. After formation of primary tumor in breast tissue, some cancer cells may also migrate and form a secondary tumor in distant region which is called as metastasis. Studies show that most of the cancer related deaths are due to metastasis which may, in turn, cause some other diseases such as organ failure [2].For metastasis process, increasing migration ability and invasiveness, cancer cells undergo some changes by which they lose their epithelial character and transit to more mesenchymal phenotype through regulation of certain genes by a process called epithelial-to-mesenchymal transition (EMT). Therefore understanding mechanism by which cancer cells migrate to distant regions and elucidating molecules that involve in their travel to other parts of the body are important for development of new treatment strategies.

1.2. Epithelial-to-Mesenchymal Transition

In cancer progression, after cells start to divide out of control, they initially form a mass of cells and proportional to increased cell number, more oxygen and nutrients are required which necessitate formation of additional capillaries around tumor core region by a process called angiogenesis [3]. Tumor cells start to invade surrounding tissue by penetrating basal membrane, normally defining tissue boundaries, by using certain protein degrading enzymes such as matrix metalloproteinases (MMP). Invasion has an important role in both formation of new capillaries and expansion of tumor to adjacent tissues and requires increased migration ability of cancer cells. During early

stages of invasion, cells lose epithelial character and convert to more mesenchymal character by a process called epithelial to mesenchymal transition (EMT) enhancing their ability for migration and invasion. EMT is also facilitated with increased tumor size which causes to oxygen and energy depletion in core region of tumor and triggers expression of other EMT-related genes, such as HIF family genes which, in turn, either activate or deactivate downstream regulators in hypoxic environment [5].For instance, changes in expression of EMT-related genes may lead to further dedifferentiation of a small number of cancer cells which, in turn, form stem cell like cancer cells. During EMT process, epithelial markers such as E-cadherin and ZO-1 are downregulated through upregulation of EMT effector genes. Snail, Slug, ZEB-1 and ZEB-2 can be considered as example of EMT effector markers. While some of these markers such as Snail directly bind to promoter region of E-cadherin to repress its expression [79], ZEB-1 and ZEB-2 mediate EMT through repression of epithelial markers and with facilitating local invasion and tumor cell dissemination which finally leads to tumor metastasis [78]. In addition to E-cadherin downregulation, mesenchymal markers are upregulated such as Vimentin, Twist and N-cadherin and cells gain more mesenchymal phenotype, increased migration and invasion ability which are critical points for tumor metastasis. Therefore, expression of mesenchymal markers are correlated to poor prognosis and advanced disease. [77][80]

Afterwards, they enter (intravasation) to blood circulation with their increased migration and invasion ability and travel through circulation until they find an appropriate site to colonize, afterwards, they exit (extravasation) circulation and form secondary tumor in distant region by a process known as metastasis [6]. Most of cancer related deaths are due to metastasis, in which EMT is one of the critical steps. Understanding EMT process and gaining new insights into mechanism are potential fields to be studied which are promising for development of more efficient strategies for cancer treatment.

1.3. Notch Pathway

Notch is an evolutionary conserved signaling pathway among multi-cellular eukaryotic organisms, ranging from worms to human, and it is a juxtacrine signaling in which two cells are affected by receptor-ligand interaction. Notch pathway is one of the

major pathways in development and homeostasis of multi-cellular organisms [7]. It is involved in normal development by regulating cell fate determination, differentiation and cell survival [8]. However, aberrant expression of Notch is also correlated to several abnormalities including tumorigenesis.

1.3.1. Notch Signaling Pathway Receptors and Ligands

Due to functional importance of Notch pathway both in development and pathogenesis, it has been deeply investigated and many receptors and ligands were identified in different organisms. Here, it is focused on Notch pathway in human. Therefore, the whole pathway will be explained through molecules involving in mammals. In mammals, Notch pathway involves four receptors (Notch 1-4) and five known ligands (Delta like 1,2 and 4 ; Jagged-1 and Jagged-2) [9]. Notch receptor is a transmembrane protein and extracellular part of it contains tandem epidermal growth factor-(EGF)-like repeats where ligand binds to receptor and three LIN12/Notch repeats which is considered to prevent ligand-independent receptor activation [10].

1.3.2. Notch Receptor Processing

Once receptor binds to ligand, extracellular part of receptor is recycled by ligand providing cell through endocytosis [11]. Then, cytoplasmic part of Notch receptor undergoes to a sequential proteolytic cleavage which starts with a cleavage at site 2 (S2) which is mediated by ADAM (a disintegrin and metalloprotease) proteases such as TACE. After this cleavage, Notch is still integrated into membrane and called as Notch extracellular truncated form (NEXT) [12]. S2 cleavage and formation of NEXT is an important step which makes Notch receptor susceptible to an additional cleavage in a S3 (site3) mediated by Presenilin-1 dependent γ-secretase complex [13]. Following third cleavage, Notch intracellular domain (NICD) becomes maturated and maturated NICD molecule goes to nucleus where it binds to promoter region of Notch target genes to turn expression of gene on or off [8][14].

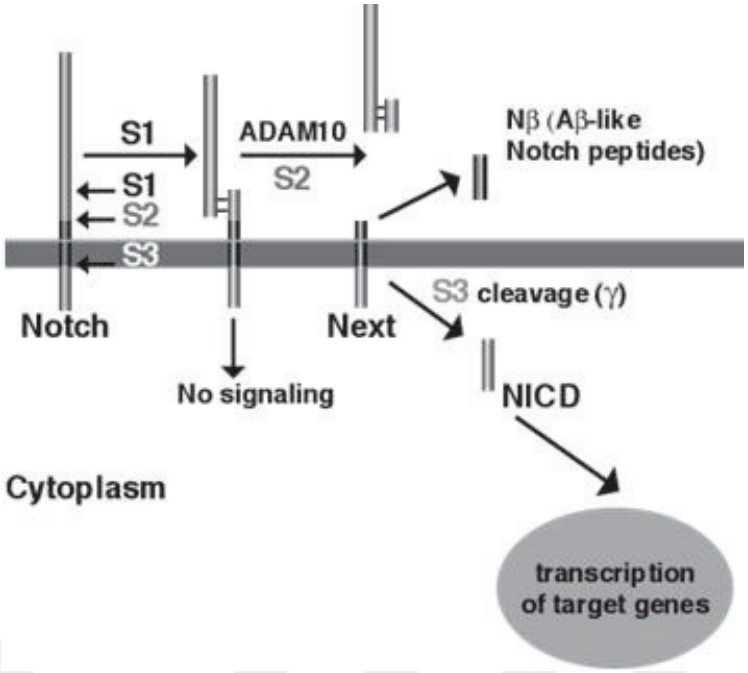

Figure 1.1.Notch Receptor Processing after activation of Notch through Ligand Binding [50]

1.3.3. Notch Target Genes

After translocation of NICD to nucleus, NICD binds to promoter site of its target genes and interacts with a transcription factor RBP-jκ (counterpart of CSL in Drosophila) which binds to consensus sequence on promoter of Notch target genes both in the absence and presence of NICD. Canonical Notch pathway depends on initial interaction of NICD with RBP-jk. In the absence of NICD, RBP- jk makes a complex with corepressors (Hairless) and recruits additional factors such as histone deacetyl transferase (HDAC) to negatively regulate Notch target genes. Once NICD enters to nucleus, it interacts with RBP-jκ which facilitates dissociation of repressor proteins and then, they form an activator complex by recruitment of additional proteins such as coactivator Mastermind-like protein (MAML1-3 in mammals) and histone acetyl transferase (HAT) to make target genes transcriptionally active. [14][15][16] Although mechanism of RBP-jκ dependent Notch target gene activation is summarized here, some studies also showed that Notch may activate its downstream genes even in depletion of RBP-jκ through the non-canonical Notch signaling pathway [17]. Hes (Hairy/enhancer of Split) and the Hey (Hairy/Enhancer of Split related with YRPW motif) are two closely related gene families and they are good example of known direct

Notch targets [18]. All known Hey members, including Hey1 and Hey2, are activated by NICD binding, however, it was shown that expression of some Hes members such as Hes6 [19] are independent of Notch activity and cannot be induced by NICD binding to the promoter region. Nevertheless, all family members have important functions during embryological development and diseases. Their encoded proteins usually act as repressor of transcription [20] and affect expression of several downstream genes. In addition to well known direct Notch targets, there are some other target genes that are directly controlled by Notch activity. Cell cycle related genes CCND1 and CDKN1A; immune regulator GATA3 are example of other Notch targets [21].When Notch signaling was first identified, it was thought that it regulates apoptosis through NICD interaction with p53 and leads to its phosphorylation [36]. However, today, increasing number of researches has also reported its oncogenic role in cancer progression. For example, some anti-apoptotic proteins such as survivin were up-regulated with over-expression of Notch [37]. In addition to its tumor promoting roles, some studies also reported that it may have anti-proliferative effect which was shown in primary breast epithelial cells [51] and two-sided effect of Notch depends on its expression level. While it has anti-proliferative effect in lower expression levels, it increases proliferation rates in higher concentrations [52]

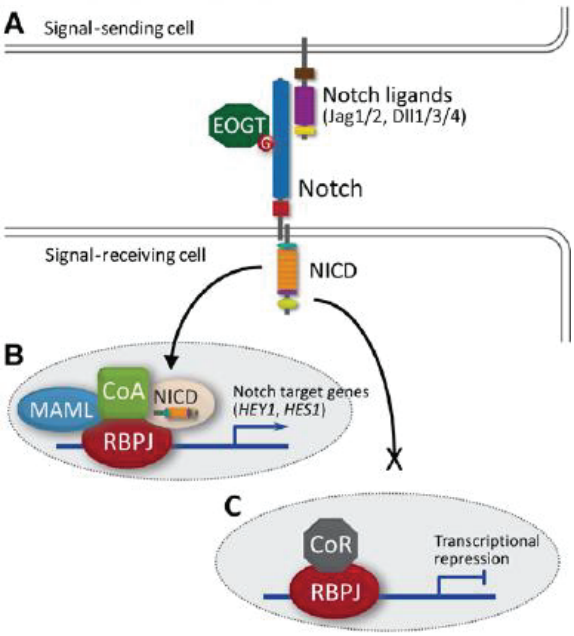

Figure 1.2.Schematic Illustration for Mechanism of Notch-mediated Target Gene Expression. In absence of NICD in nucleus, RBPJ interacts with co-repressor protein and form a complex to repress gene expression. Upon to NICD translocation into nucleus, RBPJ interacts with NICD and co-activator, forming a complex which, in turn, activates expression of Notch target genes. [53]

1.3.4. Notch in EMT and Tumor Progression

EMT is an important process during tumor progression and due to its importance, mechanism of EMT has been investigated by several research groups. Currently, many transcription factors and signaling pathways were linked to EMT. Notch pathway was shown as one of the key regulators in the induction of EMT [22][23]. Notch is highly expressed in different types of cancer and silencing of Notch leads to up regulation of epithelial markers and down regulation of mesenchymal markers in breast cancer which supports the effects of Notch on EMT process [24]. Jagged-1 stimulation in endothelial cells is also known to induce a similar mesenchymal transformation, suggesting that Jagged-1 mediated activation of Notch signaling is important during the induction of EMT [25]. Decrease in E-cadherin expression level is an important step in EMT; and, during EMT process, it is down-regulated by some other proteins such as Snail which has the ability to bind E-boxes on E-cadherin promoter, in turn, it represses E-cadherin expression [26]. Over-expression of Notch-1 in immortalized endothelial cells *in vitro* induced Snail expression which, in turn, decreases E-cadherin expression, leading to EMT through Notch-induced Snail expression [27]. Likewise, Notch inhibition in cardiac development through γ-secretase inhibitor; DAPT resulted in failure in EMT which also implies the importance of Notch signaling in EMT process [27].In addition to role of Notch in EMT, it is also attributed to increased cell motility and invasiveness during cancer progression [28][29]. Over-expression of Notch-1 increased migration and invasion in prostate cancer cell lines [30]. Notch-induced Slug expression, led to an increased invasiveness through regulation of EMT process and Slug over-expression was shown as related to poor survival rates in colorectal, breast and ovarian carcinoma [32][33].Slug is one of the known direct Notch targets [31] and similar to Snail, it acts as E-cadherin repressor and it was reported that Slug led to over-expression of mesenchymal markers such as Vimentin and Fibronectin in human esophageal carcinoma [34]. Leong et al. showed that Slug is up-regulated in Jagged-1 and Nocth-1 positive breast cancer and they also found that Notch-mediated Slug expression is also related to expression of Hey family members in breast cancer [35]. It was also reported that Notch regulates NF-κB expression through DLL-4 mediated receptor activation and it has an important role in small cell lung carcinoma metastasis to liver [38].

1.3.5. Role of Notch Pathway in Breast Cancer

Although oncogenic role of Notch was firstly identified in human pre-T-cell acute lymphoblastic leukemias (T-ALL) in which Notch-1 receptor is constitutively activated as a result of chromosomal translocation [39], aberrant Notch activity is also evident in different breast cancer subsets. Notch-1 and its ligand Jagged-1 were over-expressed after hormone therapy in MCF-7 breast cancer cell line [42] and Notch activity was also attributed to poor survival rates in breast cancer patients [43]. For example, studies in breast cancer cell lines reported that Notch 4 is aberrantly expressed in 2 of 8 cell lines while all investigated cell lines have significant Notch-1 ICD level [40]. Similarly, Notch-3 is shown as important factor for proliferation of HER2-negative breast cancer cell lines [41]. It was reported that Notch-3 silencing through RNA interference decreased the TGF-β mediated colony formation in breast cancer cells [44] while gain-of function mutations in Notch-4 was reported as reason for mammary gland carcinogenesis [45] and Notch-4 ICD over-expression in breast epithelial-like cell line, MCF10A, increased their anchorage-independent growth in soft agar assay [46]. In addition to role of Notch expression in breast cancer progression, it was also shown as an important factor for formation of breast cancer stem cells and gaining resistance to apoptosis. Chen et al. reported that ErbB2 gene, encoding for HER2, has a binding site for Notch-1[48] and with Notch-1 activation, HER2 expression increased in mammary progenitor cells and breast cancer stem cell (BCSC) populations, suggesting that Notch signaling may affect stemness of breast cancer cells by regulating their self-renewal capacity [47]. On the other hand, activated Notch also increased expression of anti-apoptotic proteins such as survivin in breast cancer cell line, MDA-MB-231. Similarly, it was shown a positive correlation between Notch-1 activity and surviving levels in basal (triple negative) breast cancer patients. [49]

1.4. Cysteine Rich Angiogenic Inducer 61 (CYR61)

CYR61 (also known as CCN1) is a member of CCN protein family, consisting of six members in mammals [54]. CCN stands for the first letters of firstly identified family members; CYR61, CTGF (connective tissue growth factor), and NOV

(nephroblastoma over-expressed). Therefore, family members are named as CCN1-6 depending on their discovery time [55].CCN family members basically consists of a common N-terminal secretory peptide, four conserved domains which have sequence homology to insulin-like growth factor-binding protein (IGFBP), thrombospondin type I repeat (TSR), von Willebrand factor type C repeat (vWC) and a carboxyl-terminal (CT) domain.

1.4.1. CYR61 in EMT and Tumor Progression

CYR61 interacts with adhesion proteins and regulates cell-cell or cell-ECM attachment [56]. Additionally, CYR61 binds to several types of integrins and triggers initiation of different signaling pathways after its secretion to ECM [57]. CYR61 has important functions both in tissue homeostasis and abnormalities. Role of CYR61 in wound healing was reported in human skin fibroblast cells [60], CYR61 expression also promoted cell proliferation and angiogenesis in endothelial cells [56][59]. However, CYR61 was mutated in several cancers [56]. CYR61 expression increases with cancer progression and its expression is attributed to poor prognosis [62]. Inhibition of CYR61 decreased cell migration, EMT and tumor progression in pancreas cancer. Ablation of CYR61 also decreased the expression of stem cell markers in pancreatic cancer cell lines. [61]

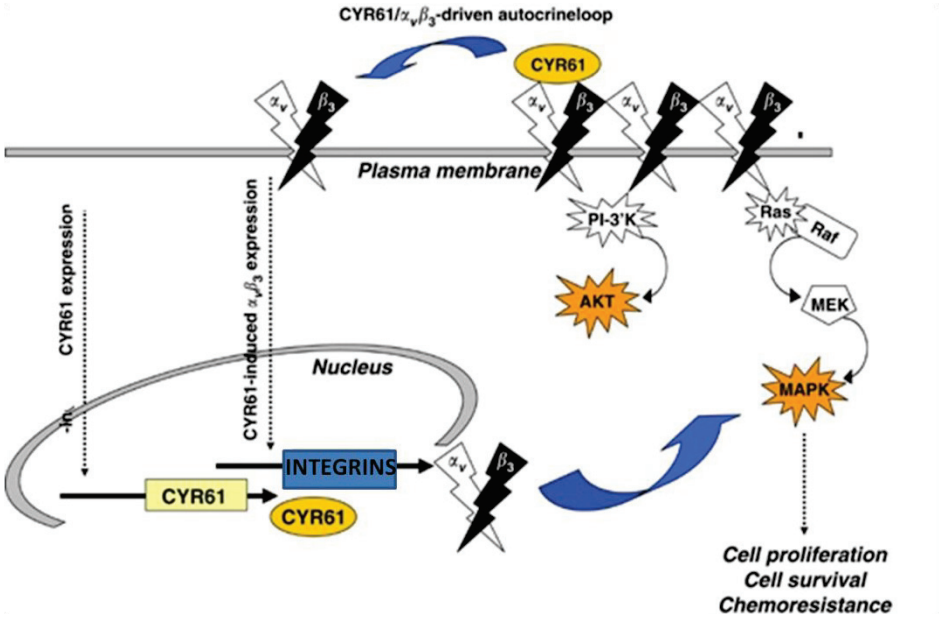

Figure 1.3.Mechanism of CYR61-integrin interaction and activation of signaling cascades.[57]

It was also reported that CYR61 stimulates angiogenesis and tumor growth in xenografts of gastric cancer cells gastric cancer tumor xenograft mice [58]. Although CYR61 has important roles in different abnormalities, particularly in cancer, several studies also reported that it has anti-tumorigenic functions. CCN1 triggered apoptosis and senescence in fibroblast cells [63]. Although CYR61 was, previously, linked to increased proliferation of prostate cancer cells, Franzen et al. showed that CYR61 enhanced apoptosis in the same cell in the presence of apoptotic TRAIL molecule [64]. It was further shown that two-sided function of CYR61 in tumorigenesis might depend on the presence or absence of certain tumor suppressor genes. For example, induction of CYR61 in hepatocellular carcinoma cells decreased their proliferation in a p53-dependent manner [65]. CYR61 over-expression also prevented tumor progression and triggered apoptosis in melanoma cells [66]. Taking everything into account, different findings suggest that CYR61 role in EMT and tumor progression may vary depending on cell type and tumor environment.

1.4.2. Role of CYR61 in Breast Cancer

CYR61 expression in breast cancer patients is associated with aggressiveness and poor prognosis. Over-expression of CYR61 in breast cancer xenografts stimulated tumor growth [67] and increased resistance to apoptosis in MCF7 breast cancer cell line by up-regulating anti-apoptotic XIAP protein [68]. CYR61 also promoted proliferation and invasion capacity of MCF7 cells [70], as well as it increased drug resistance in the same cell line [57]. It was also shown that CYR61 triggers expression of αvβ3 integrin receptor [69] and then, it uses receptor to decrease apoptosis in breast cancer cells [57]. On the other hand, inhibition of CYR61 decreased migration in MDA-MB-231 basal breast cancer cell line as well as in xenografts [71].Recently, it was shown that inhibition of CYR61 decreased the metastasis of breast cancer xenografts to lung through increased extravasation [72].

1.5. CYR61-Notch Interaction in Cancer

Tumorigenesis is a complex process and usually requires complex interaction between different proteins and signaling pathways. It is evident that CYR61 and Notch interact and facilitate tumor progression in different cancer types. For example, it was reported that CYR61 uses Notch-1 as mediator for regulation of sonic hedgehog (Shh) pathway by increasing stability of NICD in pancreatic cancer cells [73]. Besides, blocking of αv and anti-integrin β3 inhibited CYR61-mediated Notch-1 activation in pancreatic cancer cells [73] while recombinant CYR61 protein significantly increased Notch-1 receptor maturation in SGHPL-5, human cytotrophoblast cell line [74]. Furthermore, it was reported that CYR61 stimulated the transcription of Jagged-1, one of the Notch ligands, in hepatic stellate cells [75].

All these findings suggest that both Notch pathway and CYR61 are important players in tumorigenesis depending on cell type and tissue environment. Although aberrant Notch activity is associated to several malignancies, mechanism of Notch-mediated tumor progression is still poorly understood. Therefore, understanding the mechanism and identifying players in Notch-mediated tumorigenesis may hold promise to develop new approaches for treatment of different cancer.

1.6. Aim of the Project

In a previous study in our group, Notch induction in MCF10A epithelial-like breast cell line significantly increased the CYR61 expression while Notch inhibition in MDA-MB-231 basal type (triple negative) breast cancer cell line resulted in a significant decrease in CYR61 expression in both mRNA and protein levels. Although studies reported that CYR61 regulates Notch activity and affects disease progression in different cancer types, it is not known whether CYR61 and Notch interact in any way in breast cancer. Therefore, here, we aimed to understand whether Notch uses CYR61 as a mediator during EMT migration and invasion of breast cancer cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Lines and Cell Culture

Human epithelial-like breast cell line MCF10A were cultured in high glucose containing DMEM-F12 (GIBCO, Cat# 11965092) which was supplemented with 5% Horse Serum (Biological Industries, Cat# 04-004-1A), 20 ng/mL EGF (Sigma, Cat# E9644), 0.5 μg/mL Hydrocortisone (Sigma, Cat# H0888), 100 ng/mL Cholera Toxin (Sigma, Cat# C8052), 10 μg/mL Insulin (Sigma, Cat# I1882) and 1% Penicillin/Streptomycin (GIBCO, Cat# 15140-122). MDA-MB-231 breast cancer cell line and human embryonic kidney cell line, HEK 293T were cultured in high glucose containing DMEM (GIBCO, Cat# 41966-029) which was supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Cat# 10270-106) and 1% Penicillin/Streptomycin (GIBCO, Cat# 15140-122). Mouse embryonic fibroblast cell line NIH/3T3 was cultured in high glucose containing DMEM (GIBCO, Cat# 41966-029) which was supplemented with 10% New Born Calf Serum (NBCS) (GIBCO, Cat# 16010-159) and 1% Penicillin/Streptomycin (GIBCO, Cat# 15140-122). All cell lines were incubated at 37 °C, 5% CO2 in humidified incubator. For their subculturing, 0.05% Trypsin (Biological Industries, CAT # 03-053-1A) was used and cells were incubated in incubator for 1-15 minutes for different cell lines. MCF10A and MDA-MB-231 cell lines were purchased from ATCC (Virginia, USA) .HEK-293T and NIH/3T3 cell lines were kindly provided by Prof. Cathrin Brisken Lab. (EPFL, ISREC).

2.2. Virus Production

For retroviral/lentiviral gene induction and silencing, retro/lentiviral vector systems were used and virus production was performed in HEK 293T human embryonic kidney cell line. $3x10^6$ cells were seeded into 10 cm plate and they were transfected after 24-H of passaging. Transfection was performed by using FuGene (Promega, Cat #

E2311) and reagent was mixed with plasmid DNA in 3:1 ratio. For retroviral virus production, Notch1 intracellular domain (NICD) was cloned into MSCV retroviral plasmid which includes two LTR sites to integrate into host genome, and Neomycin selective marker gene sequence. For packaging of retroviral plasmid, 293-T cells were co-transfected with pcl10A vector. For lentiviral virus production, Notch1 intracellular domain (NICD) was cloned into pLENTI CMV GFP lentiviral plasmid containing Puromycin selective marker gene sequence or shRNA against CYR61 was cloned into Plko vector which contains U6 promoter for efficient shRNA expression, puromycin selective marker gene and HIV-1 RNA packaging signal between 5' LTR and 3' LTR. For CYR61 over-expression, CYR61 cDNA was cloned into psd44 lentiviral plasmid backbone.293-T cells were co-transfected with pCMV-dR8.74 and pMD2.VSVG plasmids for packaging of functional lenti viruses.

2.3. Virus Titration

To understand efficiency, virus titration was performed for each virus production. 19 x 10^4 NIH/3T3 mouse embryonic fibroblast cell was seeded one-day before infection. Then, cells were infected with dilutions of produced virus (10^{-3}, 10^{-4} and 10^{-5}). Virus was added after mixing with Polybrene (Sigma, Cat#107689) in 8 μm/ml final concentration. After 24-hours of virus addition, medium was changed with fresh growth medium and cells were splited into 10-cm plates for selection 48-hours later. For selection, 400 μg/mL Geniticin (Gibco, Cat#10131-019) or 2 μg/mL Puromycin (HyClone, Cat# SV30075) was used according to vectors. Selection was finished when all cells died in non-infected control plate. For staining of colonies, plates were washed with 1X PBS and cells were incubated with 0.5% crystal violet solution for 15 minutes and washed by 1X PBS three times for 10 minutes. Colonies were counted and compared with other viruses.

2.4. Infection of Cell Lines by Viruses

For Notch activation experiments, 2.5x10^5 MCF10A cells cultured one day before were transduced by MSCV control retro-viruses and Notch1-ICD expressing

MSCV-NICD retro-virus or pLENTI-GFP Notch1-ICD expressing lenti-virus. For Notch inhibition experiments, $3.5x10^5$ MDA-MB-231 cells cultured one day before were transduced by lenti-viruses. Infections were performed by using Polybrene (Sigma, Cat# 107689) in 8 μg/mL final concentration and medium was changed after 24 hours of virus addition with fresh growth medium. After 72 hours of transduction, cells were flash frozen in liquid nitrogen and plates were kept at -80^oC. Then, plates were used for RT-qPCR and Western Blotting.

2.5. Semi-Quantitative Real Time RT-PCR (RT-qPCR)

Total RNA of cells was isolated by using Pure-link RNA Mini Kit (Ambion, Cat#12183018A) and treated with PureLinkTM DNase (Invitrogen, Cat#12185-010) to prevent DNA contamination. After RNA isolation, complementary cDNA (cDNA) was synthesized with Fermantas First Strand cDNA Synthesis Kit (Thermo Scientific, Ca#K1622) from 1μg isolated total RNA by using random hexamer primers. cDNA was synthesized by using specific forward and reverse primers. For RT-qPCR, Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Cat# KO252) was used which includes Taq DNA polymerase, dNTPs and SYBR Green I dye in PCR buffer. PCR amplification was done by using Roche- LightCycler 96 Real Time PCR Detection System. Means of cycle threshold values (Ct) for Cyr61 ; Hey-1, Hey-2,Hes-1 Notch targets; E-cadherin, ZO-1 epithelial marker genes; ZEB-1,ZEB-2,Snail,Slug, Twist, Vimentin and N-cadherin mesenchymal marker genes were normalized to Ct values of TATA box binding protein (TBP) housekeeping gene. Primer sequences were listed in Table 2.1. Then, mRNA level were calculated with using delta-delta Ct method. Two-tailed paired student t-test method was used for statistical analysis.

Table2.1. Forward and reverse primer sequences used in RT-qPCR.

Gene	Forward	Reverse
TBP	5'-TAGAAGGCCTTGTGCTCACC-3'	5'-TCTGCTCTGACTTTAGCACCTG-3'
Hes1	5'-AACACGACACCGGATAAACC-3'	5'-TCAGCTGGCTCAGACTTTCA-3'
Hey1	5'-GGGAGGGGAACTATATTGAATTTT-3'	5'-ATTTGTGAATTTGAGATCCGTGT-3'
Hey2	5'-AAGATGCTTCAGGCAACAGG-3'	5'-GCACTCTCGGAATCCTATGC-3'
CYR61	5'-AAGGGGCTGGAATGCAACTT-3'	5'-CTGCCCGGTAACTTTGACCA-3'
E-cadherin	5'-CAGCACGTACACAGCCCTAA-3'	5'-GGTATGGGGGCGTTGTCATT-3'
ZO-1	5'-ATGGAGGAAACAGCTATATGGGA-3'	5'-CCAAATCCAAATCCAGGAGCC-3'
ZEB1	5'-CCCAGGTGTAAGCGCAGAAA-3'	5'-GTCTGGTCTGTTGGCAGGTC-3'
ZEB2	5'-ATAAGGGAGGGTGGAGTGGAA-3'	5'-GTTAATTGCGGTCTGGATCGTG-3'
Vimentin	5'-GCTAACCAACGACAAAGCCC-3'	5'-CGTTCAAGGTCAAGACGTGC-3'
Snail	5'-CTAGGCCCTGGCTGCTACAA-3'	5'-TGTGGAGCAGGGACATTCG-3'
Slug	5'-CTCCTCATCTTTGGGGCGAG-3'	5'-TTCAATGGCATGGGGGTCTG-3'
Twist1	5'-CTGTCCATTTTCTCCTTCTCTGG-3'	5'-TTCTCGGTCTGGAGGATGGA-3'
N-cadherin	5'-GACGGTTCGCCATCCAGAC - 3'	5'- TCGATTGGT TTGACCACGG -3'

2.6. Western Blot for Protein Analysis

After induction or silencing of genes of interest, cells were frozen through flash-freezing process. Then, total protein was isolated in different cell lines and expression of related genes was detected. For isolation, cells were thawed and lysate was obtained by using freshly prepared RIPA Lysis Buffer containing RIPA stock solution (containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% DOC, 5 mM EDTA) with 1 mM DTT, 1X protease inhibitor and phosphatase inhibitors 1 mM Na3VO4 and 50 mM NaF. The lysates were collected into eppendorf tubes and homogenized by passing them through an insulin syringe for 5-10 times. Lysates were incubated on ice for 20 minutes and later centrifuged at 4^{o}C, at 14000 rpm for 20

minutes. Supernatant that contains total protein was transferred into fresh eppendorf tubes and stored at -80^{o}C. For quantification of total protein, Bradford assay was performed in which Bovine Serum Albumin (BSA) (NEB, Cat # B9000S) standard samples were prepared (0.5 ,1,2,4,8 µg/µl) and then, both standards and unknown samples were added into 800 µl water and 200 µl of 5X Bradford reagent (Serva, Cat # 39222) was added to get 1X working concentration. Afterwards, samples were loaded into cuvette and concentration was measured at 595 nm in spectrophotometer. By using standard curve , unknown samples concentration were calculated and equal amount of total protein from different samples were run on SDS-PAGE for western-blot analysis. For SDS-PAGE, 5 µl loading dye, 60 µg of protein sample were mixed and filled with water up to 25 µl. Prepared samples were run on SDS-Gel consisting of 3% stacking gel and 8 or 12% resolving gel. Furthermore, 5 µL protein marker (NEB, Cat# P7712G) was loaded into one well as reference. Protein samples were run at 18 mA for stacking and 25 mA for resolving in 1X running buffer consisting of Glycine, Tris-base and SDS. Then, proteins were transferred to PVDF membrane in 1X transfer buffer consisting of Glycine and Tris-base at 40 V for 4 hours at 4 ^{o}C. Then membranes were blocked by using 5% milk powder or 5% BSA (w/v) in 1X TBS-Tween20 (TBS-T) for 2 hours at room temperature. After blocking, membranes were incubated in primary antibody solutions containing primary antibodies specific to a type of protein in optimized concentration in 5% milk powder or 5% BSA, for overnight at 4^{o}C. Next day, membranes were washed with 1X TBS-T for 5 minutes, 3 times to remove and then they were incubated in secondary antibody solution containing secondary antibodies in recommended concentrations in 1X TBS-T for 2 hours at room temperature. Then, membranes were washed again with 1X TBS-T for 5 minutes, 3 times and proteins were detected by chemiluminescence by using Vilber Fusion SL Imaging System. Intensity values of related bands were normalized to values of Beta-actin housekeeping protein. Paired student t-test was used for statistical calculations. Primary antibodies specific to a protein of interest used for western blot were anti-N-cadherin (Cell Signaling Technology, #13116S, 1/1000 in 5% BSA), anti-ZO- 1 (Cell Signaling Technology, # 8193S, 1/1000 in 5% milk powder), anti-Slug (Cell Signaling Technology, # 9585S, 1/1000 in 5% BSA), anti-ZEB1 (Cell Signaling Technology, # 3396S, 1/1000 in 5% milk powder), anti-Twist (Calbiochem, # DR1088, 1/250 in 5% milk powder), anti-E-cadherin (Cell Signaling Technology, # 3195S, 1/1000 in 5% BSA) and anti-β-actin

(Abcam, AB75186, 1/3000 in 5% milk powder). Secondary antibody was polyclonal anti-rabbit (Cell Signaling Technology, # 7074S, 1/2000 in TBS-T).

2.7. Wound Healing Migration Assay

For Notch induction in absence/presence of CYR61, $1x10^6$ MCF10A cells were seeded into each well of 12-well plates one day before the experiment. Four different conditions were used for experiment. Prior to scratch introduction, cells were treated with mitomycin C (2 μg/ml) for 2 hours in serum free medium to prevent proliferation. Then, scratch was introduced and serum free medium was added and cells were observed for cell migration under confocal microscope. For Notch inhibition in either endogenous or cDNA-mediated CYR61 over-expression, $7.5x10^4$ MDA-MB-231 cells were seeded into each well of 12-well plates one day before the experiment. Four different conditions were used for experiment. Prior to scratch introduction, cells were treated with mitomycin C (2 μg/ml) for 2 hours in serum free medium to prevent proliferation. In order to inhibit Notch activity DAPT γ-secretase inhibitor (90 μM) containing serum free medium was added and cells were observed for cell migration under Leica DMI8 confocal microscope. Cells were incubated at 37°C, 5% CO_2 in humidified environment. Images were obtained with 5X magnification. Two-tailed paired student t-test method was used for statistical analysis.

2.8. Invasion Analysis

For invasion assay, cells were incubated with green cell tracker (Thermo Fischer, Cat # C2925) for 30 min in serum free growth medium one day before the experiment. Next day, growth factor reduced matrigel was mixed with pre-cooled serum free medium in 1:1 ratio and loaded into central channel of lab-on-chip system. Until the end of this step, all processes were performed on ice. For each condition, separate chips were prepared. Then, chips were kept at 37^oC for 30 min. to solidify matrigel. Then, cells were counted and resuspended in 1x10^6 cells/ml concentration in starvation medium. Then, they were loaded into one side of the chip. Serum rich medium was added to other side: 5% horse serum containing DMEM F12 growth

medium for MCF10A cells and 20% FBS containing DMEM(1X) growth medium for MDA-MB-231 cells. Then, chips were incubated at 37 °C, 5% CO_2 in humidified incubator. Invasion of cells were observed for 4 days by using Leica DMI8 confocal microscope. Paired student t-test method was used for statistical analysis.

CHAPTER 3

RESULTS

3.1. Effect of CYR61 in Notch-Induced EMT in Normal Breast Cell Line

To understand whether Notch uses CYR61 during EMT, Notch was induced through retrovirus including Notch1 intracellular domain (N1-ICD) inside. For this purpose, MCF10A, human breast epithelial cell line, was used because it has no significant endogenous Notch activity. After infection by retro-viruses, total RNA was isolated and converted into cDNA form. Then, by using sequence specific primers for CYR61, Notch target gene; Hey-2 and EMT markers; E-cadherin, ZO-1, ZEB-1, ZEB-2, Twist, Snail, Slug, Vimentin and N-cadherin, mRNA levels were detected by RT-qPCR. Notch induction was performed either in the absence or presence of CYR61 expression. Notch activation led to 60 fold significant increase in relative mRNA expression of its target gene; Hey2 when compared to control, whereas, CYR61 expression increased 3-fold with Notch induction. Relative mRNA levels of Snail and Slug mRNA markers also significantly increased after Notch induction while CYR61 inhibition reversed Notch-induced increase in mRNA expression of mesenchymal marker genes (Snail and Slug). On the other hand, inhibition of CYR61 decreased the mRNA expression of epithelial and mesenchymal marker genes. ZEB-1 mesenchymal marker mexpression(Figure 3.1). For confirmation of RT-qPCR results, expression levels of EMT markers were also investigated by western blotting. Antibodies against to E-cadherin, ZO-1, ZEB-1, Slug, Vimentin, N-cadherin and Twist were used. Beta-actin was used for equal loading control. Notch induction significantly increased protein level of some of the mesenchymal markers (Slug), whereas, inhibition of CYR61 decreased Notch-mediated Slug stimulation. Bands were quantified after normalization to Beta-actin (Figure 3.2).

Results of relative mRNA and protein expression showed that Notch may use CYR61 as down-stream mediator during EMT through up-regulation of certain EMT markers in normal breast epithelial cell line.

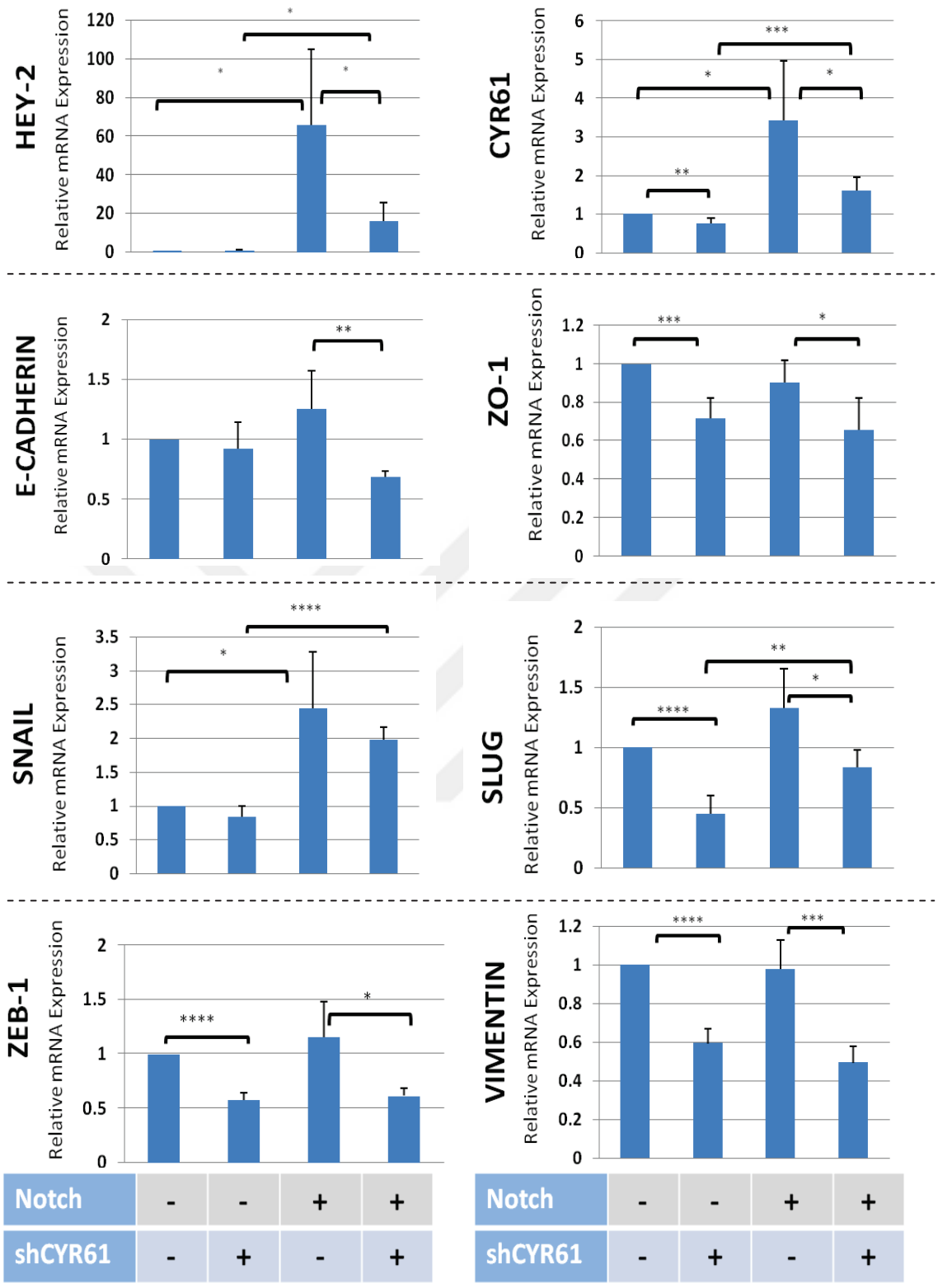

Figure 3.1. Relative mRNA expressions of EMT markers in MCF10A cells after Notch induction and CYR61 inhibition. After 48-H of infection, total RNA was isolated; cDNA synthesis and RT-qPCR were performed by using primers specific to for CYR61, HEY-2 and EMT marker genes. TBP was used for normalization. ($p<0.05$ *, $p< 0.01$ **, $p \leq 0.001$***).3 independent experiments was performed. For statistical analysis, two-tailed paired student t test was used.

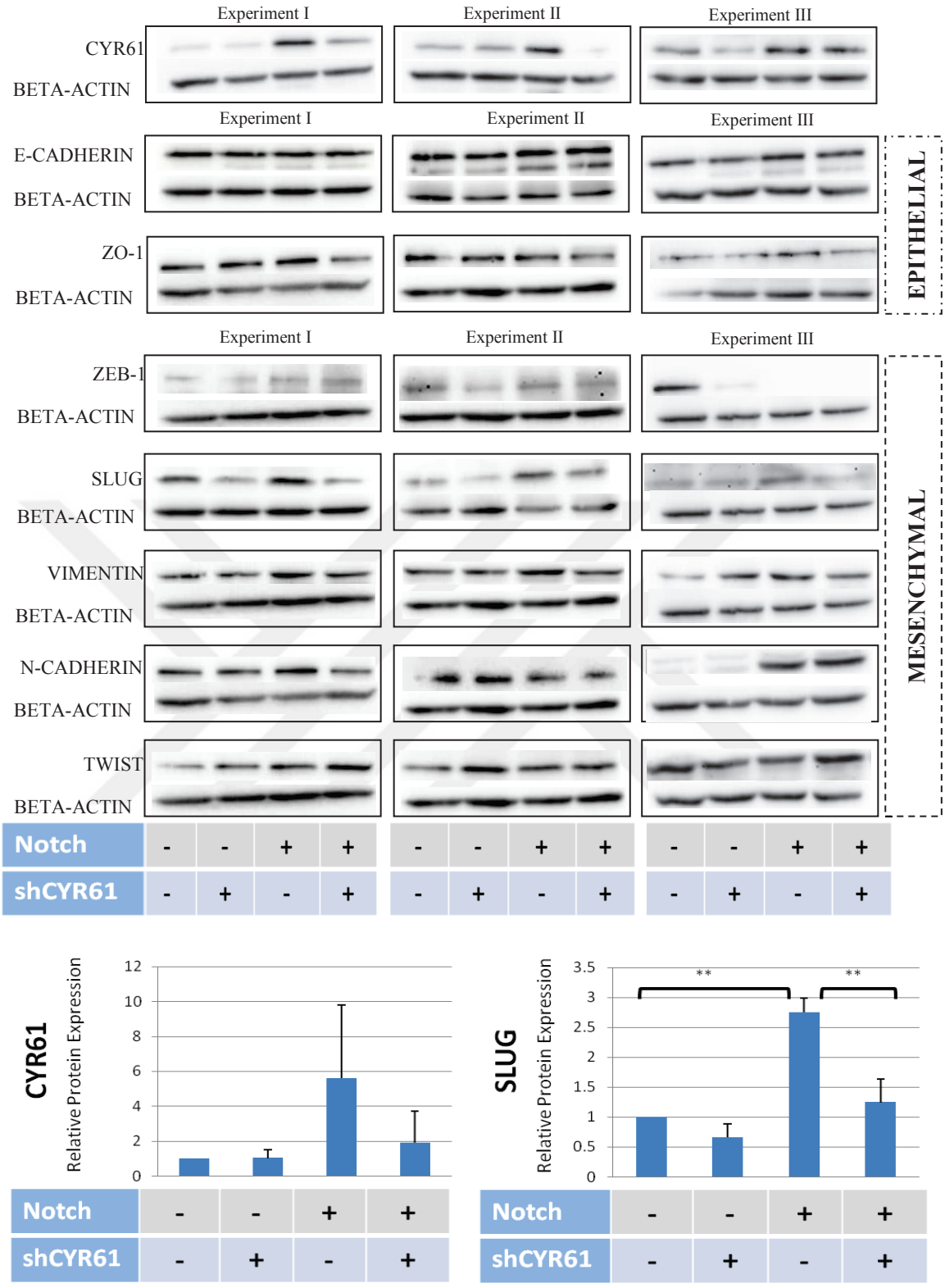

Figure 3.2. Protein expression level of EMT markers in MCF10A cells after Notch induction and CYR61 inhibition. After 48-H of infection, total protein was isolated and Western-blotting was performed by using antibodies against to E-cadherin, ZO-1,ZEB-1,Slug,Vimentin, N-cadherin and Twist. Beta-actin was used for equal loading control. For quantification, CYR61 and Slug band intensities were normalized to beta-actin, Paired t-test was used for statistical analysis. (n=3, **: $p \leq 0.01$)

3.2. Effect of CYR61 Over-expression on EMT of Breast Cancer Cell Line after Notch Inhibition

To understand whether Notch uses CYR61 during EMT, Notch was inhibited by using DAPT γ-secretase inhibitor in MDA MB 231 cell lines, which has high endogenous Notch activity. DAPT prevent final step of NICD maturation and prevent NICD translocation to nucleus for target gene activation. Concentration and timing of DAPT treatment were decided according to previous studies in the group and by considering literature. CYR61 over-expression was performed by lenti-viral infection of CYR61 cDNA in absence and presence of Notch activity. After creating CYR61 over-expressing stable cells, cells were treated with 90 uM DAPT for 48 hours to inhibit Notch cleavage or equal amount of DMSO in which DAPT was dissolved. Then, total RNA was isolated and converted into cDNA form and by using sequence specific primers for CYR61, Notch target gene; Hes-1 and EMT markers; E-cadherin, ZO-1, ZEB-1, ZEB-2, Twist, Snail, Slug, Vimentin and N-cadherin, mRNA levels were detected by RT-qPCR.

CYR61 over-expression significantly decreased Hes-1 relative mRNA expression. Similarly, Notch inhibition significantly decreased Hes-1 and CYR61 relative mRNA expression. Relative mRNA level of Snail mesenchymal marker also significantly decreased after Notch inhibition and was not rescued by CYR61 over-expression, whereas, there was no significant change in relative mRNA expression of other EMT marker genes. (Figure 3.3) For confirmation of RT-qPCR results, expression levels of EMT markers were investigated by using antibodies against to E-cadherin, ZEB-1, Slug and Vimentin. Beta-actin was used for equal loading control. Bands were quantified after normalization to Beta-actin. In contrast to mRNA analysis, Slug was significantly regulated upon treatment where Notch inhibition decreased Slug protein level and it was partially rescued by CYR61 over-expression. (Figure 3.4)

Relative mRNA and protein expression results showed that CYR61 might also be a mediator in Notch-induced EMT of breast cancer cell line.

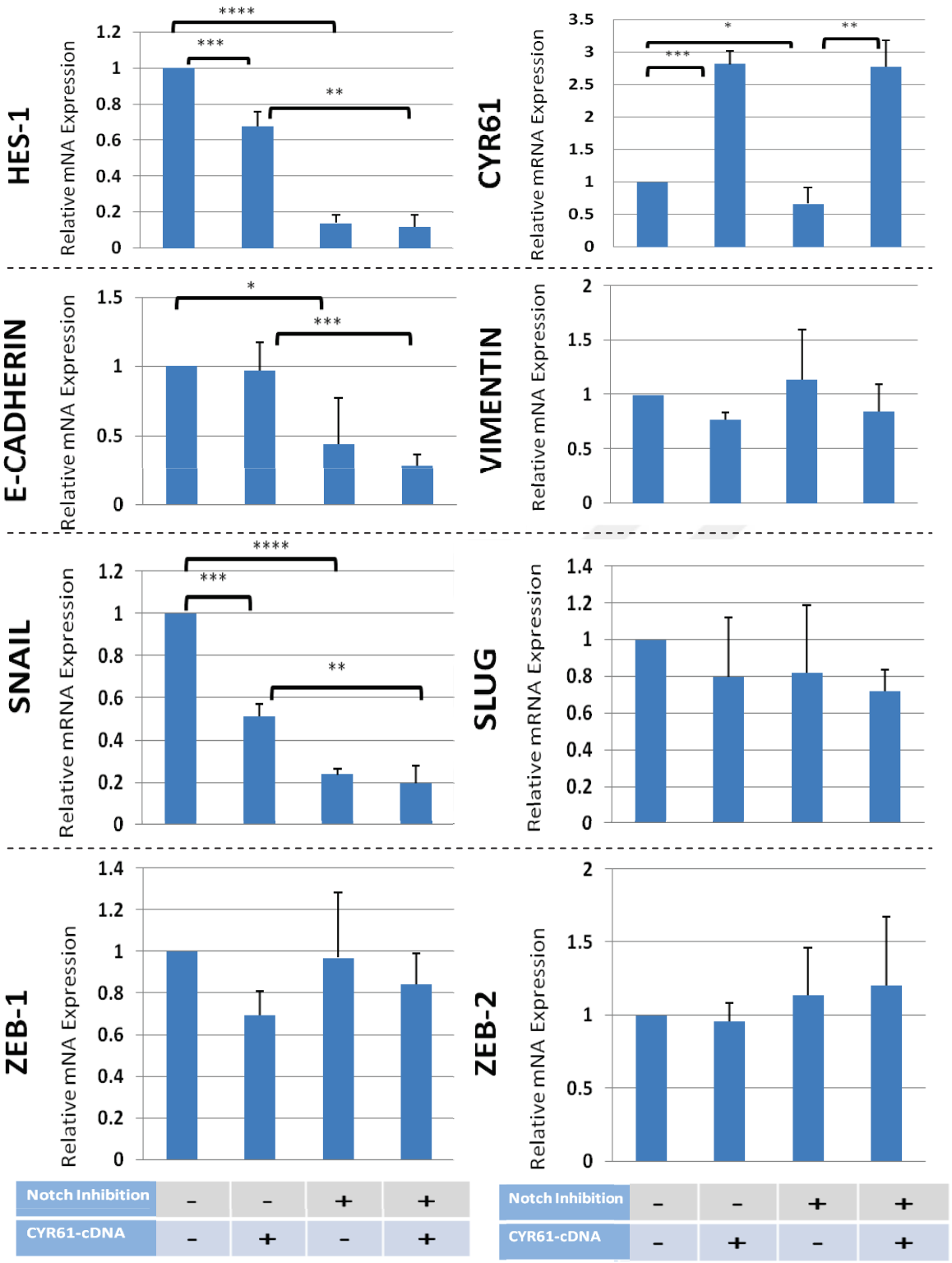

Figure 3.3. mRNA expression level of EMT markers in MDA-MB-231 cells after lenti-viral over-expression of CYR61 and Notch inhibition by DAPT. After 48-H of infection, CYR61 over-expressing stable cells were selected (2 ug/ml Puromycin). Then, stable cells were treated with 90 uM DAPT for 48-H. total RNA was isolated; cDNA synthesis and RT-qPCR were performed by using primers specific to CYR61, HEY-2 and EMT marker genes. TBP was used for normalization. Paired t-test was used for statistical analysis.
(n=3; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$)

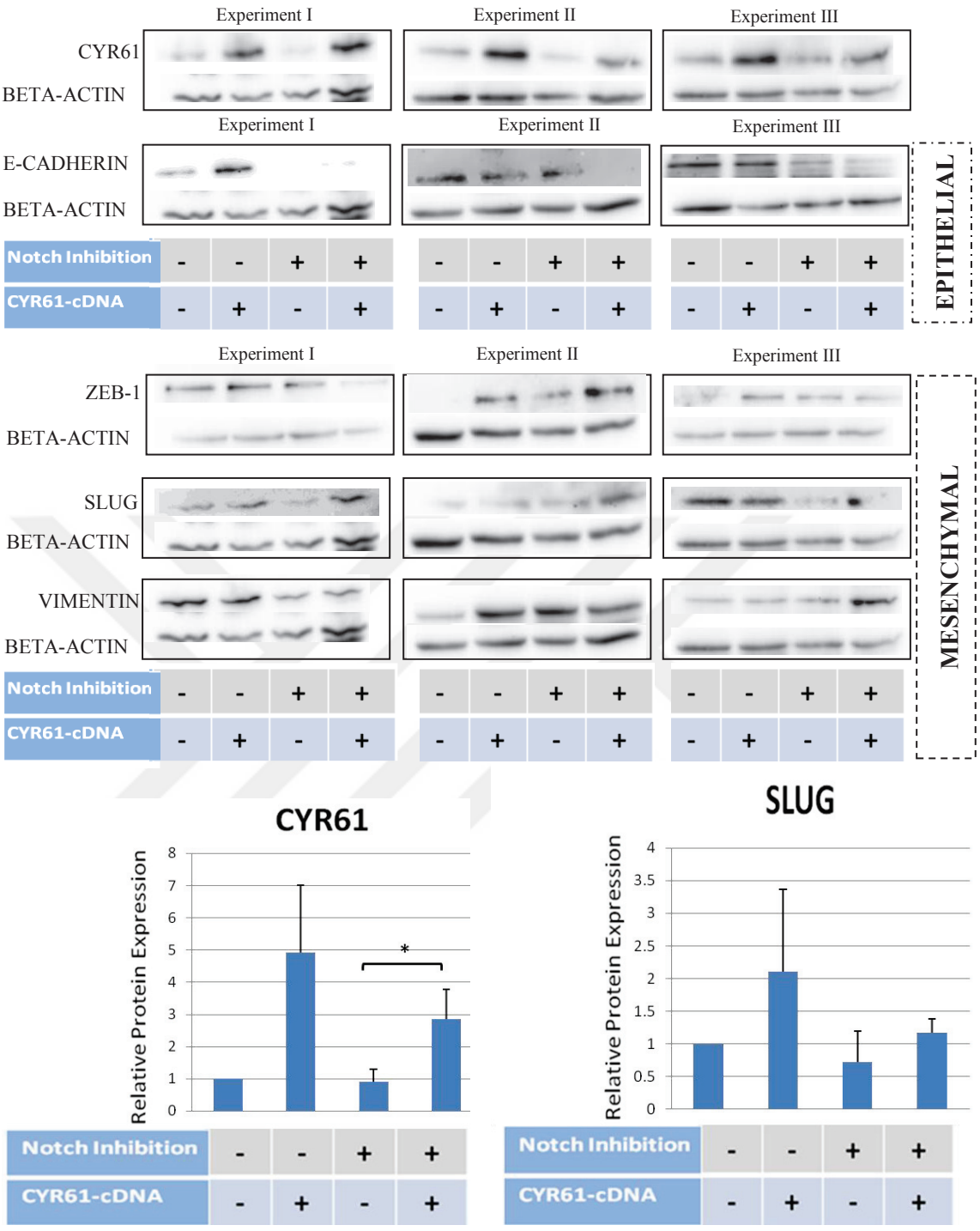

Figure 3.4. Protein expression level of EMT markers in MDA-MB-231 cells after lenti-viral over-expression of CYR61 and Notch inhibition by DAPT. After 48-H of infection, CYR61 over-expressing stable cells were selected (2 ug/ml Puromycin). Then, stable cells were treated with 90 µM DAPT for 48-H.Then, total protein was isolated and Western-blotting was performed by using antibodies against to E-cadherin, ZEB-1, Slug and Vimentin. Beta-actin was used for equal loading control. For quantification, CYR61 and Slug band intensities were normalized to beta-actin, Paired t-test was used for statistical analysis. (For CYR61 n=3 and for Slug n=2)

3.3. Effect of CYR61 on Notch-Induced Migration and Invasion of Normal Breast Cell Line

To understand effect of CYR61 inhibition on Notch-induced migration and invasion, cells were co-infected by Notch1 intracellular domain (N1-ICD) and shCYR61 lenti-viruses. After infection, stable cells were created. For this purpose, MCF10A, human breast epithelial cell line, was used because it has no significant endogenous Notch activity. Then, stable cells were seeded into 12-well plates and cells were treated with mitomycin C (2μg/ml) to prevent cell proliferation. After that, scratch was introduced and mitomycin containing medium was aspirated. Then, cells were incubated in starvation medium and migration of cells was measured under the confocal microscope for 48 hours. When migration rates were compared, CYR61 inhibition decreased migration in the first 24 hours and this significant decrease continued in the following hours of observation. On the other hand, Notch induction significantly increased migration rate in the first 12 hours and wound completely closed within 36 hours. However, Notch-induced increase in migration rate was significantly reversed by inhibition of CYR61. (Figure 3.6) For invasion analysis, lab-on-a-chip system was used and cells were seeded as in the Figure 3.5. Similarly, Notch induction significantly increased invasion ability of normal breast epithelial cell line and CYR61 inhibition through shRNA significantly reversed Notch-induced invasion capacity of normal breast cell line. (Figure 3.7)

These results show that CYR61 is a mediator of Notch during migration and invasion in normal cells suggesting that CYR61 may have a critical role in Notch-mediated tumor progression.

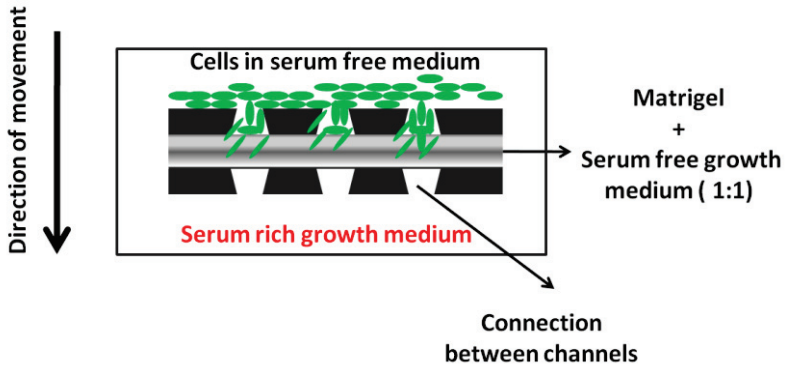

Figure 3.5. Schematic illustration of lab-on-a-chip system for matrigel invasion analysis.

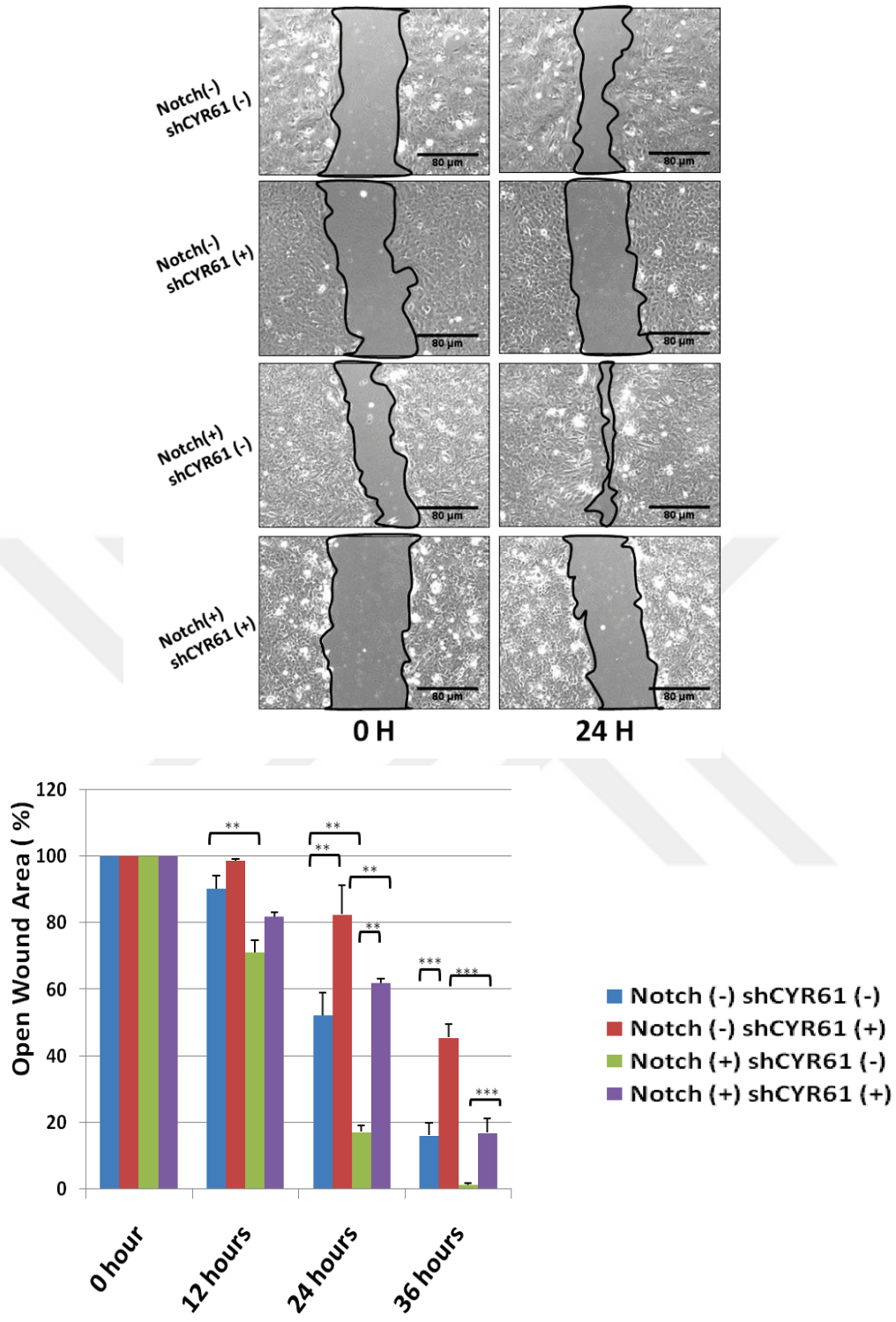

Figure 3.6. Results of wound healing - cell migration assay for MCF10A cells after Notch induction and CYR61 inhibition. Prior to scratch introduction, cells were treated with mitomycin C (2 µg/ml) to prevent proliferation. Then, scratch was introduced and serum free medium was added and cells were observed for cell migration under confocal microscope. Cells were incubated at 37 °C, 5% CO2 in humidified environment. Images were obtained with 5X magnification. Paired t-test was used for statistical analysis. (n=3, **: $p \leq 0.01$ ***: $p \leq 0.001$)

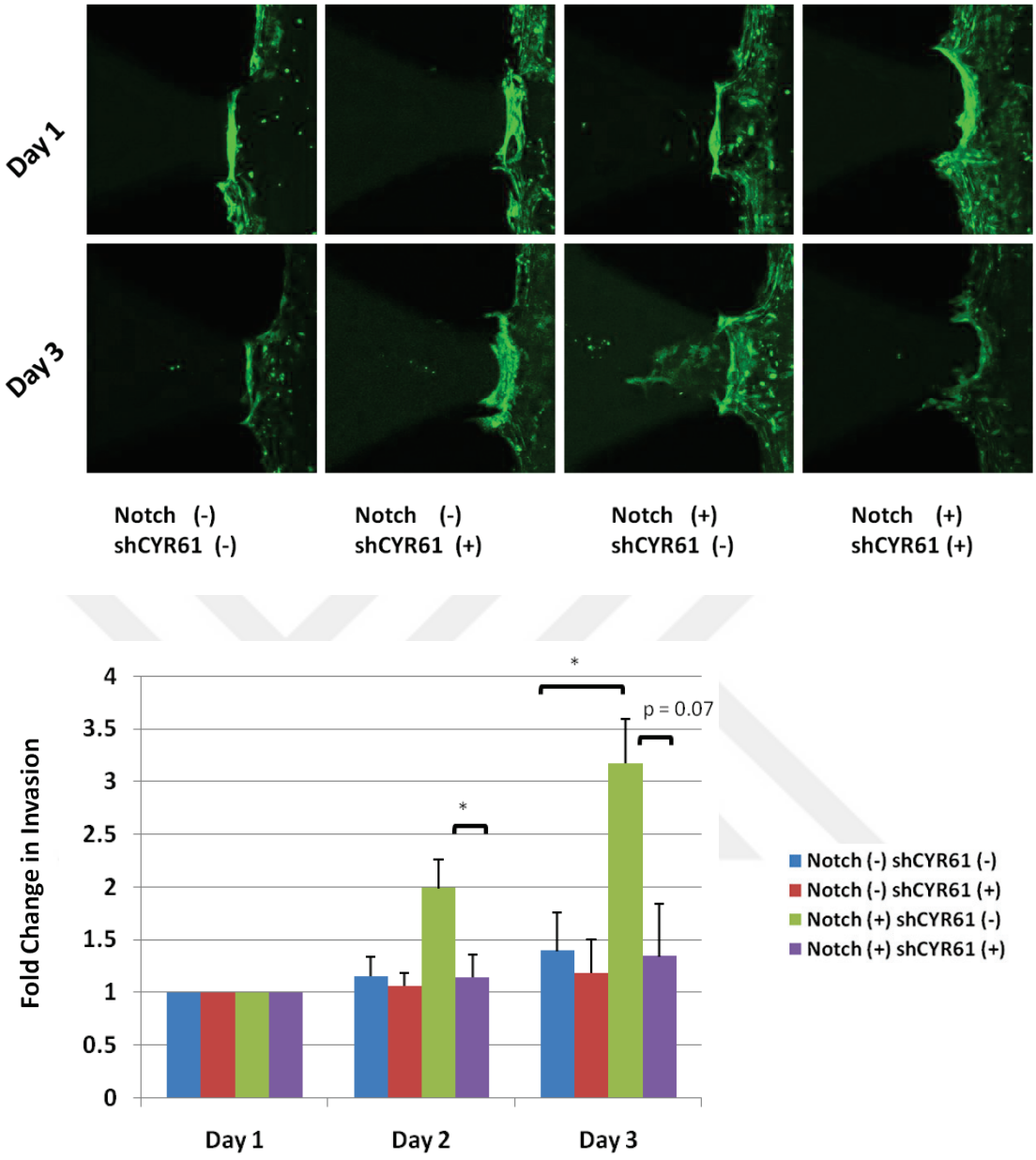

Figure 3.7. Results of invasion assay for MCF10A cells after Notch induction and CYR61 inhibition. After incubation with green cell tracker, cells were counted and resuspended in 1x10^6 cells/ml concentration in starvation medium. Matrigel was mixed with serum free medium in 1:1 ratio and loaded into central channel of lab-on-chip system. Serum rich medium was added to other side and chips were incubated at 37 °C, 5% CO2 in humidified incubator. Invasion of cells were observed for 3 days by using Leica DMI8 confocal microscope. For quantification, each condition was compared to its position on day 1. Paired t-test was used for statistical analysis. (n=3, *: $p \leq 0.05$)

3.4. Effect of CYR61 Over-Expression on Migration and Invasion of Breast Cancer Cell Line after Notch Inhibition

Although CYR61 had a migratory function in normal breast cell line and its activity is required for Notch-induced migration, CYR61 over-expression had an anti-migratory effect in breast cancer cell line.CYR61 over-expression in the presence of Notch activity significantly decreased migration in the first 18 hours. However, Notch-inhibition did not lead to a significant change in their migration with endogenous CYR61 expression. Nevertheless, when compared to CYR61 over-expression in the presence of Notch activity, Notch inhibition significantly increased anti-migratory effect of CYR61-overexpression (Figure 3.7). On the other hand, CYR61 over-expression led to an increasing trend in breast cancer cell line. Notch inhibition decreased the invasion ability of breast cancer cell line while CYR61 over-expression partially rescued decrease in invasion capacity upon Notch inhibition (Figure 3.8).

These results show that CYR61 role in Notch-induced migration might vary depending on cell type and these findings suggest that CYR61 might be a downstream mediator during migration, invasion and cancer progression.

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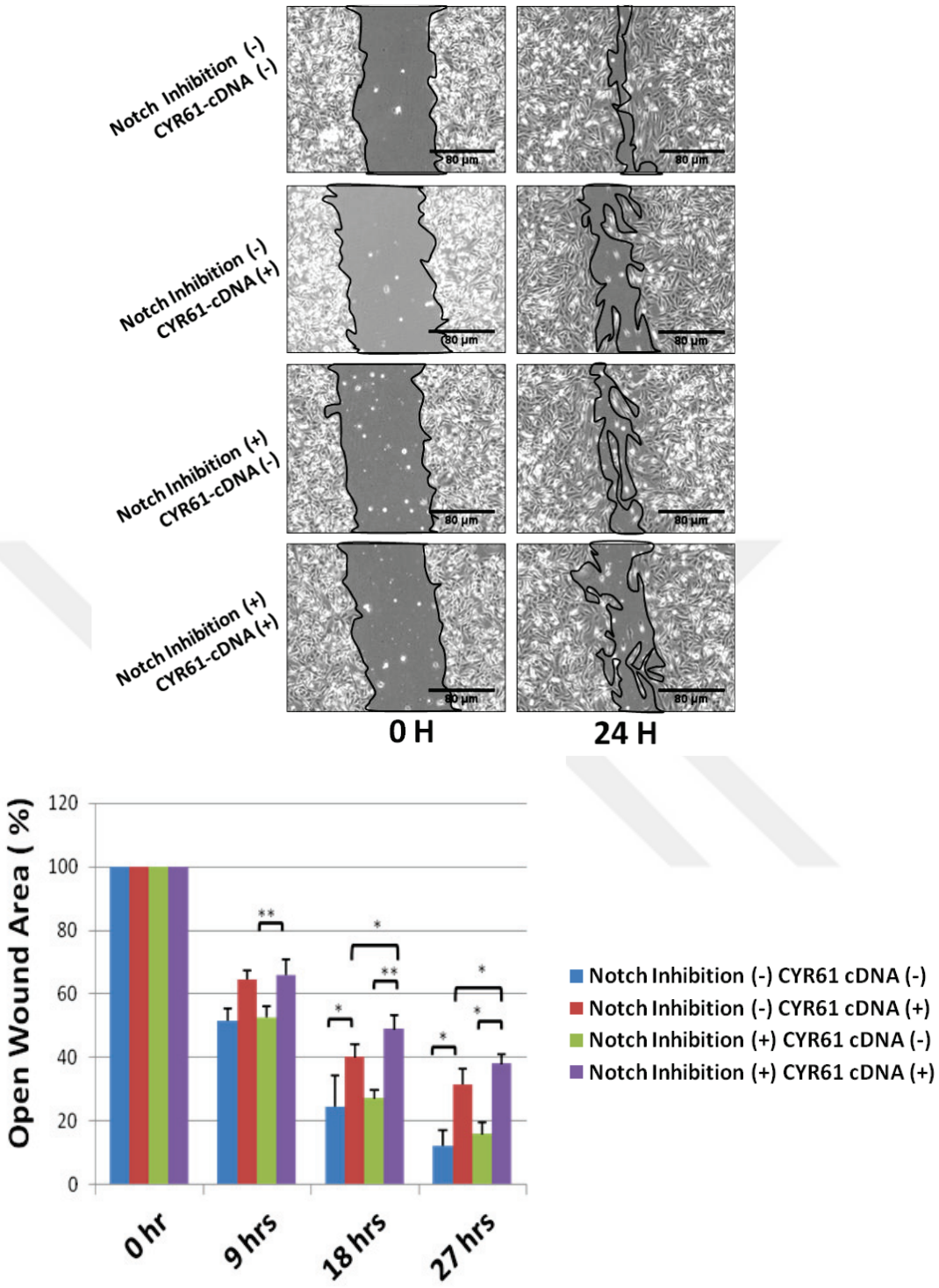

Figure 3.8. Results of wound healing - cell migration assay for CYR61 over-expressing stable MDA-MB-231 cells after Notch inhibition by DAPT. Prior to scratch introduction, cells were treated with mitomycin C (2 µg/ml) to prevent proliferation. Then, scratch was introduced and DAPT (90 µM) containing serum free medium was added and cells were observed for cell migration under confocal microscope. Cells were incubated at 37 °C, 5% CO2 in humidified environment. Images were obtained with 5X magnification. Paired t-test was used for statistical analysis.
(n=3, *: $p \leq 0.05$ **: $p \leq 0.01$)

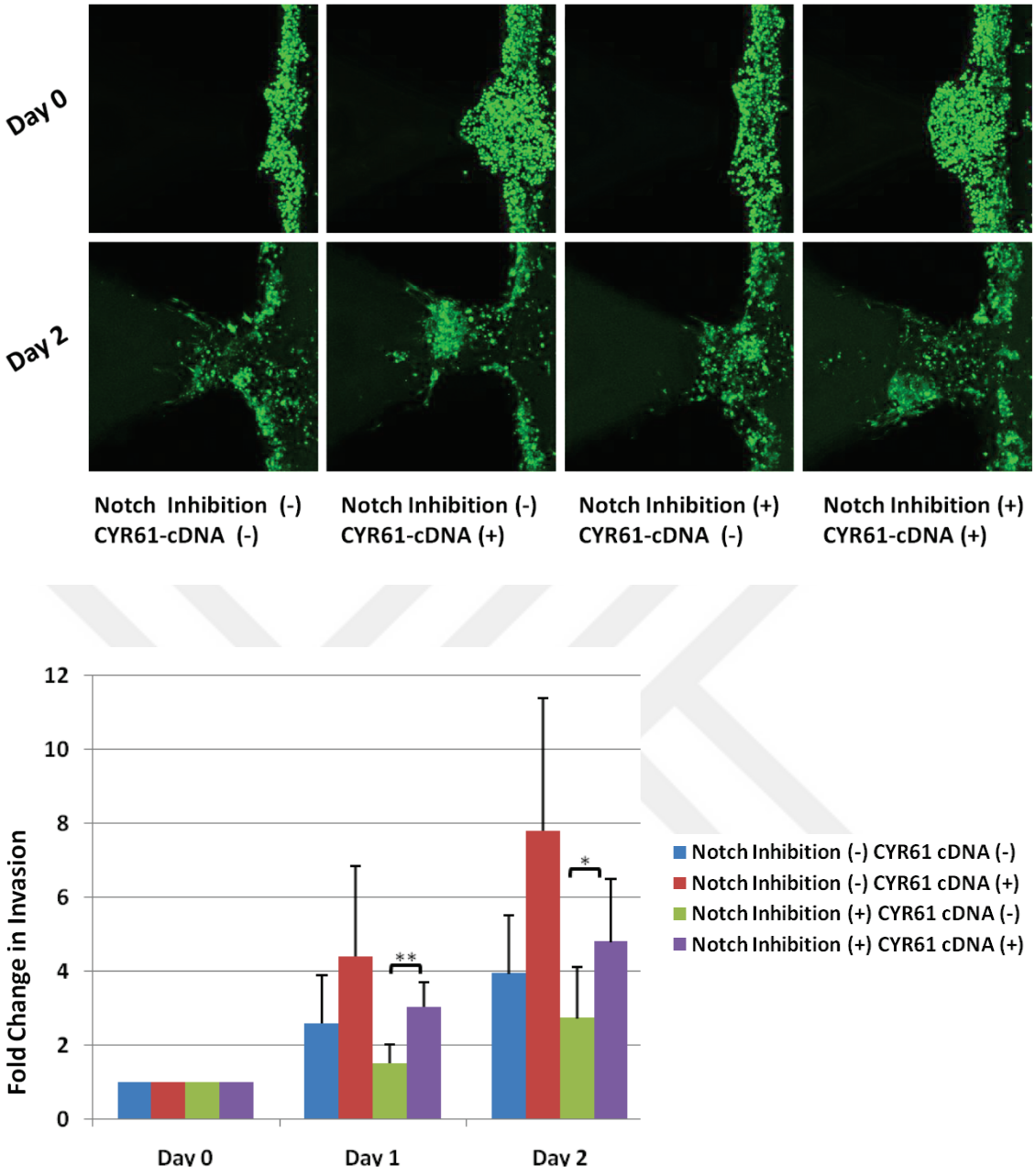

Figure 3.9. Results of invasion assay for CYR61 over-expressing stable MDA-MB-231 cells after Notch inhibition by DAPT. After incubation with green cell tracker, cells were counted and resuspended in 1x10^6 cells/ml concentration in starvation medium. Matrigel was mixed with serum free medium in 1:1 ratio and loaded into central channel of lab-on-chip system. Serum rich medium was added to other side and chips were incubated at 37 °C, 5% CO2 in humidified incubator. Invasion of cells were observed for 2 days by using Leica DMI8 confocal microscope. For quantification, each condition was compared to its position on day 0. Paired t-test was used for statistical analysis. (n=3; *: $p \leq 0.05$, **: $p \leq 0.01$)

CHAPTER 4

DISCUSSION & CONCLUSION

Breast cancer is the second leading cause of cancer related death among women [1][4]. Most of deaths are because of formation of secondary tumors through metastasis in a distant site [2]. EMT facilitates migration of cancer cells by which epithelial markers genes are down-regulated and mesenchymal markers are up-regulated. Aberrant Notch activity was correlated to increased migration, invasion and EMT in breast cancer [40][47]. However, mechanism of Notch-mediated cancer progression is poorly understood. Understanding Notch-mediated tumorigenesis has potential to develop more efficient treatment strategies. Therefore, here, our aim was to understand whether CYR61 involves in Notch-mediated EMT, migration and invasion. We hypothesized that CYR61 is a downstream mediator of Notch during EMT, migration and invasion.

To understand whether CYR61 acts in down-stream of Notch during EMT, Notch was induced. Results showed that Notch induction significantly increased expression of Notch target genes as well as CYR61 expression which support previous data in our group. Here, we also showed that Notch-induced increase in mesenchymal marker gene expression significantly reversed by CYR61 inhibition particularly, for Slug in both mRNA and protein level. As a complementary approach, Notch was inhibited by using DAPT and CYR61 was over-expressed by lenti-viral infection of CYR61 cDNA in the absence and presence of Notch activity. Results showed that Notch inhibition significantly decreased CYR61 and Notch target gene (Hes-1) expression. Notch inhibition also decreased Snail expression. Although we couldn't see a significant change in relative mRNA expression of Slug, its protein level decreased with Notch inhibition and CYR61 over-expression partially rescued its expression. When we consider change in Slug expression, results imply that Slug may act in downstream of CYR61 and seems to be a strong candidate player in Notch-CYR61 mediated EMT mechanism. Similarly, CYR61 over-expression also significantly decreased expression of Notch target genes which imply that CYR61 may also regulate Notch activity as reported by different groups [73][74]. Decrease in expression of Snail could not be rescued by CYR61 over-expression. However, there was no significant

change in expression of other EMT markers upon treatment. Relative mRNA and protein expression results suggest that CYR61 might be a mediator in Notch-mediated EMT through regulation of some of the EMT effector markers.

When migration rates were compared, activation of Notch led to a significant increase cell migration in first 12 hours and wound completely closed within 36 hours, whereas, inhibition of CYR61 significantly decreased migration in the first day and continued in the following hours of observation. CYR61 inhibition also significantly reversed Notch-induced increase in migration of normal breast cell line, MCF10A. In contrast to normal breast cell line, CYR61 over-expression had an anti-migratory effect in breast cancer cell line and this decrease became significant within 18 hours in the presence of Notch activity. However, Notch-inhibition did not lead to a significant change in their migration with endogenous CYR61 expression. Nevertheless, when compared to CYR61 over-expression in presence of Notch activity, Notch inhibition significantly increased anti-migratory effect of CYR61-overexpression. This result suggests a negative regulation by Notch on CYR61 migratory function in breast cancer cell line. However, this result might also cause from DAPT treatment. To inhibit Notch cleavage, we used DAPT γ-secretase inhibitor. Although it can inhibit NICD maturation, it may also affect other pathways because it is also required for activation of some other proteins such as CD44. One study showed that γ-secretase inhibition also blocked CD44 cleavage which is critical for tumor progression. For example, CD44 was observed in 67% of investigated breast carcinoma [75]. Therefore, this anti-migratory effect might also be related to disruption of other protein in different pathways. In addition to this possibility, Rother et al. also reported that pre-incubation with recombinant CYR61 protein decreased human monocytes migration [76] and CYR61 may act as anti-migratory protein in MDA-MB-231 breast cancer cell line. In addition to wound healing-migration assay, lab-on-a-chip invasion assay was performed in normal breast cell line. Notch activation increased invasion capacity of normal breast cell line, however, inhibition of CYR61 significantly reversed Notch-induced invasion. Similarly, Notch inhibition decreased invasion capacity of breast cancer cell line while CYR61 over-expression led to an increasing trend in invasion capacity of breast cancer cell line. Furthermore, CYR61 over-expression partially rescued decrease in invasion capacity of breast cancer cell line upon Notch inhibition.

These findings suggest CYR61 might act in downstream of Notch pathway and it might be a mediator for Notch-mediated EMT, migration and invasion in normal and

breast cancer cell lines. Although these findings suggest that Notch regulate CYR61, it should be investigated whether Notch directly regulates CYR61 at the promoter level or not. Additionally, results suggested that Slug consistently regulated by CYR61 expression in our experimental design and implies that Slug could act in Notch-mediated EMT process in downstream of CYR61. Therefore, role of Slug in Notch-CYR61-mediated EMT process should be investigated in order to gain insight into Notch-mediated cancer progression. Our preliminary data also showed that CYR61 also regulates expression of some of the direct Notch target genes suggesting possible effect of CYR61 on Notch activity. Although positive regulation of CYR61 on Notch activity is evident in different types of cancer, there is no study showing this regulation in breast cancer case. Therefore, CYR61-Notch interaction also should be investigated from this perspective.

All in all, when we consider vital importance of Notch signaling in tissue homeostasis and development and possible involvement of CYR61 in Notch-mediated EMT and cancer progression, it is possible to prevent cancer in early stages by targeting CYR61 for development of novel cancer treatment strategies.

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