ROLE OF IRF6 AS A MEDIATOR OF NOTCH SIGNALING IN BREAST CANCER CELL LINES

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ABSTRACT

ROLE OF IRF6 AS A MEDIATOR OF NOTCH SIGNALING IN BREAST CANCER CELL LINES

Notch signaling is an evolutionary conserved pathway involved in development and differentiation. Disregulated Notch signaling is involved in cancer by showing tumor supressor or oncogenic properties. Notch signaling has firstly been described in breast cancer by the activation of Notch4 locus. Expression of a constitutively active form of Notch1 induces neoplasms and high level expression of Notch1 is observed in human breast cancer which indicate that Notch is an oncogene in breast tissue. However, recently it has been shown that Notch activation can decrease cell proliferation in human breast epithelial cells. The downstream mechanisms of Notch signaling that elicit oncogenic or tumor suppressor roles remain elusive.

IRF6, a member of interferon regulatory factors, has been characterised as a novel Notch target gene involved in keratinocyte differentiation. IRF6 has been stated as a potential tumor suppressor in collaboration with Maspin in mammary epithelial cells. In this project, it was investigated whether IRF6 expression is regulated by Notch signaling in breast epithelial cells and whether it is a mediator of Notch in cell proliferation and transformation.

It was shown that activation of Notch signaling in normal breast epithelial cell line MCF10A, increased expression of IRF6, while inhibition of Notch signaling in breast cancer cell line MDA-MB-231 reduced IRF6 levels. IRF6 silencing reduced Notch induced cell transformation in MCF10A cells but did not result in significant change in proliferation. These results indicate that IRF6 is a downstream target of Notch and functions as a mediator for Notch signaling in breast epithelial cells.

ÖZET

NOTCH YOLAĞININ MEME KANSERİ HÜCRE HATLARI ÜZERİNDEKİ ETKİSİNİN DÜZENLENMESİNDE IRF6'NIN ROLÜ

Notch sinyal iletimi, gelişim ve farklılaşmada rol alan, evrimsel olarak korunmuş bir yolaktır. Notch yolağındaki bozukluk, tümör baskılayıcı veya onkogenik özellikler göstererek kanser gelişiminde yer alır. Notch sinyali meme kanserinde ilk defa Notch4 bölgesinin etkinleşmesi ile saptanmıştır. Aktif Notch1 ifadesinin tümör oluşumunu tetiklemesi ve hasta tümör örneklerinde Notch1 seviyesinin fazla olduğunun gözlenmesi ile Notch meme dokusunda onkogen olarak tanımlanmıştır. Fakat son dönemde, Notch etkinleşmesinin insan meme epitel hücrelerindeki hücre bölünmesini azaltabildiği gösterilmiştir. Notch'un onkogenik ve ya tümör baskılayıcı rollerini ortaya çıkaran alt mekanizmaları henüz tam olarak anlaşılamamıştır.

İnterferon düzenleyici faktörlerin bir üyesi olan IRF6'nın keratinosit farklılaşmasında yer alan yeni bir Notch hedef geni olduğu gösterilmiştir. IRF6, meme epitel hücrelerinde yer alan Maspin ile birlikte potansiyel tümör baskılayıcı gen olarak ifade edilmektedir. Bu tez çalışmasında, IRF6 ekspresyonunun, meme epitel hücrelerinde Notch sinyal iletimi tarafından düzenlenip düzenlenmediği ve hücre çoğalmasında ve transformasyonunda Notch'un alt bir aracı olup olmadığı araştırılmıştır.

Notch sinyal iletiminin etkinleşmesinin, MCF10A normal meme epitel hücre hattındaki IRF6 ifadesini arttırdığı; öte yandan MDA-MB-231 meme kanseri hücre hattındaki aktif Notch yolağının engellenmesinin ise IRF6 ifadesini azalttığı gösterildi. IRF6'nın susturulması, MCF10A hücre hattında Notch tarafından tetiklenen hücre transformasyonunu azaltmış fakat hücre çoğalmasında anlamlı bir değişim ile sonuçlanmamıştır. Bu sonuçlar, IRF6'nın Notch'un alt hedef genlerinden biri olduğunu ve meme epitel hücrelerinde Notch sinyal iletimi için bir aracı olarak işlev gördüğünü göstermektedir.

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CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Breast cancer has still been the most common cancer and the second most frequent cause of cancer death of women in western countries (American Cancer Society 2012). Treatment of breast cancer is dependent on the stage of the disease and can include surgery, chemotherapy, radiotherapy and hormone therapy. Chemotherapy targets rapidly dividing cancer cells such as cancer cells and stem cells in whole body which cause diverse side effects. Therefore in recent years targeted therapies which should cause fewer side effects have been designed to attack mechanisms that support cancer progression such as maintenance of stem like tumor initiating cells, invasion, metastasis, cell survival and tumor related angiogenesis. The development of more personalized targeted therapies based on characteristics of individual cancers can be more effective against many types of cancers in near future. It has been shown that pathways such as Hedgehog, Wnt and Notch which regulate developmental processes, epithelial mesenchymal transition and maintenance of somatic stem cells in adult tissues are disregulated in many cancers. And these pathways have been studied as target pathways and chemicals or antibodies have been developed against these pathways for new targeted therapies to improve breast cancer outcomes (Grandis & Egloff 2012). However, all aspects of these pathways and crosstalk between each other have not been identified well. For example, Notch inhibitors have been used in clinical trials because Notch signaling is thought as oncogene. However, Notch has also tumor suppressor roles and Notch inhibitors used in clinical trials can be harmful to patients. Therefore, Notch signaling should be identified with its all mediators and relationships in order to define cancer subtypes and develop better targeted therapies for each subgroup which target all Notch signaling or a specific Notch receptor.

1.2. Notch Signaling Pathway

Notch signaling is an evolutionary conserved mechanism used by metazoans such as worms, sea urchins, flies, mice and humans in order to control cell fate through local cell-cell interactions. Notch genes encode trans-membrane glycoprotein receptors activated by two families of trans-membrane bound ligands that transmit signals playing role in development (Artavanis-Tsakonas et al. 1999). The central events by activation of Notch signaling are also highly conserved from flies to humans.

Notch gene was identified by the description of a strain of *Drosophila* with notches at the end of their wing blades caused by a partial loss of function of what would later be identified as Notch gene (Morgan 1917). Notch gene, which was cloned firstly in 1980s by the groups of Artavanis-Tsakonas (Wharton et al. 1985) and Young (Kidd et al. 1986), encodes a cell membrane bound receptor with a single pass trans-membrane domain.

1.3. Notch Receptors and Ligands

Although a single Notch protein is present in *Drosophila* (Wharton et al. 1985), *C. elegans* has two (glp-1 and lin-12) (Greenwald 1985) and mammals such as mouse and human have four Notch proteins (Notch1-4) by the direction of gene duplication (del Amo et al. 1993; Weinmaster et al. 1992; Lardelli & Lendahl 1993; Lardelli et al. 1994; Uyttendaele et al. 1996).

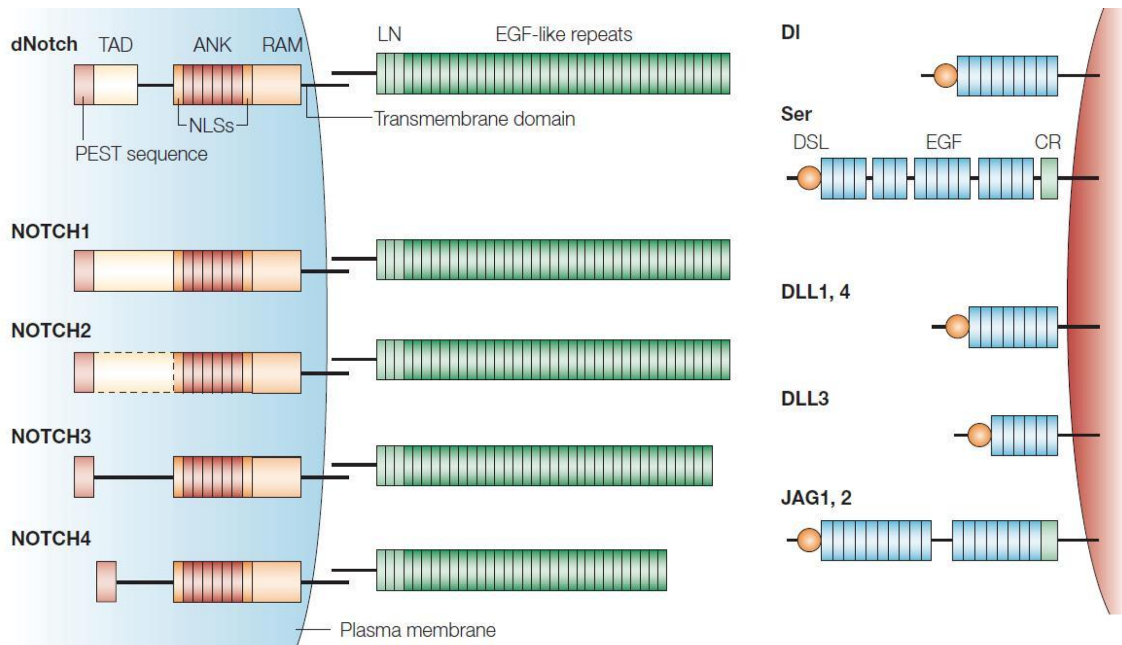


Figure 1.1. Notch Receptors and Notch Ligands in Drosophila and Human
(Source: Radtke & Raj 2003)

Each member of Notch protein family is synthesized as a single precursor protein and then it is cleaved in two at S1 site by Furin-like convertase in golgi during its transport to cell surface and exists as a heterodimeric receptor. Notch proteins consist of an extra-cellular subunit (NEC) non-covalently associated with a trans-membrane subunit (NTM) (Fiúza & Arias 2007). NEC contains multiple tandem epidermal growth factor (EGF)-like repeats which participate in ligand binding. NTM contains three cysteine-rich Notch/Lin12 (LN) repeats in extracellular part which prevent signaling in the absence of ligand, a single pass trans-membrane domain and an intra-cellular domain (NICD). NICD contains RAM domain which participate in transcription factor binding with six ankyrin repeats, two nuclear localization signals, a transcriptional activation domain (TAD) and Pro-Glu-Ser-Thr (PEST) sequence which is important for protein degradation (Greenwald 1998; Artavanis-Tsakonas et al. 1999). Although the structures of four Notch receptors are very similar, there are many differences in extra-cellular and intra-cellular parts. Notch1 and Notch2 contain 36 EGF repeats whereas Notch3 contains 34 and Notch4 contains 29 repeats in their extra-cellular parts. In their intra-cellular parts, Notch1 contains a strong TAD and Notch2 weak TAD while Notch3 and Notch4 do not contain TAD (Figure1.1) (Radtke & Raj 2003).

Notch receptors are activated by cell membrane bound Notch ligands belonging to Delta and Serrate ligand families. Notch ligands are known as DSL ligands from Delta (*Drosophila*), Serrate (*Drosophila*) and Lag-1 (*C. elegans*) ligand names. Five notch ligands have been identified in mammals so far: Deltalike1 (DLL1), Deltalike-3 (DLL3) and Deltalike-4 (DLL4) homologues of Delta ligand and Jagged1, Jagged2 homologues of Serrate ligand. Each of these ligands has multiple EGF repeats as Notch receptors have, in their extra-cellular part. All ligands have also DSL motifs together with the flanking N-terminal domain and first two EGF repeats which are necessary for binding to Notch receptors (Parks et al. 2006). However they also show differences such as Jagged1 and Jagged2 have twice number of EGF repeats and additional cysteine-rich region which is not present in Delta-like ligands (Figure 1.1) (D'Souza et al. 2008).

The affinity of Notch receptors for different ligands is controlled by glycosylation of extra cellular subunit of Notch receptors (NEC) by Manic Fringe, Lunatic Fringe and Radical Fringe glycosyltransferases. Signal strength and duration of Notch signaling are dosage dependent and modulated at multiple levels by post-translational modifications and certain proteins such as Numb, that control receptor recycling and membrane availability (Nickoloff et al. 2003).

Notch signaling is damped after transcription of target genes, by phosphorylation of NICD within PEST domain by CDK8 kinase and ubiquitination targeting NICD for proteosomal degradation by E3 ubiquitin ligases such as Sel10/Fbw7 (Kopan & Ilagan 2009).

1.4. Notch Processing and Signaling

Because of the fact that Notch receptors and their ligands are both trans-membrane proteins, Notch signal transduction can be activated between two physically adjacent cells. When a Notch-ligand binds to a Notch receptor through their EGF-like repeats, the extra-cellular subunit of Notch (NEC) dissociates from the trans-membrane subunit (NTM) (Parks et al. 2000). This dissociation is occurred by two step proteolytic cleavage of NTM at S2 and S3 sites. S2 site which is located in extra-cellular part of NTM containing LN repeats, is cleaved by a member of the metalloprotease family proteases, either TNF-α converting enzyme (TACE) or Kuzbanian (Kuz) (Brou et al. 2000; Mumm et al. 2000; Pan & Rubin 1997; Sotillos et al. 1997; Lieber et al. 2002).

This first cleavage makes NTM susceptible to second cleavage within transmembrane domain at S3 site by Presenilin-1 dependent γ-secretase complex and membrane bound Notch needs presenilin cleavage for nuclear localization and transduction of signals (Figure 1.2) (De Strooper et al. 1999).

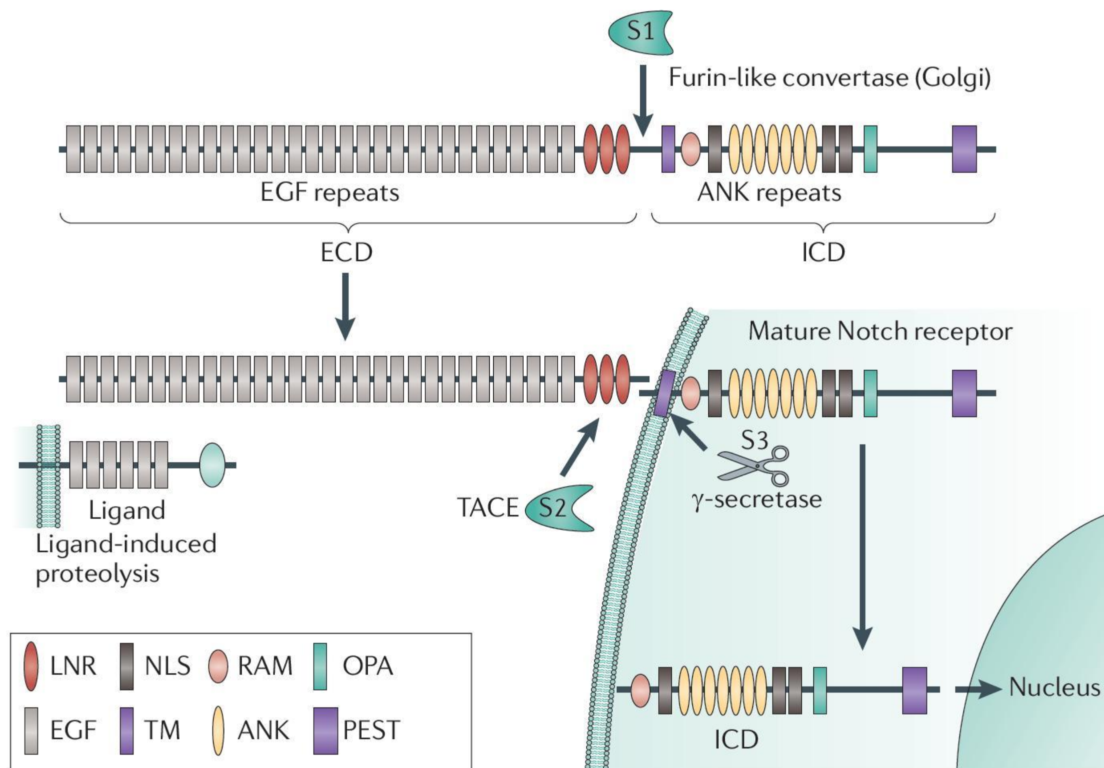


Figure 1.2. Notch Receptor Processing after Notch Ligand Binding
(Source: Ranganathan et al. 2011)

When NICD is released from the cell membrane, it translocates to nucleus directly in order to activate the expression of downstream target genes by binding to a DNA-binding transcription factor called CSL (CBF-1, RBPjK). NICD binds to CSL through its RAM domain and six ankyrin repeats. This binding converts CSL from a transcriptional repressor to an activator by dissociating it from a repressor complex containing CIR (CBF-1 Interacting Corepressor), SMRT/N-CoR, SHARP and HDAC-1 (Histone Deacetylase-1) and recruiting coactivators such as SKIP, MAML-1 (Mastermind-like Protein-1) which forms a stable multiprotein-DNA complex by binding to both CSL and NICD.

MAML-1 binding recruits another transcriptional activator p300 to this complex which has a key role in chromatin opening and initiation of transcription and histone acetyl transferases PCAF and GCN5 (Figure 1.3). This CSL dependent transcription activation of target genes is called as canonical Notch pathway. Although CSL is clearly a downstream primary target of activated Notch, there is CSL independent transduction of Notch signaling called non-canonical Notch pathway (Fiúza & Arias 2007).

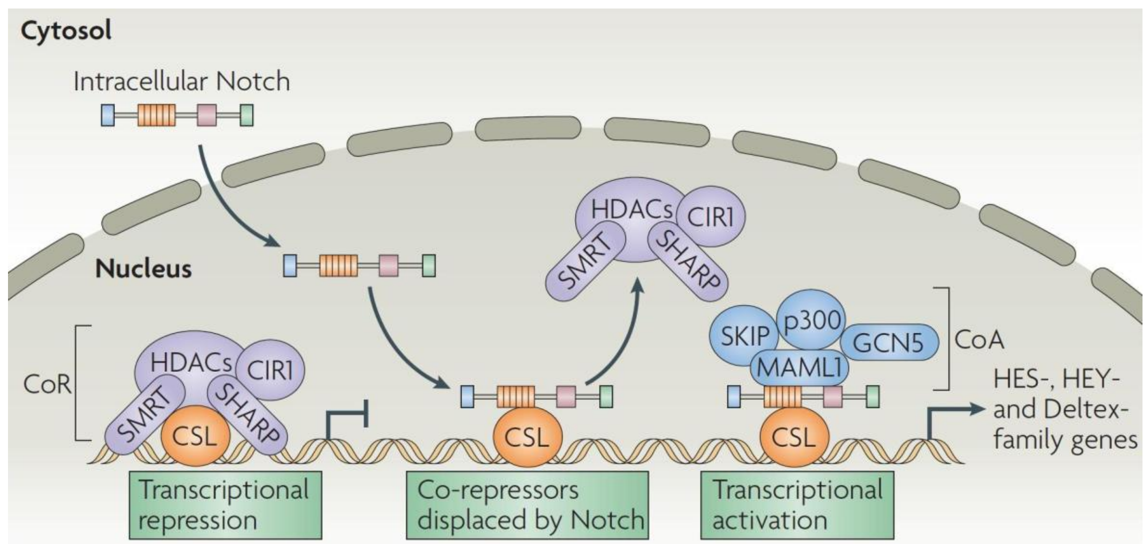


Figure 1.3. Canonical Notch Transcription Complex Formation After Notch Activation (Source: Osborne & Minter 2007)

1.5. Target Genes of Notch Signaling

Canonical Notch signaling activates transcription of target genes through translocation of cleaved NICD to nucleus and binding to CSL with other transcription factors. Many target genes such as Myc, CyclinD1, p21 which are expressed under Notch signaling in different tissues and cell types have been identified in recent years. And these Notch targets vary in both their nature and their effects on cells. Therefore function and phenotype of Notch signaling can be different from tissue to tissue even among cells in the same tissue (Hansson et al. 2004) during development and tissue homeostasis.

1.5.1. Direct Targets of Notch

The direct targets and effectors of Notch signaling pathway are Hes and Hey genes, encode a family of basic helix-loop-helix (bHLH) transcription factors which are related to Drosophila hairy and Enhancer-of-split genes (Jarriault et al. 1995; Artavanis-Tsakonas et al. 1999). The bHLH family of transcriptional regulators such as MyoD, Mash1, Myc, Max, Hes and Hey genes plays crucial roles in development of wide range of organs and cell types (Murre et al. 1994; Massari & Murre 2000; Iso et al. 2003). Hes family (Hes1, Hes2, Hes3, Hes4, Hes5, Hes6, Hes7) and Hey family (Hey1, Hey2, HeyL, HesL/Helt, Dec1, BHLHB2, Dec2/BHLHB3) members are transcriptional repressors and act as Notch effectors by negatively regulating transcription of downstream target genes such as tissue specific transcription factors (Ohsako et al. 1994; Van Doren et al. 1994; Chen et al. 1997). Although two family members share similar domains and several common features, they have differences in their structures such as specific residues and motifs affecting repression mechanisms and target sequences and therefore expression of target genes. The members of these two family bind to specific target sequences by forming different homo- or hetero-dimer complexes between each other to bind target sequences to repress their target proteins (Iso et al. 2003).

Although all members of Hey gene family can be induced by Notch signaling, only Hes1, Hes5 and Hes7 genes have been shown to be induced by Notch while Hes2, Hes3 and Hes6 appear to be independent. And less is known about Hes4 (Fischer & Gessler 2007). Furthermore, they show differential expression profile in different cell types and cancer samples such as undifferentiated embryonic stem cells express Hes1 and Hes3; Hes1, Hey1 and Hey2 are expressed in endothelial cells; Hes1, Hes4 and Hes6 are expressed in gastric cancer; Hes1 and Dec1 in pancreatic cancer; Hes1, Hes2, Hes4, Hes6 and Dec2 in colorectal cancer. Hes6 is also expressed in other tumors including brain tumors, melanoma, small cell lung cancer, retinoblastoma, ovarian cancer, and breast cancer (Katoh 2007).

1.5.2. Other Known Targets of Notch

Notch signaling through Hey and Hes genes does not show the complete output of Notch downstream responses and Notch signaling can occur through CSL but not involving Hey/Hes genes. Many genes such as cell cycle regulators cyclinD1, CDK2, Cdkn1a, p21, c-Myc; transcription factors NF-κB family and PPAR family, Nrarp (Notch regulated ankyrin repeat protein), Deltex1, pre T cell receptor-α and a ubiquitin ligase SKIP2 are downstream targets of canonical Notch signaling (Borggrefe & Oswald 2009; Hansson et al. 2004). These Notch targets can be common targets or tissue and even cell specific targets of Notch signaling (Hansson et al. 2004). And depending on the repertoire of Notch targets in specific tissue or cell lineage and transformed cells, the outcome of Notch signaling can be different.

1.6. Functions of Notch Signaling

Notch signaling plays role in a wide variety of cellular processes in development such as hematopoiesis, neurogenesis, myogenesis, vascular development, skin differentiation and the immune response, by controlling maintenance of stem cells, cell fate determination, differentiation, proliferation and apoptosis through cell-cell communication (Wu & Griffin 2004).

Notch signaling was firstly described that it controls a mechanism for the inhibition of cell differentiation therefore Notch was believed to maintain cells in undifferentiated state (Artavanis-Tsakonas S 1995). Studies in *C. elegans* and *Drosophila* development show that Notch controls the ability of the cell to respond to differentiation signals (Greenwald 1998; Artavanis-Tsakonas et al. 1999). However, in mammals Notch signaling is versatile in cellular processes because mammals express multiple Notch receptors and ligands. And Notch receptors and ligands can be expressed by different cell types or in the same type of cells in different dosages.

Although Notch signaling inhibit T cell development (Maillard et al. 2005), granulocyte differentiation (Milner et al. 1996) and myogenesis (Luo et al. 2005), Notch has been shown to direct cells toward alternate differentiation fates for instance during gliogenesis (Wang & Barres 2000) and keratinocyte differentiation (Rangarajan et al. 2001). Notch represses differentiation into neurons while directing glial cell differentiation (Pierfelice et al. 2011).

Depending on the cellular context, Notch signaling can be determinant of survival versus death, proliferation versus growth arrest, differentiation versus stem-like state and the balance between them is regulated by other signaling pathways and downstream mediators of Notch (South et al. 2012).

1.7. Notch and Cancer Relationship

Notch is related to many human diseases resulted by disregulation of Notch signaling ranging from cancer to neurodegenerative disorders. Notch pathway shows different roles as oncogene or tumor suppressor in cancer.

The connection between Notch signaling and malignancy was first recognized in human T-cell neoplasia (Ellisen et al. 1991). And then Notch disregulation has been shown in a growing number of solid tumors such as cervix, endometrial, renal, head and neck, hepatocellular carcinoma (Gao et al. 2008); lung and breast adenocarcinomas; pleural mesotheliomas and malignant melanomas; and hematological malignancies such as Hodgkin lymphomas, anaplastic large-cell non-Hodgkin lymphomas, B-cell chronic lymphoid leukemia (BCLL) and subsets of acute myeloid leukemia (Nickoloff et al. 2003).

The oncogenic role of Notch was identified firstly in T cell acute lymphoblastic leukemia (T-ALL) cells carrying a specific chromosomal translocation, t(7;9)(q34;q34.3), that attaches a portion of chromosome 7 containing Notch1 gene to chromosome 9 which includes T cell receptor β (TCR-β) gene (Reynolds et al. 1987; Ellisen et al. 1991). This translocation creates a fusion gene by attachment of carboxy terminal region corresponding to Notch1-ICD to the TCR-β promoter/enhancer region which causes permanently active expression of Notch1-ICD because of the active promoter of TCR-β gene. And this translocation that gives rise to upregulated expression of cytoplasmic part of Notch1 causes T-ALL in humans. This result is

supported by generation mouse models for T-ALL by activation of human Notch1-ICD (Pear et al. 1996). And inhibition of Notch1 by γ-secretase inhibition in pre-T-ALL cells expressing active Notch1 prevents cell proliferation (Weng et al. 2003). And similar results have been shown in transgenic mice which express either Notch1-ICD, Notch3-ICD or active Notch2 in the thymus (Robey et al. 1996; Bellavia et al. 2000; Deftos et al. 2000; Rohn et al. 1996). And Notch targets are oncogenic Myc gene, Akt and NF-κB pathways in T-ALL as in mantle cell lymphoma (MCL) (Kridel et al. 2012). Constitutive activation of Notch3 causes lymphomas through the NF-κB activation (Bellavia et al. 2000).

Notch1, Notch2 and certain Notch ligands are also highly expressed in human colon cancers (Zagouras et al. 1995). Notch1 inhibits p53 dependent apoptosis in cervical cancer through activation of Akt pathway which causes tumorigenesis (Nair et al. 2003).

Although the results of many researchs identify Notch signaling as oncogene, Notch has also tumor suppressor roles in specific tissues. For example, it is shown that in murine epidermis Notch1 has function as tumor suppressor rather than oncogene, depending on p21 mediated cell cycle arrest and growth suppression (Rangarajan et al. 2001; Nicolas et al. 2003). Furhermore, basal cell carcinomas (BCC) lack activated Notch1 consistent with its tumor suppressor role in skin and Notch inhibition causes squamous cell carcinoma (SCC) in murine skin (Proweller et al. 2006). And loss of function mutations in Notch genes preventing CSL binding of NICD or ligand binding of Notch receptors, have been found in cutaneous, lung and head and neck SCC samples and cell lines (Agrawal et al. 2011; Blacklow et al. 2011).

On the one hand, in liver, Notch can inhibit growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis; on the other hand, Notch activation causes tumor growth and proliferation in cell lines, with tumor formation in mice (Qi et al. 2003; Gao et al. 2012; Villanueva et al. 2012).

The downstream effects and mediators of Notch signaling pathway and relationships with other signaling pathways in cancer should be identified well in order to clarify the dual behavior of Notch because Notch signaling can either block or promote cell differentiation in context dependent manner through downstream mediators.

1.8. Notch in Breast Cancer

The first evidence that Notch signaling is involved in breast cancer, is demonstrated by the characterization of an insertion site of mouse mammary tumor virus (MMTV) in mouse chromosome (Gallahan & Callahan 1987). This insertion site is named as Notch4 locus and MMTV insertion causes high expression of Notch4-ICD and transforms mammary epithelial cells (Robbins et al. 1992; Uyttendaele et al. 1996).

A truncated and activated form of Notch4-ICD and Notch1-ICD accumulation with Hey1 over expression have been found in breast cancer cell lines (Imatani & Callahan 2000; Stylianou et al. 2006). Notch1 is also activated in breast cancer cells (Weijzen et al. 2002) and Notch1-ICD has oncogenic effects which are mediated through Myc upregulation (Klinakis et al. 2006). And Notch has roles in maintenance of breast cancer cells in stem cell-like properties (Kakarala & Wicha 2008; Harrison et al. 2010).

Transgenic mice that express Notch4-ICD develop mammary and salivary-gland adenocarcinomas within seven months (Jhappan et al. 1992; Gallahan et al. 1996). Notch1 and Jagged1 mRNA levels are increased in breast cancer samples from patients and related with poor prognosis, poorer overall survival and early recurrence (Reedijk et al. 2005; Dickson et al. 2007; Reedijk et al. 2008).

One of the activation mechanisms of Notch signaling is loss of Numb protein which is endogenous Notch inhibitor in almost 50% of breast cancer and Numb defective breast tumors show more aggressive phenotype and poor prognosis (Pece et al. 2004; Colaluca et al. 2008). Also Wnt1 can transform human mammary epithelial cells through increasing levels of Notch3, Notch4 and Notch ligands DLL1, DLL3 and DLL4 (Ayyanan et al. 2006). Oncogenic Ras enhances Notch1 protein levels and transcriptional activity by post transcriptional mechanisms through p38 kinase and Notch1 maintains neoplastic phenotype of Ras transformed cells (Weijzen et al. 2002). And recently, Chinnaiyan's group has found abnormal Notch mRNAs resulted from rearrangements of Notch1 and Notch2 genes and Myc is downstream target of Notch in eight breast carcinoma cell lines and primary tumors (Robinson et al. 2011). Lastly, Notch signaling can activate itself by activating one of the family members as Notch1 increases expression of Notch4 (Weijzen et al. 2002) which is one of the proto-oncogene in mammary tissue (Callahan & Raafat 2001).

Although Notch signaling pathway displays mostly oncogenic properties, the results of recent research revealed the tumor suppressor roles of Notch in breast tissue. Notch1-ICD expression in primary human breast epithelial cells decreases proliferation (Yalcin-Ozuysal Ö et al. 2010; Mazzone et al. 2010). Interestingly Notch functions in dosage dependent manner, while high Notch activity causes inhibition of proliferation, low Notch activity causes hyperproliferation (Mazzone et al. 2010).

The molecular mechanisms of downstream Notch signaling are still elusive in many tissues and cancer types. Downstream targets of Notch designate the outcome of Notch signaling in context dependent manner. Recently it has been found that Interferon Regulatory Factor (IRF) family members are possible candidates of Notch mediators playing role downstream of Notch.

1.9. Interferon Regulatory Factor (IRF) Family

The mammals possess nine different Interferon Regulatory Factors (IRF1-9) which are transcriptional regulators of such as Interferon (IFN) and Interferon inducible genes in regulation of host defense (Mamane et al. 1999; Taniguchi et al. 2001).

All IRF members include an amino (N) terminal DNA binding domain which forms helix-turn-helix domain and recognizes a consensus IRF recognition sequence (5'-AANNGAAA-3') on DNA. And carboxy terminal region of IRFs including IRF association domain (IAD) plays role in protein-protein interactions between IRF members and transcription activators or repressors (Fujii et al. 1999).

In innate immune response, against pathogens such as bacteria or viruses, IRF family plays important roles with IFNs, chemokines and proinflammatory cytokines and NF-κB pathway (Savitsky et al. 2010).

1.10. IRF Members and Their Roles

IRF1, 3, 5, 7, 8, 9 actively play role in IFN mediated innate immune response to pathogens and IRF2 positively regulates this pathway while IRF4 negatively regulates induction of proinflammatory cytokine genes. Under pathogen contamination, IRF3 and IRF7 are phosphorylated by NF-κB kinase ε (IKKε) and activate expression of IFN-α/β

genes with IRF8 while IRF5 induces proinflammatory cytokines such as Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α). IRF1 can induce IFN-γ and TNF-α mediated IFN- β genes against pathogens (Savitsky et al. 2010).

IRF family members also play roles in development of immune cells. While IRF1 and IRF2 are required for Natural Killer (NK) cell development, IRF4 and IRF8 for B cell development. While IRF2 and IRF4 are required for development of dendritic cells (DCs), IRF1 is required for T cells. IRF1, IRF2, IRF4 and IRF8 promote different subgroups of T cells (Savitsky et al. 2010).

IRF family has other critical functions in regulation of cell growth, apoptosis and oncogenesis. IRF1 can induce p21 expression and apoptosis under DNA damage through IRF1 binding sites on p21 promoter (Tanaka et al. 1996). IRF3 plays role in DNA damage induced apoptosis because IRF3 is phosphorylated and translocates from cytoplasm to nucleus under DNA damage (Weaver et al. 2001; Kim et al. 1999). IRF5 has apoptotic effects in cancer cells and overexpression of IRF5 in p53-deficient cells inhibits B cell lymphoma tumor growth (Barnes et al. 2003). IRF8 has anti-leukemic activity and lack of IRF8 causes chronic myeloid leukemia (CML) (Holtschke et al. 1996). IRF9 mediates IFN induction of p53 for tumor suppression and apoptosis (Takaoka et al. 2003). IRF2 has pro-oncogenic activity because over expression of IRF2 causes oncogenic transformation (Harada et al. 1993). IRF4 is required for survival of multiple myeloma cells and trans-activates Myc while Myc activates IRF4 (Shaffer et al. 2008).

1.11. IRF6

IRF6 is the transcription factor which is poorly understood member of IRF family and its role in immune response has not been identified. However, it has been shown that IRF6 has major roles in skin development and mutations in IRF6 gene cause Van der Woude syndrome and Popliteal Pterygium Syndrome (PPS) that include cleft lip, cleft palate, lip pits, skin folds and oral adhesions in human (Kondo et al. 2002; Kayano et al. 2003; Wang et al. 2003). And genetic variations in IRF6 are correlated with high risk for isolated cleft lip and palate occurrence in human (Zucchero et al. 2004; Blanton et al. 2005).

IRF6 deficient mice have abnormal skin, limb and craniofacial development with defects in keratinocyte differentiation and proliferation indicating that IRF6 is one of the key determinants between differentiation and proliferation switch in keratinocytes (Ingraham et al. 2006; Richardson et al. 2006). It has been recently shown that IRF6 deficient keratinocytes show abnormal phenotypes such as larger in size and irregularly shaped but retain epithelial characteristics. IRF6 deficient keratinocytes have more ability to form colonies in colony formation assay showing long term proliferating capacity. IRF6 is required for terminal differentiation by downregulating p63 (Biggs et al. 2012) which is a transcription factor required for keratinocyte (Yang et al. 1999; Mills et al. 1999). Furthermore, IRF6 depleted cells show downregulation of genes involved in epidermal development and differentiation such as keratins, transcription factor AP1, small proline-rich proteins (SPRR2A and SPRR2J) and OVOL1 which is repressor of Myc with upregulation of genes responsible for keratinocyte proliferation, angiogenesis, and cell adhesion. Genomic analysis shows that IRF6 binding sites are located in genes shown to be important in craniofacial and bone development such as TGFBR3 and MBP2 and epidermal development such as OVOL1 and EGFR.

TGF-β signaling is one of the important regulator in palate development (Iwata et al. 2011) and the relationship between IRF6 and TGF-β has been recently identified. TGF-β regulates expression of IRF6 through Smad4 during palate formation. And p21 expression is absent in both haplo-insufficiency of IRF6 and Smad4 deficient mice and same abnormal development of skin is observed. Furthermore, IRF6 reexpression rescued p21 expression in Smad4 deficient mice. Thus Smad4 and IRF6 synergistically regulate the development of skin and TGF-β mediated by IRF6 expression is responsible for degenerations (Iwata et al. 2013).

IRF6 has tumor suppressor role and is downregulated in squamous cell carcinoma (SCC) through the methylation of IRF6 promoter. And IRF6 inhibition by siRNA induces migration of SCC cells (Botti et al. 2011).

In mammary tissue, IRF6 was first identified by the group of Hendrix by using yeast two hybrid system for Maspin/SerpinB5 (mammary serine protease inhibitor) protein which is a tumor suppressor by its ability to regulate apoptosis (Jiang et al. 2002) and prevents invasion (Sheng et al. 1996). IRF6 is one of the Maspin-binding proteins and Maspin binds to IRF6 by its IRF association domain (IAD) which is regulated by phosphorylation of IRF6. Furthermore, the expression level of IRF6 is

reduced in breast cancer cells and transient re-expression of IRF6 in absence of Maspin in breast cancer cells results in elevation of N-cadherin which is related to metastasis and transformation properties of cancer cells (Bailey et al. 2005). Later the same group showed that the expression levels of IRF6 and Maspin are increased in differentiated, quiescent differentiated cells of lactating mammary gland whereas decreased in proliferating ductal and glandular epithelial cells during pregnancy. IRF6 is phosphorylated that resulted with proteosomal degradation during proliferation. The reexpression of IRF6 in breast cancer cells causes cell cycle arrest and Maspin prolongs this effect (Bailey et al. 2008; Bailey et al. 2009).

1.12. Notch-IRF6 Relationship

Notch and IRF6 relationship is a new subject because very few publications have been published in recent years. In 2009, Dixon's group showed that IRF6 and Notch ligand Jagged2 play role in skin development and development defects in convergent pathways. Both IRF6 deficient and Jagged2 deficient mice can generate cleft palate phenotype and the mice carrying heterozygous allele of both genes can form the same phenotype but with more severe situation (Richardson et al. 2009).

IRF6 expression is higher in differentiating versus proliferating keratinocyte cells parallel with differentiation markers Keratin 1 and Loricrin and conversely with basal marker Integrin β4. IRF6, Keratin 1 and Hes1 expressions are upregulated after induction of differentiation and downregulated with treatment with DAPT, a γ-secretase inhibitor. And Notch activation upregulates IRF6, differentiation marker Involucrin and Hes1. Notch activates transcription of IRF6 by CSL complex binding to CSL binding domain at -2.4 kb upstream of IRF6 transcription start site. Furthermore, IRF6 shows a role as a mediator of Notch pro-differentiation function by inducing differentiation markers and downregulation of p63 whereas p21 and Hey1 levels are not changed (Restivo et al. 2011).

These few studies show that Notch regulates development of keratinocytes with IRF6 at downstream of Notch and disregulation of them results in diseases such as cleft palate and squamous cell carcinoma. Therefore IRF6 is the best candidate to be Notch signaling mediator also in breast tissue and cancer.

CHAPTER 2

AIM OF THE RESEARCH PROJECT

In view of the fact that IRF6 is direct target of Notch signaling in skin tissue and plays role as a mediator of Notch in keratinocyte differentiation by inducing differentiation markers with suppressing basal markers, we hypothesized that IRF6 is a target and mediator of Notch signaling pathway in breast tissue which is another epithelial originated tissue. In this study, it is aimed to investigate whether IRF6 expression is regulated by Notch signaling and acts as a mediator of Notch signaling in proliferation and transformation mechanisms in breast epithelial cells.

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Lines and Cell Culture

Five cell lines were used during this project, human normal breast epithelial cell line MCF10A, human breast adenocarcinoma cell lines MDA-MB-231 and MCF7, human embryonic kidney cell line 293T and mouse embryonic fibroblast cell line NIH/3T3. 293T cell line was used for virus production while NIH/3T3 cell line for virus titration experiments. Because MCF10A cells show no Notch activity, Notch pathway is activated in this cell line while Notch inhibition experiments were performed in MDA-MB-231 and MCF7 cell lines which show high Notch activity.

Human breast epithelial cell line MCF10A were grown in special medium consisting of high glucose including DMEM-F12 (HyClone, Cat# SH30126.01) with 5% Horse Serum (HyClone, Cat# SH30074.03), 20ng/mL EGF (Sigma, Cat# E9644), 0.5 µg/mL Hydrocortisone (Sigma, Cat# H0888), 100ng/mL Cholera Toxin (Sigma, Cat# C8052), 10 µg/mL Insulin (Sigma, Cat# I1882), 1% L-Glutamine (HyClone, Cat# SH30034.01) and 1% Penicillin/Streptomycin (HyClone, Cat# SV30010).

Human breast adenocarcinoma cell lines MDA-MB-231 and MCF7 and human embryonic kidney cell line 293T were grown in medium consisting of high glucose including DMEM (HyClone, Cat# SH30285.01) with 10% Fetal Bovine Serum (FBS) (Biological Industries, Cat# 04-007-1A), 1% L-Glutamine (HyClone, Cat# SH30034.01) and 1% Penicillin/Streptomycin (HyClone, Cat# SV30010).

Mouse embryonic fibroblast cell line NIH/3T3 was grown in medium consisting of high glucose including DMEM (HyClone, Cat# SH30285.01) with 10% New Born Calf Serum (NBCS) (Biological Industries, Cat# 04-102-1A), 1% L-Glutamine (HyClone, Cat# SH30034.01) and 1% Penicillin/Streptomycin (HyClone, Cat# SV30010).

All cell lines were cultured in incubator at 37 °C, 5% CO_2 and lifted off by using 0.05% trypsin (HyClone, Cat# SV30031.01) at 37 °C, 5% CO_2 for about 3-15 minutes during cell splitting procedure.

293T and NIH/3T3 cell lines were kindly provided by Prof. Cathrin Brisken Lab. (EPFL, ISREC) while MCF10A, MCF7 and MDA-MB-231 cell lines were obtained from ATCC.

3.2. Virus Production and Titration

3.2.1. Retro-virus Production

For ectopic gene expression experiments in specific cell line, retro-viral system containing sequence of gene of interest was used. And retro-viruses were produced by using human embryonic kidney cell line 293T which shows higher transfection efficiency. Retro-viral system plasmids used for virus production were MSCV and pcl10A plasmid vectors. MSCV vector contains two LTR sites for integration into host genome, multiple cloning site for insertion of sequence of interest into vector and pgk-Neo cassette for positive selection of infected cells while pcl10A vector contains packaging genes of retro-viruses. For integration and ectopic expression of gene of interest, cDNA sequence of gene should be inserted in multiple cloning site existing between LTR sites. In order to produce viruses both virus packaging vector pcl10A and MSCV vectors should be inserted into host 293T cells by transfection.

In order to produce viruses, vectors were transfected by using transfection reagent X-tremeGENE9 (Roche, Cat# 06365787001) in optimized ratios into optimized number of 293T cells in 10cm culture dishes. As optimized, 2 μg of retro-viral MSCV vector and 2 μg of packaging vector pcl10A with 12 μL of X-tremeGENE9 as 1:3 ratios were mixed in 500 μL serum-free medium and incubated 15 minutes at room temperature. Then this mixture was added on top of the $2x10^6$ 293T cells cultured one day before in 10cm plates for transfection of both control and gene expressing virus vectors. 24 hours after transfection medium was aspirated and fresh medium was added into plates. The RNA molecules are packaged into virus by packaging proteins and produced viruses move out of cell into medium. After 48 hours and 72 hours virus

containing medium of plates were collected, aliquoted and stored at -80 ^{o}C. Before infection of target cells, virus amount in mediums was determined by virus titration experiments described below and equal amounts of viruses were used during infection.

For Notch signaling pathway activation in MCF10A cells, retro-viruses were produced by transfection of both MSCV vector including sequence of intracellular domain of Notch1 receptor which is active form of receptor (Notch1-ICD/N1-ICD) and virus packaging vector pcl10A. For Notch inhibition by DNMM, MSCV vector including sequence of DNMM and packaging vector pcl10A were transfected into 293T cells.

3.2.2. Lenti-virus Production

For RNA interference experiments, lenti-viral system was used including shRNA sequences against mRNA sequence of gene of interest. Lenti viruses were produced by transfection of shRNA containing virus vectors into 293T cells, too. Lenti-virus vectors are control plko vector which contains U6 promoter for shRNA expression, hPGK promoter and Puromycin resistance gene, multiple cloning site for insertion of shRNA sequence and HIV-1 RNA packaging signal between 5’ LTR and 3’ LTR; packaging vector pCMV-dR8.74 which contains gag gene expressing of capsid proteins and pol gene expressing reverse transcriptase through CMV promoter and envelope vector pMD2.VSVG which contains vsvg gene expressing coat protein of virus through CMV promoter. For lenti-virus production all these three vectors should be transfected together into 293T cells.

In order to produce lenti-viruses, three vectors were transfected by using transfection reagent X-tremeGENE9 (Roche, Cat# 06365787001) in optimized ratios into optimized number of 293T cells in 10cm culture dishes. As optimized, 2 µg of lenti-viral plko vector, 1.3 µg of packaging vector pCMV-dR8.74 and 0.7 µg of envelope vector pMD2.VSVG with 12 µL of X-tremeGENE9 as 1:3 ratios were mixed in 500 µL serum-free medium and incubated 15 minutes at room temperature. Then this mixture was added on top of the $2x10^{6}$ 293T cells cultured one day before in 10cm plates for transfection of three vectors. 24 hours after transfection medium was aspirated and fresh medium was added into plates. After transfection, shRNA molecules and other necessary sequences are packaged into virus by packaging and envelope proteins

and viruses move out of cell into medium. After 48 hours and 72 hours virus containing medium of plates were collected, centrifuged at 22000 rpm for 2 hours and 20 minutes, at 16 ^{o}C. The precipitations of virus were resuspended by 300 µL 1X PBS, aliquoted and later stored at -80 ^{o}C. Before infection of target cells, the amount of viruses were determined by virus titration experiments described below and equal amounts of viruses were used during infection.

For Notch signaling pathway inhibition in MDA-MB-231 cells, lenti-viruses including shRNA sequence against CSL mRNA were used. For IRF6 silencing in MCF10A cells, lenti-viruses including shRNA sequence against IRF6 mRNA were used.

3.2.3. Virus Titration

In order to determine the amount of virus and efficiency of them, virus titration was performed for each virus production. For this purpose mouse embryonic fibroblast cell line NIH/3T3 cell line was used. Virus titration was performed by serial dilutions as 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} of produced viruses and these dilutions were used to infect $75x10^{3}$ NIH/3T3 cells cultured one day before in 6 well plates. One day later medium of plates were changed with fresh medium and at third day these transduced NIH/3T3 cells were splited into 10 cm plates and selection was started by 400 µg/mL Geniticin (Gibco, Cat#10131-019) or 2 µg/mL Puromycin (HyClone, Cat# SV30075) depending on used vectors until all non-transduced control cells died. This selection killed the non-transduced cells and only virus infected cells lived and formed colonies in plate. After selection was ended, medium of plates were aspirated, washed by 1X PBS and the cells dyed by 0.5% crystal violet solution for 15 minutes and washed by 1X PBS three times for 10 minutes. Then colonies of cells were counted that gave clues about virus amount and efficiency because more colonies mean more virus or efficiency of infection. The colony numbers of each virus infection are compared in order to determine equal amount of virus for infection.

3.3. Infection of Cell Lines by Viruses

For Notch activation experiments, as optimized number of cells $125x10^3$ MCF10A cells cultured one day before were transduced by MSCV control retro-viruses and Notch1-ICD expressing MSCV-NICD retro-viruses. For Notch inhibition experiments, $125x10^3$ MDA-MB-231 cells cultured one day before were transduced by lenti-viruses including shGFP sequence as control virus and shCSL lenti-viruses or by MSCV control retro-viruses and DNMM expressing MSCV-DNMM retro-viruses. Lastly for proliferation and transformation assays, $125x10^3$ MCF10A cells cultured one day before were transduced by MSCV control retro-viruses or Notch1-ICD expressing MSCV-NICD retro-viruses with lenti-viruses including shGFP sequence as control virus or shIRF6 lenti-viruses to create four different conditions of MCF10A cells.

For all infections, polybrene (Sigma, Cat# 107689) was added into virus-medium mixture in 8 µg/mL final concentration in order to increase infection efficiency and medium was changed after 24 hours with fresh medium. At third day (72h), transduced cells were flash frozen by putting the cell plates on to liquid nitrogen after aspirating the medium and washing by 1X PBS. And these frozen plates were stored at -80 ^{o}C. As soon as possible RNA and protein isolations were performed from these frozen transduced cells for Real Time PCR and Western Blot experiments.

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

Semi-quantitative real time RT-PCR was used to determine mRNA expression levels of genes of interest. Firstly, cells were thawed for 2 minutes on ice and total RNA isolation was performed by using Pure-link RNA Mini Kit (Ambion, Cat# 12183018A) with DNase treatment (Invitrogen, Cat# 12185-010) to remove DNA contamination following the manufacturer's protocols. Later complementary DNA (cDNA) was synthesized by reverse transcription from 1 µg of isolated total RNA by using Fermentas First Strand cDNA Synthesis Kit (Thermo Scientific, Cat# K1622) using random hexamer primers. Amplification of cDNA samples was performed by using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Cat# K0252) and specific forward and reverse primers against cDNAs. SYBR Green Master Mix includes Tag

DNA polymerase, dNTPs in PCR buffer and SYBR Green I dye and mixed with forward primer, reverse primer and cDNA samples in specific optimized amounts before PCR amplification. Expression levels of targeted mRNA were determined according to fluorescence levels generated during amplification of targeted cDNA molecules by SYBR Green I which is fluorescent DNA binding dye.

In SYBR Green I based PCR amplification, free SYBR Green I dyes bind to double stranded DNA molecules during annealing and extension step and as a result emit fluorescence. Emitted fluorescence is monitored by measuring the increase in fluorescence during 40 cycle PCR amplification by BioRad IQ5 Real Time Detection System. And mRNA expressions levels were calculated by delta-delta Ct method using mean of cycle threshold values (Ct) of IRF6 and direct Notch targets such as Hey1, Hey2, Hes1, Hes5 normalized by Ct values of housekeeping gene TATA box binding protein (TBP). And student t-test method was used for statistical calculations.

Table 3.1. Forward and reverse primer sequences used for qRT-PCR.

Hey1_forward primer	5'-GGGAGGGGAACTATATTGAATTTT-3'
Hey1_reverse primer	5'-ATTTGTGAATTTGAGATCCGTGT-3'
Hey2_forward primer	5'-AAGATGCTTCAGGCAACAGG-3'
Hey2_reverse primer	5'-GCACTCTCGGAATCCTATGC-3'
Hes1_forward primer	5'-AACACGACACCGGATAAACC-3'
Hes1_reverse primer	5'-TCAGCTGGCTCAGACTTTCA-3'
Hes5_forward primer	5'-ACATCCTGGAGATGGCTGTC-3'
Hes5_reverse primer	5'-TAGTCCTGGTGCAGGCTCTT-3'
IRF6_forward primer	5'-ATCACTTGTTGCTCCCAACC-3'
IRF6_reverse primer	5'-AAACGGTGGCTGCTTCTCTA-3'
TBP_forward primer	5'-TAGAAGGCCTTGTGCTCACC-3'
TBP_reverse primer	5'-TCTGCTCTGACTTTAGCACCTG-3'

3.5. Western Blot for Protein Analysis

In order to analyze protein expression levels, western blot was performed for Notch activation and inhibition experiments.

3.5.1. Protein Isolation

Western Blot experiments were performed for detection of protein expression levels of genes of interest. Frozen cells were thawed on ice for 2 minutes and lysed by RIPA Lysis Buffer working solution fresh prepared as mixture of 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 sodium orthovanadate (Na_3VO_4) and 50 mM NaF and lysed cells were scraped with a cell scraper. The lysates were collected into eppendorf tubes and homogenized by passing them through an insulin syringe (26G) for 5-10 times. Lysates were incubated on ice for 20 minutes and later centrifuged at 4 ^oC, at 14000 rpm for 10 minutes. Supernatant that contains total protein was transferred into fresh eppendorf tubes and stored at -80 ^oC.

3.5.2. Quantification of Proteins

Protein amounts of isolated samples were determined before western blot procedure in order to load equal amount of protein in SDS-Gel. For this purpose, Bradford or BCA assays were performed. For Bradford assay equal amounts of BSA calibrator samples (0.2, 0.5, 1, 2, 4, 8 mg/mL) as reference standards and isolated protein samples were added into 100 µL 1X Bradford dye solution and put into 96 well plates as two replicates. Spectrophotometric measurement was done at 595 nm and protein concentrations of isolated samples were calculated by equation generated by BSA standard curve. BCA assay was also performed for some of the protein samples by using BCA Protein Assay Kit (Thermo, Cat# 23227) containing bicinchoninic acid as dye for protein determination. Protein samples were mixed with working reagent solution and incubated at 37 ^oC for 30 minutes to activate chemical reactions of bicinchoninic acid generating purple color product absorbs light at 562 nm wavelength measured by spectrophometer at room temperature and protein concentrations of samples were calculated by equation of BSA standard curve.

3.5.3. Western Blot Procedure

After determination of protein concentration of samples, loading sample including 5 μL loading dye, calculated amount of protein sample and water up to 25 μL was prepared for each sample. Prepared samples were loaded into SDS-Gel which consists of 5% stacking gel and 10, 12 or 15% resolving gel. Furthermore, 10 μL protein marker (NEB, Cat# P7709S) was loaded into one well as protein size determinant. Protein samples were run at 100 V for stacking and 120 V for resolving in 1X running buffer consisting of Glycine, Tris-base and SDS. And later proteins were transferred to PVDF membrane in 1X transfer buffer consisting of Glycine and Tris-base at 250 mA for 2 hours at room temperature or 30 mA for overnight 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 overnight at 4 ^{o}C. 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.

Following day, membranes were washed by 1X TBS-T for 15 minutes, 3 times to remove unbound antibodies 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 by 1X TBS-T for 15 minutes, 3 times again and later proteins were detected by chemiluminescence by using Biorad VersaDoc Imaging System (4000 MP). Protein bands show the absence or presence of a specific proteins and expression levels of them. Intensity of these protein bands were determined by using program of Biorad VersaDoc Imaging System (4000 MP) and the adjusted volume values were used for calculation. Intensity values of Hey2 and IRF6 proteins were normalized to values of housekeeping protein γ-tubulin. Student t-test method was used for statistical calculations.

Primary antibodies specific to a protein of interest used for western blot were Anti-Hey2 (Millipore, Cat# AB15562, 1:1000, in 5% milk powder), Anti-IRF6 (Abcam, Cat# ab58915, 1:400, in 5% BSA) and Anti-γ-tubulin (Sigma, Cat# T6557, 1:10 000, in 5% milk powder) for loading control. Secondary antibodies were Polyclonal Goat Anti-Mouse (Dako, Cat# P0447, 1:1000 in TBS-T) and Polyclonal Goat Anti-Rabbit (Dako, Cat# P0448, 1:2000 in TBS-T).

3.6. Growth Curve

Growth curve assay was performed for long term analysis of the proliferation effects of four different conditions of MCF10A cells. Infections were done in 6 well plates and after 72h they were split in equal number (2500 cells per well) into 12 well plates by triplicates for each condition and cultured without selection. Cultured cells were counted by using neubauer improved hemocytometer under microscope for five days after splitting and a growth curve was generated using counted numbers of infected cells. Student t-test method was used for statistical calculations.

3.7. Soft Agar Colony Formation Assay

Soft Agar Colony Formation Assay was performed in order to determine cell transformation phenotype. In this assay cells are cultured in semi-solid Difco Noble Agar (BD, Cat # 214220) and medium mixture to create 3D cell culture suitable for environment for anchorage independent cell growth which is one of the hallmarks of transformed cells. Thus, soft agar colony formation assay is used as an in vitro model to be predictor of in vivo transformation.

In this assay, cells were cultured in semi-solid layer of 0.35% noble agar and medium mixture on top of bottom layer of 0.5% noble agar and medium mixture in 6 well plates. Three days after infection, transduced MCF10A cells were split, counted and 30000 cells per well were mixed with 0.35% noble agar and medium to be cultured in soft agar wells. After cells were cultured in soft agar, medium on top of agar was changed twice a week for 8-9 weeks. Then all wells were dyed by 0.005% crystal violet solution for 1-2 hours at room temperature and washed by 1X PBS for 15 minutes 3-5 times. Colonies bigger than 30 µm in diameter were counted and colony sizes were calculated from 15 photos taken from 5 different places of well and 3 layer in depth by using Olympus CKX41 microscope with Olympus DP25 camera and DP2-BSW application software. Student t-test method was used for statistical calculations.

CHAPTER 4

RESULTS

4.1. Notch1 Activation Induces IRF6 Expression

In order to determine whether Notch signaling activation regulates IRF6 expression, we activated Notch1 signaling through expressing Notch1 Intra-Cellular Domain (N1-ICD) by retro-viral system in human breast epithelial cell line MCF10A which has no Notch activity. After infection by retro-viruses containing N1-ICD cDNA sequence, mRNA and protein levels of IRF6 were detected by semi-quantitative Real Time RT-PCR (qRT-PCR) and Western Blot techniques at third day after infection respectively.

After infection of MCF10A cells by retro-viruses, we checked whether Notch1 activation was achieved by determining the mRNA expression levels of direct target genes of Notch signaling pathway such as Hey1, Hey2 and Hes1. After Notch1 activation, relative mRNA expression of Hey2 was increased 142 fold, Hey1 was increased 3.7 fold and Hes1 was increased 1.88 fold compared to control that showed Notch activation. And IRF6 mRNA expression was increased 3.1 fold significantly after Notch1 activation (Figure 4.1).

For confirmation of qRT-PCR results, we also checked the expression levels of one of the Notch targets, Hey2 protein to show Notch activation, IRF6 protein and γ-tubulin protein for loading control by using Western Blot technique. We determined that Hey2 protein levels were increased in all samples and 1.8 fold significantly according to intensity results which showed Notch activation. After Notch1 activation, IRF6 protein levels were increased in two experiments and no change in first experiment although Hey2 was increased. Intensity results showed that IRF6 was increased 1.3 fold. Equal amounts of protein samples were loaded in each well which was showed by γ-tubulin protein levels (Figure 4.2).

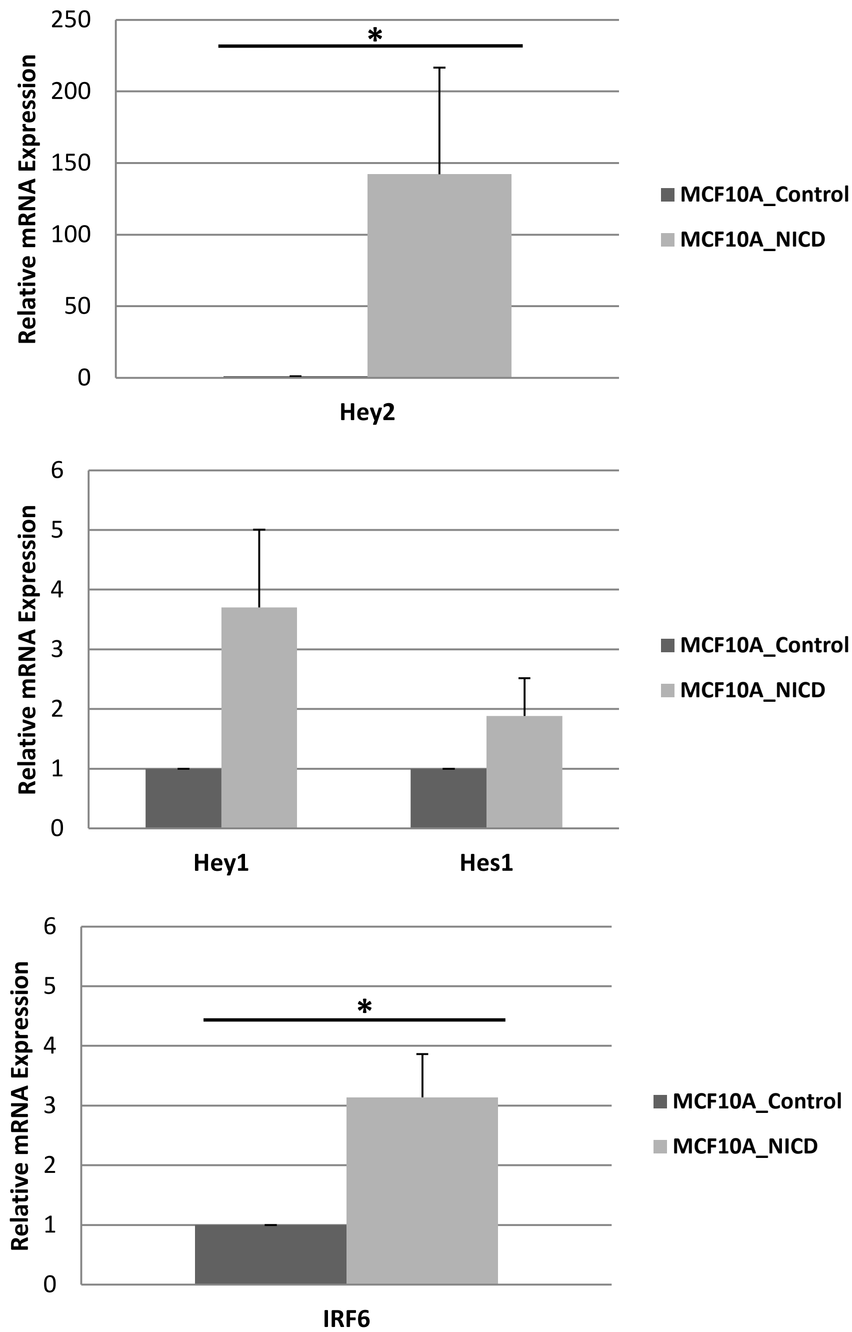


Figure 4.1. Real Time RT-PCR results of Notch1 activation in MCF10A cells. MCF10A cells were infected with retro-viruses containing N1-ICD sequence. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and qRT-PCR were performed by using specific primers against Notch targets Hey1, Hey2, Hes1; IRF6 and TBP for normalization. Three independent experiments were performed. (* $p<0.05$)

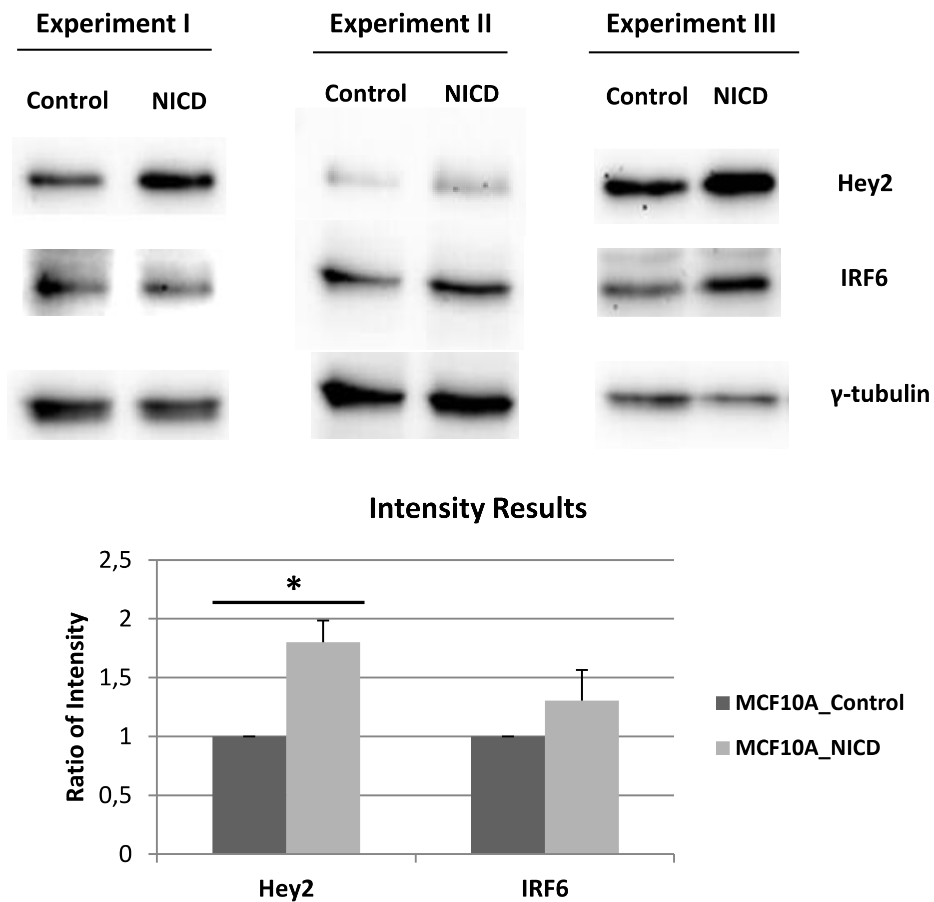


Figure 4.2. Western Blot results of Notch1 activation in MCF10A cells. MCF10A cells were infected with retro-viruses containing N1-ICD sequence. At third day of infection cells were lysed and protein isolation and immunoblotting were performed by using protein specific antibodies against Notch target Hey2, IRF6 and γ-tubulin for loading control. Three independent experiments were performed. (* $p<0.05$)

Real Time RT-PCR and Western Blot results show that Notch1 activation by expression of active Notch1-ICD in MCF10A cells upregulates IRF6 expression in both mRNA and protein levels.

4.2. Notch Inhibition Reduces IRF6 Expression

In order to conclude that Notch regulates IRF6 expression, it must also be shown that IRF6 is downregulated when Notch signaling pathway is inhibited. Therefore, we inhibited Notch signaling in human breast adenocarcinoma cell lines MDA-MB-231 and MCF7 which have constitutively active Notch signaling by using three methods and checked the expression levels of Notch targets and IRF6.

4.2.1. Notch Inhibition by Using γ-secretase Inhibitor

One of the methods to inhibit Notch signaling is using a γ-secretase inhibitor such as DAPT in culture of target cells. Because intra-membrane protease γ-secretase is essential for release of NICD after activation of Notch receptor bound to Notch ligand, γ-secretase inhibitor DAPT can inhibit Notch signaling pathway by avoiding release of NICD and thus activation of Notch signaling cascade.

Firstly, DAPT concentrations and treatment time in culture were optimized to find best condition for inhibition of Notch signaling. Different DAPT concentrations and time points (5 μM, 50 μM and 100 μM for 2 hours, 6 hours and 12 hours; 7.5 μM, 15 μM, 30 μM, 60 μM, 90 μM and 180 μM for 24 hours and 48 hours) used by other research groups in literature were tested to inhibit Notch signaling in MDA-MB-231 and MCF7 cell lines by checking mRNA expression levels of Notch target gene Hey2 by Real Time RT-PCR (Figure 4.3).

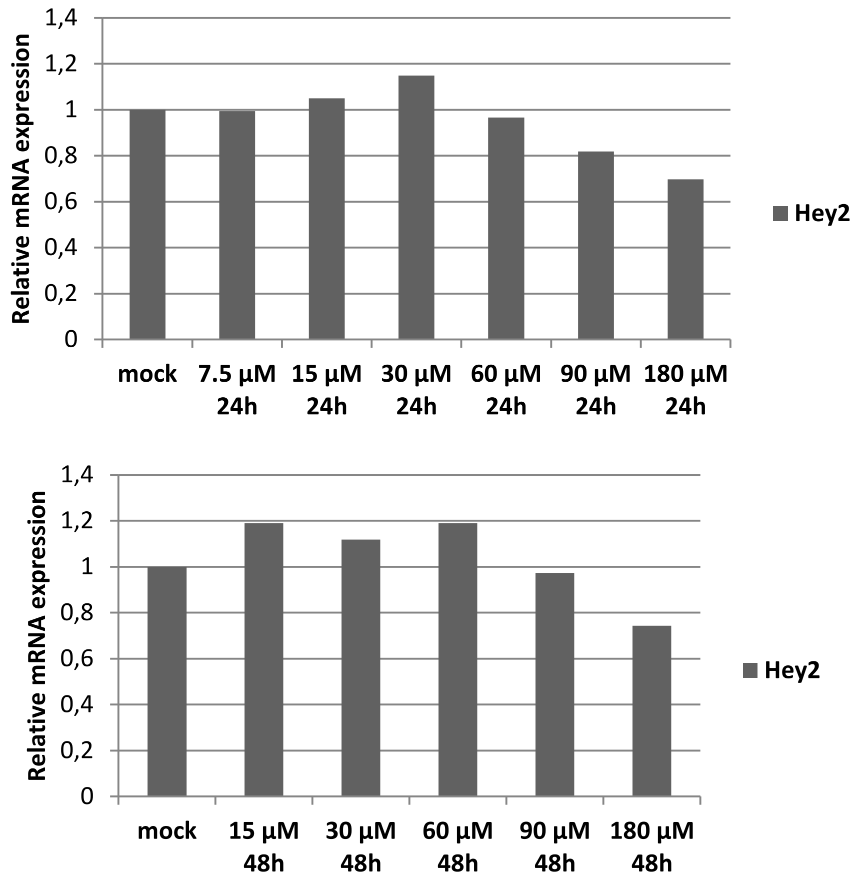


Figure 4.3. Real Time RT-PCR results of optimization of Notch inhibition in MCF7 cells by DAPT. MCF7 cells were treated with DAPT in 7.5 µM, 15 µM, 30 µM, 60 µM, 90 µM and 180 µM for 24 hours and 48 hours. After these specific time points RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch target Hey2 and TBP for normalization. One experiment was done for each condition.

Different DAPT concentrations (7.5 µM, 15 µM, 30 µM, 60 µM, 90 µM and 180 µM) and time points (24 hours and 48 hours) were tried to find which condition can inhibit Notch signaling in MCF7 cells. Only 90 µM and 180 µM concentrations of DAPT treatment for both 24 hours and 48 hours could inhibit Notch signaling but slightly which is not suitable for inhibition experiments (Figure 4.3). Therefore MCF7 cells were not used for Notch inhibition experiments by using DAPT treatment.

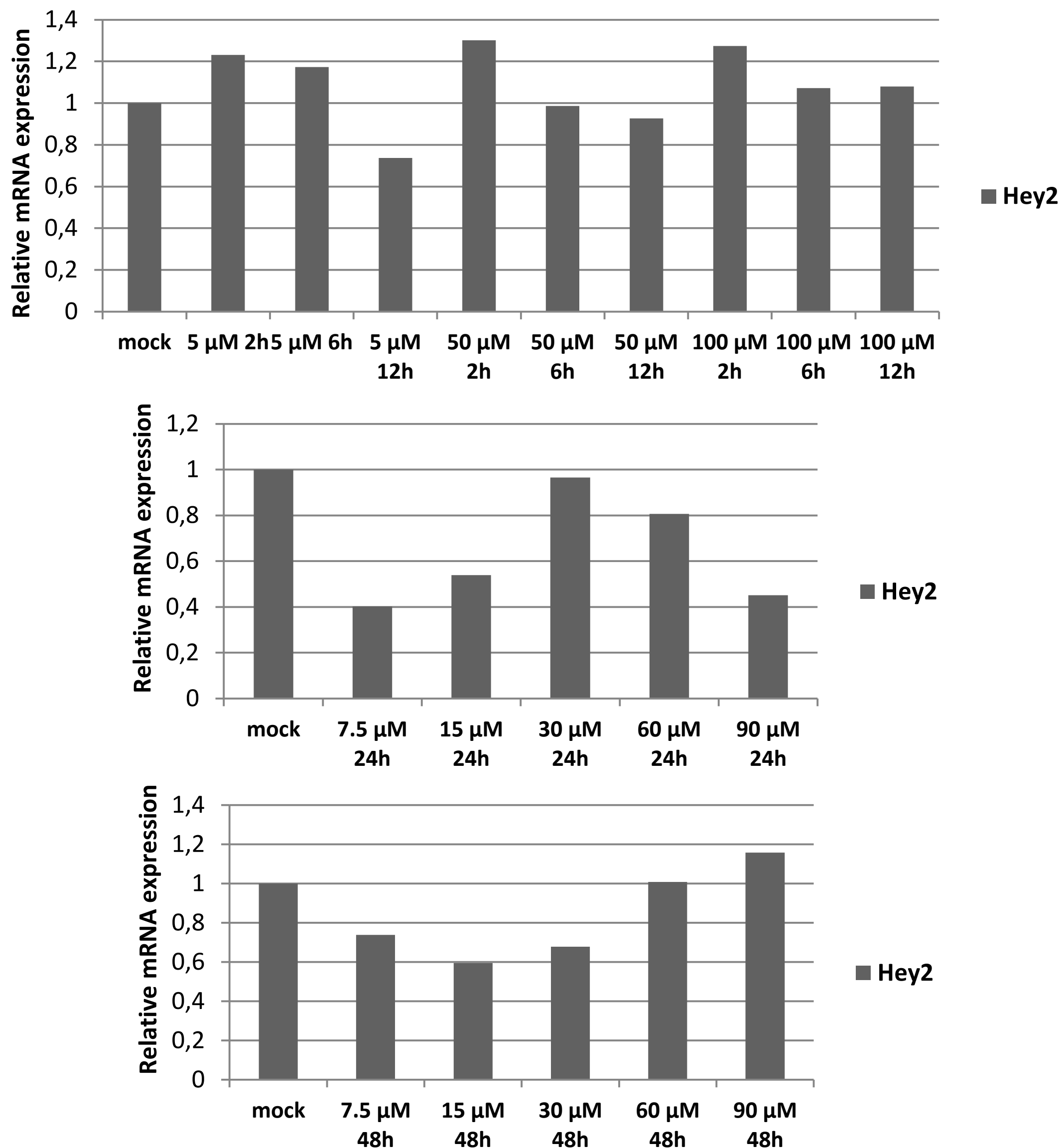


Figure 4.4. Real Time PCR results of optimization of Notch inhibition in MDA-MB-231 cells by DAPT. MDA-MB-231 cells were treated by DAPT in 5 µM, 50 µM and 100 µM for 2 hours, 6 hours and 12 hours; 7.5 µM, 15 µM, 30 µM, 60 µM and 90 µM for 24 hours and 48 hours. After these specific time points RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch target Hey2 and TBP for normalization. One experiment was done for each condition.

Optimization experiments for Notch inhibition in MDA-MB231 cells showed that Notch inhibition was successful by DAPT treatment in 7.5 µM, 15 µM and 90 µM for 24 hours and also 15 µM for 48 hours by generating more than 40% inhibition in MDA-MB-231 cells. Thus 90 µM concentration of DAPT treatment for 24 hours was chosen for Notch inhibition experiments in MDA-MB-231 cells (Figure 4.4).

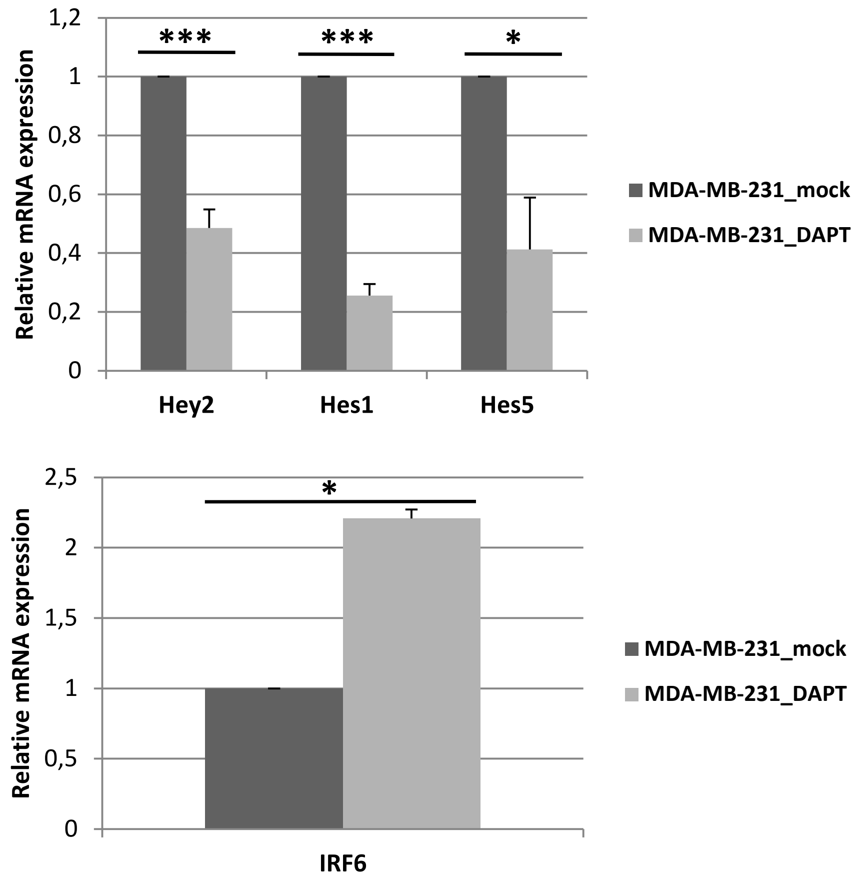


Figure 4.5. Real Time RT-PCR results of Notch inhibition in MDA-MB-231 cells by DAPT. MDA-MB-231 cells were treated by 90 µM DAPT for 24 hours. After DAPT treatment RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch targets Hey2, Hes1, Hes5 and IRF6 and TBP for normalization. Three independent experiments were performed. (* $p<0.05$, *** $p\leq0.001$).

When DAPT treatment was performed in 90 µM concentration for 24 hours in culture medium of MDA-MB-231 cells, mRNA expression levels of Notch targets were decreased. Hey2 was decreased 51%, Hes1 was decreased 74% and Hes5 59% significantly which shows that Notch inhibition was generated. However, mRNA expression of IRF6 was upregulated 2.2 fold significantly under Notch inhibition (Figure 4.5).

On the other hand, Western Blot results showed unexpected results. Protein levels of Hey2 were increased although Hey2 mRNA expression was decreased with increased protein expression of IRF6 under Notch inhibition by DAPT (Figure 4.6).

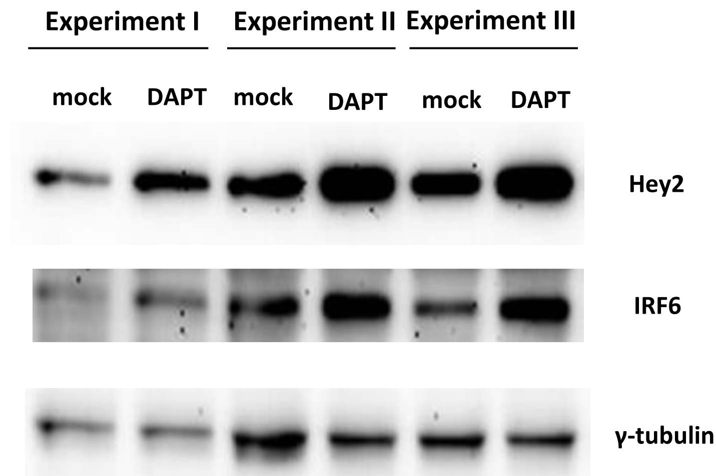


Figure 4.6. Western Blot results of Notch inhibition in MDA-MB-231 cells by DAPT. MDA-MB-231 cells were treated by 90 μM DAPT for 24 hours. After DAPT treatment cells were lysed and protein isolation and immunoblotting were performed by using antibodies against Notch target Hey2, IRF6 and γ-tubulin as loading control. Three independent experiments were performed.

These results showed that 90 μM DAPT treatment of MDA-MB-231 cells for 24 hours could inhibit Notch signaling showed by downregulation of Notch targets. However Hey2 protein expression showed opposite results by increasing after DAPT treatment. In these cells, IRF6 was upregulated in both mRNA and protein expression.

All in all, DAPT treatment experiments showed that Notch inhibition was generated in mRNA levels of Notch targets Hey2, Hes1 and Hes5 but not in protein levels of Hey2 in MDA-MB-231 cells. Furthermore there was no significant Notch inhibition in MCF7 cells. Therefore, other two methods such as knock down of CSL and dominant negative regulation of MAML-1 transcription factors were used for Notch inhibition and IRF6 mRNA and protein levels were determined in MDA-MB-231 cells by Real Time RT-PCR and Western Blot.

4.2.2. Notch Inhibition by Knock Down of CSL Transcription Factor

CSL is one of the main transcription factors of canonical Notch transcription complex because after activation and translocation into nucleus, NICD binds to CSL and then recruits other transcription factors. Therefore CSL is necessary for transcription of Notch targets and if CSL protein is silenced, Notch transcription complex can not be built and transcription is blocked.

In order to inhibit Notch signaling pathway in MDA-MB-231 cells, lenti-viruses including shRNA sequence against CSL mRNA (shCSL) or shRNA sequence against GFP mRNA (control) as unspecific control because there is no GFP expression in that cells normally, were used. At first, we determined which shRNA sequence inhibited Notch signaling efficiently by using shCSL#4 or shCSL#5 with shGFP lenti-viruses. After infection, mRNA expression levels of Notch targets Hey1, Hey2, Hes1 and Hes5 were determined by Real Time RT-PCR. Expressions of Notch targets Hey2 and Hes5 were increased while Hes1 was decreased slightly in cells infected with shCSL#4 lenti-viruses. However shCSL#5 lenti-viruses decreased mRNA expressions of Notch targets Hey1, Hey2 and Hes1 which showed high Notch inhibition in MDA-MB-231 cells (Figure 4.7). Therefore shCSL#5 lenti-viruses were used for Notch inhibition experiments in MDA-MB-231 cells.

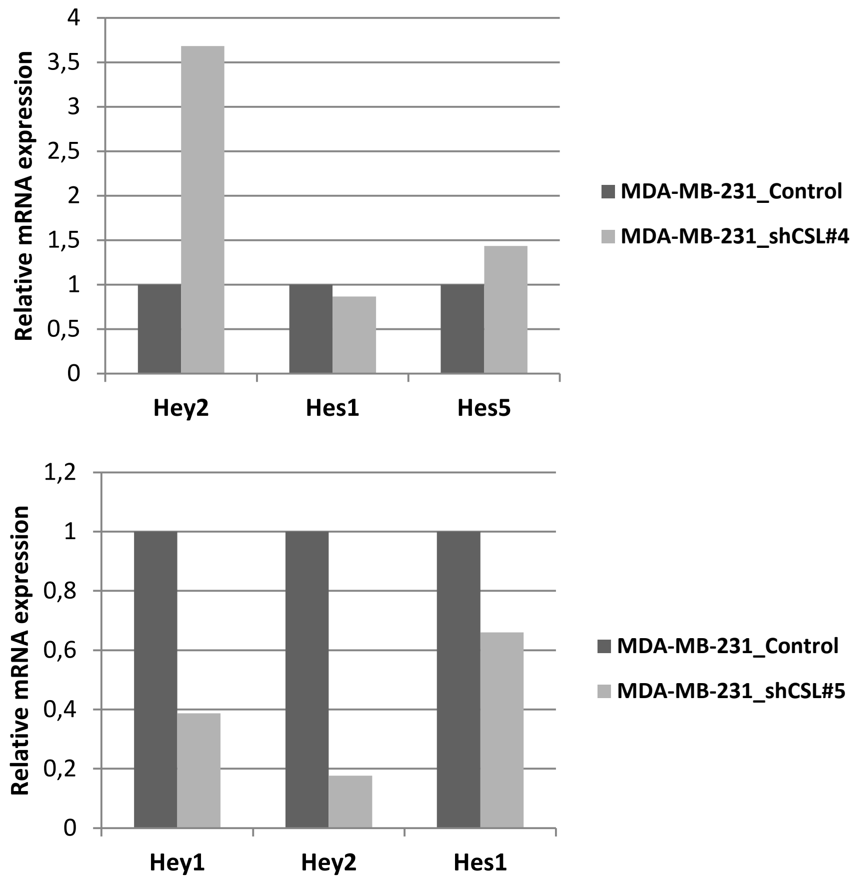


Figure 4.7. Real Time RT-PCR results of optimization of Notch inhibition in MDA-MB-231 cells by shCSL viruses. MDA-MB-231 cells were infected with lenti-viruses containing shRNA sequence numbered 4 or 5 against CSL mRNA sequence. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch targets Hey1, Hey2, Hes1 and Hes5 and TBP for normalization. One experiment was done for each construct.

Notch was inhibited in MDA-MB-231 cells by using shCSL#5 lenti-viruses with shGFP control lenti-viruses. Then mRNA and protein expression levels of direct Notch targets Hey1 and Hes1 to show Notch inhibition and IRF6 were determined. Real Time RT-PCR results showed that Hey1 mRNA expression was decreased 62% significantly, Hey2 mRNA expression was decreased 80% significantly and Hes1 mRNA expression was decreased 22% that shows Notch was inhibited successfully. And IRF6 mRNA expression was decreased 55% significantly after inhibition of Notch signaling (Figure 4.8).

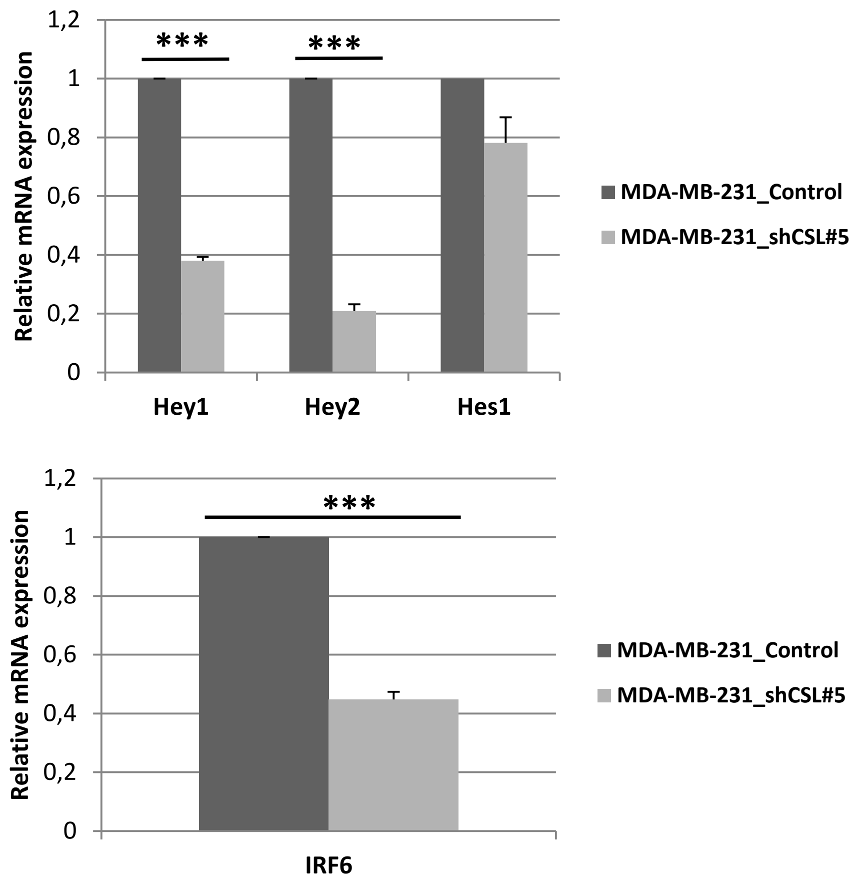
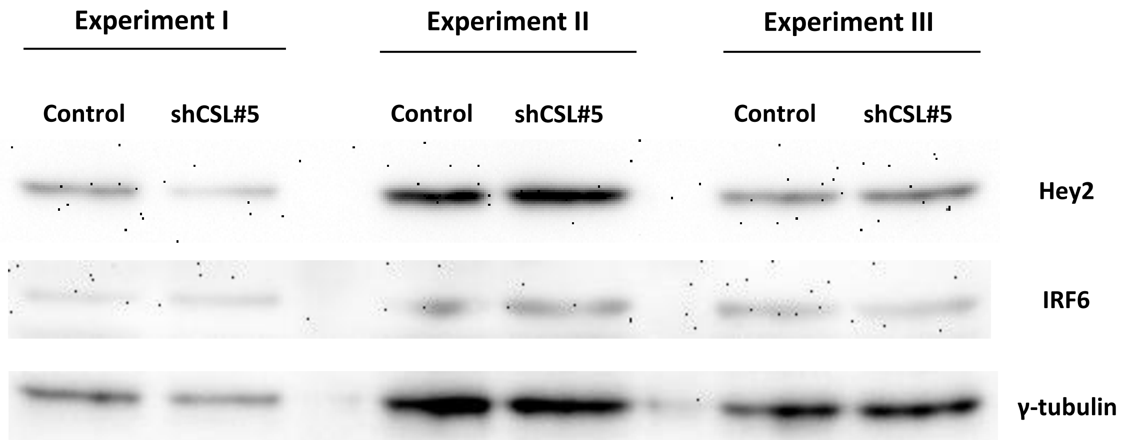


Figure 4.8. Real Time PCR results of Notch inhibition in MDA-MB-231 cells by knock down of CSL protein. MDA-MB-231 cells were infected with lenti-viruses containing shRNA sequence 5 against CSL mRNA sequence. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch targets Hey1, Hes1; IRF6 and TBP for normalization. Three independent experiments were performed. (*** $p \leq 0.001$)

Western Blot was performed for protein samples from MDA-MB-231 cells infected with lenti-viruses containing shRNA sequence against CSL mRNA to block Notch dependent transcription complex and inhibit Notch signaling. Protein levels of Notch target Hey2 protein, IRF6 and γ-tubulin were checked. It was determined that protein levels of both Notch target Hey2 and IRF6 were not decreased in Notch inhibited cells according to control cells clearly. And the intensity results showed that there is no reduction of Hey2 with induction of IRF6 showing high amount of standard deviation and these results were not significant (Figure 4.9).



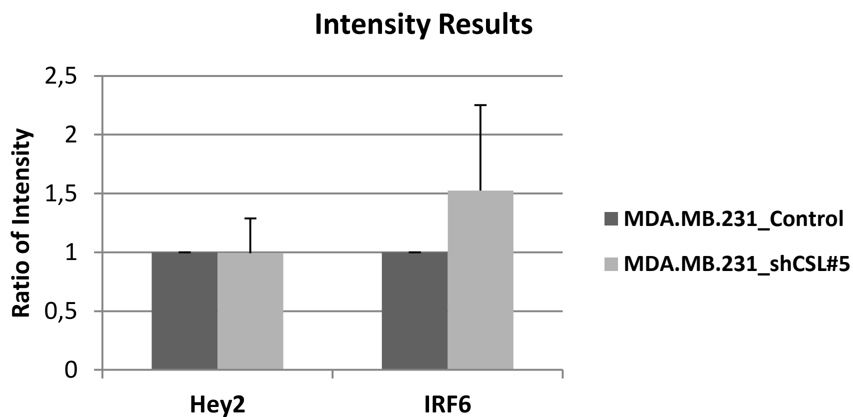


Figure 4.9. Western Blot results of Notch inhibition in MDA-MB-231 cells by knock down of CSL protein. MDA-MB-231 cells were infected with lenti-viruses containing shRNA sequence against CSL mRNA sequence. At third day of infection cells were lysed and protein extraction and western blot analysis were performed by using antibodies against Notch target Hey2 protein, IRF6 and γ-tubulin as loading control. Three independent experiments were performed.

Notch inhibition by knock down of CSL protein expression cause downregulation of IRF6 in mRNA levels significantly which could not be shown clearly in protein level. Thus, IRF6 is regulated transcriptionally by Notch signaling pathway through CSL dependent transcription complex.

4.2.3. Notch Inhibition by Dominant Negative Regulation of Mastermindlike-1

As confirmation of CSL knock down experiments, another method was performed to inhibit Notch signaling pathway by expression of Dominant Negative Mastermindlike-1 (DNMM) which is truncated mutant form of MAML-1 which is another transcription factor which is necessary for building of transcription complex with NICD and CSL. Deletion mutant form of MAML-1 protein (DNMM) lacks essential domains responsible for binding to DNA and other transcription factors and competes against MAML-1 to bind NICD. Thus binding of DNMM to NICD avoids building of NICD directed transcription complex that shows similar phenotype with loss of Notch function (Helms et al. 1999).

MDA-MB-231 cells were infected with retro-viruses including DNMM sequence or retroviruses consisting of empty vector as control. Then, Real Time RT-PCR and Western Blot were performed to detect mRNA and protein levels of Notch targets and IRF6. It was determined that mRNA expression of Notch target Hey1 was decreased 52% significantly, Hey2 was decreased 38% and Hes1 45% fold which shows Notch inhibition. And IRF6 mRNA expression was downregulated as 35% significantly under Notch inhibition compared to control cells (Figure 4.10).

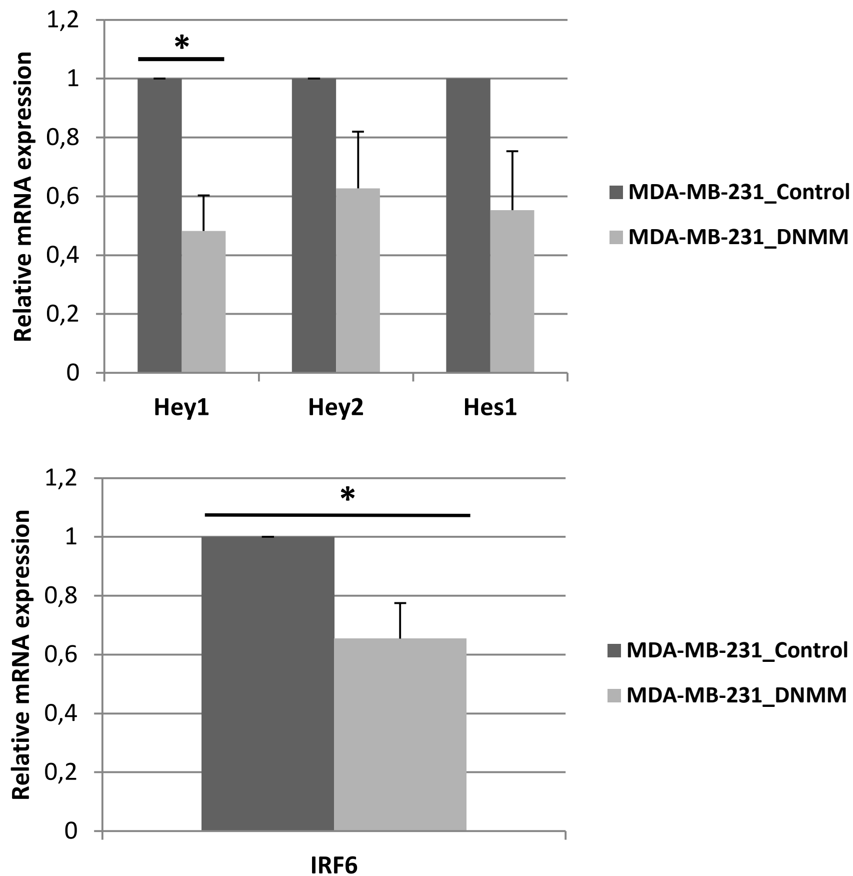
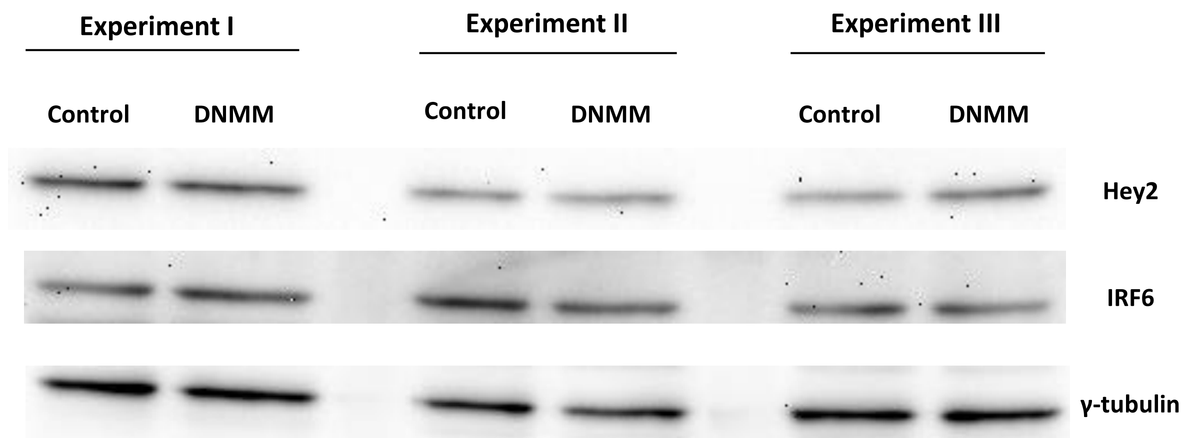


Figure 4.10. Real Time PCR results of Notch inhibition in MDA-MB-231 cells by DNMM. MDA-MB-231 cells were infected with retro-viruses containing DNMM sequence. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Notch targets Hey1, Hey2; IRF6 and TBP for normalization. Three independent experiments were performed. (* $p<0.05$)

Protein levels of Notch target Hey2, IRF6 and γ-tubulin were detected by Western Blot in Notch inhibited MDA-MB-231 cells by expression of DNMM. In Notch inhibited cells, protein levels of Notch target Hey2 and IRF6 were not decreased clearly compared to control cells. And intensity results showed no significant difference of Hey2 and IRF6 protein levels between control and Notch inhibition samples (Figure 4.11).



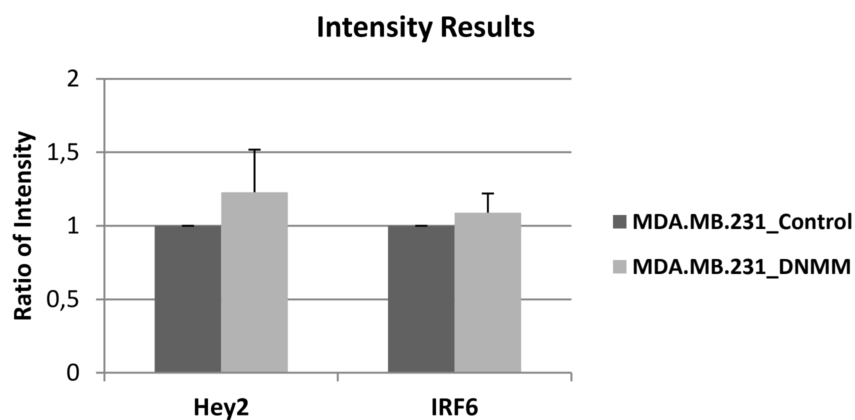


Figure 4.11. Western Blot results of Notch inhibition in MDA-MB-231 cells by DNMM. MDA-MB-231 cells were infected with retro-viruses containing DNMM sequence. At third day of infection cells were lysed and protein extraction and western blot analysis were performed by using antibodies against Notch target Hey2 protein, IRF6 and γ-tubulin as loading control. Three independent experiments were performed.

Notch inhibition by expression of DNMM in Notch positive MDA-MB-231 cells cause downregulation of mRNA expression of Notch targets such as Hey1, Hey2 and Hes1 with downregulation of IRF6 while no significant difference in protein levels of Hey2 and IRF6.

All in all, the upregulation of IRF6 under active Notch1 signaling and downregulation of IRF6 mRNA levels when canonical Notch signaling is inhibited by either knock down of CSL or dominant negative regulation of MAML-1 shows that IRF6 is regulated transcriptionally by canonical Notch signaling pathway in human breast epithelial cells.

4.3. IRF6 has no Effect on Cell Proliferation under Notch1 Signaling

As IRF6 is regulated by Notch signaling pathway, IRF6 becomes powerful candidate to be Notch signaling mediator in mammary tissue. Thus in order to investigate whether IRF6 is a mediator under Notch signaling in proliferation mechanism, growth curve assay was performed in human breast epithelial cells.

In order to identify the role of IRF6 under Notch signaling pathway, IRF6 was knocked down in Notch activated or normal MCF10A cells as control. IRF6 silencing was optimized in IRF6 expressing MCF10A cells independent of Notch activation. Thus, four different shRNA sequences against IRF6 (shIRF6) were checked with viral infection to find which one knocks down IRF6 better by determining mRNA expression levels of IRF6 in MCF10A cells. And the mRNA expression of IRF6 was downregulated by shIRF6#4 as 67%, shIRF6#6 as 43% and shIRF6#7 as 66% (Figure 4.12). Therefore shIRF6#7 was chosen to silence IRF6 expression in Notch activated or normal MCF10A cells for both growth curve assay for cell proliferation and soft agar colony formation assay for cell transformation.

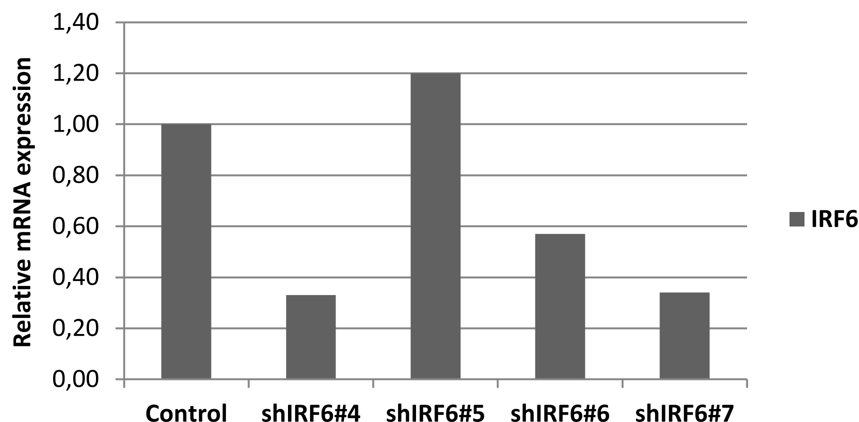


Figure 4.12. Real Time PCR results of optimization of IRF6 silencing in MCF10A cells. MCF10A cells were infected with lenti-viruses containing different shIRF6 sequences. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and Real Time PCR were performed by using specific qPCR primers against IRF6 and TBP for normalization. One experiment was done for each construct.

In order to find the role of IRF6 under Notch signaling during the proliferation and transformation mechanisms, IRF6 was knocked down in Notch activated or normal MCF10A cells. Therefore, four different conditions with proper controls were generated as following:

1. Notch -, IRF6+ : MSCV (control virus) + shGFP (control virus)
2. Notch -, IRF6- : MSCV (control virus) + shIRF6#7 (IRF6 knock down virus)
3. Notch +, IRF6+ : MSCV-N1-ICD (Notch activation virus) + shGFP (control virus)
4. Notch +, IRF6 - : MSCV-N1-ICD (Notch activation virus) + shIRF6#7 (IRF6 knock down virus)

After transduction of MCF10A cells by control or Notch activation virus with control or shIRF6 virus, four different conditions were performed and controlled by determining mRNA expressions of Notch targets Hey2, Hes1 and IRF6. While mRNA expressions of Notch targets Hey2 and Hes1 were upregulated in both NICD/control and NICD/shIRF6 samples, IRF6 was knocked down at cells including shIRF6 virus and upregulated in Notch activated cells significantly (Figure 4.13).

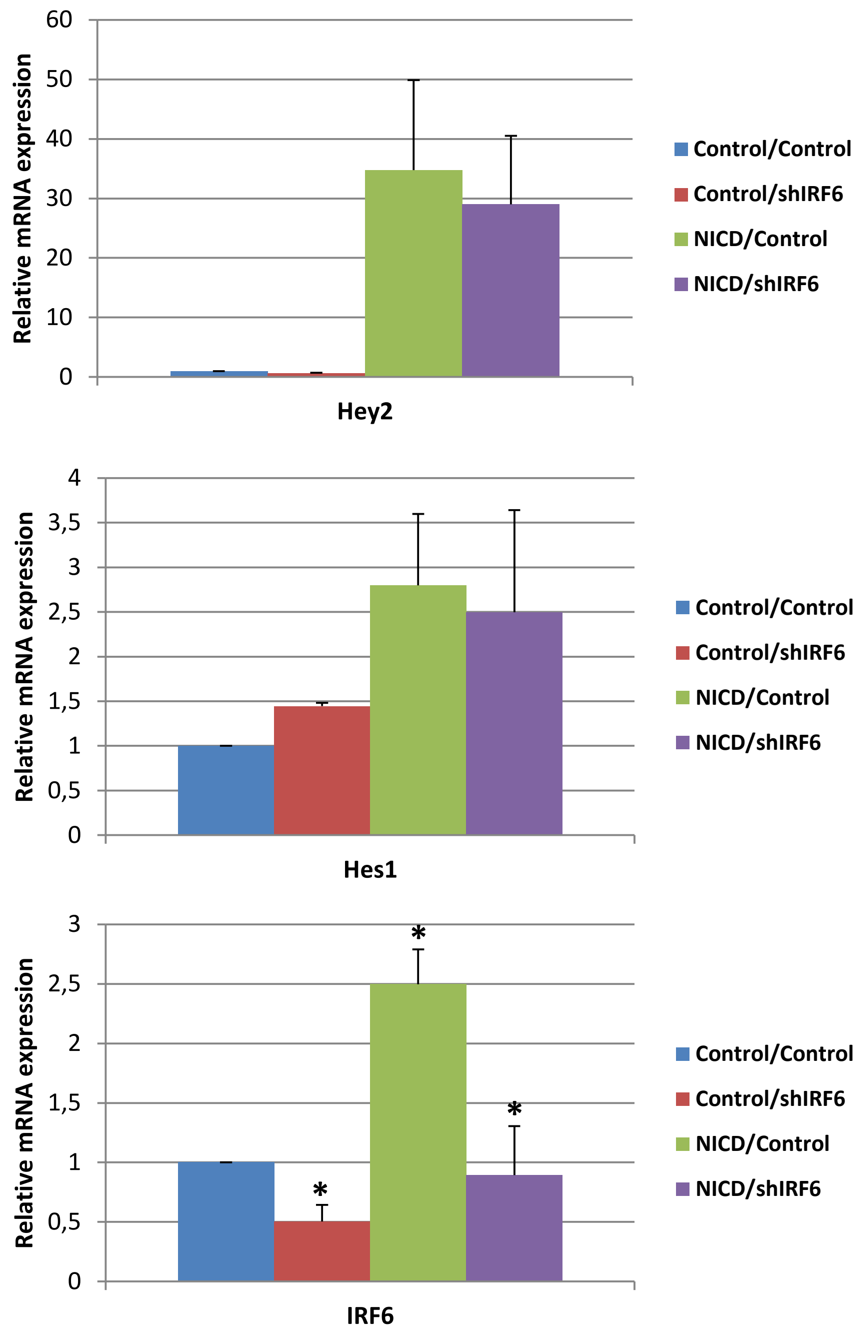


Figure 4.13. Real Time PCR results of Notch1 activation and IRF6 knock down in MCF10A cells. MCF10A cells were infected with control or N1-ICD viruses with control or shIRF6 viruses. At third day of infection cells were lysed and RNA isolation, cDNA synthesis and Real Time RT-PCR were performed by using specific primers against Hes1, IRF6 and TBP for normalization. Three independent experiments were performed. Hey2 results were calculated from two experiments.

The proliferation effects of these four different conditions were determined by growth curve assay. MCF10A cells were transduced by control or Notch activation virus with control or shIRF6 virus in 6 well plates and after 72h they were split in equal number into 12 well plates as triplicates for each condition and cultured without selection (day 0). Cultured cells were counted for five days (day 3-7) after infection and a growth curve was drawn depending on the counted numbers of infected cells. The results of growth curve showed that Notch activated cells did not show significant difference in proliferation according to control cells. There was a tendency to increased cell proliferation in IRF6 knocked down cells and Notch activated with IRF6 silenced cells compared control cells but they were not significant (Figure 4.14).

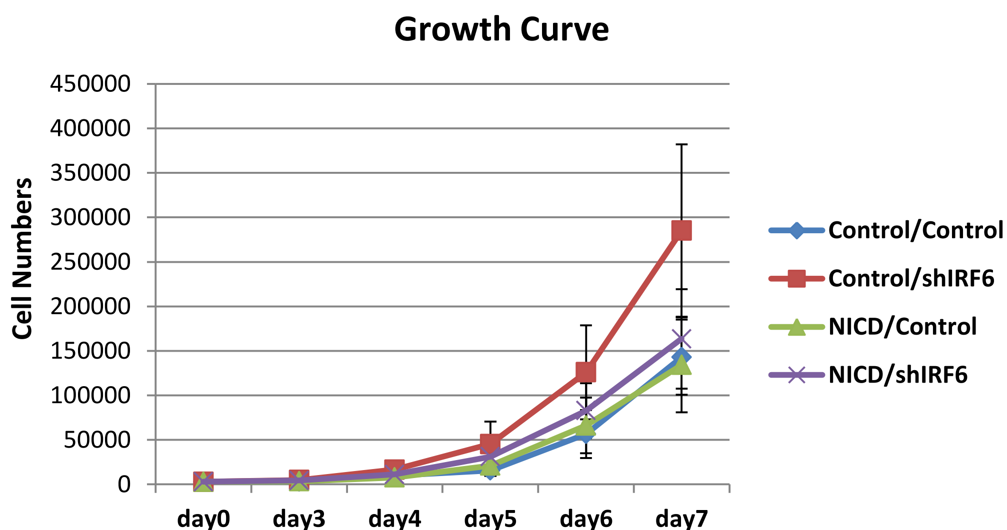


Figure 4.14. Growth curve assay of four different conditions of MCF10A cells. Transduced MCF10A cells were split into 12 well plates in equal amounts at third day after infection (day 0) and counted for five days during one week (day 3-7) to generate growth curve. Three independent experiments were performed.

All in all, IRF6 does not affect outcome of Notch1 signaling in MCF10A cells for regulation of cell proliferation because there is no significant difference between NICD/Ctrl and NICD/shIRF6 samples. And also there is no effect of Notch1 activation on cell proliferation of MCF10A cells. Thus the effect of IRF6 under Notch activation could not be determined for cell proliferation mechanism.

4.4. IRF6 is Required for Notch1 Induced Cell Transformation

In order to determine whether IRF6 has an effect on cell transformation under Notch signaling, Soft Agar Colony Formation Assay was performed. In this assay cells were cultured in semi-solid soft agar-medium mixture to create 3D cell culture suitable for environment for anchorage independent cell growth. This type of growth is one of the hallmarks of cell transformation because neoplastic transformation causes cell growth independently of cell-matrix attachment while normal cells must attach to solid surface for cell growth and cell division. Thus, soft agar colony formation assay is one of the best in vitro models to be predictor of in vivo transformation.

In this assay, cells were cultured in semi-solid layer of 0.35% noble agar and medium mixture on top of bottom layer of 0.5% noble agar and medium mixture in 6 well plates. Transduced MCF10A cells were split and cultured in soft agar wells in equal number (30 000 cells per well) three days after infection. After cells were cultured in soft agar, medium on top of agar was changed twice a week for 8 weeks. Then cell colonies were dyed by 0.005% crystal violet solution and colony numbers with colony sizes were determined.

Soft agar colony formation assay results showed that Notch activated cells formed much more and bigger colonies compared to normal cells and IRF6 knock down in Notch activated cells showed decreased colony number (Figure 4.15). Colony numbers were increased in Notch activated cells significantly and decreased with knock down of IRF6 in Notch activated cells significantly. However, there was no significant difference in IRF6 silenced cells without Notch activation compared to normal cells. On the other hand, Notch1 activated cells formed bigger colonies whether IRF6 was knocked down or not (Figure 4.16). Furthermore, IRF6 knock down without Notch activation did not show any effect on both colony number and colony size in soft agar, although this condition showed tendency to induce cell proliferation in growth curve.

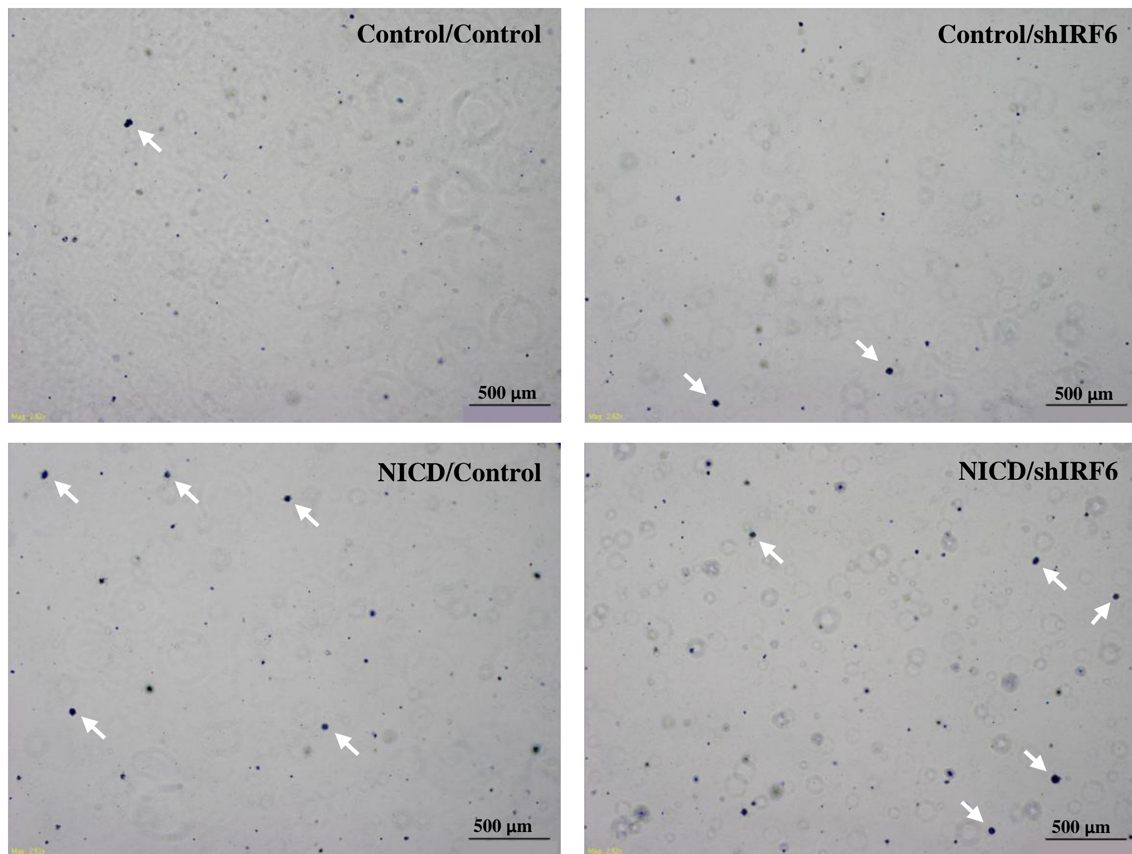


Figure 4.15. Soft Agar Colony Formation Assay representative images. Transduced MCF10A cells were split and cultured in noble agar and medium mixture. Arrows show representative colonies.

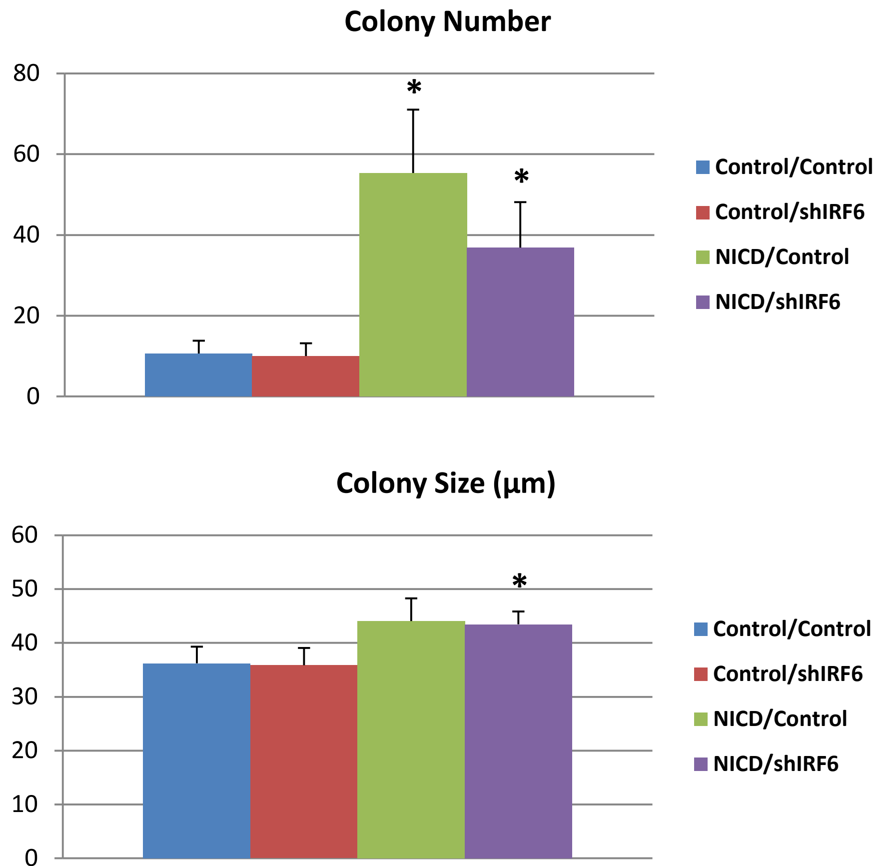


Figure 4.16. Soft Agar Colony Formation Assay results. Colony numbers and sizes were calculated for three independent experiments. (* $p<0.05$).

All in all, soft agar colony formation experiments show that Notch1 activation increases colony number and knock down of IRF6 reduces this effect. And Notch1 activation increases colony size although not significant and this outcome is not affected by IRF6 knock down. Thus, IRF6 has a mediator role under Notch signaling in cell transformation.

CHAPTER 5

DISCUSSION

After first evidence that constitutive expression of active variant of Notch1 causes human T-ALL (Reynolds et al. 1987), Notch disregulation has been found in many cancer types. Notch shows mostly oncogenic roles however Notch has tumor suppressor roles in skin. In breast tissue, it has been firstly shown that MMTV insertion in Notch4 locus which results in upregulation of Notch4-ICD resulted in mammary adenocarcinomas (Robbins et al. 1992; Uyttendaele et al. 1996). Many other evidences have been found and designate that Notch signaling has oncogenic properties. However, recently it has been found that Notch activation can repress cell proliferation in human breast epithelial cells (Yalcin-Ozuysal Ö et al. 2010; Mazzone et al. 2010). It has not been identified clearly how Notch signaling mediates oncogenic or tumor suppressor roles. Notch can regulate expression of some genes such as cyclinD1, c-Myc, CDK2, Cdkn1a, p21 transcription factors; NF-κB family and PPAR family members; Nrarp (Notch regulated ankyrin repeat protein), Deltex1, pre T cell receptor-α and a ubiquitin ligase SKIP2 (Hansson et al. 2004). However, these genes are not targets of Notch in all tissue types and some are oncogene such as Myc and CyclinD1 or others are tumor suppressors such as p21. Furhermore, Notch signaling shows oncogenic or tumor supressor roles depending on the tissue type and its expression dosage. The broad repertoire of Notch mediators and different outcomes of Notch signaling suggest that different mediators are expressed under Notch signaling in different conditions of cell and depending on which mediators are expressed the outcome of Notch can be different. Thus, dual behaviour of Notch can be regulated by different set of mediators and relationships with other signaling pathways through these mediators. In order to enlight the complex Notch signaling network, new mediators and relationships between each other and with other cellular pathways should be identified.

Recently it has been shown that Notch directly regulates the transcription of IRF6 in keratinocytes (Restivo et al. 2011). Therefore, IRF6 is a strong candidate to be Notch mediator also in breast tissue and it was aimed to find whether IRF6 expression is regulated by and a mediator of Notch signaling in proliferation and transformation mechanisms in breast epithelial cells.

Immortalized normal human breast epithelial cells (MCF10A) were infected with a recombinant retro-virus expressing constitutive active form of Notch1 (N1-ICD) versus control virus and expression levels of direct Notch targets and IRF6 were determined. When Notch1 is activated showed by activation of Notch targets Hey1, Hey2 and Hes1, IRF6 was upregulated which is consistent with previous observations (Restivo et al. 2011). This regulation is also supported by inhibiton of active Notch signaling in human breast adenocarcinoma cell line MDA-MB-231 by knock down of CSL and dominant negative regulation of MAML-1 by DNMM (Dominant Negative MAML-1). When CSL transcription factor which is essential for canonical transcription events of Notch Intracellular domain (NICD) is knocked down by shRNA including lenti-viruses, expressions of Notch target genes Hey1, Hey2 and Hes1 were downregulated with IRF6 expression. In addition, dominant negative reagulation of MAML-1 by expressing DNMM which blocks NICD leading transcription complex including CSL, decreases the expression levels of direct Notch targets Hey1, Hey2 and Hes1 and IRF6. However, treatment of MDA-MB-231 cells with DAPT which is general Notch signaling inhibitor, showed confusing results. Although DAPT treatment could reduce mRNA expressions of Notch targets Hey2, Hes1 and Hes5, IRF6 expression was induced. And Western Blot results showed higher expression of Hey2 protein opposite to mRNA levels of Hey2 while IRF6 protein expression was increased consistent with upregulation of IRF6 mRNA levels. This situation can be attributed to Notch independent events. Because DAPT plays role in cleavage of other proteins such as Src, ErbB4, CD44, LDL-receptor related protein and E-cadherin (Lammich et al. 2002) that may generate Notch independent regulation of Hey2 or IRF6. As γ-secretase inhibitors are used in clinical trials but their molecular and pharmacologic properties on cells should be investigated well. All in all, these results show that expression of IRF6 is regulated by canonical Notch signaling through CSL and MAML-1 transcription factors.

As Notch signaling pathway has both oncogenic and tumor suppressor roles in skin and breast tissue, and IRF6 expression is regulated by Notch, IRF6 has possibility to be a critical mediator directing oncogenic or tumor suppressor roles. Therefore, the effect of IRF6 under Notch signaling was studied for proliferation and transformation mechanisms of breast epithelial cells. In order to determine role of IRF6 under Notch, it was determined how absence or presence of IRF6 in Notch activated cells affects the proliferation and transformation by generating four different conditions with proper controls. These four different conditions were generated by double infection by using control or Notch activation virus with control or shIRF6 virus. Thus, as MCF10A cells show Notch-, IRF6+ phenotype, the conditions of these infections were Notch-,IRF6+; Notch-,IRF6-; Notch+IRF6+ and Notch+,IRF6-. Results showed that Notch1 activation did not affect cell proliferation whether in presence or absence of IRF6 expression in control cells. IRF6 knock down generated a tendency to increase the cell growth not significantly which correlates with tumor suppressor role of IRF6. Because Notch has been shown to induce cell proliferation and transformation in MCF10A cells (Mazzone et al. 2010; Stylianou et al. 2006; Rustighi et al. 2009), induced cell proliferation was expected in Notch activated cells. However, Notch activation did not result any significant induction in cell proliferation and the role of IRF6 under Notch signaling could not be determined. Notch activation increased the number of floating cells because Notch activation induces cell detachment triggered by a reduction of Integrin β1, Integrin β4, Laminins and E-Cadherin which play role in cell adhesion (Mazzone et al. 2010; Stylianou et al. 2006). And there were less attached and proliferating cells in Notch activated wells after splitting and re-culture of infected cells which resulted opposite results in growth curve. Therefore, alternative cell proliferation assays should be tried to see Notch1 activation effect in cell proliferation and role of IRF6 under Notch1 such as BrdU assay or determining levels of proliferation markers such as PCNA, Ki67 and CyclinD1. Because, in these methods splitting and re-culturing of transduced cells are not needed and so at third day after infection the proliferation phenotype of cells can be determined without observing floating cells.

Cell transformation situation of four different conditions of transduced MCF10A cells was determined by using soft agar colony formation assay which supply semi-solid agar-medium mixture so anchorage independent environment for single cells. If a cell is transformed and has stem-like and cancer phenotype, it can survive, proliferates and

forms colony. Soft agar colony formation assay results showed that Notch1 activated cells formed much more colonies with higher colony size compared to normal cells, which shows Notch induced transformation. This outcome is consistent with previous observations that Notch activation causes cell transformation in MCF10A cells (Stylianou et al. 2006; Mazzone et al. 2010). And IRF6 knock down in Notch activated cells showed decreased colony number significantly without affecting colony size which is inconsistent with tumor suppressor role of IRF6 and unexpected for soft agar assay (Bailey et al. 2008; Bailey et al. 2009). Furthermore, there was no significant difference in IRF6 silenced cells without Notch activation compared to normal cells which is also inconsistent with tumor suppressor role of IRF6 and more colonies were expected for these cells (Bailey et al. 2008; Bailey et al. 2009). Thus, IRF6 has a mediator role under Notch signaling for Notch induced transformation.

If we consider that Notch pathway shows tumor supressor roles in keratinocytes (Rangarajan et al. 2001; Nicolas et al. 2003; Proweller et al. 2006) and IRF6 also shows tumor supressor roles (Bailey et al. 2008; Botti et al. 2011; Restivo et al. 2011), it can be hypothesized that IRF6 mediates the outcome of Notch in skin. In breast tissue, IRF6 also shows tumor suppressor roles (Bailey et al. 2008) and therefore it was hypothesized that IRF6 directs oncogenic or tumor suppressor roles of Notch signaling (Mazzone et al. 2010; Yalcin-Ozuysal Ö et al. 2010). In our research IRF6 showed oncogenic role under oncogenic Notch1 signaling in cell transformation which conflicts with tumor supressor role of IRF6. And the possible relationship between Notch, IRF6 and other Notch mediators on cell growth and differentiation should be identified to clarify Notch pathway in breast tissue and cancer. And also it will be important to determine why IRF6 is required for Notch signaling.

On the other hand, there are four different Notch receptors and their roles can differ which confuses the issue. Notch1 activation was studied while Notch inhibition was performed unspecifically for all Notch receptors through knock down of CSL or dominant negative regulation of MAML-1 in this project. Therefore, IRF6 relationship with other Notch receptors should also be identified to draw clear pathway of Notch signaling in breast cancer.

CHAPTER 6

CONCLUSION

After it has been shown that MMTV insertion in Notch4 locus which results in upregulation of Notch4-ICD and causes breast adenocarcinomas, many other evidences have been found designated that Notch signaling has oncogenic properties. However, recently it has been found that Notch activation can decrease cell proliferation in human breast epithelial cells. It has not been identified clearly how Notch signaling mediates oncogenic or tumor suppressor roles. Thus, dual behavior of Notch can be regulated by different set of mediators under Notch signaling. In order to clarify the complex Notch signaling network, new mediators and their connections should be identified. Both Notch and IRF6 play role in development and differentiation of keratinocytes. Recently it has been shown that Notch directly regulates the transcription of IRF6 in keratinocytes (Restivo et al. 2011). Thus IRF6 is one of the strong candidates to be Notch mediator also in breast tissue. It was aimed to determine whether IRF6 expression is regulated by and is a mediator of Notch signaling in proliferation and transformation mechanisms in breast epithelial cells.

Notch signaling was activated in human normal breast epithelial cells (MCF10A) and expression levels of direct Notch targets and IRF6 were determined. When Notch1 was activated, mRNA and protein expressions of both Notch targets Hey1, Hey2 and Hes1 which shows activation of Notch signaling and IRF6 were upregulated. This regulation was also supported by inhibiton of active Notch signaling in human breast adenocarcinoma cell line MDA-MB-231 by knock down of CSL and expressing DNMM. When CSL transcription factor which is essential for canonical transcription events of Notch Intracellular domain (NICD) was silenced by shRNA including lenti-viruses, expressions of Notch target genes Hey1, Hey2 and Hes1 were downregulated with IRF6 expression. In addition, dominant negative reagulation of MAML-1 by expressing DNMM which blocks NICD directed transcription complex, decreased the expression levels of direct Notch targets Hey1, Hey2 and Hes1 with IRF6. However treatment of MDA-MB-231 cells by DAPT which is general Nocth signaling inhibitor through inhibition of γ-secretase, showed confusing results. Although DAPT

treatment could reduce mRNA expressions of Notch targets Hey2, Hes1 and Hes5 as expected and reduce mRNA expressions of IRF6. Protein results showed higher expression of Hey2 protein conversely with mRNA levels of it while IRF6 protein expression was consistent with upregulation of IRF6 mRNA levels. All in all, these results show that expression of IRF6 is regulated by canonical Notch signaling through CSL and MAML-1 transcription factors.

Furthermore, in order to check the mediator role of IRF6, the effect of IRF6 under Notch signaling was studied for proliferation and transformation outcomes of breast epithelial cells. Results showed that Notch1 activation did not affect cell proliferation independent of IRF6 expression. IRF6 knock down also did not change proliferation significantly although there is a tendency to increase cell proliferation. In soft agar colony formation assay, there was significant increase in colony numbers with increased colony size in Notch1 activated cells which shows Notch induced cell transformation and IRF6 knock down decreased the colony number in Nocth1 activated cells. Thus, IRF6 is required for Notch induced cell transformation and has a mediator role under oncogenic Notch signaling.

All in all, IRF6 expression is regulated by canonical Notch signaling in breast epithelial cells. IRF6 is required for Notch induced cell transformation and shows mediator role under Notch1 signaling in cell transformation.

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