ROLE OF IRF6 IN NOTCH REGULATED APOPTOSIS, CELL CYCLE AND DIFFERENTIATION IN BREAST EPITHELIAL CELLS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

In Molecular Biology and Genetics

by
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December 2015
İZMİR

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ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and thanks to my supervisor Assist. Prof. Dr. Özden YALÇIN ÖZUYSAL for his patience, encouragement, understanding, guidance, support and helps during my graduate studies. I would like to thank to Cansu KÜÇÜKKÖSE and Burcu FIRATLIGİL for their laboratory help and opinions about my work. I also want to thank to Hande AYPEK, Melis OLCUM UZAN, Öznur BASKAN and Özge KARADAŞ for their social environment and geniality.

Furthermore, I would like to thank to Assist. Prof. Dr. Gülistan MEŞE ÖZÇİVİCİ and Assoc. Prof. Dr. Engin ÖZÇİVİCİ for their helps. I would like to also thank to Prof. Dr. Ahmet KOÇ and Assoc. Prof. Dr. Çağlar KARAKAYA for let me to use their laboratory resources and Scientific and Technological Research Council of Turkey (TÜBİTAK) for financial support (project number 110T895).

I would like to thank to Özgür OKUR and other staff and facility of Izmir Institute of Technology, Biotechnology and Bioengineering Research and Application Center for their facility support and great helps.

Lastly,I would like to thank my thesis committee members Assist. Prof. Özden YALÇIN ÖZUYSAL, Assist. Prof. Dr. Gülistan MEŞE ÖZÇİVİCİ and Assoc. Prof. Dr. Hatice Güneş ÖZHAN for their help guidance.

I am also grateful to my family for their support all over my life and moral during my education. I would like to thank all my friends for their help, support and social environment.

ABSTRACT

ROLE OF IRF6 IN NOTCH REGULATED APOPTOSIS, CELL CYCLE AND DIFFERENTIATION IN BREAST EPITHELIAL CELLS

Notch pathway, an evolutionarily conserved signaling, controls development, differentiation and proliferation. Notch1 and Notch4 activation caused mammary tumor formation showing an oncogenic effect. Overexpression of Notch1 can also supress proliferation in breast epithelial cells depending on dosage and cell type. Thus, Notch can act as an oncogene or tumor suppressor in breast.

IRF6, a member of interferon regulatory factor family, has a role in development and differentiation of the epidermis, downstream of Notch signaling. IRF6 overexpression induces cell cycle arrest in breast cancer cells showing a tumor suppressor role. It was recently identified that IRF6 is a mediator of Notch in proliferation and transformation of breast epithelial cells. In this study, it was aimed to identify whether IRF6 has any effect on cell cycle regulation, apoptosis and breast cancer stem cell population (BCSCs) under Notch and whether IRF6 has a role in expression of luminal and basal markers in breast cell lines.

Our results showed that IRF6 knockdown in normal breast epithelial cell line, MCF10A, reduced percentage of cells in S-phase, which was increased by Notch activation. IRF6 knockdown induced early apoptosis and reduced BCSCs, however it has no effect downstream of Notch in these processes. On the other hand, IRF6 did not play an essential role on expression of luminal and basal markers.

In conclusion, our previous observation was supported that IRF6 is a mediator of Notch in cell proliferation. Furthermore, these data showed that IRF6 has a novel role on early apoptosis and stem cell population independent of Notch signaling.

ÖZET

IRF6'NIN EPİTEL MEME HÜCRELERİNDE NOTCH İLE DÜZENLENEN APOPTOZ, HÜCRE DÖNGÜSÜ VE FARKLILAŞMASINDAKİ ROLÜ

Evrimsel olarak korunan Notch yolağı, gelişim, farklılaşma ve çoğalmayı kontrol etmektedir. Meme tümör oluşumuna neden olan Notch1 ve Notch4 aktivasyonu, kanser yapıcı etki gösterir. Notch1'in fazla aktivasyonu, meme epitel hücrelerinde doz ve hücre tipine bağlı olarak çoğalmayı önleyebilmektedir. Bu nedenle memede Notch, onkogen ve tümör baskılayıcı olarak görev alabilmektedir.

Interferon düzenleyici faktör ailesinin bir üyesi olan IRF6, epidermisin gelişimi ve farklılaşmasında, Notch sinyalinin alt mekanizmasında görev almaktadır. IRF6'nın fazla ekspresyonu, meme kanseri hücrelerinde hücre döngüsünü durdurarak, tümör baskılayıcı özellik gösterir. Son zamanlardaki çalışmalarda, IRF6, Notch sinyalinin, meme epitel hücrelerinin çoğalma ve transformasyonunda mediyatörü olarak gösterilmiştir. Bu çalışmada, IRF6'nın Notch altında, hücre döngüsü regülasyonu, apoptoz ve meme kanser kök hücre popülasyonunda herhangi bir etkisi olup olmadığını ve ayrıca IRF6'nın meme hücre hatlarındaki lüminal ve basal işaretleyicilerin ekspresyonunda rolünü belirlemek amaçlanmıştır.

Sonuçlarımız, normal meme epitel hücre hattında susturulan IRF6 ekspresyonunun, Notch aktivasyonuyla artış gösteren S fazındaki hücre yüzdesini azalttığını göstermiştir. IRF6 ekspresyonunun susturulması, erken apoptozu indüklemiş ve meme kanser kök hücre populasyonunu azaltmıştır. Ancak bu süreçlerde, Notch sinyalinin alt aracı olarak hiç bir etki göstermemiştir. Buna karşılık, IRF6, lüminal ve basal işaretleyicilerin ekspresyonunda önemli bir rol oynamamıştır.

Sonuç olarak, IRF6'nın Notch sinyalinin bir mediyatörü olduğuna dair geçmişteki gözlemlerimiz desteklenmiştir. Ayrıca sonuçlarımız, IRF6'nın Notch sinyalinden bağımsız olarak, erken apoptoz ve kök hücre popülasyonunda özgün rol oynadığını göstermiştir.

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CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Breast cancer is the most common cancer type among woman in developed and developing countries (Ferlay et. al. 2010, Jemal et. al. 2011, American Cancer Society 2015). Breast cancer is the second leading cause of cancer death in women, after lung cancer (American Cancer Society 2015).

Breast cancer can metastasize to many organs in the body including especially bone, brain and lung. Metastasis is the major reason of deaths from breast cancer (Weigelt et al. 2005). In metastasis, tumour cells and host cells have to make dynamic interactions for separating tumour cells from primary site and placing in another organ (Echardt et al. 2012). In order to detect metastasis of breast cancer, different methods including biopsies of affected organs, radiological evaluation, imaging methods and serum tumor markers are used.

Different therapy approaches including surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy are applied to treat breast cancer (American Cancer Society, 2014). Different therapies are applied depending on the stage, type and metastatic status of breast cancer. Although, chemotherapy affects rapidly dividing cancer cells, it causes side effects in body. Therefore, in recent years, targeted therapies causing fewer side effects are applied for breast cancer treatment. There are many steps in cancer progression, which include invasion, metastasis, survival in circulation, tumor related angiogenesis. For each of these steps, proper targeted therapy method has been applied. It has been found that targeted therapy is more effective in primary and secondary tumours then metastatic tumor cells (Echardt et al. 2012). Targeted therapies against the ER and epidermal growth factor receptor 2 (ERBB2) are effectively used in clinic (Echardt et al. 2012).

1.1.1. Breast Cancer Subtypes

Breast cancer has several distinct pathological and molecular subtypes. Pathological subtypes include lobular carcinoma in situ (LCIS), invasive lobular carcinoma (ILC), Ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC) and medullary carcinoma (MC).

Breast cancer can be staged as 0-I-II-III-IV. Stage 0 is in situ class, Stage I and II is in early invasive class, Stage III is in locally advanced class and Stage IV is in metastatic class (Horner et al. 2009, American Cancer society 2010). While 5 year relative survival rate is 100% at stage 0, it is 22% at stage IV (American Cancer Society, 2015).

Recently, DNA microarray has been used to identify breast cancer subtypes based on gene expression levels. 496 intrinsic gene have been used to separate molecular subtypes of breast cancer (Carey et. al. 2006) (Figure 1.1). Breast cancer is divided into five different molecular subtypes including Her2 enriched known ERBB2, luminal A, luminal B, basal like, and unclassified (normal breast-like) subtypes (Banerji et.al. 2012) (Figure 1.1).

Luminal A is oestrogen receptor(ER) and /or progesteron receptor (PgR) positive, HER2 negative and Ki-67 low. Luminal B is divided two types that the first type is ER and /or PgR positive, HER2 negative and Ki-67 high. Second type of luminal B is ER and /or PgR positive, HER2 positive/or amplified and any Ki-67. HER2 enriched (ERBB2 overexpression) is ER and /or PgR absent, HER2 over expressed/or amplified and non luminal. Basal-like is as triple negative, ER and PgR absent and HER2 negative (Nielsen et al. 2004, Cheang et al. 2009). Normal breast-like is ER negative/or positive, PgR unknown and HER2 negative (Bernard & Parker et al. 2009) (Figure 1.1).

Breast cancer is a heterogeneous and complex disease. When breast cancer has been examined in different studies, pathologic, clinical, epidemiologic and molecular differences have been found. Therefore, breast cancer is divided into different pathologic and molecular subtypes. On the other hand, breast cancer stages were determined through cancer progression in clinic. When molecular and pathologic subtypes are thought, breast cancer is one of the very complex cancer types (Weigelt et al. 2010).

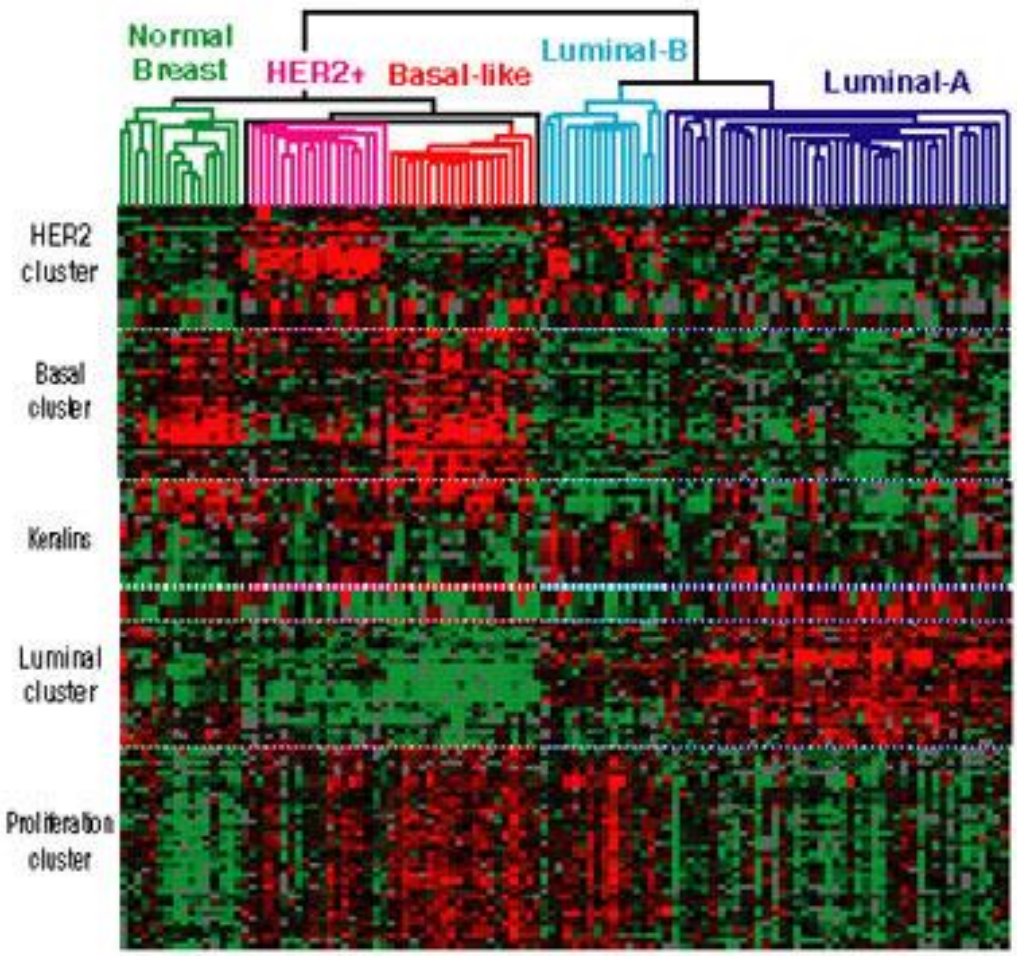


Figure 1.1. Diversity of Molecular Breast Cancer Subtypes
(Source: Carolina Center for Genome Sciences, Perou, 2015)

1.2. Breast Epithelium

The mammary gland composes of epithelial, adipose and other stromal cells and important aim of them is milk production during nursing (Benjamin et. al. 2011).

The human epithelium is ductolobular system including a dual layer of epithelia; luminal epithelial cell and myoepithelial cell layers. Luminal epithelial cell layer is the inner layer of bilayered ductolobular system and myoepithelial cell layer is the outer layer resting on a basement membrane. These layers make branching ductal networks in a collagenous stroma (Thorarinn et. al. 2005) (Figure 1.2).

Luminal cells connect with each other with tight junctions and single luminal epithelial cell layer is generated. Several cell markers are expressed at high levels such as cytokeratin 18 (CK18) and heat-stable surface marker CD24.

Luminal cells produce hormone receptors including nuclear hormone receptors, estrogen receptor-α (ERα), and progesterone receptor. They play a role as sensor cells to make detection of steroid hormones and to convert systematic stimulus into local autocrine/paracrine signals (Jones et al. 2004, Sleeman et al. 2006, Clarke et al. 1997, Tanos et al. 2008).

Basal cells including myoepitheial cells and progenitors produce high levels of cytokeratin 14 (CK14), adhesion molecules β4 (ITGB4) and α6 (ITGA6) (Jones et al. 1991, Raaij-helmer et al. 1991).

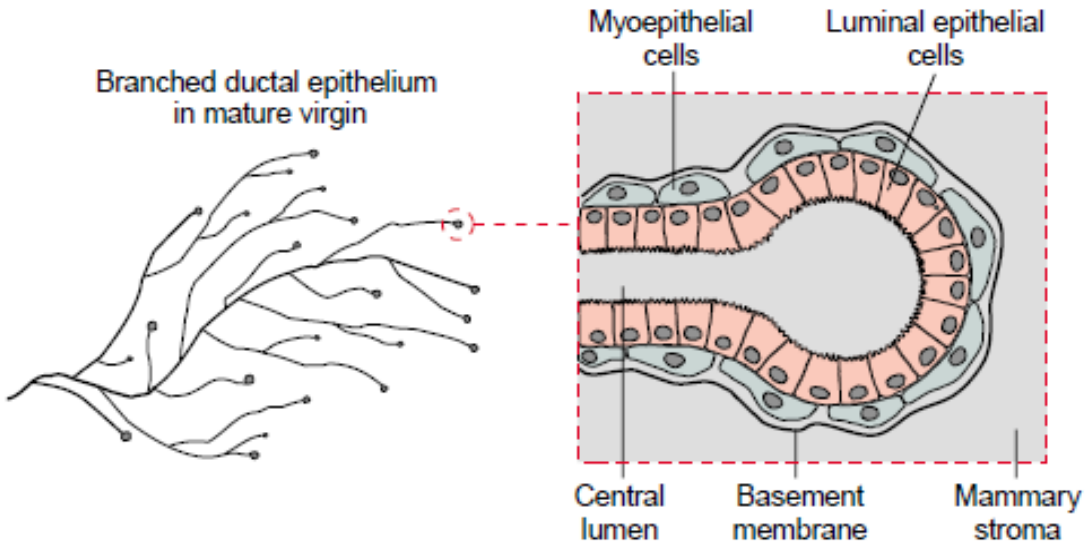


Figure 1.2. Structure of Breast Epithelium consisting luminal epithelial and myoepithelial cells (Source: Radisky & Derek et al. 2003)

1.3. Cancer Stem Cells (CSCs)

Until the early 1990s, it was thought that cells proliferated, were grouped together and then, initiated cancer. But along with technology and research, leukemia stem cells were discovered and then, it has been found that hematologic malignant tumors includes two different cell populations that are cancer stem cells (CSCs) and others. Neoplastic tissues have been found to have CSCs, which are similar to somatic stem cells that can self-renew or produce non-cancer stem cells (Mani et al. 2008). There are a few CSCs in tumors and these cells have to form tumor in new seeding tissue. Therefore, another name of CSCs is tumor initiating cells (Reya et al. 2001). Other subpopulation cells including non-cancer stem cells (non-CSCs), and CSC-derived progenitors cannot induce tumor growth when they are seed to a new place (Reya et al.2001).

In a recent study, nontumorigenic human MCF10A mammary epithelial cells were reprogrammed by using induced pluripotent stem cells (iPSC) technology. Non-tumorigenic human immortalized mammary epithelial MCF10A cells were infected by using retroviral-mediated induction and iCSCL-10A or CSC-like-10A cells, a tumorigenic $CD44^{+}/CD24^{-}$ cells, were generated. It was seen that these cells have CSCs properties. Stem cell markers including CD44, ABCG2 and small amount of SOX2 are expressed by iCSCL-10A cells. Generation of multilineage tumors contained differentiated cells of bone and muscle was observed in an immunosuppressed mouse models (Nishi et al. 2014). This paper showed that cells showing tumorgenic and CSC-like properties can be generated from normal human cells, such as mammary epithelial cell line MCF10A by induction of reprogramming factors.

1.3.1. Breast Cancer Stem Cells (BCSCs)

Origin of breast cancer stem cells (BCSCs) is still unknown. The tumorigenic cancer stem cells can be distinguished from non-tumorigenic cells by using CD24 and CD44 cell surface markers; $CD44^{+}/CD24^{-/LOW}$ was determined as BCSCs population (Al-Hajj et al. 2003, Snyder et al. 2009). Human mammary epithelial cells have two different cell populations including luminal and myoepithelial cell types and also including their

putative progenitors and basal cells as we mention it (Figure 1.3). These cells can be separated from each other by using specific surface markers (Smalley, et al. 2003).

While $CD44^{+}/CD24^{-/LOW}$ cells are enriched in basal-like breast cancer stem cells, $CD44^{-}/CD24^{+}$ cells are enriched in luminal breast cancer cell lines. Breast cancer stem cells (BCSCs) have common cell surface profile with basal cells (Fillmore et al. 2008, Sheridan et al. 2006). CD44 expression shows characteristic features of stem cell-like while CD24 expression is related to epithelial differentiation (Park et al. 2010).

It is considered that human BCSCs was derived from basal cells (Figure 1.3). After mutations in immature basal cells that are stem cells and their progenitors existing in the basal membranes of alveolar units of mammary glands, BCSCs are generated. These cells resist to anticancer therapy more than non-CSCs (Kai et al. 2010).

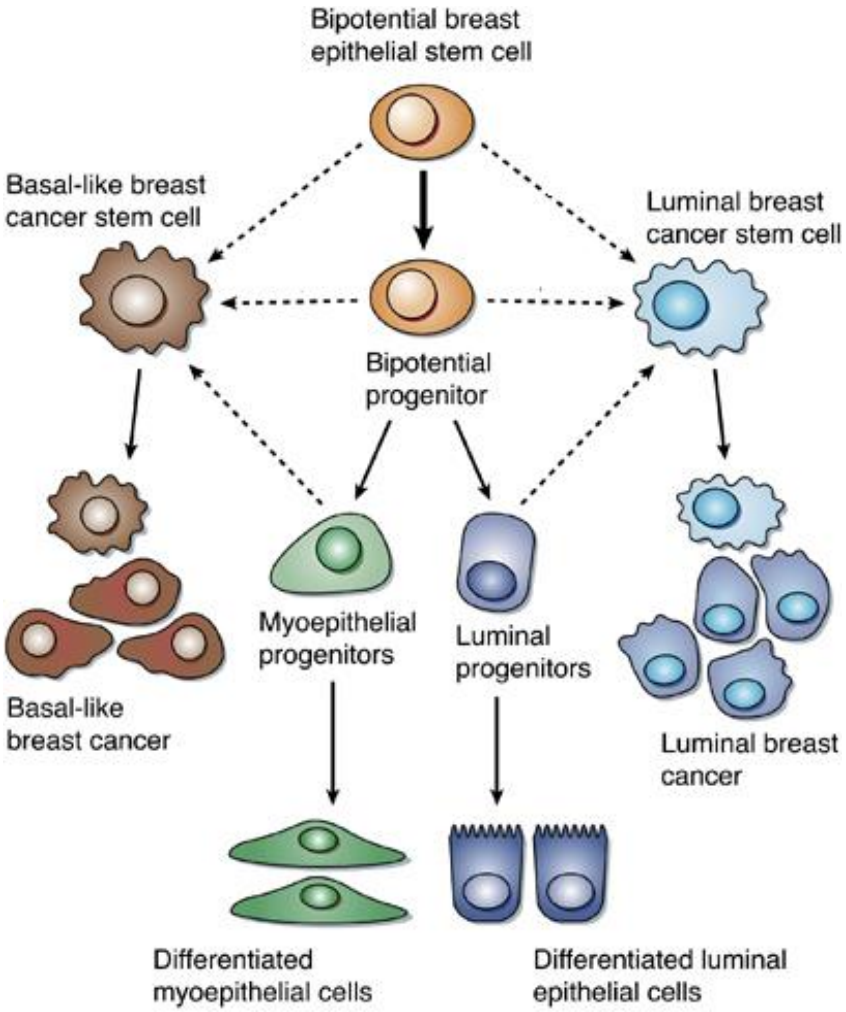


Figure 1.3. Breast Cancer Stem Cells Differentiation Pathway
(Source: Shipitsin et al. 2008)

1.4. Notch Signaling Pathway

Notch signaling pathway is an evolutionary conserved pathway from invertebrates to mammals and has roles in many processes including determination of cell fate, tissue patterning and morphogenesis, cell differentiation, proliferation and death. Notch family proteins are single-pass type 1 trans membrane proteins having two roles that are cell surface receptors and nuclear transcription regulators (Blaumueller et al. 1997).

Notch gene was discovered at Drosophila Melanogaster when a mutant strain of *Drosophila* was descripted. Notch gene was identified, after partial loss of function gene was seen to cause notches at the end of their wing blades (Morgan et al. 1917). The Notch gene was cloned firstly in 1985 (Wharton et al. 1985).

1.4.1. Notch Receptors and Ligands

A number of Notch receptors and ligands shows differences from invertebrates to mammals. There is a single Notch protein in *Drosophila* (Wharton et al, 1985), are two Notch proteins in *C.elegans* (glp-1 and lin-12) (Greenwald 1985), and are four Notch receptors in mammals (Noutch1-4) (Lardelli& Lendahl et al. 1993, Uyttendaele et al. 1996).

Each Notch receptor protein is synthesized as a single precursor protein. When these precursor proteins are being transported to cell surface, they are cleaved at S1 site by Furin-like convertase in Golgi apparatus. They are seen as a heterodimeric receptors. Each Notch receptor is used for different functions in cells (Blaumueller et al. 1997, Borggrefe et al. 2009). Notch protein family contain extracellular, transmembrane and intracellular domains. In Notch extracellular domain (NEC), 29-36 EGF (Epidermal growth factor) tandem array-like repeats, conserved negative regulatory region (NRR or LNR) including three cystein-rich-Notch Lin12 repeats (N/Lin 12) and a domain of heterodimerization (HD) are found, respectively. Number of EGF repeats differ in different Notch receptors but structures are the same while EGF-like repeats provide physical binding of Notch receptors with specific ligands, NRR or LNR protect from ligand-dependent and independent signaling. In intracellular domains of Notch receptors, DNA binding (RBP-Jk associated molecule/RAM) domain, flanked by two nuclear

localization signals (NSL), transactivation domain(TAD) and PEST domain including rich proline, glutamine, serine and threonine residues are found, respectively (Kopan et al. 2009, Okuyama et al. 2008, Tien et al. 2009) (Figure 1.4).

In mammals, there are five different Notch ligands including Jagged1-2, Delta-like (DII) 1, 3, and 4 that are type I trans membrane proteins. Notch ligands coming from Delta and JAG/Serrate ligand families activate Notch receptors. Based on their domain, Notch ligands can be divided into two different subgroups; canonical DSL (Delta/Serrate/LAG-2) ligands, with or without DOS (Delta and OSM-11-like domains) domain (Figure 1.4). JAG1, JAG2 and DII-1 have Delta/Serrate/LAG-2 (DSL), and "Delta and OSM-11-like domains" (DOS) consisting of specialized tandem EGF motifs. The members of subgroup, which contains DSL but not DOS, are DII-3 and DII-4 (Cordle et al. 2008, Komatsu et al. 2008). DSL and DOS domains provide physical binding between ligands and Notch receptors. On the other hand, it was shown that other cell surface proteins including membrane-tethered and non-canonical ligands that do not contain DSL and DOS domains can also activate Notch signaling pathway (D'Souza et al. 2008, Albig et al. 2008, Vaccari & Lu et al. 2008).

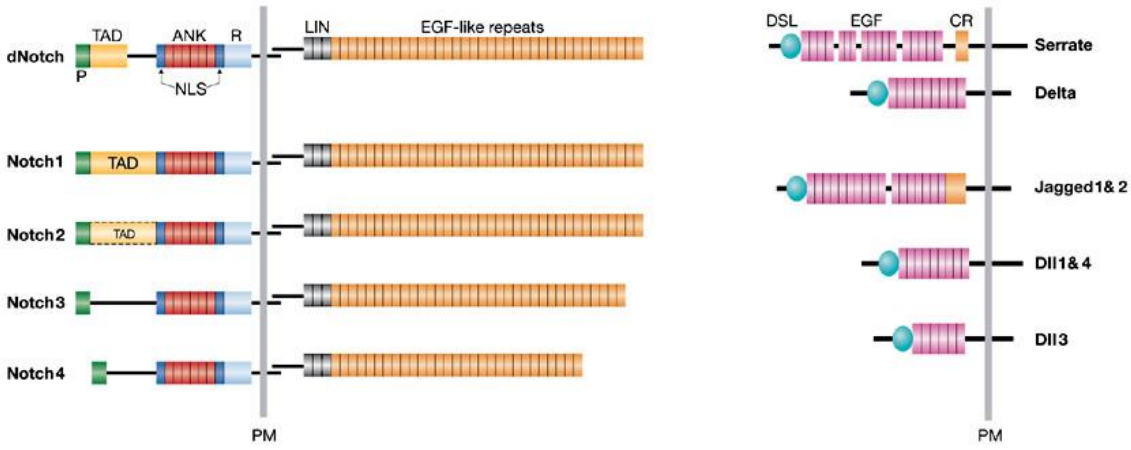


Figure 1.4. Notch Receptors and Notch Ligands in Drosopila and Human (DSL Domain: Delta, Serrate and Jag2, P: PEST sequence, TAD: Transactivated domain, NLC: Nuclear localization signals, R:RAM domain, CR: Cysteine-rich domain, PM: Plasma membrane) (Source: Radtke et al. 2005)

1.4.2. Notch Processing

Notch signaling pathway is activated when mature Notch receptor bind to ligand on adjacent cell. First of all, this interaction between receptor and ligand make conformational change and two proteolytic cleavages occur in Notch receptors. The first cleavage takes place at the extracellular domain (S2), is made by metalloprotease (ADAM17 (a disintegrin and metalloprotease)/TACE(TNFα converting enzyme)) to cleave transmembrane subunit. The next cleavage (S3) takes place at the transmembrane domain, is made by γ-secretase that have five-subunit complex including presenilin1-2, nicastrin, Pen-2 and Aph1. After these cleavage steps, Notch intracellular domain (NICD) is separated from Notch receptor to cytoplasm and then NICD is translocated from cytoplasm to nucleus to activate the transcription of Notch target genes (Guo et al. 2011, Yin et al. 2010) (Figure 1.5).

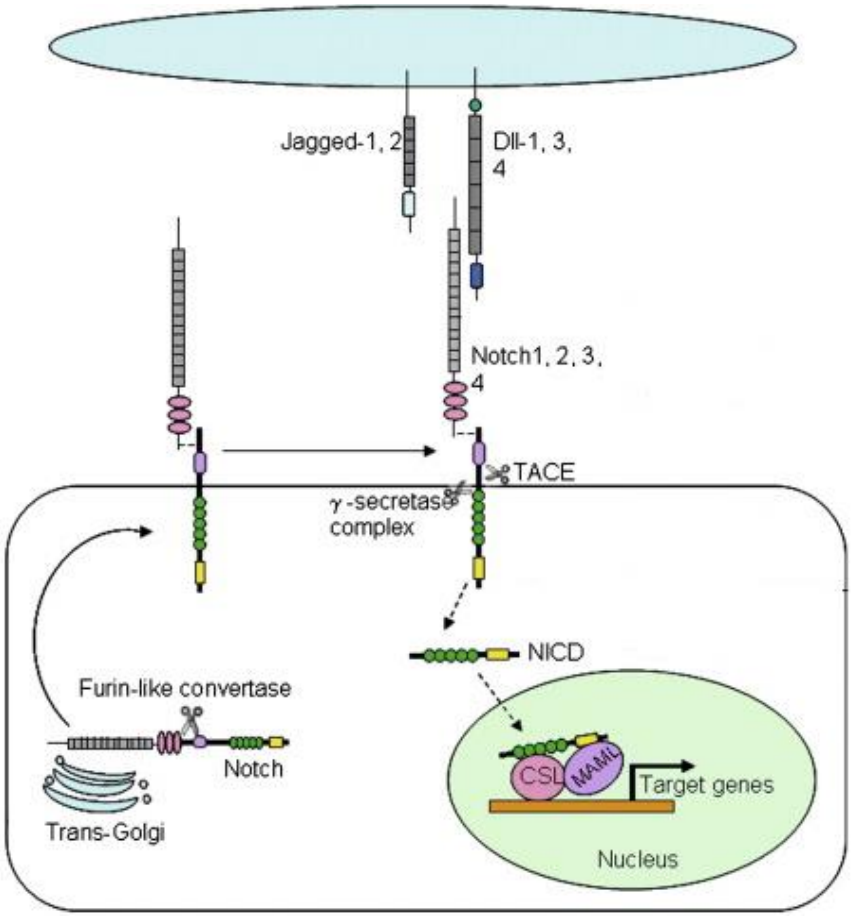


Figure 1.5. The Notch Signaling Pathway (NICD: Notch intracelluar domain, CSL: Recombination signal binding protein for immunoglobulin kappaJ (RBP-Jk) or CBF1/Su(H)/Lag-1 family of transcription factors, MAML: mastermind-like, TACE: TNFα converting enzyme) (Source: Yin et al. 2010)

After translocation, NICD interacts with DNA binding proteins, and transcriptional repressor CSL (recombination signal binding protein for immunoglobulin kappaJ (RBP-Jk) or CBF1/Su(H)/Lag-1 family of transcription factors), co-repressor complex is replaced with NICD and then, activated NICD-CSL complex recruits coactivators (co-A) as mastermind-like (MAML), p300 and Notch target gene transcriptions are mediated. If NICD is absent in nucleus, ubiquitous co-repressor (Co-R) and histone deacetylases (HDACs) interact with CSL and transcriptions of Notch target genes are blocked. There are a lot of Notch target genes including Hey1 (hairy/enhancer-of-split related with YRPW motif), Hes-1 (hairy enhancer of split), cyclinD1, p21, NF-kB (Guo et al. 2011, Yin et al. 2010) (Figure 1.5).

1.5. Notch in Cancer

In embryonic development and patterning, Notch signaling pathway has important roles. In many studies, it has been shown that organs development have a similar mechanism with tumorigenesis. Therefore, development pathways such as Notch, Wnt and Hedgehog are thought to be very effective on tumor formation and progression. It was observed that Notch signaling pathway in highly aggressive tumor cells is used to promote their survival (Wang et al. 2006, Santagata et al. 2004, Balint et al. 2005).

Notch can be either oncogene or tumor suppressor depending on organs and tissues. It is a mystery that Notch activation causes two opposite results in different cell types. This paradox was explained that Notch signaling pathway can turn on or off specific genes in different tissues. Notch signaling effect can be determined by downstream pathways (Nicolas et al. 2003). It was known that Notch signaling pathway is used in a wide range of cancer types and Notch activation and overexpressed NICD cause hematological tumors including leukemias, lymphomas and multiple myeloma, and solid tumors including breast cancer and lung cancer (Yin et al. 2010).

Many different cancer types can occur by Notch activation. For example, in B-chronic lymphocytic leukemia (B-CLL), Notch1-2 receptors and their ligands were overexpessed. In B-CLL, mutation on PEST domain of Notch2 increased its expression. By this mutation Notch2 gained oncogenic function in B-cell lymphoma (Lee et al. 2009). In human colon cancer, overexpression of Notch 1, Notch2 and their ligands were observed. p53 induced apoptosis is inhibited by Notch1 which causes tumorigenesis (Nair et al. 2003).

Although, many studies have showed that Notch signaling pathway have an oncogenic role, in some tissues, Notch has a role as tumor suppressor. When Notch1 was inhibited in murine skin, highly effective basal cell carcinoma (BCC)-like tumors was observed (Nicholas et al. 2003). This suggested that active Notch pathway protects cells from tumor formation. Inhibition of Notch signaling by dominant negative-MAML1 (DN-MAML1) causes cutaneous squamous cell carcinoma (SCC) and this showed that Notch signaling pathway protects epidermal skin cells from forming cutaneous SCC (Proweller et al. 2006). In keratinocytes, CSL also binds to p21 promoter by Notch1 and then p21 mediates cell cycle arrest and tumor suppression. Therefore, Notch is tumor suppressor in this tissue (Rangarajan et al. 2001, Nicholas et al. 2003).

1.5.1. Role of Notch in Breast Cancer

Tumorigenic effect of Notch in breast cancer was discovered in mouse models. Mouse Mammary tumor virus (MMTV) was inserted into Notch4 locus of mouse chromosome (Gallahan et al. 1987). In two other studies, overexpression of truncated Notch4 (Notch4-ICD) protein that is constitutively active was observed and this MMTV insertion was shown to transform mammary epithelial cells (Robbins et al. 1992, Uyttendaele et al. 1996).

Besides Notch4, insertion mutation at Notch1 locus occurred by using MMTV, and resulted in overexpression of active truncated Notch1 (Notch1-ICD). These mice developed mammary tumors. In the mammary carcinoma development, Notch1 acts as an oncogene (Dievart et al. 1999). In murine models, relationship between mammary tumors and Notch signaling is identified well but relationship between human breast cancer and Notch signaling is identified less. But recently variety of studies showed high level of Notch was expressed in human breast cancers.

Notch receptors have different effects on breast cancer (Clarke et al. 2005, Parr et al. 2004, Reedijk et al. 2005). While increased Notch1 expression level was observed in differentiated breast tumors, Notch2 over expression correlated with increased survival. These suggest that, Notch2 might have a tumor suppressor role while Notch1 might have a tumor promoting role in human breast cancer (Par et al. 2004).

Wide variety of studies illustrated that Notch signaling pathway interacts with other signaling pathways and they can cause together breast cancer. It has been known that Wnt signaling deregulation causes breast cancer. Wnt1 expression activate Notch signaling pathway through expression of Notch ligand DII-3 and DII-4. Therefore, Wnt1 can make transformation of human mammary epithelial cells through Notch activation (Prosperi et al. 2010, Ayyanan et al. 2006). Deregulated oncogenic Ras signaling can cause development of breast cancer. Notch1 levels and activity are increased by oncogenic Ras activity. Notch ligand DII-1, and presenilin-1 that is used for Notch processing are upregulated by using p38-mediated pathway. It was seen that Notch1 is the downstream effector of Ras signaling (Weijzen et al. 2002, Gustafson et al. 2009).

Notch signaling pathway shows mostly oncogenic properties, but in recent studies, it has been illustrated that Notch can play a tumor suppressor role in breast tissue. Proliferation is reduced by overexpressed N1-ICD in primary human breast epithelial

cells (Yalcin-Ozuysal et al. 2010, Mazzone et al. 2010). On the other hand, dosage of Notch activation changes Notch effect on proliferation. When Notch activity is high, proliferation is inhibited but when Notch activity is low, proliferation is increased (Mazzone et al. 2010).

Notch activity has been investigated in breast cancer stem cells (BCSCs). Recent studies showed that Notch expression level is high in BCSCs. Some stem cell surface markers such as CD44, CD24, and CD29 have been used to identify BCSCs and $CD24^{+}/CD29^{HIGH}$ and $CD44^{+/HIGH}/CD24^{-/LOW}$ population were shown to be enriched in breast cancer stem cells (Al Hajj et al. 2003). Overexpression of Notch1 ICD in mammary cells increases $CD24^{+}/CD29^{HIGH}$ population (Ling et al. 2010).

High level of Notch1 expression in luminal cells of normal breast epithelium (Raouf et al. 2008) and Notch4 expression in basal cells of normal breast epithelium and BCSC enrich population are observed (Harrison et al 2010). These results show that Notch1 and Notch4 affect different subpopulation of breast cells and play different roles in BCSCs (Harrison et al 2010).

Notch1 knockdown decreased $CD44^{HIGH}/CD24^{LOW}$ phenotype expression, macrometastasis formation and micrometastasis formation. Notch1 in 231-BR cells was inhibited by using DAPT and then, these cells were injected into the immunodefficient mice. To confirm this, shRNA were used to inhibit Notch1. CSC markers expressions ($CD44^{HIGH}/CD24^{LOW}$) were reduced with Notch1 inhibition. These results suggest that Notch1 activation is important for BCSC population and induced brain metastasis development of breast cancer (Steeg et al. 2011).

1.6. Apoptosis-Notch Relationship

Notch signaling regulates apoptosis in different cancer types via different mechanisms that are p53 inhibition, Foxo3a or c-Jun NH2-terminal kinase (JNK) function and Akt activation (Kim et al. 2005, Kim et al. 2007, Palomero et al. 2007, Sade et al. 2004, Mandinova et al. 2008).

In breast epithelial cells, Akt is activated by Notch signaling through an autocrine signaling loop. Akt activation is essential to protect from apoptosis in Notch induced normal breast epithelial cells. In breast cancer cells, Notch inhibition reduces Akt phosphorylation and induces apoptosis (Meurette et al. 2009).

It has been known that Notch signaling is increased and NICD is accumulated in human breast carcinomas. When Notch is activated in normal breast epithelial cells, these cells are protected from drug-induced apoptosis and apoptosis was inhibited. When RBP-Jk dependent Notch signaling is increased in normal breast epithelial cells, transformation is seen and apoptosis is suppressed (Stylianou et al. 2006).

1.7. Interferon regulation Factors (IRFs) Family

Interferon regulatory factors (IRFs) family including nine human members (IRF1-9) are transcription factors. IRFs family members have essential roles in antiviral defense, immune defense, regulation of cell growth, apoptosis and stress response because IRFs are virus inducible, bacteria inducible and IFN inducible proteins (Savitsky et al. 2010).

IRFs have two conserved functional domains that are amino (N)-terminal DNA binding domain and carboxy (C)- terminal protein interaction domain. N-terminal DNA binding domain (DBD) was identified by five well-conserved tryptophan repeats that forms helix-loop-helix domain. IFN-stimulated response element (ISRE) that is IRF recognition (5'AAN NGAAA 3') on DNA sequence is recognized by three repeats of this domain (Fujii et al. 1999, Escalante et al. 1988). C-terminal region of IRFs include IRF associated domain (IAD) that mediates homomeric or heteromeric interaction with other IRFs or transcription factors or co-factors (Mamane et al 1999, Taniguchi et al 2001).

1.7.1. IRF Family Members and Their Functions

There are nine members of IRF family. IRF family members also play roles in immune response to pathogens, immune cells development, cell growth, apoptosis and oncogenesis. Each member of IRF family has different roles in different cell types.

The first IRF family member discovered is IRF1 by studies of the transcriptional regulation of IFN-B gene. IRF1 together with p53 tumor suppressor activates cyclin-dependent kinase (CDK) inhibitor p21 and so, apoptosis with DNA damage is induced by IRF1 (Tanaka et al. 1996). Expression level and protein stability of IRF1 regulate expressions of DNA repair protein BRIP1 and Fanconi anemia gene J (FANCJ) (Frontini et al. 2009).

Effects of IRFs in the regulation of oncogenesis firstly were seen at transformed NIH3T3 cells overexpressing IRF2. Oncogenic transformation is caused by over expression of IRF2 so it was identified that IRF2 has pro-oncogenic activity (Harada et al. 1993).

Four IRFs that are IRF1, IRF3, IRF5 and IRF7 play significant role as positive regulators of transcription of type I IFN gene (Takaoka et al, 2005).

Study of gene targeting showed that IRF5 is not important to induct type I IFN gene by using viruses or TLR agonists while expression of inflammatory cytokine genes such as inteleukin (IL)-12 or TNF-α is regulated by IRF5 (Takaoka et al, 2005).

IRF3 shows high homology with IRF7 and they are antiviral IRFs. IRF3 and IRF7 play important roles to regulate expression of type I IFN genes including IFNα and IFNβ. Because of pathogen contamination, IRF3 and IRF7 phosphorylations by NF-κB kinase induce promoter activation of INFα and INFβ genes (Akira et al. 2006, Honda et al. 2006, Marie et al. 1998, Ronco et al. 1998, Au et al. 1998). IRF3 has a role in DNA damage. Phosphorylated IRF3 translocates from cytoplasm to nucleus when DNA is damaged and it induces apoptosis (Weaver et al. 2001, Kim et al. 1999).

IRF4 and IRF8 are highly homologous and their expressions are high level in lymphocytes, macrophages, B cells and dendritic cells (DCs) (Eisenbeis et al. 1995, Politis et al. 1992). There are important roles of IRF4 in maturation of B and T cells. IRF8 interacting with IRF1 and IRF2 binds to DNA and is required for development of B cells (Savitsky et al. 2010).

There is few information about IRF9. IRF9 works as an essential protein in the antiviral effect of type I IFN (Veals et al. 1992). IRF9 increases p53 onco proteins via IFN for tumor and so it mediates tumor suppression and apoptosis (Takaoka et al. 2003).

1.7.2. IRF6

IRF6, the poorly understood member of IRF family is a transcription factor and plays a role as regulator for normal development and differentiation of the epidermis (Kondo et al. 2002).

In humans, relationship was found between Van der Wounde Syndrome and mutation in *Irf6* gene locus. Novel mutation in IRF6 causes Van der Wounde Syndrome (VWS) including cleft lip and cleft palate (Kondo et al. 2002, Mostowska et al. 2005).

Mutation of IRF6 causes Popliteal Pterygium Syndrome (PPS) including skin folds, genital abnormalities and oral adhesion in human (Kondo et al. 2002).

In mice, because of loss of IRF6, defects in development of limb and skin are shown such as abnormal skin, short forelimbs and lack of ears, hind lips and tails. Loss of IRF6 in mice can be lethal, or can cause morphological and skeletal defects. An important change that was observed in IRF6 deficient mice was abnormal stratified epidermis because keratinocytes continued to proliferate and failed in differentiation (Ingraham et al. 2006). They show that IRF6 is essential regulator to switch from differentiation to proliferation of keratinocytes (Ingraham et al. 2006, Richardson et al 2006). IRF6 deficiency in keratinocytes causes abnormal phenotypes including large size, and irregular shape but interestingly epithelial cell properties were continued. In long term proliferative capacity, IRF6 deficiency increases cell number and bring ability of more colony formation in keratinocytes (Biggs et al. 2012). It has been recently shown that IRF6 deficient keratinocytes are larger and more spread but interestingly this deficiency increases stress fiber network and active RhoA. IRF6 deficiency decreases expression of Arhgap29 that activates Rho GTPase. These data show that cell migration is regulated by IRF6 through RhoA pathway (Biggs et al. 2014).

Tumor suppressor activity of IRF6 has been shown in squamous cell carcinoma (SCC). Expression of IRF6 in SCC cells causes tumor suppression, inhibition of cell invasion and inhibition of cell migration. IRF6 inhibition causes SCC cells migration (Botti et al. 2011). Expression of IRF6 in renal cell carcinoma (RCC) cells is low because of methylation compared to normal cells. Expression of IRF6 was restored in RCC cells then, reduction of invasion and migration of RCC cells was observed. These data suggest that IRF6 could be important to inhibit invasion and migration in RCC cells (Wei et al 2015).

Irf6 null mutant mice showed evagination of incisor epithelium suggesting that it has an essential role to regulate invagination of epithelial tissue in tooth development (Blackburn et al. 2012).

It has been known that TGF-β signaling also regulates palate development (Iwata et al. 2011). Relationship between IRF6 and TGF-β was shown during palate formation (Iwata et al. 2013). Expression of IRF6 is regulated by TGF-β through Smad4. Both IRF6 haplo-insufficiency and Smad4 deficiency in mice causes inhibition of p21 expression. Therefore, abnormal development of skin was observed. Then, when IRF6 was reexpressed in Smad deficient mice, p21 expression was observed again. Thus, it is said

that combined effect of IRF6 and Smad4 is seen in development of skin. TGF-β signaling mediated by IRF6 has a role in degenerations (Iwata et al. 2013).

In recent study, adult rat traumatic brain injury (TBI) model was established. After TBI, IRF6 was upregulated in ipsilateral brain cortex compared to sham brain cortex and co-localization of IRF6 with neurons was observed. Additionally, active caspase-3 which is apoptosis marker, and IRF6 were co-localized in neurons while IRF6 knock down in PC12 cells reduces active caspase-3 levels and increases expression level of Bcl-2 and p-Akt. According to these results, it could be concluded that after TBI, IRF6 expression induces neuronal apoptosis through inhibition of Akt phosphorylation (Lin et al. 2015).

In mammary tissue, firstly, Hendrix group identified IRF6 by using yeast hybrid system for Maspin/Serpin5 (mammary serine protease inhibitor) protein. Maspin/Serpin5, a tumor suppressor protein, regulates apoptosis (Jiang et al. 2002) and inhibits invasion (Sheng et al.1996). IRF6 is a binding protein of maspin and IAD domain of IRF6 regulated by phosphorylation was identified as the interaction side with maspin protein. In breast cancer, IRF6 expression was reduced when maspin is absent. Reduction and transient re-expression of IRF6 increases expression level of N-cadherin which is expressed in metastasis and transformation in cancer cells (Bailey et al. 2005). Later, IRF6 and maspin showed to be important for mammary epithelial cell differentiation (Bailey et al. 2008). While expression level of IRF6 and maspin was decreased in ductal and glandular epithelial cells during pregnancy, it was increased in quiescent differentiated cells of lactating mammary gland (Bailey et al 2008, Bailey et al. 2009). IRF6 phosphorylation in mammary epithelial cells causes IRF6 degradation, ubiquitination signals and proteasomal degradation (Bailey et al. 2008). In these studies, it was shown that reexpression of IRF6 in breast cancer cells increases cell cycle arrest and presence of maspin increases this response with IRF6 (Bailey et al. 2008).

1.8. Notch-IRF6 Relationship

Relationship of Notch and IRF6 was shown in few studies. Showing that IRF6 deficiency causes cleft palate phenotype similar to JAG2 deficiency in mice (Jiang et al. 1998), relationship between IRF6 and Notch was identified in 2009 (Richardson et al. 2009).

Direct interaction between IRF6 and Notch signaling was illustrated in keratinocytes. IRF6 is expressed high level in the differentiating versus proliferative Keratinocytes.When differentiation is induced, expression of IRF6, Keratin 1 and Hes1 are upregulated. When DAPT, a γ-secretase inhibitor, treatment is applied, these protein levels are reduced in keratinocytes. Endogenous Notch activation induces expressions of IRF6, Notch target gene Hes1 and differentiation marker involucrin. IRF6 is induced by Notch through CSL-dependent canonical pathway because CSL complex protein bind to CSL binding domain at -2.4 kb upstream transcription site of IRF6 in keratinocyte differentiation. As a result of this study, it was shown that IRF6 has a role as a mediator of Notch in keratinocyte differentiation (Restivo et al. 2011).

We have shown that IRF6 is also a mediator of Notch signaling in breast cells (Zengin et al. 2015). IRF6 is upregulated along with Notch target gene Hey1 and Hey2 by Notch activation in normal breast epithelial cells, MCF10A, whereas IRF6 is down regulated by Notch inhibition via DAPT and silencing of RBPjk in breast cancer cell line, MDA MB 231. Notch signaling has a role in regulation of IRF6 expression. Furthermore, IRF6 has an essential role in Notch induced proliferation and transformation in breast epithelial cells. IRF6 knockdown in Notch induced cells reduces cell viability and proliferation. Therefore, proliferation of Notch induced cells requires expression of IRF6 (Zengin et al. 2015). Notch act as an oncogene in MCF10A cells and induces transformation (Stylianou et al. 2006). Notch activation increases colony number in soft agar assay. IRF6 knockdown in Notch induced cells, reduced colony number in soft agar assay. Therefore, IRF6 expression is important for Notch induced transformation. These results showed that IRF6 plays a role as a downstream target of Notch signaling and has an important role as a mediator of Notch signaling in proliferation and transformation of normal breast epithelial cells (Zengin et al. 2015).

These studies showed that IRF6 under Notch signaling affects keratinocytes development and IRF6 is a player regulated by Notch signaling in differentiation of

keratinocytes. On the other hand, IRF6 is a mediator of Notch in proliferation and transformation in breast epithelial cells. IRF6 effect under Notch in cell cycle regulation, apoptosis and differentiation is not known in breast. The aim of this study is to investigate whether IRF6 has a role under Notch in cell cycle regulation, apoptosis and stem cell population in normal breast epithelial cells and whether IRF6 play a role in differentiation of luminal or basal epithelial cells.

CHAPTER 2

AIM OF THE RESEARCH PROJECT

In previous studies, it was shown that IRF6 is a player of Notch signaling in keratinocyte differentiation and proliferation and transformation mechanisms of breast epithelial cells. In this study, it is aimed to investigate whether IRF6 has a role under Notch in cell cycle regulation, apoptosis and stem cell population in normal breast epithelial cells and whether IRF6 play a role in differentiation of luminal or basal epithelial cells.

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Line and Cell Culture

During this project, five cell lines were used that are human normal breast epithelial cell line MCF10A, human breast adenocarcinoma cell line MCF7 and MDA-MB-231, human embryonic kidney cell line 293T and mouse embryonic fibroblast cell line NIH3T3.

Special medium were prepared for each cell line. Human normal breast epithelial cell line MCF10A medium includes high glucose DMEM-F12 (Gibco, Cat# 31-330-038), 5% donor horse serum (BI, Cat# 04-004-1B), 20ng/mL EGF (Sigma, Cat#E9644), 0.5 µg/mL Hydrocortisone (Sigma, Cat# H0888), 100 ng/mL Cholera Toxin (Sigma, Cat# C8052), 10µg/mL Insulin (Sigma, Cat#I1882), and 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B).

Human breast adenocarcinoma cell lines MDA-MB-231 and MCF7 and human embryonic kidney cell line 293T medium include high glucose DMEM (Gibco, Cat# 41966-029), 10%Fetal Bovine Serum (FBS) (BI, Cat# 04-007-1A), and 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B).

Mouse embryonic fibroblast cell line NIH3T3 medium includes high glucose DMEM (Gibco, Cat# 41966-029), 10% New Born Calf Serum (NBCS) (Biological Industries, Cat# 04-102-1A) , and 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B).

These cell lines were cultured in incubator at 37^0C, 5% CO_2.

3.2. Plasmids

In order to transfect cells and produce viruses, several plasmids were used.

Notch1 intracellular domain (N1-ICD) was expressed using retroviral plasmid MSCV-NICD. MSCV vector includes two LTR sites to integrate into host genome, multiple cloning site to insert sequence of interest into vector and pgk-Neo cassette for positive selection of infected cells. Empty backbone vector (MSCV) was used for control condition.

pcl10A which contains packaging genes of retro-viruses, was used to produce retro-viruses.

IRF6 expression was silenced by expressing shRNA in Plko backbone. Plko vector contains U6 promoter for shRNA expression, hPGK promoter and puromycin resistance gene and HIV-1 RNA packaging signal between 5' LTR and 3' LTR. Plko-shIRF6 (clone 14837) plasmid, which contains shIRF6 was used for suppression. Plko-shGFP plasmid including shRNA against GFP sequence was used fo control condition.

pCMV-dR8.74 and pMD2.VSVG plasmids were used for packaging. pCMV-dR8.74 vector containing gag gene that expresses capsid protein and pol gene that expresses reverse transcriptase through CMV promoter is package vector. pMD.VSVG contains VSVG gene that expresses virus coat protein through CMV promoter is envelope vector.

pCMV6-XL4-IRF6 mammalian expression plasmid, which contains human IRF6 cDNA is used to overexpress IRF6 in cells.

3.3. Lenti and Retro-Viruses Production

Retro-viruses and lenti-viruses were produced by using human embryonic kidney cell line 293T, which has high transfection efficiency. $3X10^6$ 293T cells were cultured in 10cm plate. After 24 hours, to produce viruses, 293T cells were transfected with vectors by using transfection reagent X-tremeGENE9 (Roche, Cat# 06365787001) or Fugene HD. (Promega, Cat#E2311) Transfection reagent and vectors were mixed in 1:3 ratio in serum free medium.

To produce retro-viruses for control and gene expressing virus vectors, 2μg of retro-viral MSCV or MSCV-NICD vector and 2μg of packaging vector pcl10A were mixed with 12μL of X-tremeGENE9 or Fugene HD in 500μL serum free medium. To produce lenti-viruses, 2 μg of lenti-viral plko vector and 1.3 μg of packaging vector pCMV-dR8.74 and 0.7 μg of envelope vector pMD2.VSVG were mixed with 12 μL of X-tremeGENE9 or fugene HD in 500 μL serum free medium. This mixture was incubated at room temperature for 30 minutes. Then, mixture was added drop by drop on 293T cells. 24 hours after transfection, transfection medium was changed with fresh medium. The RNA molecules are packaged into virus by packaging proteins and produced viruses move out of cell into medium. 48 and 72 hours after transfection, medium containing viruses were collected. Viruses was spun down at 2000 rpm for 5 minutes to separate dead cells. Viruses were filtered on 50% PEG solution through 0,45 μm filter and final concentration was made 10%. This 10% PEG-virus mixture was incubated at 4^0C, in the dark for 4 days. After incubation, PEG-virus mixture was spun down at 2500 rpm for 20 minutes. Supernatant was aspirated but ~100μl liquid was left at the bottom. Again pellet was spun down at 1200 rpm for 5 minutes to aspirate as much liquid as possible. Then, viruses pellet was resuspended in PBS by pipeting. Viruses were aliquoted and stored at -80^0C.

3.4. Virus Titration

In order to determine virus efficiency, virus titration was performed for each virus. Mouse embryonic fibroblast cell line (NIH/3T3 cell) was used for this experiment. At first day, $15X10^4$ NIH/3T3 cells were plated in 6-well plate. At second day, virus titration was done with infection by using polybrene (FINAL CONCANTRATION) by serial dilutions as 10^{-3}, 10^{-4} and 10^{-5} of produced viruses. At third day, medium in plate was changed with fresh medium. 72 hours after infection, infected cells were split from 6-well plate into 10cm plates and selection was done by 400μg/mL Geneticin (Gibco, Cat#10131-019) or 2μg/mL puromycin (Hyclone, Cat#SV30075). Until non-infected control cells (mock) died, selection was maintained. After selection was finished, selected medium on cells was aspirated and cells were washed with 1X PBS. Then, cells were stained by 0.5% crystal violet solution for 15 minutes on shaker and washed by 1X PBS three times for 15 minutes. 1 day after wash, cell colonies were counted. At the end of

experiment, colony number of each viruses were compared to determine virus efficiency. Virus with similar efficiency were used for experiments.

3.5. Transfection of MCF7 Breast Cancer Cell Line

$5X10^5$ MCF7 cells were plated in 6 well plate one day before transfection. MSCV (neo) as a control and pCMV6-XL4-IRF6 overexpression plasmids were used to transfect MCF7 cells. Transfection medium mixture including serum free medium, transfection reagent FuGENE HD (Promega, Cat#E2311) and 4μg plasmid was prepared. One day after cells were plated, mixture was put on cells drop by drop. 24 hours after transfection, medium was changed with fresh medium. Then, 72 hours after transfection, medium was aspirated, transfected cells were washed by 1X PBS. Transfected cells were flash frozen by putting the cell plates on to liquid nitrogen to make RNA isolation and Real Time RT-PCR.

3.6. Infection of MCF10A Cell Line

$2X10^5$ MCF10A cells were plated one day in 6 well plate before infection. MSCV (control), Notch1-ICD expressing MSCV-NICD plko-shGFP (control), and plko-shIRF6 shRNA viruses were used. MCF10A cells were infected by these viruses and four different conditions were created that were MSCV-plko-shGFP (control-control), MSCV-plko-shIRF6 (control-shIRF6), MSCV-NICD-plko-shGFP (NICD-control) and MSCV-NICD-plko-shIRF6 (NICD-shIRF6).

To infect cells, medium mixture including viruses, MCF10A medium and polyprene (Sigma, Cat#107689) at the final concantration of 8μg/mL, was prepared. 24 hours after cells were plated, medium was aspirated and virus-medium mixture was added on cells. Medium was changed with fresh MCF10A medium 24 hours after infection. 72 hours after infection, medium was aspirated, infected cells were washed by 1X PBS.

Infected cells were flash frozen by putting the cell plates on to liquid nitrogen to make RNA isolation and Real Time PCR. Infected cells were harvested from plate to make cell cycle analysis, Annexin-V assay and stem cell determination.

3.7. Semi-Quantitative Real Time RT-PCR (qRT–PCR)

To determine mRNA expression levels of genes of interest, semi-quantitative real time RT-PCR was performed. Frozen cell plate was thawed on ice for 2 or 3 minutes, total RNA of cells was isolated by using Pure-link RNA Mini Kit(Ambion, Cat#12183018A) with PureLink^{TM} DNase (Invitrogen, Cat#12185-010) removing DNA contamination. After RNA isolation, complementary cDNA (cDNA) was synthesized by using Fermantas First Strand cDNA Synthesis Kit (Thermo Scientific, Ca#K1622) from 1μg isolated total RNA and random hexamer primers. cDNA was amplified by using specific forward and reverse primers and Maxima SYBR Green qPCR Master Mix (Thermo Scientific, Cat# KO252) including Taq DNA polymerase, dNTPs and SYBR Green I dye in PCR buffer. PCR amplification was done by using Bio-Rad IQ5 Real Time PCR Detection System.

Means of cycle threshold values (Ct) of IRF6, Notch target Hey2, basal markers; CD24 and K18, luminal markers; ITGA6, ITGB4 and K14 were normalized by Ct values of housekeeping gene TATA box binding protein (TBP). Then, mRNA level were calculated with using delta-delta Ct method. Student t-test method which was unpaired was used for statistical analysis.

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

Hey2_forward primer	5'-AAGATGCTTCAGGCAACAGG-3'
Hey2_reverse primer	5'-GCACTCTCGGAATCCTATGC-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'
ITGA6_forwad primer	5'-AAAGCAACCATTTCCCATTG-3'
ITGA6_reverse primer	5'-AAAGCAACCATTTCCCATTG-3'
ITGB4_forward primer	5'-ACTGACCCGCTCAGAACACT-3'
ITGB4_reverse primer	5'-CTCCTGCCAGCTCACTCTG-3'
K18_forward primer	5'-CACAGTCTGCTGAGGTTGGA-3'
K18_reverse primer	5'-GAGCTGCTCCATCTGTAGGG-3'
K14_forward primer	5'-CACAGCCACAGTGGACAATG-3'
K14_reverse primer	5'-GGCTCTCAATCTGCATCTCC-3'
CD24_forward primer	5'-CTGCTGGCACTGCTCCTAC-3'
CD24_reverse primer	5'-ACCACGAAGAGACTGGCTGT-3'

3.8. Propidium Iodide Stain

In order to determine cell cycle phases, Propidium iodide stain was performed. 1 hour before experiment was started, 1X PBS and 100% ethanol were put at -20^{0}C. At first day, cells were harvested by using trypsin from plates and collected in falcons. Falcons were centrifuged at 1200 rpm for 10 minutes. Then, supernatants were aspirated and falcons were placed on ice. 1 ml cold PBS was added in each falcons, and mixed by pipetting very gently. After that, 4 ml 100% cold ethanol was added in each falcon and again mixed by pipetting very gently. Falcons were placed at -20^{0}C for at least overnight, and up to at most 1 month. At second day, cells were spun down at 1500 rpm, for 10 minutes at 4^{0}C and at 2000 rpm, for 1 minute. Supernatants were aspirated and 1 ml PBS were added in each falcon, mixed by pipetting well. Then, solutions in falcons were

transferred to eppendorf tubes. Tubes were spun down at 1500 rpm, for 10 minutes at 4^0C. Supernatants were aspirated. 200µl 0,1% triton X-100 PBS and 20µl Rnase A (200µg/ml) were added to each tube and mixed by pipetting well. Cells were incubated at 37^0C for 30 minutes. After that, 20µl PI (1mg/ml) were added on cells and mixed by pipetting well. Cells were incubated at room temperature for 15 minutes in the dark. Cells were analyzed by using BD original software with BD FACS Canto flow cytometry. Unpaired student t-test method was used for statistical analysis.

3.9. Annexin-V Assay

In order to determine apoptotic cells, Annexin-V assay was performed for Notch activation and IRF6 knock down experiments in MCF10A. Cells were harvested by using trypsin from plates and collected in falcons. Falcons were spun down at 800 rpm for 5 minutes and supernatants were aspirated. Then, 1X cold PBS were added on cells and mixed by pipetting well. Cells were spun down at 800 rpm for 5 minutes and supernatants were aspirated.1X Annexin-V binding buffer were added on cells and cells were resuspended well in solution. Annexin V Assay kit (BD Pharmingen, Cat#559763) was used and cells were stained by Annexin-V and 7-AAD dyes. Cells were incubated with dyes for 15 minutes at room temperature in the dark. 1X binding buffer were added on cells and mixed well. Cells were analyzed by using BD original software with BD Facs Canto flow cytometry. Paired student t-test method was used for statistical analysis.

3.10. Stem Cell Markers

Cells were harvested by using trypsin, washed with PBS-CS including New Born Calf Serum (Gibco, Cat#16010-159) and spun down at 1400 rpm for 2 minutes. 500.000 cells were resuspended in 1X PBS. Cells were mixed with CD44 antibody (Thermo, Cat# MA1-10229, FITC conjugate, 2µg/ml) and CD24 antibody (Thermo, Cat# MA1-10154, PE conjugate, 20µl/10^6 cells) and incubated at room temperature in the dark for 30 minutes. At 15^{th} minutes of incubation and end of the incubation, solutions were mixed by pipetting well. Then, cells were washed with 1X PBS and were spun down at 1400

rpm for 2 minutes. Supernatants were removed. Cells were fixed with PBS containing 1% paraformaldehyde. Then, cells were analyzed by using BD original software with BD Facs Canto flow cytometry. Unpaired student t-test method was used for statistical analysis.

CHAPTER 4

RESULTS

4.1. Notch Activation and IRF6 Knockdown was Achieved in Experimental Conditions

To see IRF6 effect under Notch signaling, two different groups were constructed. In the first group, Notch which induces IRF6 expression was activated in normal breast epithelial cells. In the second group, IRF6 was inhibited in Notch induced cells to see whether absence of IRF6 has any effect on Notch induced cellular processes.

In order to determine whether IRF6 has an effect on cell cycle regulation, apoptosis and differentiation under Notch signaling, four different conditions were prepared with double infections and different assays were applied. The first condition is control in which MCF10A cells were infected with control retro-viruses (MSCV) and control lenti-viruses (plko-shGFP); control-control. In the second condition, MCF10A cells were infected with control retro-viruses and shIRF6 lenti-viruses that encodes shRNA against IRF6 (plko-shIRF6); control-shIRF6. In these cells, IRF6 was knockdowned. In the third condition, these cells were infected with MSCV-NICD retro-viruses that activates Notch1 signaling through expressing Notch1 Intra-Cellular Domain and control lenti-viruses; NICD/control. So Notch1 was activated. In last condition, cells were double infected with MSCV-NICD retro-viruses and plko-shIRF6 lenti-viruses; NICD-shIRF6. So in these cells, Notch1 was activated while IRF6 was knockdowned.

In order to test whether the Notch activation and IRF6 knock-down were achieved in experimental conditions, mRNA analysis was done 72 hours after infection. IRF6 and Notch direct target gene Hey2 were controlled by semi-quantitative Real Time RT-PCR (qRT–PCR).

mRNA level of Notch target Hey2 were increased in both NICD/control and NICD/shIRF6 conditions by 180 fold and 40 fold, respectively (Figure 1.1 A). When NICD/control and NICD/shIRF6 were compared with each other, Hey2 expression level was reduced 4,5 fold in NICD/shIRF6, however this decrease is not significant.

According to Hey2 results, Notch activation was achieved in MCF10A cells by using NICD virus.

When control/control and control/shIRF6 were compared with each other, mRNA level of IRF6 was decreased more than 3 folds in control/shIRF6. On the other hand, mRNA expression of IRF6 was increased nearly 2 folds in NICD/control, as expected. When NICD/shIRF6 was compared with NICD/control, IRF6 expression was significantly reduced by 5 folds. (Figure 4.1 B) According to this result, IRF6 silencing was achieved by using shIRF6 virus.

All in all, expression levels of Hey2 and IRF6 results showed that we successfully generated the experimental conditions.

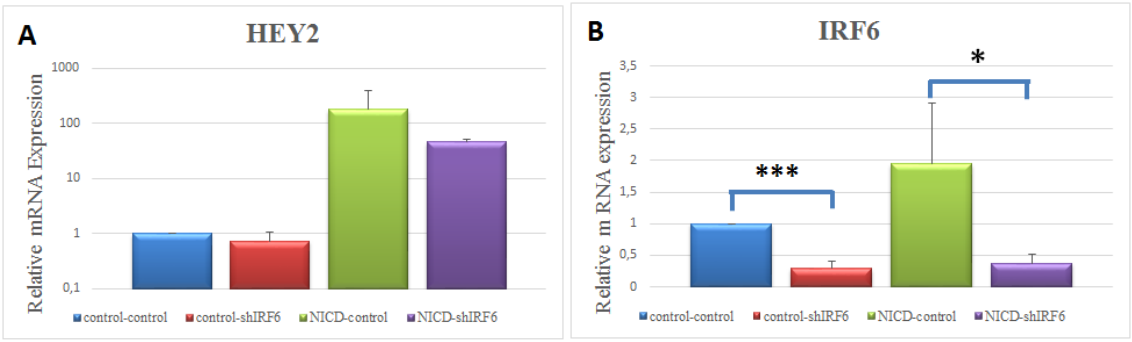


Figure 4.1. Real Time RT-PCR results of Notch1 activation and IRF6 knock-down in MCF10A cells. At 72 hours after infection, mRNA expression levels of A) Hey2 and B) IRF6 were measured. Three independent experiments were performed. (* $p<0.05$, *** $p<0.005$)

4.2. Notch Induced Cell Proliferation is Reduced by IRF6 Knockdown in MCF10A

In order to determine the role of IRF6 on cell proliferation under Notch signaling pathway, IRF6 was knocked down in Notch activated and normal MCF10A as explained in section 4.1.

In order to determine cell proliferation capacities, Propidium iodide stain assay were performed. Percentages of cells in G1, S, and G2/M phases were measured. To see effects of Notch activation and IRF6 inhibition on cell proliferation, infected MCF10A cells were stained 72 hours after infection.

When control/control was compared with NICD/control, the percentage of cells at S phase increased in NICD/control condition from 12% to 15% (Figure 4.2 A). When control/shIRF6 and control/control conditions were compared with each other, in control/shIRF6, cell percentage at S phase was reduced from 12% to 8,5% (Figure 4.2 A). Percentage of cells at S phase decreased from nearly 15% to 11% in Notch/shIRF6 compared with Notch/control significantly.

Percentage of cells at G1 increased by IRF6 knockdown while decreased by Notch activation (Figure 4.2 B). When control/shIRF6 was compared with control/control, percentage of cells at G1 phase significantly increased in control/shIRF6 from 72% to 78%. When NICD/control was compared with control/control, cell percentage at G1 phase decreased from 72% to 69%. When NICD/control and NICD/shIRF6 conditions were compared, percentage of cells at G1 phase increased significantly in NICD/shIRF6 condition from 69% to 76% (Figure 4.2 B).

In control/shIRF6 condition, percentage of cells at G2/M phase reduced significantly from 15.5% to 12.5% when compared with control/control condition. When NICD/control and NICD/shIRF6 were compared, percentage of cells at G2/M phase reduced in NICD/shIRF6, but it did not reach to significant levels. (Figure4.2 C)

Propidium iodide stain showed that Notch activation induced cell proliferation while IRF6 knockdown reduced cell proliferation. Results especially at S and G1 phases shows that IRF6 under Notch activation in MCF10A cells is important in cell cycle progression and cell proliferation.

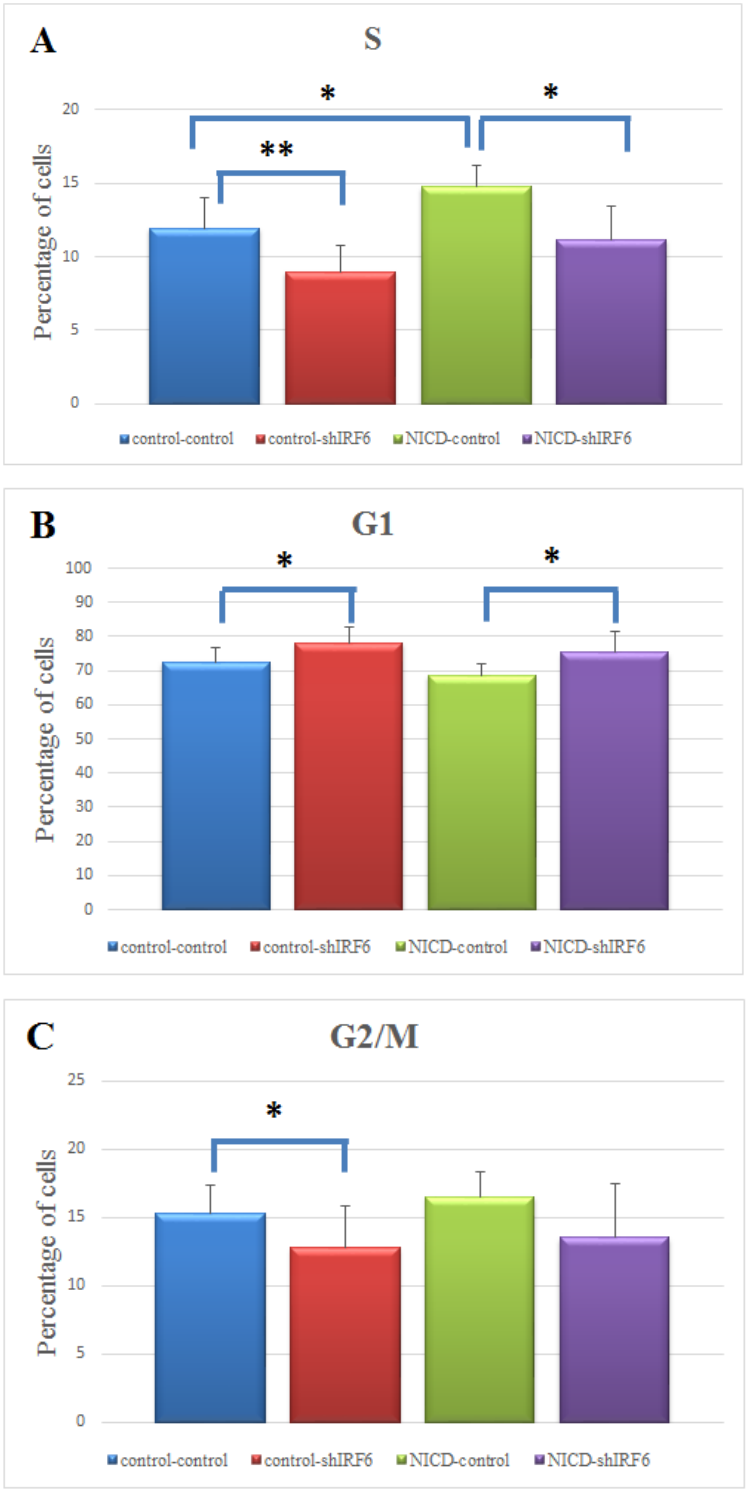


Figure 4.2. Results of cell cycle analysis. At 72 hours after infection, infected cells were stained with propidium iodide for cell cycle phase determination. Percentage of cells at (A) S phase (B) G1 phase and (C) G2/M phase were shown. Four independent experiments were performed. (* $p<0.05$, ** $p<0.01$)

4.3. Notch Activation and IRF6 Knockdown Induce Apoptosis in MC10A Cells

In order to determine whether IRF6 has an effect on apoptosis under Notch signaling, IRF6 was knockdown in Notch activated and normal MCF10A cells. 72 hours after infection, apoptosis assay was performed. Cells were stained by using PE-conjugated Annexin-V stain and 7-AAD stain.

Percentage of early apoptotic cells was significantly increased in control/shIRF6 and NICD/control conditions by 1,2 fold and nearly 2 fold respectively (Figure 4.3 A). Notch activation and IRF6 knockdown cause early apoptosis in MCF10A cells. IRF6 knockdown in Notch induced MCF10A cells increased number of early apoptotic cell number when it was compared with Notch induced MCF10A cells but this change was not significant (Figure 4.3 A). According to these results, IRF6 could not be said to have an effect on early apoptosis down stream of Notch.

Number of late apoptotic cells was increased in control/IRF6 and NICD/control conditions by 1,1 fold and 1,4 fold. These increases were not significant (Figure 4.3 B). When NICD/shIRF6 was compared with NICD/control, increase was not observed significantly (Figure 4.3 B). These data showed that Notch activation or IRF6 knockdown has no effect on late apoptosis.

When NICD was activated in MCF10A, percentage of necrotic cells was decreased dramatically by nearly 2 fold (Figure 4.3 C). On the other hand, IRF6 knockdown increased necrotic cell number in MCF10A from 1% to 1,3% (Figure 4.3 C). When NICD/shIRF6 was compared with NICD/control, percentage of necrotic cell increased insignificantly by 1,5 fold in NICD/shIRF6 (Figure 4.3 C). These results illustrates that IRF6 in Notch induced MCF10A cells can have an effect on the protection from necrosis.

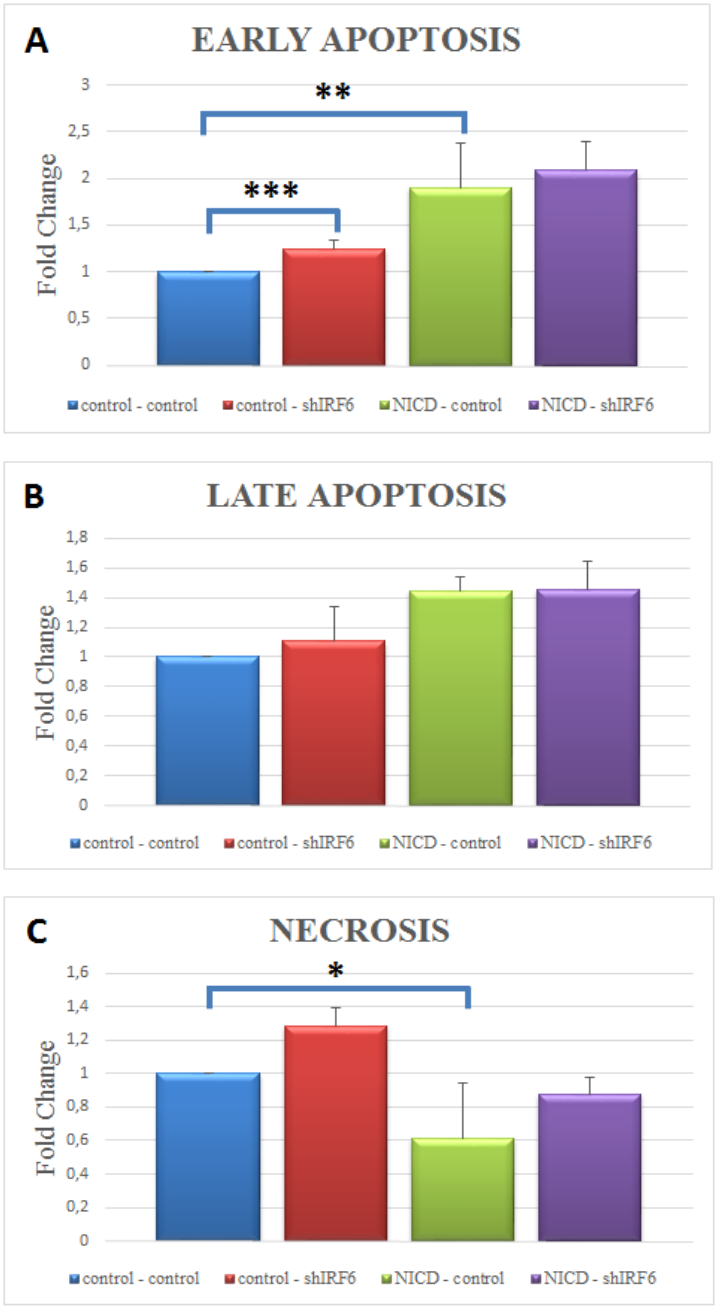


Figure 4.3. Apoptosis Assay results. 72 hours after infection, cells were harvested and apoptosis was analyzed using PE-conjugated Annexin-V. Four independent experiments were performed. A) Early apoptotic B) late apoptotic and C) necrotic cell population percentages were shown. (* $p<0.05$, ** $p<0.01$, *** $p<0.005$)

4.4 IRF6 Knockdown Reduce Stem Cell Population in MC10A Cells

In order to identify whether IRF6 knockdown in Notch activated and normal MCF10A cells plays a role on stem cell population, CD24 and CD44 stem cell surface markers were used (Figure 4.4 A). $CD24^{-}/CD44^{HIGH}$ population is determined as cancer stem cell population in breast (Al Hajj et al. 2003, Farnie et al. 2010).

$CD24^{-}/CD44^{HIGH}$ population was decreased significantly from 16% to 10% in control/shIRF6 (Figure 4.4 B). In control/NICD, it was increased from 16% to 20%, but this increase did not reach to significant levels. In NICD/shIRF6, 2% reduction was observed compared to NICD/control but this change was not statistically significant.

The percentage of $CD24^{-}/CD44^{HIGH}$ population was reported between 2% - 1,83% in MCF10A cells (Yu et al. 2013,Gonzales et al. 2014). Our $CD24^{-}/CD44^{HIGH}$ population was much higher than this ratio. As a second analysis, we determined another region, which has the highest CD44 expression (R2 in Figure 4.4 B). This population (labeled as stem cells) was decreased from 2.3% to 1% in control/shIRF6 significantly and to 2% in NICD/control, which was not statistically significant. In addition, there was no difference between NICD/control and NICD/shIRF6 (Figure 4.4 B).

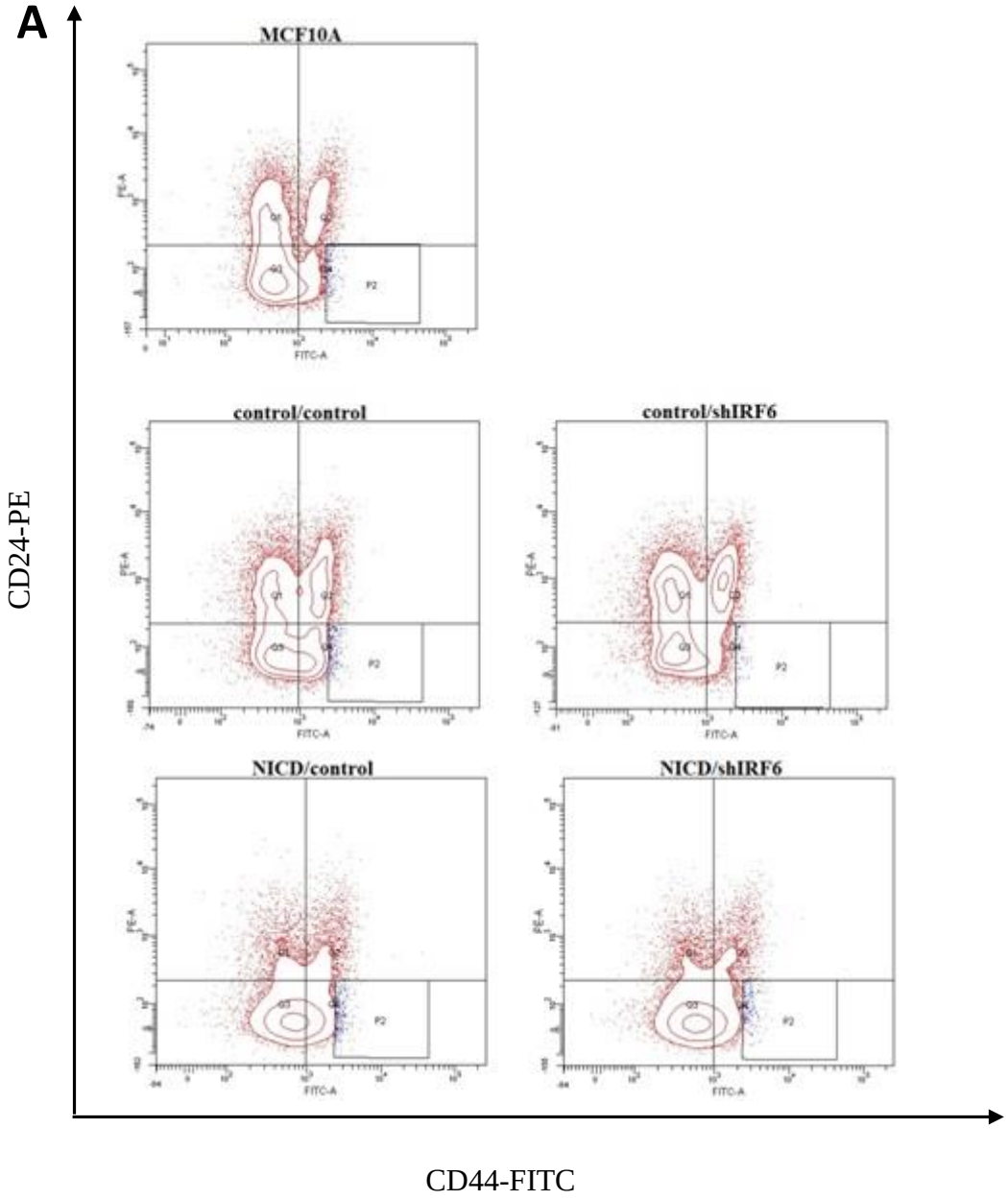
Two different regions, R2 and $CD24^{-}/CD44^{HIGH}$ gave similar results. In conclusion, IRF6 has essential significant reducing effect on stem cell population in MCF10A cells. However, no significant effect of IRF6 downstream of Notch could be determined.

Notch activation significantly increased $CD24^{-}/CD44^{LOW}$ population from 26% to 45% (Figure 4.4 B). IRF6 knockdown increased these cell numbers but has not significantly. When NICD/shIRF6 was compared with NICD/control, percentage of $CD24^{-}/CD44^{LOW}$ cell population increased from 45% to 54% but this was not significant change. This population could be considered as less differentiated population, since it has lower expression of both luminal CD24 and basal CD44. Notch activation in MCF10A increased undifferentiated cell number significantly (Figure 4.4 B).

Percentage of double positive population, $CD24^{+}/CD44^{HIGH}$, was decreased in control/shIRF6, NICD/control and NICD/shIRF6. Notch activation significantly reduced $CD24^{+}/CD44^{HIGH}$ population from 27.70% to17.57% (Figure 4.4 B). This cell population could be considered as bipotential.

Percentage of more luminal-like cell population; $CD24^{+}/CD44^{LOW}$ was increased from 30% to 37.33% in control/shIRF6 but decreased in NICD/control when compared with control/control (Figure 4.4 B).

All in all, IRF6 knockdown reduces cancer stem cells, and increases luminal-like cell population. Notch activation reduces luminal-like cell and double positive populations while Notch activation has a positive effect on undifferentiated cells formation. IRF6 has slight effects downstream of Notch on cancer stem cells, basal-like cells, luminal-like cells and undifferentiated cells but these result values were not found to be significant. Therefore, it could be said that IRF6 does not have any function downstream of Notch in this context.



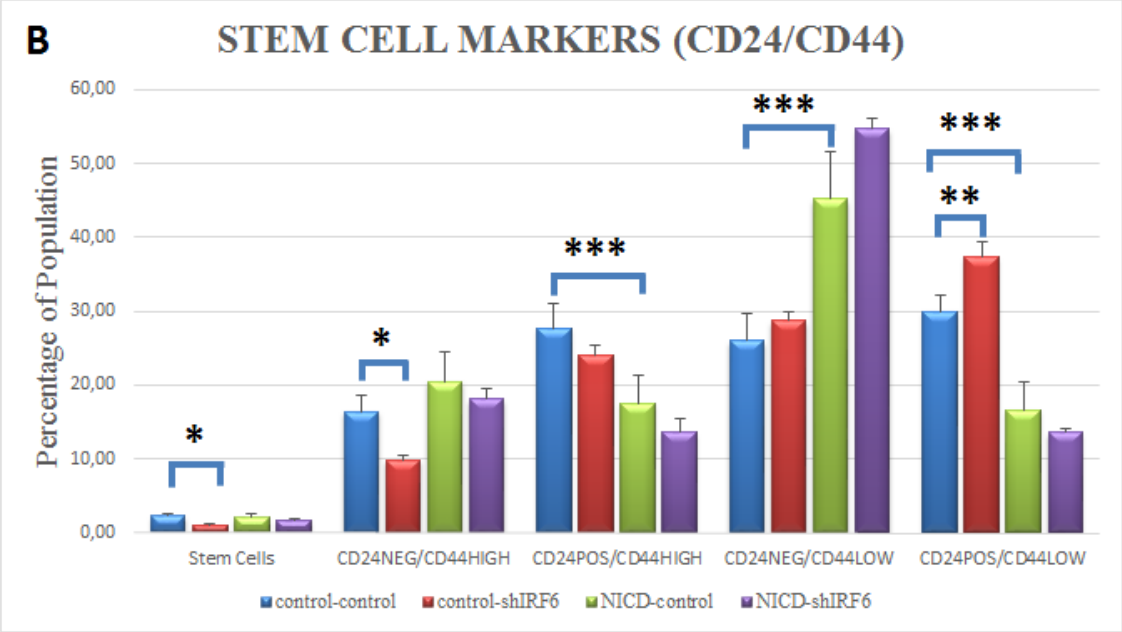


Figure 4.4. Results of CD24 and CD44 stem cell surface. 72 hours after infection, infected cells were treated with antibodies against CD24 and CD44 stem cell surface markers to detect breast cancer stem cell population. A) FACS analysis to measure CD24 and CD44 expressions in incefted cells B) Percentage of stem cells, $CD24^{-}/CD44^{HIGH}$, $CD24^{+}/CD44^{HIGH}$, $CD24^{-}/CD44^{LOW}$and $CD24^{+}/CD44^{LOW}$ cells were shown. (* $p<0.05$, ** $p<0.01$, *** $p<0.005$)

4.5. IRF6 Knockdown Alters Expressions of Basal and Luminal Markers in MCF10A

In order to determine whether IRF6 has an effect on luminal and basal cell characteristics, IRF6 was knocked down in MCF10A. MCF10A, normal breast epithelial cell line, shows basal-like properties and expresses IRF6, so it was used in this experiment (Bailey et al. 2008).

72 hours after infection, total mRNA were isolated and RT-PCR were performed. ITGB4, ITGA6 and K14 were used as basal cell markers, while CD24 and K18 were used as luminal markers.

In basal markers, expression of ITGA6 and K14 basal cell markers were induced in IRF6 knocked down MCF10A cells by 0,5 fold and 5 fold respectively (Figure 4.5 A).

On the other hands, expression of ITGB4 basal cell marker was reduced by nearly 2 fold in IRF6 knocked down MCF10A cells (Figure 4.5 A). These are not significant values.

When luminal markers expression level were measured, expression of K18 luminal marker was reduced in IRF6 knocked down MCF10A but expression of CD24 luminal marker was induced more than two folds significantly (Figure 4.5 B).

IRF6 expression level was decreased nearly 5 folds, and this change was significant (Figure 4.5 C).

All in all, although IRF6 has a significant role for expression of CD24 luminal markers, it cannot be said IRF6 has an effect on the maintenance of basal character or differentiation into luminal character in MCF10A.

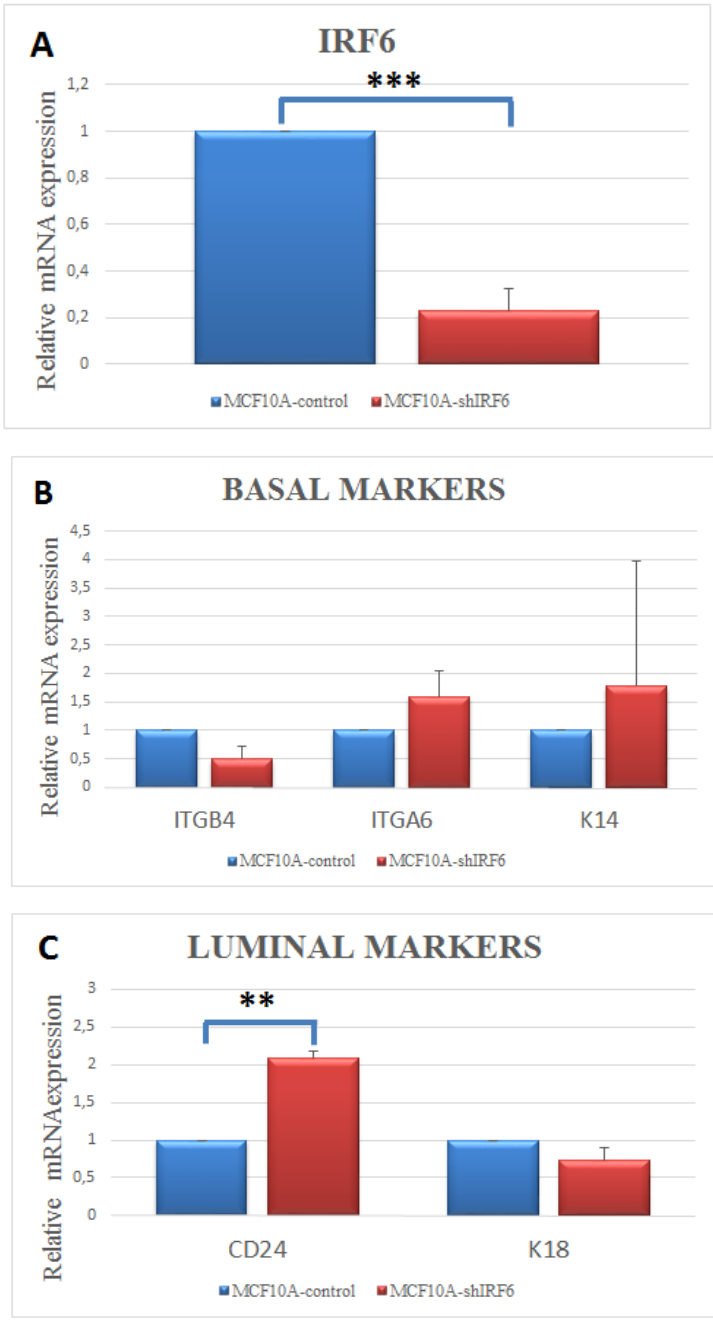


Figure 4.5. Real Time RT-PCR results of IRF6 knock-down in MCF10A cells. MCF10A cells were infected with control and shIRF6 lenti-viruses. 72 hours after infection mRNA A) for basal markers; ITGB4, ITGA6 and K14 B) for luminal markers; CD24 and K18 C)IRF6 were analyzed by Real Time RT-PCR. Three independent experiments were performed. (** $p<0.01$, ***$p<0.001$)

4.6. IRF6 Overexpression Alters Expressions of Basal and Luminal Marker Levels in MCF7

In order to determine whether IRF6 has an effect on luminal and basal cell characteristics in MCF7, IRF6 was overexpressed in MCF7 by transfection with PCMV-XL4-IRF6 overexpression vector. MCF7 breast cancer cell line is used because it is a luminal-like cell line and does not express high levels of IRF6 (Bailey et al. 2005).

72 hours after transfection, total mRNA were isolated and qRT-PCR were performed. ITGB4, ITGA6 and K14 were used as basal cell markers, while CD24 and K18 were used as luminal markers.

According to RT-PCR results, IRF6 expression level was increased 6000 folds, as expected, and this change was significant. Levels of luminal and basal markers expression in IRF6 over expressed MCF7 changed very small amount and these changes were not significantly (Figure 4.6 A, B).

All in all, although, MCF7 cells were transfected with pCMV6-XL4-IRF6, it was seen that IRF6 did not significantly affect levels of luminal and basal cell markers in MCF7 cell line.

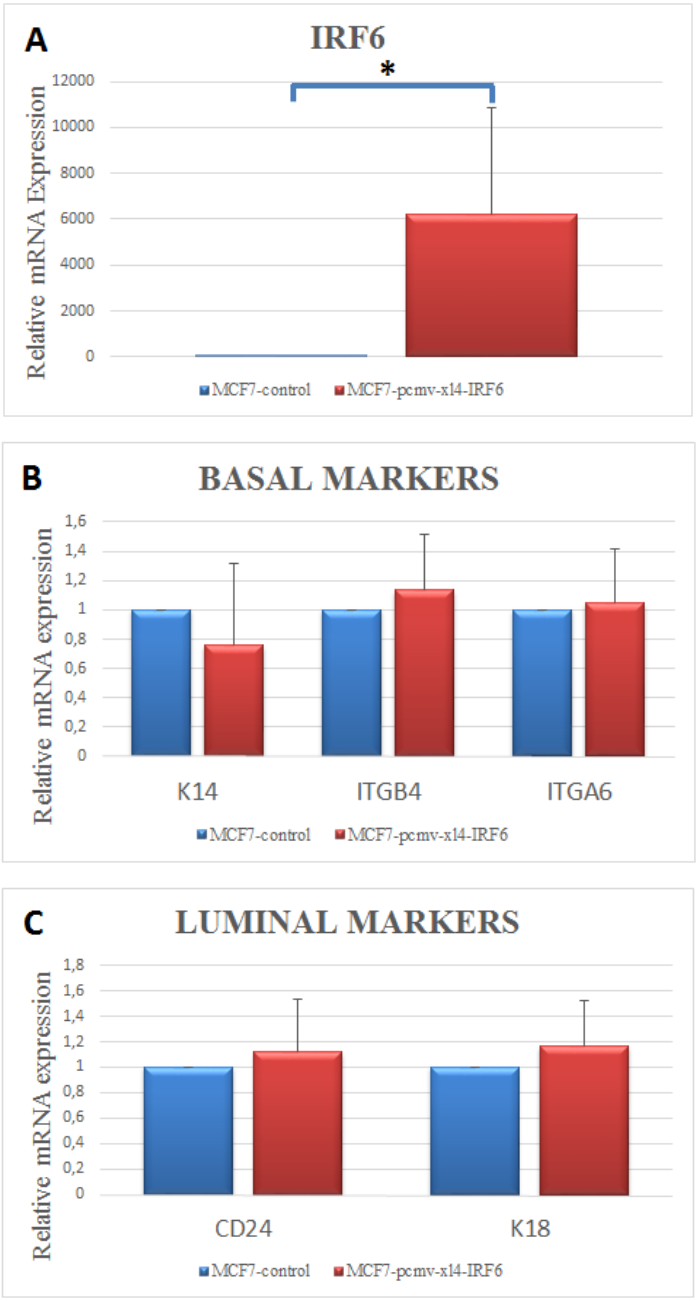


Figure 4.6. Real Time RT-PCR results of IRF6 overexpression in MCF7 cells. MCF7 cells were transfected with control and pcmv-xl4-IRF6 plasmids. 72 hours after transfection mRNA expression levels A) for basal markers; ITGB4, ITGA6 and K14 B) for luminal markers; CD24, K18 and C) IRF6 were analyzed by Real Time RT-PCR. Five independent experiments were performed. (* $p<0.05$)

CHAPTER 5

DISCUSSION & CONCLUSION

Notch signaling pathway is active and NICD is over expressed in highly aggressive tumor cells for their survival (Wang et al. 2006, Santagata et al. 2004, Balint et al. 2005). It has been firstly found that abnormal Notch expression is observed in many cancer types since active Notch1 expression was seen to cause human T-ALL (Reynolds et al. 1987). In addition to its oncogenic role in many cancer types, Notch also has a role as tumor suppressor in skin. In breast cancer, first evidence was found with MMTV insertion in Notch4 locus that shows over expression of truncated active Notch4 proteins in mammary adenocarcinomas because of this insertion (Robbins et al. 1992, Uyttendaele et al. 1996). On the other hand, it was shown that Notch activation can have a negative effect on cell proliferation in human breast epithelial cells (Yalcin-Ozuysal et al. 2010, Mazzone et al. 2010). Different cell lines can be affected differently by Notch signaling. Notch signaling roles have not been found yet clearly that it work as tumor suppressor or oncogenic on which cell lines and conditions. However, it has been known that oncogenic or tumor suppressor roles of Notch signaling change depending on cell types and dosage of Notch expression (Yalcin-Ozuysal et al. 2010, Mazzone et al. 2010).

Notch signaling has different downstream effectors acting on different cellular processes in breast cancer cells. Myc (Palomero et al. 2006) and Cyclin D1 (Cohen et al. 2010) are mediators of Notch induced cell proliferation and survival. PI3K/Akt pathway (Uemura et al. 2010) and Survivin (Lee et al. 2008) are activated by Notch signaling to protect cells from apoptosis. Identification of Notch and its mediator relationship is important to identify clearly Notch signaling pathway.

It has been shown that IRF6 expression is directly regulated by Notch and is involved in Notch induced differentiation in keratinocytes (Restrivo et al. 2011). Then, we showed that IRF6 expression is regulated by Notch signaling and it is a mediator of Notch in proliferation and transformation in breast epithelial cells (Zengin et al. 2015). Therefore, in this study, it was aimed to find whether IRF6 is a player regulated by Notch in cell cycle regulation, apoptosis and stem cell population in normal breast epithelial cells.

To determine IRF6 effect under Notch, conditions of absence or presence of IRF6 in Notch activated cells were prepared. Therefore, four different conditions were generated by double infections.

Firstly, expression level of IRF6 and Notch target gene Hey2 were measured to see whether infection were done proper or not. Notch1 activation was determined with induced Notch target gene Hey2 expression and IRF6 knock down was determined with reduced IRF6 expression. According to these results, infections work properly and expected experimental models, which reflect conditions with active Notch signaling in the presence or absence of IRF6, were generated.

IRF6 knockdown reduced expression level of Hey2 in normal and Notch induced MCF10A cells but these data were not significant. IRF6 could be upstream of Notch, regulate Notch signaling activity or IRF6 could independently regulate expression of Hey2. It was not expected result because in the literature, there is no data showing that IRF6 can act upstream of Notch signaling or has an independent effect on Hey2. It was not observed in our previous experimental sets as well (Zengin et al. 2015). To understand the reason of this, other Notch target genes can be checked whether there is similar significant change or it can be checked whether IRF6 can independently bind and activate Hey2 promoter.

Notch has a role in induction of cell proliferation and cell transformation in MCF10A cells (Mazzone et al. 2010, Rustighi et al. 2009, Stylianou et al 2006) and Notch activation increases cell proliferation in breast (Guo et al. 2011). When IRF6 was activated in breast cancer cells, cell cycle arrest was observed (Bailey et al. 2008). Propodium iodide stain results showed that Notch activation significantly increases while IRF6 knockdown in these cells significantly decreases percentage of cells at S-phase in MCF10A. In parallel cell percentage at G1 phase is increased in Notch activated cells, when IRF6 was silenced. This data is in parallel with our previous observations showing that BrdU incorporation was reduced in the same conditions (Zengin et al. 2015). Thus, we confirmed our conclusion that IRF6 is required downstream of Notch signaling to induce cell proliferation.

Propidium iodide stain results showed that IRF6 silencing alone significantly reduced cell percentage at S-phase and G2/M phase and increased cell population at G1 phase. IRF6 was shown to induce cell cycle arrest in breast cancer cells. It was suggest that IRF6 has a tumor suppressor role in breast cancer (Bailey et al. 2008). According to these results, IRF6 effect in MCF10A cells does not compatible with result of Bailey

group. It could be concluded that IRF6 has a tumor promoting role in MCF10A cells because IRF6 knockdown decreased cell proliferation. IRF6 dosage could be an effect on cell proliferation. In our study, IRF6 knockdown effect was measured at short time after infection. We demonstrated that IRF6 has an oncogenic effect in short term studies we performed, but it is possible that we could observe tumor suppressor activities of IRF6 if we perform long time experiments.

Apoptosis assay was applied for four conditions to determine IRF6 role under Notch signaling on apoptosis. When results of NICD-control and NICD-shIRF6 conditions were compared with eachother, there were not significant differences in rates of early or late apoptosis and necrosis between NICD-control and NICD-shIRF6 conditions. Thus, we can conclude that IRF6 has no specific role downstream of Notch in apoptosis.

Interestingly, Notch activation increased early apoptosis significantly. Notch activation was shown to reduce apoptosis through transactivation of p53 target genes or upregulation of survivin (Lee et al. 2008, Kim et al. 2007). It has been shown in a few studies that Notch activation can increase or decrease proliferation of cells depending on activation dosage or cell type (Mazzone et al.2010, Yalcin-Ozuysal et al. 2010). Similarly, in our experimental conditions the level of Notch activation could have induced apoptosis in some cells. On the other hand, Notch activation increases percentage of cells significantly at S phase. It could be that high dose NICD causes DNA damages in S phase. It is also known that Notch activation decreases cell adhesion molecules ITGA6, ITGB1 and ITGB4 (Yalcin-Ozuysal et al. 2010), which may indirectly induce apoptosis.

IRF6 knockdown also increased early apoptotic cells. This data was parallel with pro-tumorigenic role.

According to some previous studies, cell proliferation with Notch activation depending on its dosage is increased (Mazzone et al. 2010) while cell proliferation by IRF6 expression is decreased (Bailey et al. 2008). Therefore, it is expected that Notch activation and IRF6 knockdown decrease necrotic cell number in MCF10A cells. According to our results, Notch activation decreased significantly necrotic cell percentage while IRF6 knockdown increased. IRF6 knockdown in Notch induced MCF10A cells induced necrosis compared to Notch induced MCF10A cells but this data was not significant. These results showed that IRF6 has an oncogenic role in cells and necrosis is decreased by IRF6 in MCF10A cells. On the other hand, role of IRF6 under Notch could not be determined in necrosis.

Surface marker profile of breast cancer stem cells (BCSCs) are $CD24^{-/LOW}/CD44^{+}$. In recent studies, in BCSCs, high Notch activation was observed (Dontu et al. 2004).

Notch4 activity was increased in BCSCs enrich population in breast cancer cell lines, while Notch1 activity was less (Harrison et al 2010). Inhibition of both Notch1 and Notch4 signaling were shown to inhibit BCSC activity in vivo (Harrison et al. 2010). In our experiments, we observed that Notch1 activation did not make a significant change in stem cell population in MCF10A cells. Relevant receptor could be Notch4 and there is more data showing that Notch4 is functionally important for BCSC activity, but not Notch1. IRF6 knockdown in MCF10A reduces significantly stem cell population, which suggests that IRF6 play a role as oncogene in MCF10A cells. It can be involved in maintenance of BCSCs but IRF6 role in BCSCs formation should be examined in different breast cancer cell lines.

Notch activation in MCF10A increases significantly percentage of undifferentiated cell population ($CD24^{-}/CD44^{LOW}$) while decreasing significantly percentage of bipotential ($CD24^{+}/CD44^{HIGH}$) and luminal cell ($CD24^{+}/CD44^{LOW}$) populations. These results showed that Notch activated cells are kept in undifferentiated state parallel with its function in progenitor population.

Role of IRF6 in keratinocytes is identified, according to in vivo and in vitro experiments, IRF6 suppresses cell growth and induces keratinocytes differentiation. (Moretti et al. 2010, Thomason et al. 2010). To determine IRF6 role in expression of luminal and basal marker in breast, IRF6 was knocked down in MCF10A and IRF6 overexpressed in MCF7. Breast epithelium include two different cell types; basal and luminal. While CD24 and cytokeratin 18 (K18) are luminal cell markers, ITGB4, ITGA6 and K14 are basal cell markers (Jones et al. 1991, Sonnerberg et al. 1991).

Except CD24 increase in IRF6 silenced MCF10A cells, there was no significant effect on expression of luminal and basal markers. This CD24 expression increase is parallel with flow cytometry data. IRF6 knockdown significantly increased $CD24^{+}/CD44^{LOW}$ population. These data shows that IRF6 knock down in MCF10A causes induction of expression of CD24 luminal cell. But it could not be said whether IRF6 has a significant role on cell fate determination in MCF10A cells.

IRF6 has a tumor suppressor role in breast tissue (Bailey et al. 2008). Therefore, it was thought that IRF6 effect on oncogenic and tumor suppressor features of Notch signaling (Mazzone et al.2010, Yalcin-Ozuysal et al. 2010). In keratinocytes, it has been found that IRF6, as a tumor suppressor, is a mediator of Notch signaling (Restivo et al.

2011). Then, we have shown that IRF6 is a mediator of Notch signaling in proliferation and transformation in normal breast epithelial cells (Zengin et al. 2015).

In our research, IRF6 have an oncogenic activity in MCF10A cells that does not correlate with tumor suppressor role. It has a role downstream of Notch in cell cycle regulation and proliferation. A novel role of IRF6 was identified in apoptosis and stem cell population independent of Notch.

To determine clearly oncogenic role of IRF6, new experiments should be done in different breast cancer cell lines by over-expressing or silencing IRF6 and examining BCSCs or apoptosis rates.

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