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Ph.D. THESIS

EFFECTS of NKILA SILENCING on HSP90, NF-κB, and β-CATENIN in MCF-7 CELLS

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
%	: Percentage
ºC	: Celsius degree
C	: C terminal domain
CO_2	: Carbondioxide
Δ	: Delta
κ	: Kappa
M	: Molar
mM	: Milimolar
m/v	: Mass/volume ratio of the concentrations in the solution
NH_2	: Amino radical group
P	: Phosphor
S	: Serine
T	: Threonine
Y	: Tyrosine
µ	: Micron
µl	: Microliter
U	: Units
v/v	: Volume/volume ratio of the concentrations in the solution

Abbreviation	Explanation Akt
	: Protein kinase B
ANOVA	: Analysis of variance
APS	: Ammonium persulphate
ATCC	: American type culture collection
BC	: Breast cancer
BCA	: Bicinchoninic acid assay
bp	: Base pair
CCAT2	: Colon cancer-associated transcript 2
circRNA	: Cicular RNA

CK1α : Casein kinase 1α
Ct : Cycle threshold
DMEM F-12 : Dulbecco's modified Ham's F-12
DNA : Deoxyribonucleic acid
E-cadherin : Epithelial cadherin
EDTA : Ethylenediamine tetra-acetic acid
EMT : Epidermal to mesenchymal transition
ER : Estrogen receptor
FADD : Fas-associated protein with dead domain
GAPDH : Glyceraldehyde 3-phosphate dehydrogenase
GSK-3β : Glycogen synthase kinase 3 β
HCl : Hydro chloric acid
HOTAIR : HOX antisense intergenic RNA
HOX : Homeobox gene
HSP : Heat shock protein
HSP90 : Heat shock protein 90
IC_{50} : Half maximal inhibitory concentration
Ig : Immunoglobulin
IKK : IκB kinase
IκB : Nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor
JAK/STAT : Janus kinase/signal transducer and activator of transcription
LEF : Lymphoid enhancing binding factor
Log : Logaritm
kDa : Kilodalton
LncRNA : Long non-coding rna
MALAT : Metastasis-Associated in Lung Adenocarcinoma Transcript 1
mRNA : Messenger ribonucleic acid
min : Minute
miRNA : Micro RNA
MAPK : Mitogen activated protein kinase
MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide
N : N terminal domain
NF-κB : Nuclear factor of kappa b
NKILA : NF-κB interacting lncRNA

nM : Nanomolar
nm : Nanometer
NTC : No template control
ORF : Open reading frame
PCR : Polymerase chain reaction
pH : Power of hydrogen
PI3K : Phosphatidylinositol-3-kinase
piRNA : PIWI-interacting RNA
PKA : Protein kinase A
PMSF : Phenylmethylsulfonylfluoride
PVDF : Polyvinylidene difluoride
RHR : Rel homology domain
RNA : Ribonucleic acid
RT-qPCR : Real time quantitative polymerase chain reaction
SD : Standart deviation
SDS : Sodium dodecyl sulphate
SDS-PAGE : Sodium dodecyl sulphate polyacrylamide gel
siRNA : Small interfering RNA
snRNA : Small nuclear RNA
STAT-3 : Signal transducer and activator of transcription-3
TBS-T : Tris-buffered saline tween-20
TCF : T-cell factor
TEMED : Tetramethylethylenediamine
TNF : Tumor necrosis factor
TNFR : Transmembrane protein tumor necrosis factor receptor
TR : Transfection reagent
TRAF : TNFR-associated factor
Tris-HCl : Trizma-hydrochloride
TLR : Toll-like receptor
Ver : Version
VIM : Vimentin
Wnt : Wingless-related integration site

ÖZET

MCF-7 HÜCRELERİNDE NKILA SUSTURUMUNUN HSP90, NF-*κ*B ve *β*-KATENİN ÜZERİNDEKİ ETKİLERİ

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Meme kanseri günümüzde halen kadınlar arasında en sık rastlanan kanser tipi olup, ölüm oranları oldukça yüksektir. Normalde, kanser tedavisinde onkojenik proteinlere yönelik hedefli terapilere ağırlık verilmiGtir. Ancak hedefli terapilerin baGarısı kanser hücre hattına, hücre yüzeyi markırlarına ve hücrede apoptoz direncinin geliGimine bağlı olarak düGebilir. Yapılan son çalıGmalar sadece proteinlerin değil, kodlamayan-RNA'ların da tüm kanser tiplerinde yer alabileceğini ortaya koymuGtur. LncRNA'lar 200 den fazla baz çiftine sahip, kodlamayan RNA'lardır, mRNA'lara benzemekle beraber onlardan farklı olarak yapılarında ORF dizileri içermezler. Hücrelerdeki rolleri çok çeGitlilik göstermektedir ve birçoğunun proteinlerle etkileGime geçtiği bilinmektedir. NF-κB önemli bir transkripsiyon faktörü olarak kanser geliGimi ile direkt iliGkili bir proteindir ve bir lncRNA türü olan NKILA tarafından kontrol edilebilmektedir. NF-κB'nin negatif düzenleyicisi olarak tanımlanan NKILA hastalarda kötü prognoz ile iliGkilendirilmektedir. Günümüze değin, NKILA'nın çok az sayıda

sinyal molekülü ile iliGkisi ortaya konulmuGtur. Ancak, NKILA'nın diğer onkojenik proteinlerden HSP90α ve β-katenin ile iliGkisi henüz ortaya konulmamıGtır. NF-κB ile bağıntılı olarak çalıGan bu iki molekülün NKILA ile iliGkisinin aydınlatılması, kanser ilerleyiGi hakkında önemli ipuçları sunabilir. NF-κB, Wnt sinyali ve ısı-Goku yanıt sistemi kanser ilerleyiGi için birbiri ile korelasyonlu çalıGabilir, çünkü NF-κB bu iki sinyal ağında da çakıGma proteinleri vasıtasıyla etkili olabilir. Normalde, NF-κB HSP90 ile pozitif korelasyon gösterirken β-katenin ile olan bağıntısında çeliGkili durumlar söz konusudur.

Bu tez çalıGmasında NKILA'nın daha önce incelenmemiG HPS90 ve β-katenin proteinlerinin anlatımları üzerindeki etkileri Western blot ve RT-qPCR yöntemleri kullanılarak araGtırılmıGtır. Elde ettiğimiz sonuçlara göre NKILA inhibisyonu, NF-κB protein kompleksini oluGturan alt ünitelerin ve HSP90 proteinlerinin ekspresyonlarında artıGa, β-katenin ekspresyon seviyesinin ise azalmasına neden olmuGtur. Bu bağlamda, NKILA'nın NF-κB gibi HSP90 ekspresyonu üzerinde de baskılayıcı bir rolü olduğunu söyleyebiliriz. β-katenin ekspresyonundaki azalma ise NF-κB seviyelerindeki artıGtan kaynaklanıyor olabilir. Literatüre daha önce sunulmamıG bu yeni bilgiler bize NKILA'nın moleküler mekanizmasını aydınlatmamızda önemli ayrıntılar verebilir ve ayrıca kanser tedavisi için yararlı yeni hedefler sunabilir.

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Anahtar kelimeler: NKILA, HSP90, NF-κB, β-katenin, MCF-7.

SUMMARY

EFFECTS of NKILA SILENCING on HSP90*α*, NF-*κ*B, and *β*-CATENIN in MCF-7 CELLS

Ph.D. THESIS

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Breast cancer is still a prevalent cancer type among women and has a very high mortality rate. Normally, cancer treatments rely on targeting the oncogenic proteins. However, targeted therapy success can be very low depending on the cancer cell type, cell surface markers difference, and resistance to apoptosis of the cancer cells. Recent discoveries showed that not only proteins but non-coding RNAs are also involved in signalling networks in all cancer types. LncRNAs are one type of non-coding RNA and longer than 200 bp, they are similar to the mRNAs but lack ORF in their structure accordingly does not involve codes for the protein synthesis. However, their role in the cells is so variable but most of them can interact with the proteins. NF-κB is another important transcriptional factor directly related to cancer progression and regulated by NKILA which is another lncRNA. NKILA is defined as a negative regulator of the NF-κB, and NKILA inhibition has been shown as related to poor

prognosis in cancer patients. Up to date, its relation with only a few signals transducing proteins has been elucidated. Nevertheless, NKILA association with other oncogenic proteins HSP90 and β-catenin never shown before and should be enlightened to clarify the role of the transcript which may give us important clues about the cancer prognosis. NF-κB, Wnt signalling, and the HSR system might collaborate to develop cancerous progress because of the strict correlation of NF-κB with the HSP90 and β-catenin. In this case, NKILA might affect the β-catenin and HSP90 expression profiles. β-catenin is a controversial protein because it can collaborate with NF-κB proteins or can be antagonistic to the NF-κB at the same time, but NF-κB is positively associated with the HSP90.

Thus, we aimed to study the association of NKILA with two of these crosstalking molecules using western blot and RT-qPCR methods. Indeed, we found that NKILA inhibition resulted in to increase of NF-κB and HSP90 but a decline in β-catenin levels. We detect the NF-κB and HSP90 upregulation but decline in the β-catenin levels. Therefore, we suggest that NKILA also has a regulatory role on the HSP90 as well as NF-κB, but the decrease of β-catenin can be explained by the catenin- NF-κB cross-regulation during the cell cycle. These novel findings are important to reveal the NKILA mechanism in the cancer cell which can be a helpful therapeutical target.

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Keywords: NKILA, HSP90, NF-κB, β-catenin, MCF-7.

1. INTRODUCTION

1.1. BREAST CANCER

Breast cancer (BC) presents serious challenges to cure, specifically among women. Not only genetic factors, but also environmental factors, and life choices (smoke, nightlife, and alcohol consumption) increase the risk of breast cancer. Researchers have focused on the different treatments of breast cancer but chemotherapeutic approaches are particularly promising (Eccles et al., 2013; Hashemi et al., 2014; Lei et al., 2015).

Most common types of BC are caused by mutations of *BRCA1* and *BRCA2* genes, especially for the hereditary BC cases. Some studies indicate family history importance to determine the risks of BC (Mehrgou and Akouchekian, 2016; Brewer et al., 2017). Also, progesterone receptor (PR) gene variants are commonly seen in metastatic BC patients. Additionally, estrogen receptor (ER) negative and positive forms are other common types of BC (Yip et al., 2014). Understanding these mutations is vital to understand the nature and tumoral development of breast cancer (Fowler et al., 2020). Prognosis and predicting a cancer case is another challenging factor because diagnostic approaches according to the stage of cancer might affect the therapeutical approaches. Meanwhile, socio-demographic approaches might attenuate the prediction model and consequently survival ratio (Barclay et al., 2021). In 2020, not-surprisingly breast cancer showed the highest frequency among the other type of cancer for the women while this was prostate cancer in the male. According to this study, 30% of all cancer types were breast cancer for women in 2020 (Siegel et al., 2020). Today, women from most countries are suffering from breast cancer with 10 years of survival rate. Even though survivors of this disease can live up to 10 years, breast cancer is still a major risk among the other cases in most countries which is compelling.

Breast cancer cell types are discriminated according to their cell surface receptors [PR, ER, human epidermal growth factor receptor 2 (HER2)] and classified as triple-negative and positive which can be determining factors to specify treatment methods for the patients. For instance, receptor tyrosine-protein kinase erbB-2 receptor (HER2-receptor) positive breast cancer types are recommended to treat by hormonal therapy while HER2-receptor negative

types are subjected to chemotherapy (Hildebrant et al., 2019; van Geelen et al., 2020). The receptor profile of the primary cancer cells can be an indicative factor of prognosis and metastatic state for the course of the disease and the proliferative properties of the cancer cells. Moreover, cell surface markers such as HER2 and PR can change their expression profiles through the metastatic state within the patient and this might affect the risk of death (Kroigard et al., 2016). The elemental mechanism of the invasion and metastasis are extensively studied properties in breast cancer cases. Within this scope, it is well known that the molecular mechanism of breast cancer involves a highly complicated molecular network and pathways as in the other cancer types. Majorly, transcription factors, tumor suppressor proteins, growth factors, and cell adhesion molecules direct, control and change the proliferative and metastatic properties of breast cancer cells (Zheng et al., 2017). However, all of these protein types are associated with non-coding RNAs within the cytoplasm and the nucleus of the cell.

1.2. NON-CODING RNAs (ncRNAs)

Only 3 billion base pair of the 1.5% of the human genome belongs to the protein-coding but the remaining portion is responsible for the non-coding transcripts. This ratio is solely enough to implicate the importance of the ncRNA molecules within the organism. Structurally ncRNAs do not contain translated open reading frame (ORF), consequently, they never involve protein synthesis and are generally not polyadenylated (Gardiner, 1995; Lander et al., 2001; Wang and Chang, 2011). Most of them transcribed from homeobox (*HOX)* clusters of the chromosome. Plus to their gene regulation function, they also can play epigenetic regulator role within the organisms and can be responsible for the chromosomal regulation and subsequently gene regulation (Rinn et al., 2007).

Today, there is a considerable amount of study focus on non-coding RNAs to reveal their governing mechanism in breast cancer cells. Besides, an important class of ncRNAs can be given as following examples: exon formalizing "circular RNAs" (circRNAs), genome protector "PIWI-interacting RNAs" (piRNA), the epigenetic and expressional controller "micro RNAs" (mRNAs), retrotransposon silencing RNA (rasiRNAs), post-translational modificators small CB-specific RNA (scaRNA) and small nucleolar RNAs (snoRNA), transducers in cell stress tRNA-derived stress-induced RNA (tiRNA) and finally multiple-

functional long non-coding RNAs (lncRNAs) are the main examples of non-coding RNA family (Table1.1).

Table 1.1: Types of ncRNAs in human cells.

Name	Function
Circular RNAs (circRNAs)	Exon forming
Long non-coding RNAs (lncRNAs)	Multiple-roles in the cells
Micro RNAs (miRNAs)	Epigenetic or transcritpional regulations
PIWI-interacting RNAs (piRNAs)	Genomic regulations
rasiRNA	Retrotransposon silencing
small CB-specific RNAs (scaRNA)	Post-translastional modifications
small nuclear RNAs (snoRNAs)	Post-translastional modifications
tRNA-derived stress-induced RNAs (tiRNAs)	Signal transducing in cell stress

The mechanism behind the metastatic and proliferative features of the breast cancer cell cannot be merely explained by protein interaction or protein pathways but non-coding RNA's are also primarily involved in these tangled molecular networks. These molecules are highly effective in basic molecular mechanisms and the functioning of leading protein molecules in proliferation and metastasis (Shipz et al., 2009; Klinge, 2018; Deryusheva et al., 2019; Tao et al., 2020).

1.3. LONG NON-CODING RNAs (LncRNAs)

As an important member of the ncRNA, lncRNAs are described as non-coding RNA molecules that are longer than 200 nucleotides. Diagnostic and prognostic features of lncRNAs are yet to be understood but today there are ascending numbers of studies that identify and reveal the basic mechanism of these molecular types especially in breast cancer. As transcript molecules, lncRNAs can involve in transcriptional and post-transcriptional regulation in addition to their protein binding properties (Dykes and Emanueli, 2017).

Up to date, nearly 60,000 lncRNAs have been recorded (Ruan et al., 2020). Their expression levels depend on cell type and may found both in the nucleus and cytoplasm. LncRNAs can involve in transcription and post-transcriptional processes and also in epigenetic regulations. Due to these features, any failure in the lncRNA mechanism can associate with many types of cancer. LncRNAs affect the normal metabolism of the cells, thus they can be a key aspect for

many diseases especially cancer (Serviss et al., 2014; Hajjari and Salavaty, 2015). Although thousands of lncRNA molecules have been recorded to genetic databases to date, authors mostly focus on their underlying mechanisms recently. If we consider the fact that only %1.5 of the human genome is a coding sequence for proteins, but a great part of the human genome contains codes for non-coding transcripts, we may foresee the investigations needed to clarify their role in cancer metabolism. (Hauptman and Glavac, 2013; Kornfeld and Brüning, 2014).

Briefly, their mechanism of action can be reviewed in these three cooperation types; chromatin interaction, protein interaction, and RNA interaction. These interaction types are enough to summarize the activity of lncRNAs in cells because their molecule targeting properties render them pivotal interfering molecules. They are capable of regulating chromosomal modifications, transcriptional arrangements and directing the molecular signalling within the cytoplasm and nucleus. Even, they can involve some epigenetic regulations. However, these functions of lncRNAs are also valid for cancer cell. In cancerous cells, lncRNAs highly involves vital functions such as proliferation, growth suppression, motility, immortality (apoptotic regulations), angiogenesis, and viability (Schmitt and Chang, 2016; Peng et al., 2017a).

Moreover, their expression profiles might change according to cell surface markers among the breast cancer cell types. Meta-analyses lncRNA expression profiles exhibit a significant biomarker utility for the BC diagnosis. According to this, pooled data showed that metastasis-related lncRNAs were significantly increased in 835 patients from 10 different studies. This may present a good example of how lncRNAs are significant to diagnose BC patients and giving some critical clues to take advantage of these critical points in medical approaches (Yu et al., 2018).

1.4. LncRNAs IN CANCER

In the last decade, many studies have shown the significance of lncRNAs in cancer prognosis and metastasis. More importantly, some lncRNAs can be used as a marker to diagnose cancer or clinic pathology of cancer (Tian et al., 2018). Some examples for the important lncRNA which effective on breast cancer metastasis can be given as follows:

- HOTAIR (HOX antisense intergenic RNA) has been shown that effective on phosphatidylinositol-3-kinase (PI3K/AKT) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways. HOTAIR expression levels were elevated in breast cancer cells and trastuzumab resistance was increased. Thus, HOTAIR is suggested to provide resistance against therapeutic agents in breast cancer cells (Chen et al., 2019). Another study again approves the significance of HOTAIR for the cancer prognosis. Xue and co-workers (2016) have found that HOTAIR overexpression stimulates the tamoxifen resistance in the MCF-7 cell line by ER signalling and led to ER translocation from cytoplasm to nucleus. As a result, even in the estrogen depleted conditions, ER can translocate to the nucleus and plays a role as a transcription factor which is effective in proliferation.
- Metastasis-associated in lung adenocarcinoma transcript 1 (MALAT1) is a confounding example of lncRNA because it might switch its action according to cancer cell type; for instance, MALAT1 induces cell survival and proliferative activities in renal carcinoma and lymphoma cell while suppressing these properties in colorectal and breast cancer cells (Chen et al 2020). MALAT1 also can be a prognostic biomarker for triple-negative breast cancer (Ou et al., 2019).
- H19 is another regulator of lncRNA in breast cancer cells. It can regulate the epidermal to mesenchymal transition (EMT) process through interacting excess of miRNA within the cytoplasm of the breast cancer stem cells and provide a negative feedback loop to regulate this mechanism. H19 overexpression is associated with proliferation and tumor growth (Peng et al., 2017b). Furthermore, lncRNA H19 expression associates with estrogen and significantly contributes to the cancer cell growth *in vitro*, and as well as responsible for the doxorubicin resistance in breast cancer (Sun et al., 2015; Wang et al., 2020).
- Colon cancer-associated transcript 2 (CCAT2) shows its oncogenic effects through the Wnt/β-catenin pathway in colon cancer and BC. CCAT2 overexpression has been found as associated with poor prognosis of BC patients and important for clinical predictions (Redis et al., 2013; Sarrafzadeh et. al., 2017).

Revealing the biomarkers for the cancer is pivotal to develop genuine treatment targets and advance the prognosis conditions during treatment. Furthermore, to advance in breast cancer therapies transcriptomics would help to comprehensively understand the mechanism of breast

cancer progression. Thus, investigating the lncRNAs is significant for the therapy and diagnosis of BC (Richard and Eichorn, 2018). The most particular signaling pathways that lncRNAs are playing an important role in breast cancer are p53, PI3K/Akt, Notch, and finally NF-κB pathways (Peng et al., 2017a).

1.5. NF-κB STRUCTURE AND ROLE IN THE CANCER CELLS

NF-κB is a protein complex and it has been presented to the literature first time by Sen and Baltimore as an immunoglobulin (Ig) expression enhancer in 1986 (Sen and Baltimore, 1986). Today, there are hundreds of studies that imply its distinct role in cancer development. NF-κB is a complex transcription factor that regulates the proliferation and metastatic features of cancer cells. Many researchers have focused its role on cancer progression and prognosis since its discovery in 1986. This molecule can induce many proteins directly related to the anti-apoptotic and drug response mechanism. Therefore, it is a very important molecular marker for cancer diagnosis (Liu et al., 2020).

1.5.1. NF-κB Structure

Normally, NF-κB complex splits into two different classes; NFκBI [NF-κB1 (also called p50), NF-κB2 (also called p52)] and NFκBII [RelA (also called p65), RelB and c-REL)]. p105 and p100 are the precursor proteins for the p50 and p52 respectively and contain ankyrin-repeat domains to bind their subjected proteins p65 is another part of dimeric structure (Huxford and Ghosh, 2009), (Figure 1.1). Dimerization of these proteins forms the active NF-κB protein complex to bind the DNA (Fan and Maniatis, 1991; Kohl et al., 2019). The production p50 subunit of the NF-κB is conducted by p105 proteolytic activities within the cytoplasm which makes the p105 precursor of the p50 and this mechanism is regulated by nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) kinases (Lapid et al., 2017). However, p105 might involve other physiologic changes in the cells such as mitogen-activated protein kinase (MAPK) regulation, thus it is expressed constitutively (Tan et al., 1994; Waterfield et al., 2003). p50 contains DNA binding domains while p65 manages the IκB interaction to be progressed toward NF-κB formation (Ghosh et al., 1990). At the clinical level, p65 protein is associated with the BC and plays as a marker for the BC development (Jones et al., 2011). For the assembling of the NF-κB complex, firstly IκB should be degraded to free the p65 subunit of the complex. IKK (IκB kinase) is responsible for this degradation.

Canonical or non-canonical (inducible) NF-κB signaling depends on this IκB phosphorylation by the IKK complex and resulting transcriptional activation. In both ways, Rel family proteins are taking a central role to drive this mechanism through the nucleus and transmembrane protein tumor necrosis factor receptor (TNFR) interacts with TNFR-associated factor (TRAFs), Fas-associated protein with dead domain (FADD) stimulated by tumor necrosis factor (TNF) (Jost and Ruland, 2007; Oeckinghaus et al., 2011) (Figure 1.2).

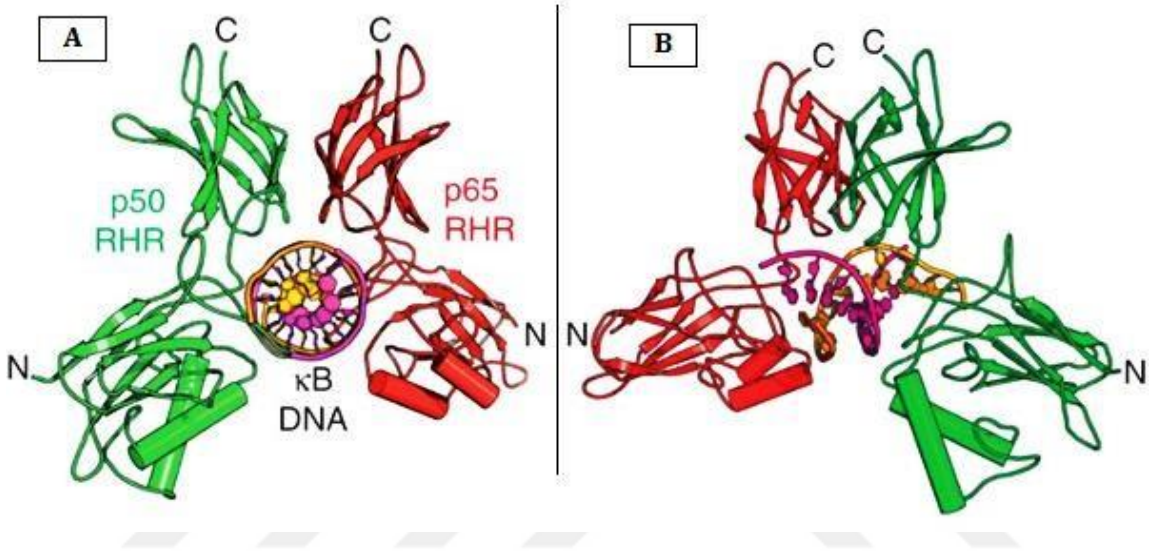

Figure 1.1: Subunits of the NF-κB complex by Huxford and Ghosh (2009). p50 and p65 Rel Homology Regions (RHR) provides attachment to the κB units of the DNA. N=Amino terminal domain, C= Carboxy terminal domain. A panel (left) front view of DNA and NF-κB interaction, B panel (right) side view of DNA and NF-κB interaction.

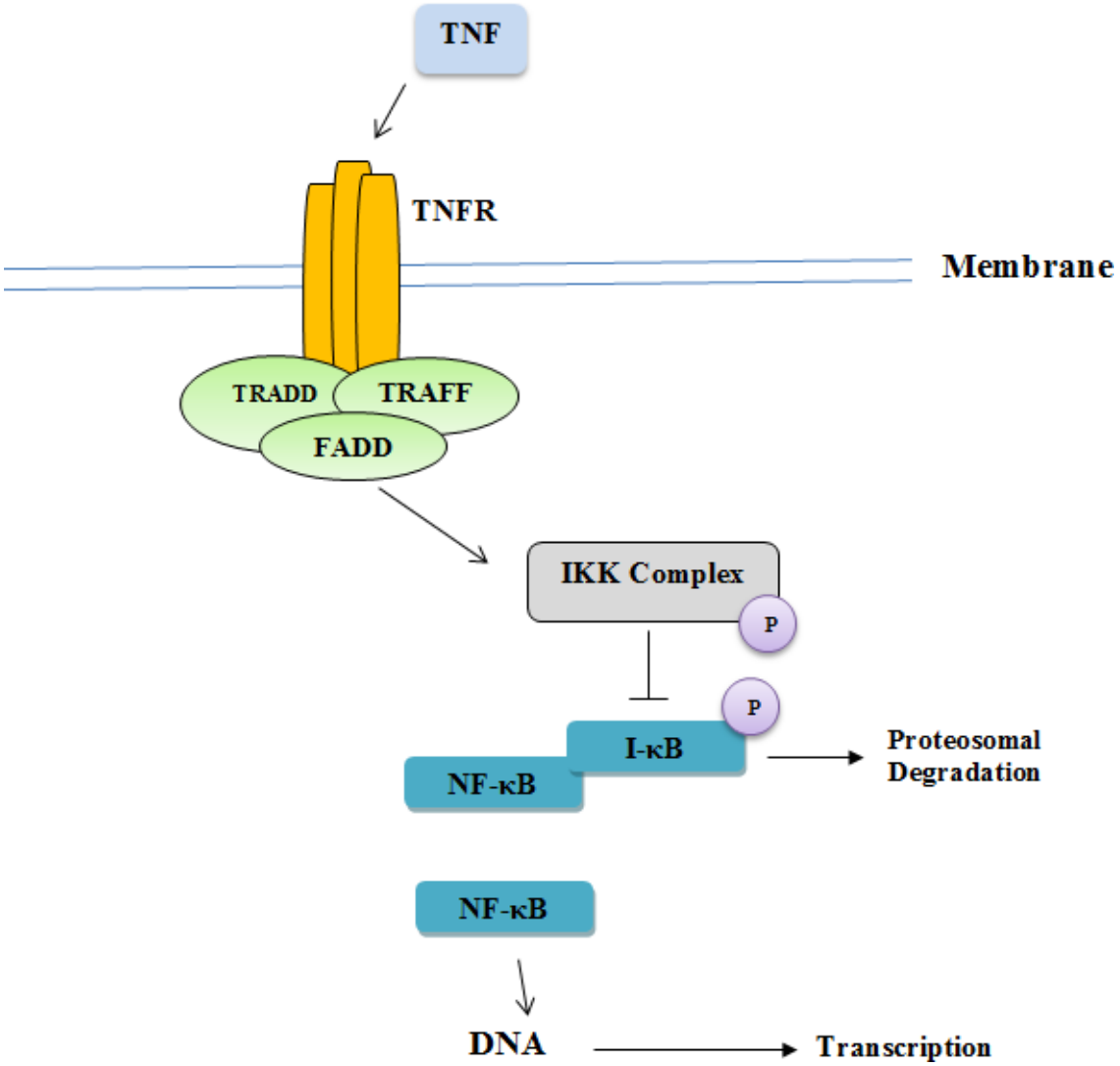

Figure 1.2: The schematization of canonical activation of the NF-κB signaling by Oeckinghaus et al., (2011). After TNF stimulation, transmembrane protein TNFR interacts with TRADD, TRAFF, and FADD to activate the IKK complex which is responsible for the phosphorylation of the IκB. NF-κB complex involves and κB domain-containing Rel family proteins and locates to the nucleus after its activation. The phosphorylation and ubiquitination process of the kinases are central control mechanisms in this cascade which is provided by ubiquitination after phosphorylated (P on the schema).

1.5.2. HSP90 and NF-κB

Heat shock proteins (HSPs) are a group of multifamily proteins and help organisms survive during stress conditions. In cancer cells different HSP families have prognostic values and a significant effect on the cell cycle (Buttacavoli et al., 2021). HSP90 is a chaperon protein and has two major isoforms as HSP90α and HSP90β. HSPα isoform is upregulated under stress conditions while HSP90β isoform fundamentally expressed in the cytoplasm (Baginear et al., 2017). HSP90 involvement in the apoptosis inhibition may be modulated by Akt signaling and NF-κB transcriptional activation (Arya et al., 2007). It is not surprising that HSPs might involve or intervene in the NF-κB pathway. For instance, inhibition of NF-κB reduces the HSP90α levels and also HSP90α can interact with the promoter region of the NF-κB complex (Ammirante et al., 2008). Moreover, HSP90 is responsible or the proliferation of the BC cells and its expression levels directly affect the drug resistance of the cancerous cells. There are some specific drugs to inhibit HSP90 because this chaperone diverts the stress conditions and increase survival rate of not only healthy cells but also cancer cells (Zagouri et al., 2012).

Normally, controlling the NF-κB proteins is provided by the IκB molecules in the cytoplasm (Gerondakis et al., 1993). However, upregulation of this complex might depend on other molecules which are crossing with the apoptotic trafficking such as HSPs as mentioned. In some cases, NF-κB transcriptional activation can be intact with stress proteins. Besides, HSP90 can affect the NF-κB signaling by directly binding to its promoter region but also able to interfere by rendering the NF-κB as its client protein. This might explain the association between stress proteins –especially Hsp90- and the NF-κB complex. NF-κB is shown to be inhibited after HSP90 inhibition (Thangjam et al., 2012). This is also another indication of the relation between NF-κB and HSP90 molecule (Rong et al., 2018). Moreover, this might be alone enough to explain, the importance of HSP90 and NF-κB co-operational mechanism in the cancer cells.

Even though many studies mentioned above approve the cooperation between the NF-κB and HSP90, their relation might depend on the physiological conditions or cell type. For instance, induced HSP90 under the oxidative stress conditions triggers the NF-κB/p65 activation and causes apoptosis in the neural stem cells. Normally, HSP90 associates and upregulates the NF-κB but consequences may differ from the cancer cells by leading to cellular death instead

of proliferative process activation of the cells (Liu et al., 2016). Plus, HSP90 is a chaperon which means that this molecule intervenes the proteins to fix misfolding in their structures as NF-κB misfolding response in the Hodkin lymphoma cells. For these reasons, HSP90α is a regulator of the NF-κB signaling pathway (Broemer et al., 2004). Under these circumstances, it is very reasonable to investigate the NKILA effect on the HSP90α. However, of course, NF-κB can be affected by β-catenin levels in the cytoplasm too. Many research proved their interaction and correlation between them. In colon and breast cancer cells, β-catenin can interact with NF-κB and responsible for the crosstalking between Wnt/β-catenin and NF-κB signalling pathways. This cross-regulation is suggested to be caused by Wnt signalling inhibition. After this inhibition, β-catenin levels are increased in the cytosol and this excess of catenins interacting with the p65 and the p50 subunit of the complex. A high level of catenin was reported to associate with reduced levels of NF-κB upstream proteins (Deng et al., 2002). This crosstalk between these two critic pathways can be very prevalent. For instance, an increase of NF-κB activation caused Wnt/catenin signalling inhibition and resulted in induce of bone diseases in rats (Pei et al., 2017). The main crosstalk point between these molecules is glycogen synthase kinase 3β (GSK-3β). Additionally, targeting cross-regulation between catenin and NF-κB might bring new therapeutical approaches (Chandrakesan et al., 2013). Nonetheless, NF-κB/Wnt pathway cross-regulation can be differential according to tissue or cell type. In a study, researchers showed that canonical NF-κB signalling positively correlated and leading to tumorigenesis *in vivo* (Vlantis et al., 2011). Toll-like receptor (TLR) proteins are involved in the upstream signalling of both canonical and non-canonical NF-κB signalling and Wnt/β-catenin signalling. However, β-catenin itself can interact and block the TLR membrane proteins and negatively affect the NF-κB pathway. TLR molecules hence, constitute a crosstalk point between these oncogenic signalling pathways (Oeckinghaus et al., 2011; Ma and Hottiger, 2016; Baizabal-Aguirre, 2017). Targeting the HSP90 can inhibit the catenin complex although catenin was capable of interact and establish crosstalk with many pathways. In lung cancer cell lines, HSP90 inhibition caused degradation of the β-catenin and cell cycle arrest (Huang, 2020). Also, HSP90 overexpression leads to Wnt/β-catenin activation and contributes to the epithelial-mesenchymal transition (EMT) (Wang, 2019). This is another result that supports the fact β-catenin is another client protein of the HSP90 as well as NF-κB.

All these literature results indicate the importance of the β-catenin and its broad-scale target protein through proliferation and metastasis. Especially, HSP90, NF-κB, and β-catenin signalling pathways have a major effect on carcinogenic development, and these three molecules tangled each other by various crosstalking molecules.

1.6. NKILA ASSOCIATION WITH NF-κB

NKILA is a lncRNA that negatively regulates by binding the p65 subunit with its secondary structures (Figure 1.3) and NKILA inhibition has a leading factor in cancer prognosis (Liu et al., 2015, Li, 2020). NKILA masks the IKK phosphorylation sites and inhibits IκB phosphorylation together with this p65-IκBα complex cannot be formed. As a result, NF-κB complex formation is blocked by this non-coding transcript. Since NF-κB is responsible for the proliferation and cancer prognosis in patients with breast cancer, NKILA has a vital role to prevent or decelerate the cancer prognosis as a repressor of the NF-κB transcription factor. Furthermore, NKILA can be responsible for the inhibition of proliferation and cancer progression in laryngeal cancer cells (Yange et al., 2018), hepatocellular carcinoma (Yu et al., 2018), esophageal squamous cells (Ke et al., 2018), tong squamous cells (Huang et al., 2016) and malignant melanoma (Bian et al., 2017) cells. In addition to cancer, NKILA inhibition can be responsible for neurodegeneration as related to NF-κB activation (Jia et al., 2018). For this reason, NKILA can be announced as a negative regulator of the NF-κB molecule. Decreasing of NKILA in cancer cells indicated its prevalence in the cancer prognosis (Dijkstra and Alexander, 2015). As supportive to this, metastasis suppressing effect of NKILA on cancer cells were shown (Bian et al., 2017, Huang et al., 2016). Additionally, it has to suppress the effect not only on the Rel family protein complex but also taking an effective role on the inflammation cascade in the lung cancer cells (Cho et al., 2019). However, there is no adequate information about NKILA association with the other molecules positioned on crosstalk points in Wnt/β-catenin signalling because of its novelty. Especially, the relation between NKILA, HSP90α, NF-κB and β-catenin has not shown before although there is a crosstalking possibility between NKILA and these proteins.

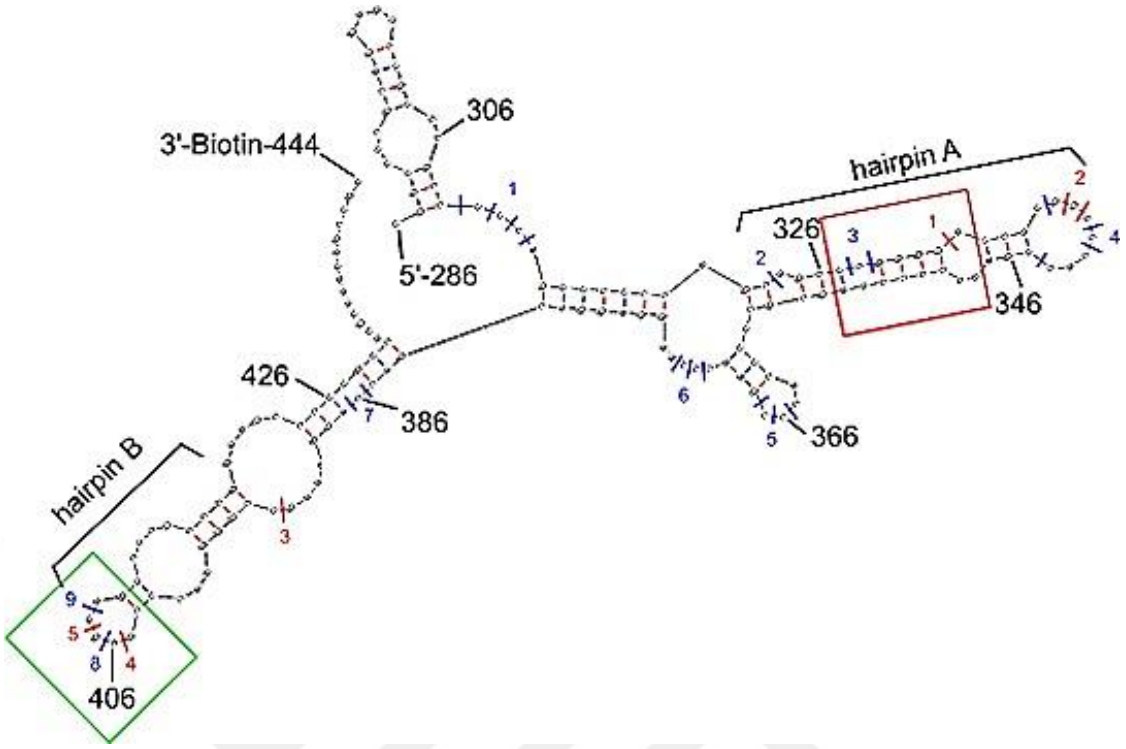

Figure 1.3: Predicted part of the 3'-Biotin labeled NKILA by Liu et al., (2015). Different colored numbers indicate RNAase digestion sites. Hairpin structures here contribute to binding to NF-κB, because these sites contain binding motifs which are similar to κB domains.

1.7. OTHER CLOSE RELATED REGULATORY PROTEINS PERTAINING TO β-CATENIN AND NF-κB SIGNALLING

Vimentin, actin, lymphoid enhancing binding factor (LEF), Akt, and e-cadherins are also related to the NF-κB signalling pathway primarily and can cause crosstalk with the Wnt-β catenin signalling (Figure 1.4). Vimentin is an intermediate protein that mediates motility of the cancer cells and involves in cytoskeletal regulation and also a good target for the Akt signalling in the migration (Zhu et al., 2011). Akt can be also responsible for the free β-catenin levels in the cytoplasm which directly affect the transcriptional activity of the cancer cells towards oncogenic proteins (Fukumoto et al., 2001). LncRNAs have a wide variety of mechanisms in the cells and one of these roles is to interact and directing the cell skeleton structure. In various studies, researchers showed the lncRNA has a vital role in the manner of taking part in the cytoskeletal mechanism by interacting with actin filament (Zeng et al.,

2015) and vimentin (Huang et al., 2013). After activation by Wnt signal proteins β-catenin cooperates with LEF and can interact with the adherence junction protein e-cadherin (Prieve and Waterman, 1999; Conacci-Sorrel, 2002).

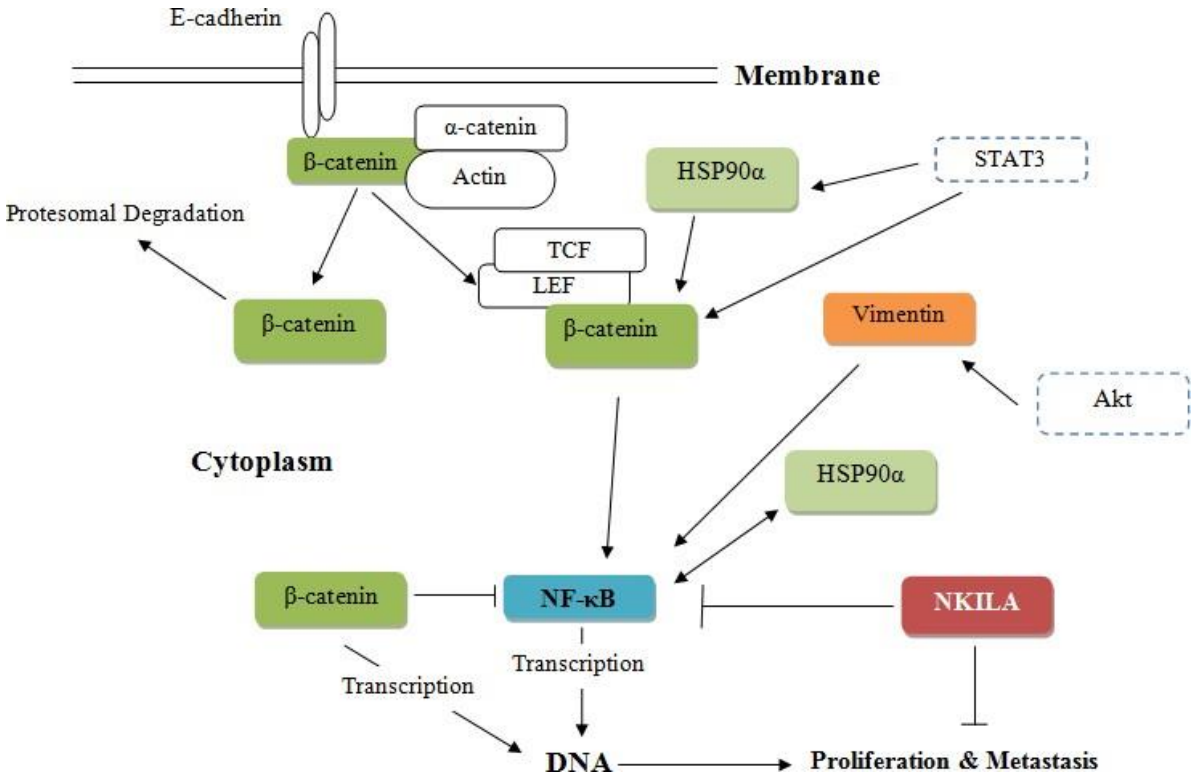

Figure 1.4: NF-κB and β-catenin molecular crosstalking with other cancer-related molecules. This might give an idea about the importance of the NKILA by directly regulating the NF-κB and consequently has the potential to affect other signalling pathways. HSP90 and NF-κB reciprocally intervene with each other. Intricately, β-catenin may cooperate with NF-κB on HSP90 in some cases but also it may downregulate the transcription of the same complex. Vimentin, actin, TCF/LEF, and α-catenin are other important molecules concert to this complicated association. Akt and STAT3 proteins are other signal transductions that are crosstalking with this confounding pathway.

β-catenin has many binding regions (Figure 1.5) and interacts with many target proteins which are crucial for cancer cell development. One of the most important target proteins of the catenin is epithelial cadherins (e-cadherin). Wnt-β catenin signalling pathway provides signal trafficking by the TCF/LEF (T-Cell Factor/ lymphoid enhancing binding factor) complex on the DNA (Kolligs et al., 2002). Activation of the Wnt signal pathway results in e-

cadherin inhibition while vimentin expressions increase and lead to cell metastasis (Gong et al. 2014). Normally β-catenin taking part in the e-cadherin mediated cell to cell adhesion as a substitute but Wnt (+) signal disrupt this complex by phosphorylation of β-catenins inside the cytosol subsequently this free β-catenins may interact with the TCF on DNA and leads to metastatic process (Gilles et al., 2003; Clevers, 2006; Matsuda et al., 2009; Tian et al., 2011). β-catenin interacts and forms a complex with the e-cadherin (Figure 1.4) to control the cellular polarization and growth and essential to cell-cell adhesion (Ma and Hottiger, 2016). Normally, β-catenin might involve cancer prognosis with its oncogenic promoter binding ability but the e-cadherin/β-catenin complex prevents this process because cadherins provide cell-cell adhesion. As such, adhered cells do not manage to progress towards metastasis which requires a departure from other cells and concomitantly individual cellular motility, because of this cadherin function of cell-cell adhesion (Heuberger and Birchmeier 2010; Kaszak et al., 2020). Therefore, e-cadherins are known to be down-regulated to start the metastatic processes in the cancerous cells. Besides, e-cadherins were showed to intact with the actin and α-catenin molecules which are important regulatory molecules of the cytoskeleton (Conacci-Sorrel 2002; Wang et al., 2020). As supportive to this, e-cadherin inhibition increased β-catenin accumulation in cytoplasm and localization to the nucleus, subsequently attenuate the oncogenic properties of the breast cancer cells by Wnt independent β-catenin signalling (Yang, 2001). Cell-cell adhesion also pertinent to NF-κB signalling thus, we may involve the NF-κB in the e-cadherin/β-catenin related signalling cascade because the involvement of the NF-κB complex to the adherent protein transcription cascade plus the survival genes can be seen in the cancer cells (Cowell et al., 2009) (Figure 1.4). Oppositely, mutated e-cadherin proteins did not affect the Wnt-catenin signalling pathway in the breast cancer cells in a study (Wetering, 2001) which might bring the question of "Are the crosstalk proteins other than adherence junction proteins able to activate constitutive (canonical) Wnt signalling?"

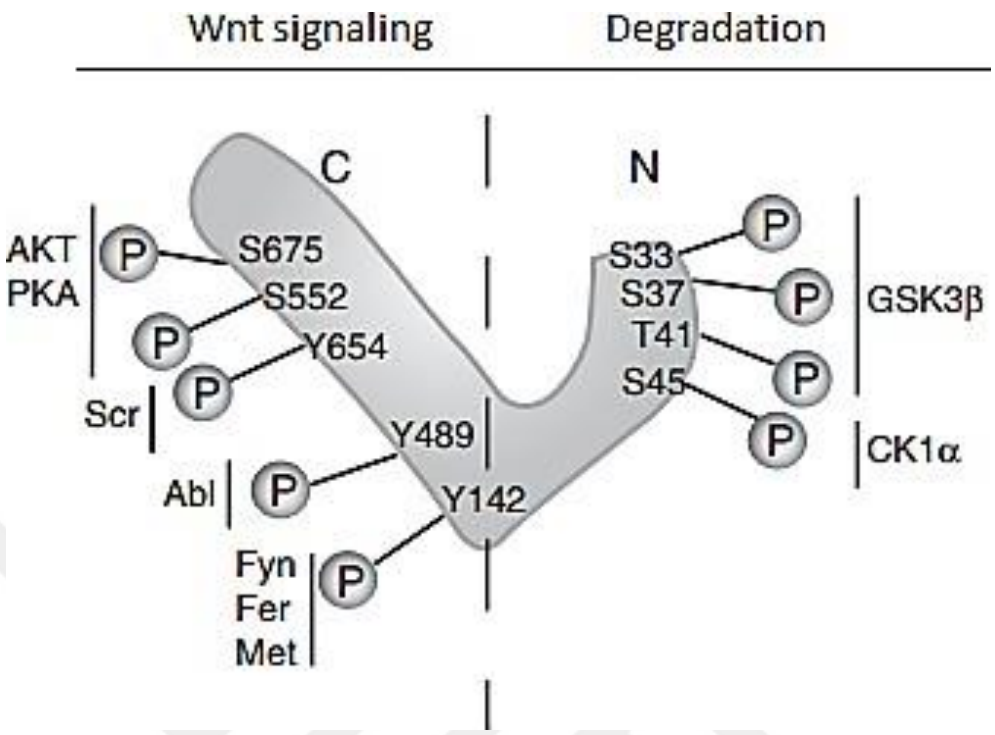

Figure 1.5: Multiple phosphorylation sites of the β-catenin by Heuberger and Birchmeier (2010). As seen here, these multiple phosphorylation sites might explain the reason why β-catenin molecule interplay crosstalk between many signalling pathways and can interact with various proteins. Both activation and degradation sites of the catenin are presented here. C=C-terminal domain, N=N terminal domain. AKT and protein kinase A (PKA) have promoting effect on catenin while GSK-3β and Casein kinase 1α (CK1α) act as deregulation of the catenin. Numbered S (serine), Y (tyrosine), T (threonine) letters indicate specific sites on the aminoacid sequence of the catenin.

1.8. POSSIBLE RELATION BETWEEN NKILA, NF-κB, HSP90α, and β-CATENIN

Due to the mentioned aspects above, HSP90 is closely associated with the NF-κB transcription complex as well as β-catenin. However, the suggested NKILA relation with these crosstalk molecules has never been shown before. Hereby, for the first time, cross-regulation between these molecules was investigated in this doctoral thesis. The molecular mechanism of the NKILA on the NF-κB has been clearly shown in the limited number of

studies but its relation with other important signalling pathway proteins has not been adequately reported yet. We assume that any transcriptional changes in the NKILA levels might give some clues about its influence on the NF-κB and Wnt signalling cascades. These intricate signalling pathways contain so many crosstalking points especially by β-catenin which has a wide variety of binding domains. In this manner we can suggest the NKILA effect on these molecules that are playing strategic points for each other to control the cellular growth mechanism. Accordingly, NKILA can affect the HSP90, NF-κB, and β-catenin molecules through the p50 and the p65 proteins which have never been shown before. Wnt/β-catenin and NF-κB signalling pathways have major crosstalking points against each other. Additionally, HSP90 has some client proteins in both pathways. This makes the NKILA more interesting to reveal its mechanism in these complicated signal networks because NKILA has a crucial role in NF-κB signalling.

Indeed, we suggest that investigating these crucial molecules by considering the effect of NKILA might bring new questions to be answered; in other words, shed light on the tangled molecular pathway of the metastatic cancer types. Due to the close association between these vital signalling cascades, it is very plausible to investigate their association between these molecules by considering the effect of NKILA in these manners. Therefore, we aim to reveal these possible effects of NKILA on the HSP90 and β-catenin and contribute to divulge the molecular mechanism of the NKILA. Besides, this might help to present new therapeutic targets for cancer treatments and bring new information to the literature.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

MCF-7 cell line was cultured in DMEM-F12 (Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient Mixture) (GibcoTM) medium supplemented with 10% FBS (Fetal Bovine Serum) (GibcoTM) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) (PANTM Biotech). The cell line was maintained in a humidified air atmosphere with 5% CO_2 at 37ºC. Cells (Figure 2.1) were subcultured two or three times weekly.

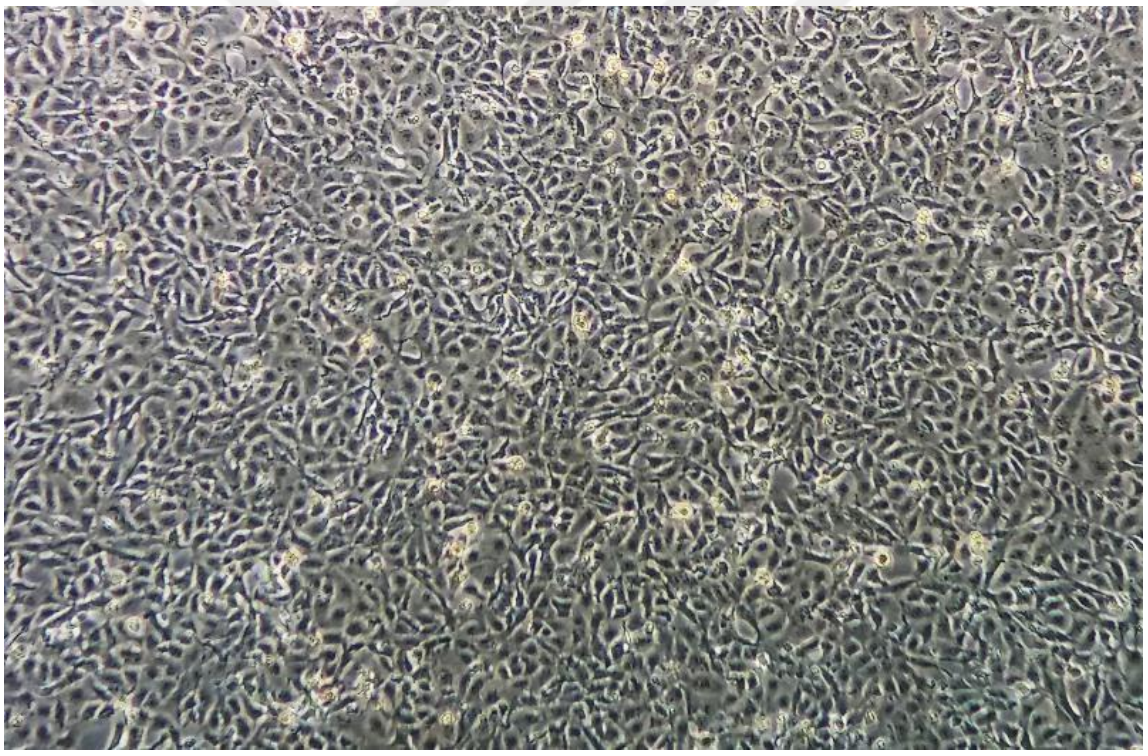

Figure 2.1: Confluent MCF-7 cell line under the inverted light microscope (100x magnification).

2.2. DETERMINING THE CELL GROWTH RATES

To determine the doubling time and cell growth rate of the MCF-7 cell lines, 24 well plates were used to incubate. MCF-7 cells were cultured in $1.0x10^5$ cell/ml and $0.5x10^5$ cell/ml

concentrations to each well and cells were counted daily by using hemocytometry for ten days. Cell counts were performed by using Neubauer hemocytometer and cell number calculated by the equation mentioned below:

$$\text{Cell number/ml} = N \times 10^4 \quad (2.1)$$

"N" indicating the total number of cells counted on the Neubauer slide (Freshney, 1983).

Population doubling time was calculated according to following equation:

$$\text{Population doubling time (hours)} = \frac{\text{Log (2)}}{\text{Log(B)} - \text{Log (A)}} \quad (2.2)$$

Where the Log (A) is the initial cell count at the beginning of the incubation; and the Log (B) is the final cell count at the end of the logarithmic phase (Moat, 2002).

Growth rates from cell counting results and related trend lines were calculated with ANOVA test by "Graphpad Prism" (ver. 7.0.1) software.

2.3. EXPERIMENTAL LAYOUT AND STUDY GROUPS

All the experimental groups have consisted of a control, only transfection reagent exposed group "Mock", only scrambled siRNA (non-target siRNA or negative control), and NKILA silenced "NKILA-siRNA" exposed group. Experimental results were gained through statistical comparing with the control group. However, before the experiments, toxicity levels of the transfection reagent, scrambled and targeting siRNA groups were performed at different concentrations for 48 hours in order to determine the non-toxic levels of these compounds. Especially, non-toxic siRNA concentrations are needed to specify the effective concentration otherwise NKILA-siRNA can show its detrimental effects on the cells rather than showing effective silencing.

2.4. 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYL-2H-TETRAZOLIUM BROMIDE (MTT) ASSAY

MTT assay was performed to the MCF-7 cell line in order to detect non-toxic levels of the preferred NKILA-siRNA, and transfection reagent concentrations (Suto et al., 1989). To do

this first, 50 nM siRNA with 12 µl and 15 µl transfection reagent (TR) (Lipofectamine 2000™) and 25 nM siRNA with 12 µl and 15 µl TR concentrations were combined and respectively experimented on MCF-7 cells with below-mentioned siRNA protocol. After siRNA exposure at defined concentrations, the cells were incubated for 48 hours. Following the incubation period, cell mediums were removed and 30 µl MTT was added to each well. After incubation, formazan crystals were observed under an inverted microscope. At the end of this period, 150 µl DMSO was added to each well to solve the formazan crystals and then the optical density (O.D.) was measured at 540 nm wave lenght to determine the cell viability by using EonTM Microplate Reader (PowerWave HT™). Cell viability percentages were calculated according to the following equation:

$$\text{Cell viability percentage} = \frac{\text{Blank exluded optical density of exposed cells}}{\text{Blank exluded optical density of control group}} * 100 \qquad (2.3)$$

2.5. siRNA TRANSFECTION

After determining the non-toxic levels of the siRNA concentrations, cells were incubated in 60x15 mm Petri dishes by grouping as control, NKILA-siRNA, Mock, and Scrambled. In order to achieve gene silencing of NKILA transcript by siRNA molecule (SISEL custom siRNA 5 nM, Ambion^{TM}).

Custom designed NKILA-siRNA sequences were:

Sense: 5’-GGACUGCGAGAAUUAAAUAtt-3’

Anti-sense: 5’-UAUUUAAUUCUCGCAGUCtg-3’

MCF-7 cells were transfected with 25 nM NKILA-siRNA by using 12 µl Lipofectamine 2000 reagent (transfection reagent, ThermoFisher). The cells were exposed to siRNA for 4 hours and siRNA containing cell medium refreshed by normal DMEM-Ham's F-12 medium at the end of this transfection period. By following the medium refreshment, cells were incubated in a normal medium for 48 hours.

2.6. TOTAL RNA ISOLATION AND cDNA PRODUCTION

Control and transfected cells were collected after 48 hours. RNA isolated from collected cells by using RNA Lysis buffer (GeneMarkbio Lab). Total RNA concentration was measured by NanoDrop 2000^{TM} Spectrophotometer (ThermoFisher). Before the quantitative analyses of gene amplification by RT-qPCR, RNA samples were separated and 18S and 28 ribosomal-RNA bands were visualized in denaturing 1.5% agarose gel to check the RNA integrity.

cDNA was prepared from this obtained RNA samples by using High Capacity cDNA Reverse Transcription Kit^{TM} (Applied Biosystems). Primers for PCR were prepared by using gmPFU buffer and DNA polymerase and then PCR was performed in 35 cycles and stages were 25^{o}C for 10 min., 37^{o}C for 2 hours, 85^{o}C for 5 min. and 4^{o}C for infinite hold.

2.7. REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR)

After synthesis of the related cDNA from total RNA samples, NKILA transcript levels were determined by RT-qPCR (BioRad CFX96 Touch^{TM}) using Power SYBER^{TM} Green Master Mix. First, an RT reaction mix was prepared which contains nuclease-free water, SYBER green master mix, and reverse and forward primers and added to each well of the plate. And then related RNA content added to each well (20 µl of total volume in each well) and no template control (NTC) also prepared with the three technical replicates. Then, plates were placed to sample block and RT-qPCR assay was performed by appropriate (two-step RT-qPCR) thermal-cycling conditions (Table 2.1) (Saiki et al., 1985). β-actin was used as the housekeeping gene to normalize the data.

Gene-specific primer sequences for NKILA transcript were as follows:

Forward; ACCTACCCACAACGCTTGAC,

Reverse; GCGCCAGAGACCACTAAGTC

Gene-specific primer sequences for β-actin were as follows:

Forward; TGCGCAGAAAACAAGATGAG

Reverse; GTCACCTTCACCGTTCCAGT

Cycle threshold (Ct) values after qPCR process were used to calculate "fold change" of related genes by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2.1: Stages for two step RT-qPCR to detect the NKILA transcript levels.

Phase	Temperature	Duration	Cycles
Initial denaturation	95ºC	2 Minutes	1
Denaturation	95ºC	30 Seconds	40
Annealing	55ºC	45 seconds	
Extension of each primer	72ºC	45 Seconds	
Final reading	65ºC	5 Seconds	1
	95ºC	5 Seconds	1

2.8. PROTEIN ISOLATION AND PROTEIN CONCENTRATION DETECTION

Once we analyse the fold change of the expresion levels of the NKILA gene, soluble protein was extracted from the groups (control, mock, scrambled and siRNA groups). Protein lysis buffer which contains trizma-hydrochloride (Tris-HCl; pH 6.8), ethylenediamine tetra-acetic acid (EDTA), Triton-X 100, complete EDTA-free tablet, and phenylmethylsulfonyl fluoride (PMSF) (Table 2.2) were added to all samples. After pipetting and sonication of the samples, they centrifuged at 20.000 rpm for 20 minutes, and then the supernatant was transferred to another tube for the total protein concentration measurement. Protein concentration measurement was performed with a bicinchoninic acid assay (BCA) (Smart™ BCA Kit, Intron Technology). According to this kit, working solution A was mixed with solution B at a 50:1 ratio. Total solution volume is calculated upon the total sample number. 200 µl mixed (solution A and the B) solution was added to each well of 96-well plate and then 25 µl 1:5 diluted protein samples added to the well, and pipetted gently. These steps also were repeated for three technical replicate. After pipetting, the plate was incubated at 37ºC for 30 minutes. After incubation, colorimetric changes were measured at 562 nm wavelength by using Eon™ Microplate Reader (PowerWave HT), and O.D. values were used to calculate total protein

concentration. Standard curve was drawn and it was used to calculate the total protein concentration according to the manufacturer's instructions (Figure 3.6).

Table 2.2: Lysis buffer ingredients.

Ingredients	Volume Ratio in the Solution
Tris-HCl (pH; 6.8)	0.02 %
EDTA	0.04%
Triton X-100	1%
Complete EDTA-free Proteinase	1 tablet
PMSF	Added to mix according to volume

2.9. IMMUNOLOGICAL ANALYSES

Western blot technique was used in order to detect the expression profile of the mentioned proteins. Gel electrophoresis, transfer, blocking, primary antibody incubation, secondary antibody incubation and finally protein detection steps were followed respectively.

2.9.1. Gel Electrophoresis

After isolation of total protein, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins by their electrophoretic mobility (Walker, 1996). First, 10% resolving gel solution prepared using the volumes in Table 2.3. After gel polymerisation is completed, 5% stacking gel solution (Table 2.3) is added to the gel cassette. Protein samples were mixed with loading dye which contains glycerol, β-mercaptoethanol, and bromophenol-blue (Table 2.4), and heated at 95^{o}C for 3-7 min. Then, protein samples mixed with the loading dye were transferred to the stacking gel and electrophoresis was performed.

Table 2.3: Gel ingredients for casting of resolving and stacking gel in one gel-cassette.

Ingredients	Resolving Gel (pH 8.8)	Stacking Gel (pH 6.8)
Tris-HCl	1 ml	250 µl
dH_2O	2.262 ml	1.875 ml

Acrylamide (30%)	1.662 ml	337.5 ml
SDS (10%)	50 µl	25 µl
APS (10%)	25 µl	12.5 µl
TEMED	1.75 µl	3.5 µl

Table 2.4: Loading dye ingredients and their volume ratio in the buffer solution.

Ingredients	Volume Ratio in the Solution
Tris-HCl (0.6 M, pH; 6.8) (v/v)	5%
SDS (m/v)	1%
Glycerol (m/v)	5%
β-mercaptoethanol (v/v)	0.5%
Bromophenol-blue (v/v)	5% (0.5% in stock solution)

2.9.2. Electroblotting of the Proteins

Protein electroblotting is performed by wetblot system (BioRad^{TM}). After the separation of protein samples by their molecular weight, a mini-gel is placed between paper and polyvinylidene difluoride (PVDF) membrane and foam pad. This "pad-paper-membrane-gel-paper-pad" sandwich was placed in a buffer tank and conductive transfer solution (Table 2.5) was added into the tank with cooling apparatus. Then, transfer was performed at 100 V for 1-1.5 hours in the transfer buffer.

Table 2.5: Transfer buffer ingredients.

Ingredients	Concentration or Volume ratio in the Solution
Tris-Base	25 mM
Glycine	192 mM
SDS (m/v)	0.1%
Methanol (v/v)	20%

2.9.3 Blocking and Antibody Incubation

After transferring the proteins to the PVDF membrane (BioRad), the membrane was submerged in the 5% non-fat skimmed milk (BioRad) and rinsed for 1-1.5 hours to block the membrane with random proteins. Then, the membrane was exposed with referred antibodies (Table 2.6) which are anti-HSP90α (ThermoFisher), anti-β-catenin (ThermoFisher), and anti-NF-κB (p105/p50) (ThermoFisher) and NF-κB p65 (Boster) primary antibodies and incubated overnight at 4^{o}C. The following day, membranes were washed with Tris-buffered saline with 1% tween-20 (TBS-T) for 5 minutes and this washing process was repeated 5 times. After washing was completed, membrane incubated in secondary anti-rabbit (ThermoFisher) antibodies for beta-catenin (ThermoFisher) and anti-NF-κB (p105/p50) and secondary anti-mouse antibody for the anti-HSP90α and anti-β-catenin primary antibodies for 2 hours at room temperature. After secondary antibody incubation, membranes were washed with the same process and incubated chemiluminescent (Pierce^{TM} ECL Western Blotting Substrate, ThermoFisher) for 1-5 minutes and finally, membrane protein band images were detected by using an imaging device (Bio-Rad ChemiDoc^{TM}).

Table 2.6: Antibodies used for immunochemical analyses.

Type	Brand	Cataloge	Concentration
NF-κB p50 primary antibody	ThermoFisher	MA141314	1:1000-1:200
NF-κB P105 primary antibody	ThermoFisher	MA141314	1:1000
NF-κB P65 primary antibody	Boster	A00284-1	1:1000
HSP90 Alpha primary antibody	ThermoFisher	MA3010	1:1000
Beta Catenin primary antibody	ThermoFisher	PA516762	1:1000
GAPDH primary antibody	Boster	A0027	1:2000
Goat Anti-Mouse, secondary antibody	ThermoFisher	31430	1:2000
Goat Anti-Rabbit secondary antibody	Boster	BA1054	1:2000

Primary and secondary antibodies were removed by stripping procedure after the aforementioned protein imaging. Hence, the membrane was incubated in striping solution (Table 2.7) at 55-60ºC for 30-40 minutes and washed with dH_2O until β-2-mercaptoethanol odor removed and then washed with TBS-T for 5 minutes and repeated five times. After stripping process, membrane incubated with 5% non-fat skimmed milk for 1.1-5 hours again and this time incubated in anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary and related secondary antibody for the normalization of the subjected proteins band intensities.

Table 2.7: PVDF-Membrane stripping solution ingredients.

Ingredients	Concentration in the Solution
β-2-mercaptoethanol	100 mM
SDS	2% (m/v)
Tris-HCl (pH; 6.7-6.8)	62.5 mM

All the band images were evaluated and normalized with band images of a loading control (GAPDH) by using ImageLab (ver. 6.0.1) software. After normalization, band intensities were presented as a percentile.

2.10. STATISTICAL ANALYSES

Data from MTT assay, RT-qPCR fold-change comparisons, and protein expression comparisons with their control groups were analyzed and expressed as mean±standard deviation (SD). Student t-test was used to detect the silencing efficiency of NKILA-siRNA in comparison with the control group. Variance homogeneity was measured with the F test parallel to the t-test. On the other hand, the One-way ANOVA test was used to determine significant differences between the groups. Data that shows $p<0.05$ were accepted as significantly different from the control group. Variance homogeneity of the multiple groups was measured with Bartlett's Test as parallel to ANOVA tests. All the statistical analyses were performed by GraphPad Prism (ver. 7.00) software.

3. RESULTS

3.1. CELL GROWTH RATES

In order to determine the doubling time and cell growth rate of MCF-7 cells, 24 well plates were used for incubation. The cells were incubated at different concentrations ($1.0x10^5$ cell/ml and $0.5x10^5$ cell/ml) to each well and everyday cells were counted by using hemocytometry and this process was repeated for ten days. Growth rates from cell counting results and related trend lines were gained through GraphPad software. According to results; the MCF-7 cell line was reached the logarithmic stage after 24 hours and reach the plateau phase at 72nd hour. Accordingly, the doubling time of the MCF-7 and were detected as 37 hours (Figure 3.1). This result is compatible with the doubling time of the MCF-7 presented by ATCC's manual.

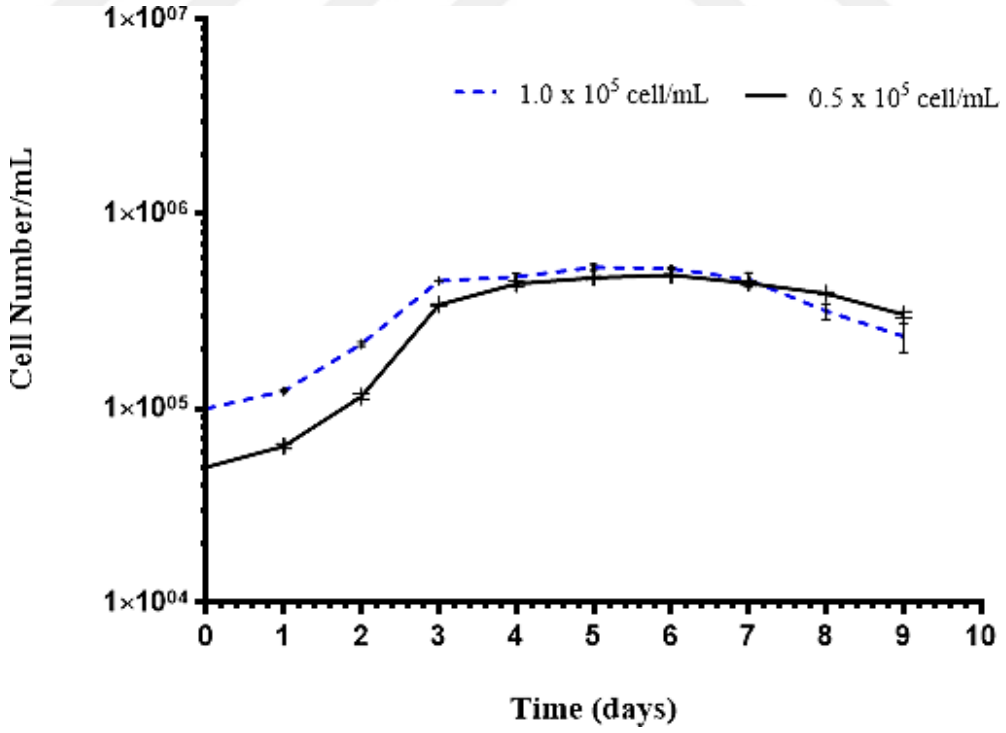

Figure 3.1: Cell growth curve of MCF-7 cell line. The cell line grasps the plateau phase between 4th and 7th days. After the lag phase within the first 24 hours exponential phase were detected between 24th and 72nd hours of incubation

period, after 72 hours of incubations cell reached to plateau phase. After this stage, cell numbers significantly declined ($p<0.05$).

3.2. OPTIMIZATION OF siRNA CONCENTRATIONS FOR THE NKILA SILENCING IN THE MCF-7 CELL LINE

Prior to exposure cells with NKILA-siRNA at different concentrations MCF-7 cells confluency was observed under inverted microscope (Olympus CKX31) 24 hours after the culturing at DMEM-F12 cell medium. We approved that cells did not reach to the confluency and single cell layer did not cover the all surface area of the culture flask after 24 hours of culturing (Figure 3.2). This confluency result indicated the proper time for the transfection.

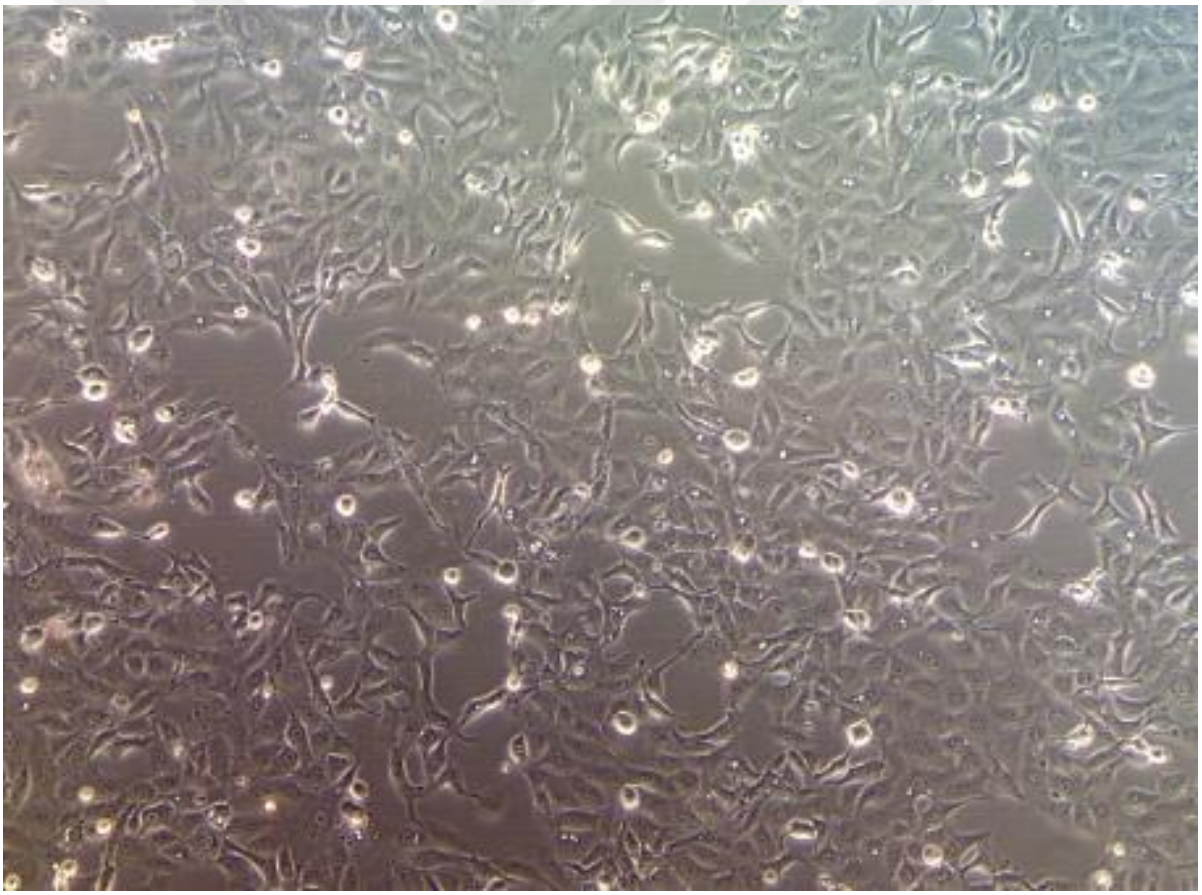

Figure 3.2: Image of MCF-7 cells under the invert microscope after 24 hours of culturing (100x magnification).

For the optimization, the cells were exposed to 12 µl or 15 µl transfection reagent (TR) combined with 25 nM or 50 nM siRNA respectively, and MTT assay was performed (section

2.4). We determined that MCF-7 cells showed high viability under the effect of 12 µl TR and 25 nM NKILA-siRNA. 25 nM NKILA-siRNA combined with 15 µl transfection reagent and 50 nM NKILA-siRNA combined with 12 µl or 15 µl transfection reagent exposures caused high toxicity as the cell viability were below 80% (Figure 3.3). Therefore, we decide to use 25 nM NKILA-siRNA and 12µl transfection reagent combination for the experiments.

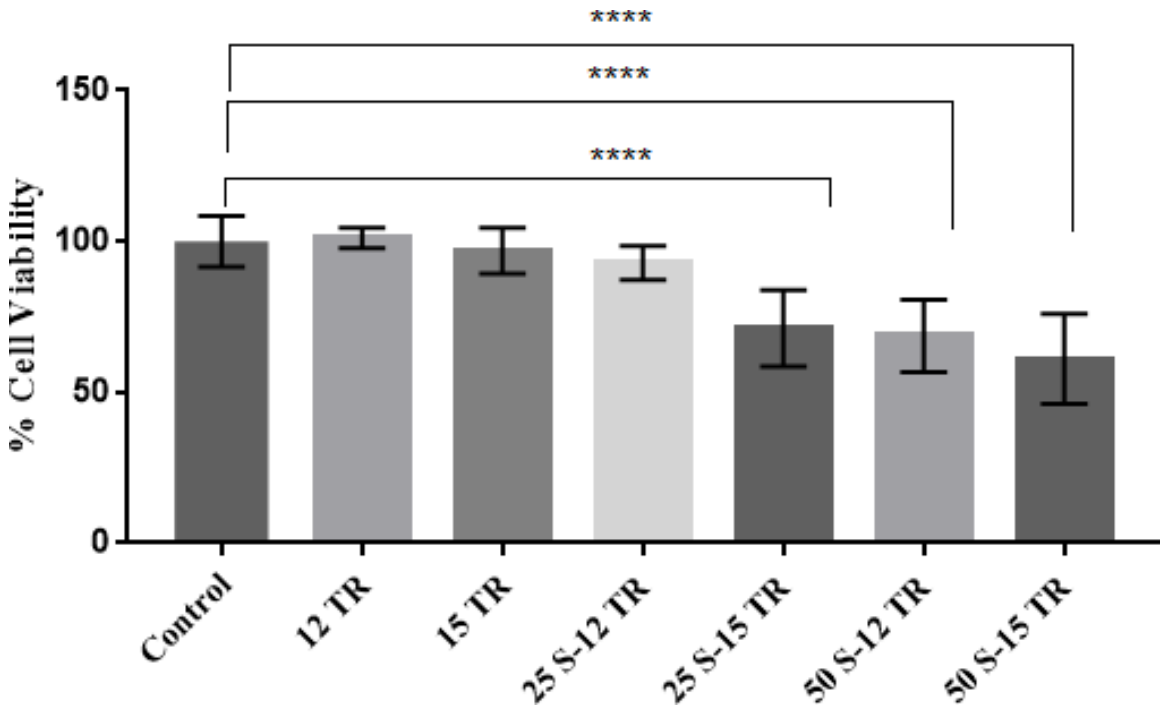

Figure 3.3: Toxicity levels of 25 nM and 50 nM siRNA concentrations combined with 12 µl or 15 µl transfection reagent respectively. Control: non-treated group, 12 TR: 12 µl transfection reagent treated group (mock group), 15 TR: 15 µl transfection reagent treated group (mock group), 25S-12 TR: 12 µl transfection reagent and 25 nM siRNA treated group, 25S-15TR: 15 µl transfection reagent and 25 nM siRNA treated group, 50S-12TR: 12 µl transfeciton reagent and 50 nM siRNA treated group, 50S-15 TR: 15 µl transfection reagent and 50 nM siRNA treated group.

To detect the cell viabilities of the control, mock, scrambled and siRNA groups were also performed MTT assay. The viability of control group was accepted 100%. Cell viability ratios of 12 µl transfection reagent (mock), 12 µl transfection reagents and 25 nM scrambled siRNA (negative control), 12 µl transfection reagent and 25 nM NKILA-siRNA were 101.1%,

95.82% and 92.78% respectively (Figure 3.4). There was no statistical difference between the experimental groups.

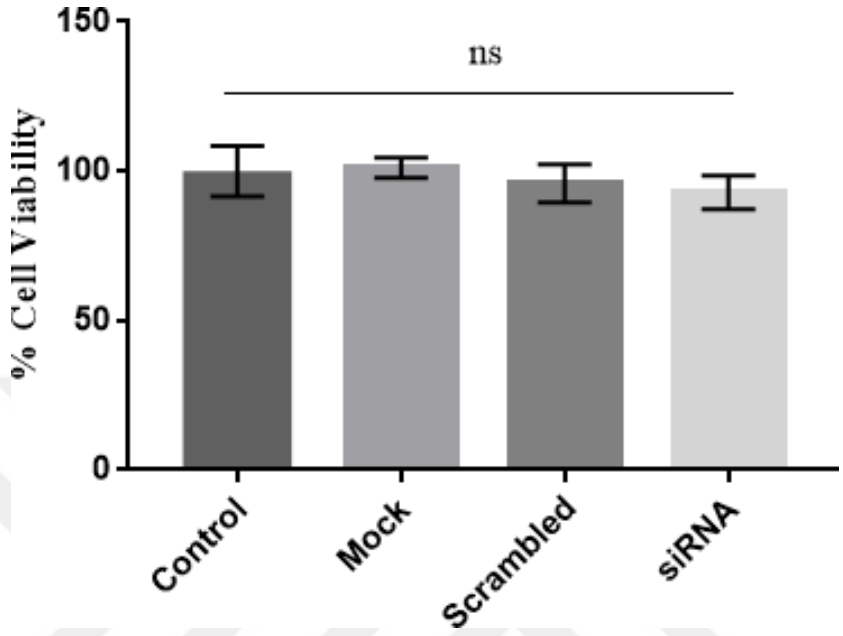

Figure 3.4: Toxicity levels of preferred concentrations for the transfection of the MCF-7 cells. Control: Non-treated group, Mock: 12 µl TR treated group, Scramble: 12 µl TR and 25 nM scramble siRNA treated group (as negative control), siRNA: 12 µl TR and 25 nM NKILA-siRNA treated group.

3.3. SILENCING EFFICIENCY OF THE NKILA-siRNA WITHIN THE MCF-7 CELL LINE

As confirmed by $2^{-\Delta\Delta Ct}$ method, after exposure of 25 nM NKILA-siRNA by the 12 µl transfection reagent, NKILA transcription levels were significantly decreased ($p<0.0001$) by 0.38±0.09 as fold change by comparing to the control group. Statistically, there were no difference of NKILA transcription between the mock, scrambled (negative control) and the control groups. NKILA transcription levels were 90% and 89% of the mock and scrambled groups by comparing to control respectively ($p>0.05$). Thus, we specify the silencing efficiency as 62% after the set of NKILA silencing (Figure 3.5).

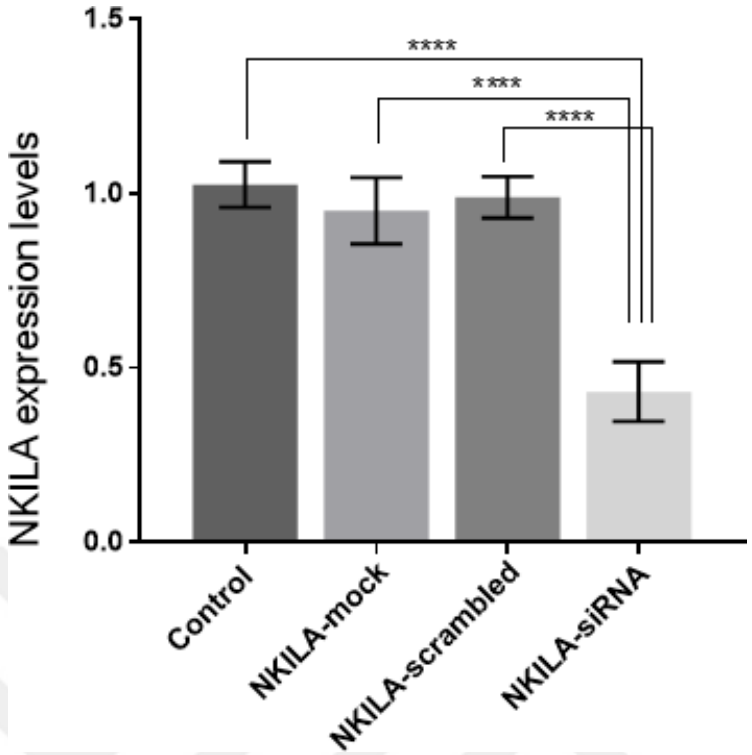

Figure 3.5: Silencing efficiency of the NKILA-siRNA. Mock (no-siRNA) and Scrambled (non-target siRNA as negative control) groups in the MCF-7 cells, ($p<0.0001$). After siRNA transfection, NKILA expression levels decreased 62% in comparison to control. Mock (no-siRNA) and scrambled (non-target siRNA as negative control) groups showed no any significant difference in comparing to control as expected. Control: Non-treated group, Mock: 12 µl TR treated group, Scramble: 12 µl TR and 25 nM scramble siRNA treated group (as negative control), siRNA: 12 µl TR and 25 nM NKILA-siRNA treated group. Stars indicates significancy degree ($p<0.0001$).

3.4. TOTAL PROTEIN CONCENTRATIONS

Total protein concentration of the experimental groups was determined by BCA assay and concentrations were calculated from O.D. values by using the standard curve formula mentioned below (Figure 3.6). The standard curve was drawn according to the manufacturer's

directions. Calculated total protein concentrations varied between 2000-4000 µg/ml for each experimental group. Concentrations were calculated with an equation (correlation coefficient "R-value" of 0.992 which is determined by the regression curve).

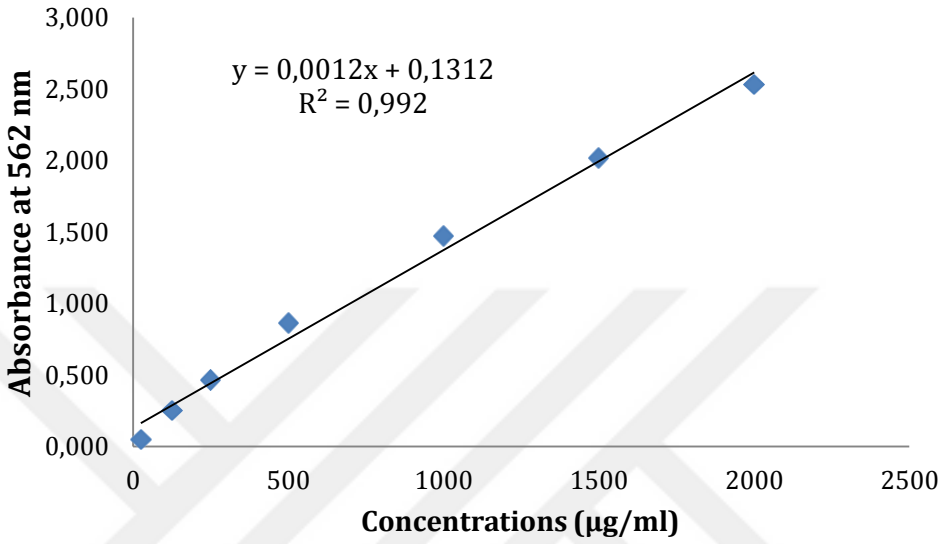

Figure 3.6: BCA assay standart curve of concentrations versus absorbance. Total protein concentration of the cells varied between 2000 and 4000 µg/ml according to BCA assay. Concentrations were calculated according to equation (R value of 0.992 which is determined by the regression curve).

3.5. HSP90α, NF-κB AND β-CATENIN EXPRESSION LEVELS AFTER NKILA-siRNA EXPOSURE

3.5.1. Relative expression levels of HSP90α

In NKILA-siRNA group HSP90 showed a significant increase as predicted in our study, and first time shown by us. According to this experiment, after 62% inhibition of the NKILA caused an significant upregulation of the HSP90 by 54.2±5.9% (Figure 3.7, $p<0.001$). Significancy degree was relatively high compared with control group ($p<0.001$).

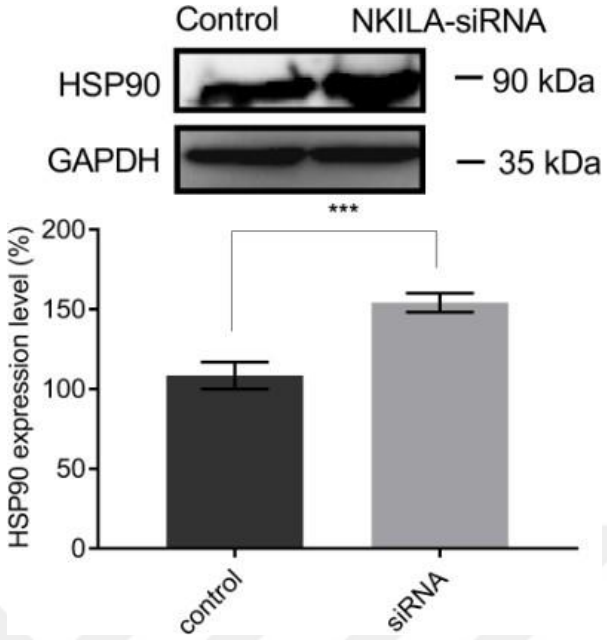

Figure 3.7: Relative expression levels of HSP90 protein. Band intensities (above the graph) and expression level. Stars indicate significancy level ($p<0.001$).

3.5.2. Relative expression levels of NF-κB/p105

NF-κB/p105 is a precursor molecule of the p50 subunit, thus it is effective on the NF-κB expressions in the cytoplasm (Huxford and Ghosh, 2009). Therefore it is normal to have an increased expression profile of the p105 under effect of NKILA-siRNA. We detected significant upregulation of the p105 precursor and this presented to us compatible results with induced levels of the p50 and p65. This result was supportive the general term of NF-κB/p105 expression but there is no other literature result to approve this finding. p105 acts in accordance with the NF-κB transduction. As proof of this, we detected an increase of the relative p105 levels by 33±9.7% ($p<0.001$) in comparing to control groups (Figure 3.8).

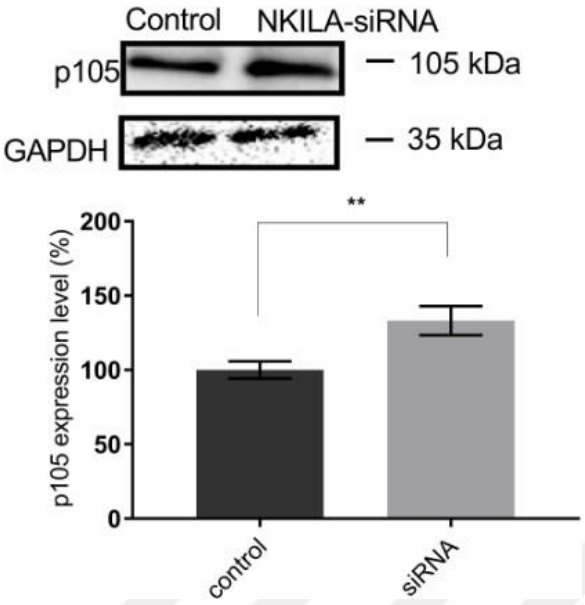

Figure 3.8: Relative expression levels of NF-κB/p105 protein. Band intensities (above the graph) and expression level. Stars indicate significancy level ($p<0.01$).

3.5.3. Relative expression levels of NF-κB/p50

NF-κB/p50 is another protein which increased its relative expression levels significantly ($p<0.001$). According to our findings; p50 was upregulated by 44.3±14.3%. As one of the main subunits of the NF-κB, NKILA inhibition by siRNA resulted in of p50 relative expression increase (Figure 3.9). Our results support the literature (Liu et al., 2015).

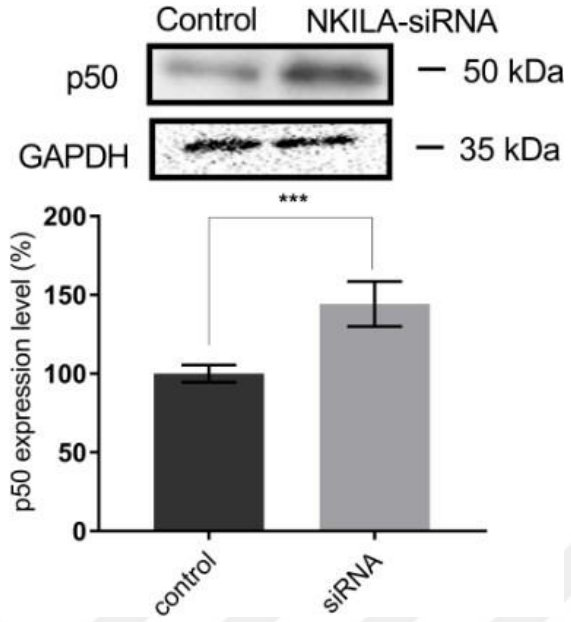

Figure 3.9: Relative expression levels of NF-κB/p50 protein. Band intensities (above the graph) and expression level. Stars indicate significancy level ($p<0.001$).

3.5.4. Relative expression levels of NF-κB/p65

As compatible with the p50 levels, p65 is another NF-κB subunit which increased its relative expression profile after the NKILA-siRNA. According to this, p65 was upregulated by 33±10.4% as significant in comparing to control group ($p<0.01$), (Figure 3.10). However its significancy levels were not high as p50 and p105. Nevertheless, this was also supportive the literature finding (Liu et al., 2015).

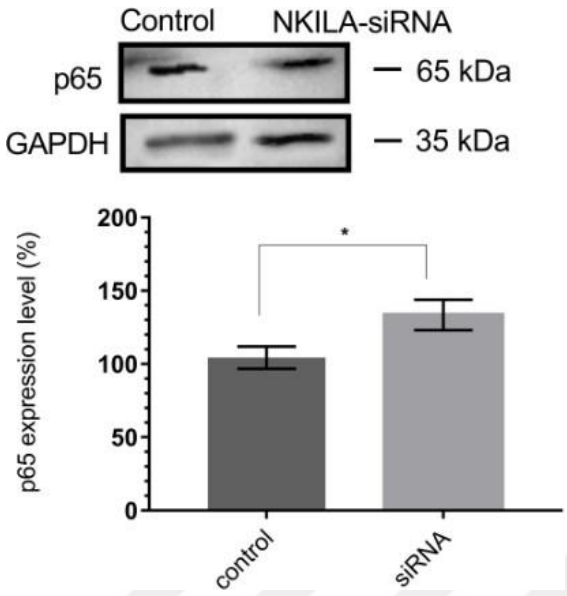

Figure 3.10: Relative expression levels of NF-κB/p65 protein. Band intensities (above the graph) and expression level. Stars indicate significancy level ($p<0.01$).

3.5.5. Relative expression levels of β-catenin

After inhibition of NKILA transcript expressions by 62% of the control levels, β-catenin expression level decreased to 28.51±11.13% of the control groups. We detect a negative correlation between inhibited NKILA and β-catenin expression levels that might be associated through NF-κB pathway. There was significant decrease of the β-catenin expression profile (Figure 3.11).

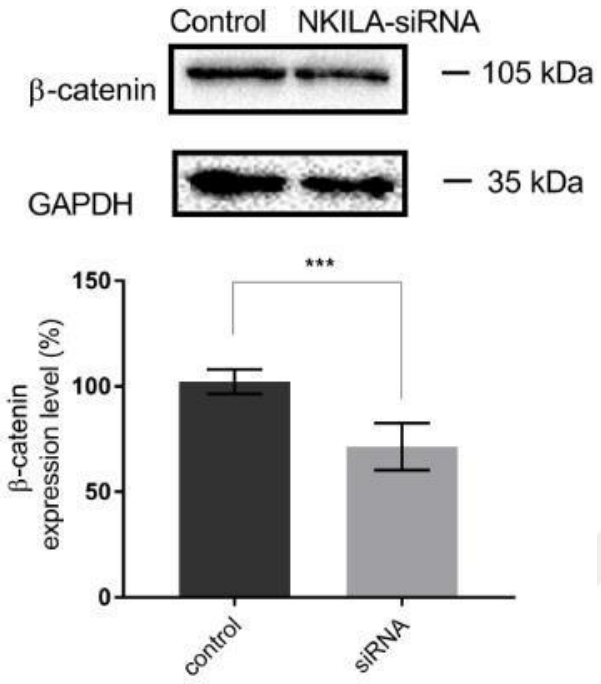

Figure 3.11: Relative expression levels of β-catenin protein. Band intensities (above the graph) and expression level. Stars indicate significancy level ($p<0.001$).

4. DISCUSSION

NF-κB has relation with more than 500 genes which play role in invasion, survival, metastasis and angiogenesis of breast cancer cells, and highly affect these proteins (Wang et al., 2015). Therefore, NKILA show its significance as a negative regulator of the NF-κB, and directly associates with the poor prognosis of cancer patients (Chen et al., 2020). In accordance with this fact, revealing the role of NKILA with other oncogenic proteins is important to enlighten the metastatic and invasive properties of the breast cancer cells.

In this dissertation study, there was a negative association between p105-p50-p65 and NKILA transcript, and NF-κB subunits were significantly ($p<0.001$) increased in the NKILA-siRNA group. Inhibition of the NKILA transcript caused an increase of NF-κB. In this case, this result is expected, because NKILA is a suppressor of NF-κB in the cancer cells (Calderwood, 2010, Liu et al., 2015; Wu et al., 2018). In this study, negative association has been also shown between p105-p50-p65 and NKILA. Moreover, p105 is a precursor protein and it is degraded by the proteasome to form p50 (Fan et al., 1991). p105 involves various cellular mechanisms through the NF-κB signalling (Moorthy et al., 2006). Besides, p105 may have a different role aside from forming the p50 such as involvement in MAP kinase regulation (Waterfield et al., 2003) and it is expressed constitutively (Tan et al., 1994; Zheng et al., 2011). Normally, NF-κB is a complex transcription factor that directly binds to DNA, associated with tumor development, proliferation, and metastatic properties of the cancer cells (Zheng et al., 2011). p105 subunit of NF-κB protein is known to be a precursor of the p50 subunit (NFκB1 homodimer). Additionally, p52, RelA, RelB, and c-Rel are the other parts of this complicated transcription factor. Nevertheless, p105 has a crucial role in the p50 function within the nucleus of the cancer cells (Ishikawa et al., 1998; Cartwright et al., 2016). Rather than NF-κB subunits p65, p50, and p52 homodimers, p105 has independent roles from the NF-κB complex and is related to more signalling pathways within the cancer cells (Ma and Hottiger, 2016). Lin and co-workers, found that NF-κB subunits p65, p105, and p50 showed discrepant expression profiles in non-small cell lung cancer (NSCLC) (Lin et al., 2018). However, p65 and p105 are a marker for the poor prognosis in the NSCLC (Ishikawa et al., 1998).

Our results suggest that NKILA expressions in MCF-7 cells suppress the NF-κB subunits and downregulates catenins. However, HSP90 levels also increased which is suggested as HSP90 might associate with NF-κB transcription levels under the effect NKILA transcript. Due to the blocking effect of NKILA on the NF-κB transcription complex, it seems HSP90 levels were affected by this non-coding protein which normally associates with NF-κB molecule as indicated by literature. Therefore, our findings provide supportive data for the literature and showed the NF-κB levels can be attenuated in the case of NKILA inhibition which is a pivotal molecule for the cancer cell prognosis as shown by several studies.

Moreover, we approve that HSP90α levels increase their expression levels in the siRNA treated group which means HSP90 and NKILA levels can be negatively correlated as with the NF-κB subunits. HSP90α is known as stress protein and provides a necessary response against stress conditions but also attenuates cancer progression (Calderwood, 2010). In this case, after inhibiting the NKILA HSP90 levels may increase its activation as a response to inhibition of an inhibitory molecule NKILA because the NKILA transcript normally suppresses the activity of NF-κB as mentioned above.

HSP90 is responsible for drug resistance, and inflammatory response in cancer. These conditions were also related to the NF-κB which is known as a poor prognosis marker in cancer patients (Thangjam et al., 2012). Under these circumstances, our findings suggest that HSP90 levels might increase as a response to NKILA inhibition because of direct relation with the NF-κB subunits. This brings the question such as NKILA alone can trigger or block the stress response in breast cancer.

β-catenin was significantly decreased ($p<0.001$) in the NKILA-siRNA treated group which can be a sign of the relation between NF-κB and β-catenin. Because, a study showed that catenin and NF-κB negatively correlated because catenin molecules within the cytoplasm can bind to the NF-κB promoter regions (Deng et al., 2002), and that cause antagonistic effect, but also there is another study which found a positive correlation between β-catenin and NF-κB subunits (Cooper et al., 2011). However, in our study, we only showed that β-catenin and NF-κB protein expression levels altered oppositely under the effect of NKILA inhibition. In a study, they detected an association between HSP90 and β-catenin levels as related to the GSK3-β signalling pathway in the MCF-7 cells (Cooper et al., 2011). Another study indicated

a negative correlation between these two proteins. Deng and co-workers showed that β-catenin can interact and inhibit the NF-κB transcription factor in colon and breast cancer cells. Additionally, high levels of β-catenin within the cytoplasm decline the NF-κB activation (Deng et al., 2002).

NKILA also negatively affects the interleukine-11/signal transducer and activator of transcription 3 (IL-11/STAT3) signalling in lung cancer cells (Liu and Shi, 2019). This means that it is normal to have significant expression changes of the HSP90 and catenin other than the NF-κB because STAT3 signalling has also cross-regulation with the Wnt (Alshaer et al., 2019) and one of the client proteins of the Hsp90 in BC (Cho et al., 2019), and responsible for the mortality of the patients (Ernst et al., 2008). Concomitantly, crosstalk relation with copious signal transduction renders the NKILA pertinent to many signalling pathways. In our study, HSP90 levels increased as catenin levels declined in the NKILA-siRNA group. We suggest that this may be caused by the possible negative correlation between HSP90 and β-catenin in the cytoplasm but this suggestion requires another study that focused on catenin and HSP90 relation. Nevertheless, after inhibition of NKILA increase of HSP90 with β-catenin decreasing suggests the idea of a negative correlation between these two molecules. We suggest that NKILA inhibition indirectly upregulated the HSP90α in the MCF-7 cells (Figure 4.1).

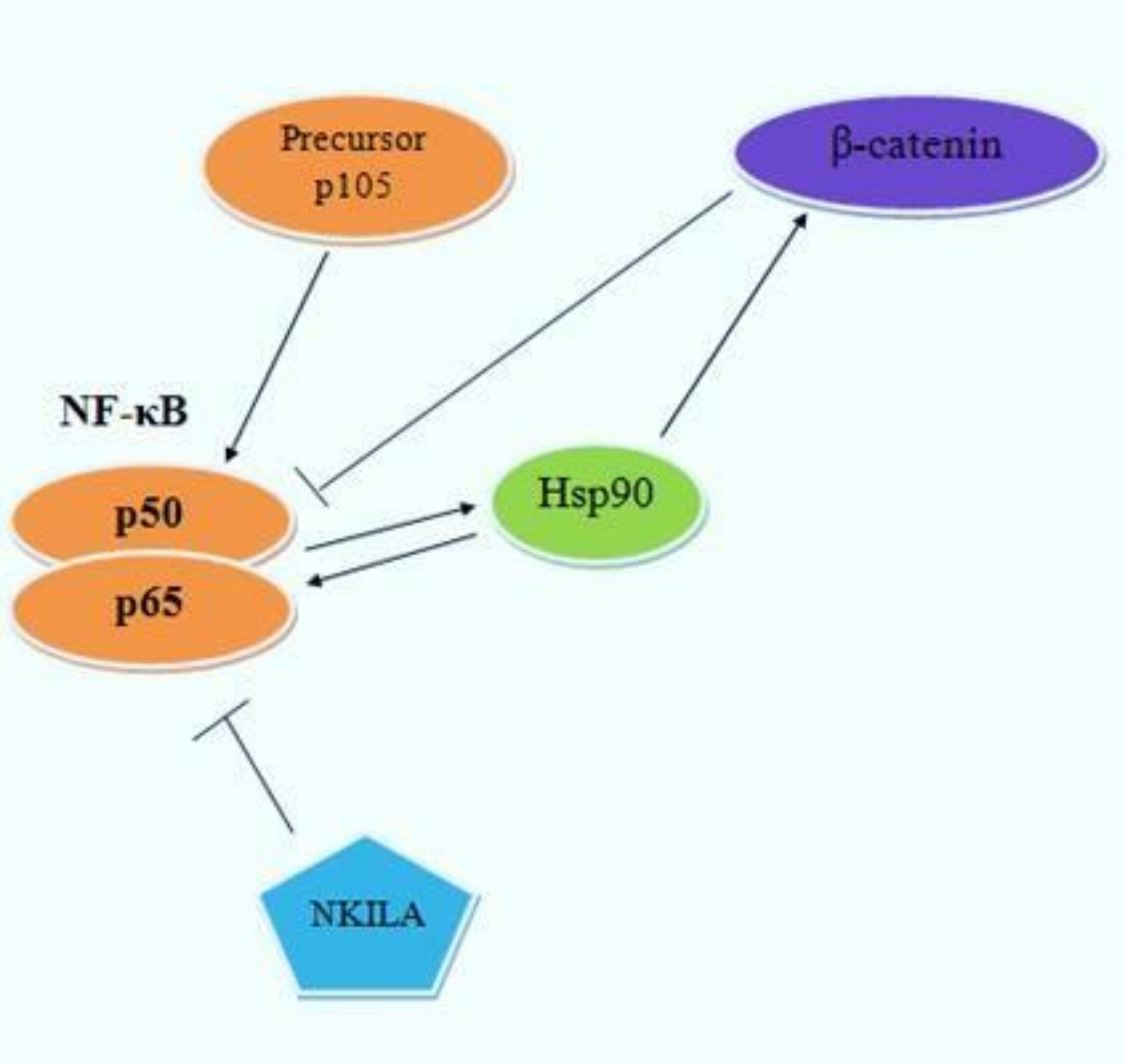

Figure 4.1: Possible association between NKILA, HSP90α, β-catenin and NF-κB proteins. Our findings support the literature which indicates a negative association between β-catenin and NF-κB due to crosstalks between their relevant signalling pathways. Plus, as a stress protein HSP90 might involve this association as a response to NF-κB emerging after NKILA inhibition which is a natural NF-κB suppressor. HSP90 was shown to be positively regulated by NF-κB in various signalling pathways or by directly binding to HSP90 promoter in literature. We suggest NKILA inhibition is indirectly upregulated HSP90 and β-catenin in MCF-7 cells.

We detected the increase of p50, p65, and p105 levels as with a decrease of β-catenin in NKILA-siRNA treated group. Most probably, NF-κB increased its expression in the

cytoplasm as a result of after inhibition of NKILA which is a negative regulator of the NF-κB by silencing then showing its antagonistic effect. Further studies are needed to approve this implication but decline of catenin levels while increasing of NF-κB can be suggested in this way. However, the association between β-catenin and NKILA transcript has never been shown before and there are no other data to compare our results recently. Thus, future studies again are needed to clarify the relation between these molecules in the manner of relevant signalling pathways.

We can suggest that this opposite direction of β-catenin and NF-κB expression levels after NKILA inhibition may result due to the Wnt/β-catenin signalling pathway which normally shows crosstalk with canonical NF-κB signalling pathway. β-catenin and p50 might show a negative correlation (Cooper, 2011) but in another study, it was positively associated with β-catenin in glioblastoma cells (Zhang et al., 2012). Generally, NF-κB crosstalking and competes with the catenin signalling pathway in breast cancer cells (Wang et al., 2015). Therefore we can suggest that increase of NF-κB as with the decline of β-catenin levels might be related to crosstalk of these proteins.

These results suggest that NKILA expressions in MCF-7 cells suppress the NF-κB subunits and down-regulates catenins. However, HSP90 levels also increased which is suggested as HSP90 might associate with NF-κB transcription levels under the effect NKILA transcript. Due to the blocking effect of NKILA on the NF-κB transcription complex, it seems HSP90 levels were affected by this non-coding protein which normally associates with NF-κB molecule as indicated by literature.

5. CONCLUSION AND RECOMMENDATIONS

Today, efficient therapeutic approaches are still challenging due to the intricate and complex total structure of the signalling network which involves in BC development. Furthermore, molecular therapeutic approaches such as targeting HSP proteins, inhibiting the oncogenic gene regions, or combined drugs are giving hope to improve survival rates of BC patients. Nevertheless, cancer is a major health problem all over the world and especially, breast cancer protects its high mortality rate among women. Therefore, it is possible to argue that therapeutic approaches or combinatory experiments are not enough to significantly inhibit the BC with the low level of side effects on the patients. We assume that targeted therapies might not be significant until lncRNAs are not considered as possible cancer-related regulators of the oncogenic proteins such as HSPs, NF-κB, catenins, etc. Many of the proteins within the cytoplasm and nucleic environment have unique regulators from the non-coding RNA family. Protein-protein interactions or targeted therapies which aim to inhibit oncogenic proteins cannot be solely enough to inhibit cancer sufficiently because of these hidden players in the molecular realm. They are responsible to control, inhibit, upregulate or downregulate the proteins in the cells and capable of intervening in the transcriptional process during cellular development. Therefore, molecular targeting therapies should consider all these non-coding especially, lncRNAs to reach an optimum level of cancer inhibition. Because lncRNAs can divert the signalling mechanisms by acting as a substructure of the signalling pathways and other related molecular networks in all cell types. Aiming of these hidden players of the molecule world is giving promising results to understand and cure the complicated cancer mechanism.

Consequently, the first time we found that HSP90 and NF-κB subunits expression levels were increased but β-catenin levels decreased after NKILA inhibition in MCF-7 breast cancer cells. Thus, we suggest that these molecules might in a vanishing point as associated with NKILA because this transcript has a direct effect on the NF-κB protein subunits which is one of the main crosstalk molecules in the cancer cells. Today, an increasing number of studies focusing on the noncoding RNA-protein association to enlighten and reveal the mechanism of cancer development in molecular biology and clinic science (Deng, et al., 2002; Guo, et al., 2019). Thus, more detailed studies about non-coding RNA molecule involvement in various

signalling pathways should be investigated. However, there is no other information in the literature about HSP90α and β-catenin association with the NKILA transcript. Therefore, further studies are needed to clarify and/or approve the role of this transcript molecule on these protein molecules which are on the crosstalk points between various signalling pathways. Besides, regulatory effects of NKILA transcript on the other signalling pathways different than NF-κB pathway might reveal interesting key points to explain the dual role of NF-κB subunits. With all these aspects, these aforementioned future studies might be pivotal for a better understanding of cancer cell development and targeted therapies.

, Highlights, Limitations and Future Aspects:

- The first time we showed that NKILA is associated with a stress protein (HSP90α) and β-catenin.
- An increase of NF-κB after NKILA inhibition is a supportive finding for the studies in the literature about NKILA effects on NF-κB.
- These findings may present useful and novel data for the NF-κB targeted cancer therapies.
- We had some limitations about laboratory materials and shortage of some consumables. Therefore, we had to cancel some of the experimental works. Due to these kinds of limitations, we have restricted our research content to minimize the expenditure of consumables.
- We need to show other related stress proteins and Wnt/β-catenin pathway proteins to clarify the NKILA roles in cancer development in addition to NF-κB negative regulation.
- There is no other study that showed the HSP90α and β-catenin relation with NKILA which might involve rudimental information, thus Stress Response-Wnt signalling and NKILA association is needed to be investigated in more detail.
- Other related lncRNAs should be investigated to understand the crosstalk mechanism between Wnt and NF-κB signalling pathways in the cancer cells. Because these cells are in the center of cancer development almost every type of cancer cell.

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