MOLECULAR CHARACTERIZATION OF THE GTF2A-1 ANTISENSE LONG NON-CODING RNA

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by
Murat Caner YARIMÇAM

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İZMİR

We approve the thesis of **Murat Caner YARIMÇAM**

Examining Committee Members:

____

Assoc. Prof. Dr. Bünyamin AKGÜL
Department of Molecular Biology and Genetics, Izmir Institute of Technology

____

Assoc. Prof. Dr. Özden YALÇIN ÖZUYSAL
Department of Molecular Biology and Genetics, Izmir Institute of Technology

____

Prof. Dr. Ayşe Semra KOÇTÜRK
Department of Medical Biochemistry, Dokuz Eylül University

22 December 2017

____

Assoc. Prof. Dr. Bünyamin AKGÜL
Supervisor, Department of Molecular Biology and Genetics,
Izmir Institute of Technology

____________________________	__________________________
Prof. Dr. Volkan SEYRANTEPE	**Prof. Dr. Aysun SOFUOĞLU**
Head of the Department of Molecular Biology and Genetics	Dean of the Graduate School of Engineering and Sciences

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ABSTRACT

MOLECULAR CHARACTERIZATION OF THE GTF2A-1 ANTISENSE LONG NON-CODING RNA

One of the essential events in cell regulation and normal development of an organism is apoptosis. The dysregulation of apoptosis is associated with diseases such as cancer. Apoptosis induction can kill cancer cells without harming the individual. For this purpose, new methods are developed to fight the cancer cells. One of the novel approaches is based on long non-coding RNAs (lncRNAs). LncRNAs are differentially expressed in cancer cells and they regulate and interact essential pathways. The ones related to apoptosis are the targets. In this study, target lncRNA was determined based on RNA-Seq data. Then apoptosis was induced in HeLa cells with cisplatin and qRT-PCR was performed with isolated RNAs from the cells to validate the data with regard to upregulation of GTF2A-1 anti-sense lncRNA in apoptosis. Then GapmeR specific to target lncRNA was designed and transfected into HeLa cells in order to induce apoptosis. After induction of apoptosis, total RNA and protein were isolated from the cells. qRT-PCR was performed to validate the RNA-Seq data. Western blotting was performed in order to characterize the target lncRNA by controlling its effects on different apoptosis pathways. Western blotting results are showing resemblance between GTF2A-1 anti-sense lncRNA silencing-induced apoptosis and cisplatin-induced apoptosis. The western blotting result of Cytochrome c is interesting because its amount is decreased in GTF2A-1 anti-sense lncRNA silencing-induced apoptosis. The candidate, GTF2A-1 anti-sense lncRNA, is directly regulating the apoptosis in HeLa cells and in this study, some of the pathways that are regulated with this lncRNA were shown.

Keywords: apoptosis, long non-coding RNA, GTF2A-1, anti-sense

ÖZET

GTF2A-1 ANTİSENS UZUN KODLAMAYAN RNASININ MOLEKÜLER KARAKTERİZASYONU

Apoptoz, kontrollü hücre ölümü demektir ve hücre içi dengelerin sağlanması ve hücrelerin normal gelişimi konusunda çok önemli roller oynar. Apoptoz mekanizmasının bir şekilde düzgün çalışmadığı durumlar genel olarak hastalık durumlarıdır. Bu hastalıklardan biri de kanserdir. Apoptozun indüklenmesi, bu kanser hücrelerini bireydeki diğer dokulara zarar vermeden öldürebilir. Bu sebeple, kanser hücreleri ile mücadelede apoptozun indüklenmesi temelinde yeni metotlar geliştirilmektedir. Geliştirlen yeni metotlardan birisi uzun kodlamayan RNAları (ukmRNA) kullanarak apoptozu indüklemektir. Bazı ukmRNAlar kanser hücrelerinde apoptozu kontrol ederler. Hedefler apoptoz ile ilişkili ukmRNAlar arasından seçilir. Bu ukmRNAların karakterizasyonu ve apoptozla olan ilişkilerinin bulunması, kansere karşı savaşta yeni yöntemler bulunmasını sağlamıştır ve sağlayacaktır. Bu çalışmada, hedef ukmRNA önceki çalışmadan gelen RNA-Sek datası kullanılarak bulunmuştur. İlk olarak HeLa hücrelerinde sisplatin kullanılarak apoptoz tetiklenmiştir. Sonra bu hücrelerden elde edilen RNAlarla yapılan qRT-PCR deneyinde elde edilen sonuç RNA-Sek datasını doğrulamak için kullanılmıştır. Sonrasında hedef ukmRNAya spesifik GapmeR dizayn edilmiştir ve apoptozu tetiklemek için HeLa hücrelerine transfekte edilmiştir. Apoptozun tetiklenmesinden sonra, hücrelerden total RNA ve protein izole edilmiştir. Sonrasında ukmRNA-Sek datasını doğrulamak için qRT-PCR yapılmıştır. Hedef ukmRNAnın farklı apoptoz yolakları ile ilişkisini anlamak için western blot yapılmıştır. Western blot sonuçları GTF2A-1 anti-sens ukmRNAsının susturulması ile indüklenen apoptoz ile sisplatin uygulaması ile indüklenen apoptoz arasında benzerlikler göstermektedir. Western blot sonuçları, sitokrom c proteininin GTF2A-1 anti-sens ukmRNAsının susturulması ile indüklenen apoptoz koşullarında azaldığını göstermesi sebebiyle ilgi çekici bir yerde durmaktadır. Yapılan bu çalışmada; seçilen adayın, yani GTF2A-1 anti-sens ukmRNAsının HeLa hücrelerinde apoptozu kontrol ettiği ve apoptozu hangi yolaklar üzerinden kontrol ettiği, birkaç farklı yolak kontrol edilerek gösterilmiştir.

Anahtar Kelimeler: apoptoz, uzun kodlamayan RNA, GTF2A-1, anti-sens

You mere mortals

You are useless

Everything in the universe is

Completely meaningless

But of course

There is hope for us

Who live their short lives

Seeking their dreams

But a poem is never actually finished

It just stops moving

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CHAPTER 1

INTRODUCTION

1.1. Apoptosis

Programmed cell death (PCD) is a phenomenon in which a cell or the cells of an organism commit "suicide" in the presence of pathological events or in the development process of said organism in order for that organism to survive. There are three major forms of PCD and these are apoptosis, autophagy and programmed necrosis. Apoptosis was the first in this group of PCDs to be discovered in 1972. The word apoptosis comes from a Greek word meaning "falling off", like leaves from a tree (Ouyang et al., 2012). Apoptosis is an essential process for the development process of multicellular organisms as well as normal cell turnover, normal immune system development and function, hormone-dependent atrophy and cell death induced by chemicals. If apoptosis goes out of control, it may cause many disorders including cancer in humans (Taylor, Cullen & Martin, 2008).

The process of apoptosis affects the morphology of cells and these changes can easily be observed by light microscope. In the early stages of apoptosis, cell shrinkage and chromatin condensation occur. Then, the membrane of the cell gets "blebbed" and nuclear fragmentation and budding occur. In the process of budding, fragments that consist of cytoplasm and organelles of cells are tightly packed and are called "apoptotic bodies". These bodies are then phagocytosed by macrophages, neoplastic or parenchymal cells and are degraded by phagolysosomes (Hollville & Martin, 2016). There are also biochemical changes that are happening during apoptosis in addition to morphological changes and the most important one of these changes is the fragmentation of DNA (Jayakiran, 2015). Apoptosis is an important field of study for scientists because it can be used to cure diseases. The ability to send suicide signals to harmful cells is a method that is and will be developed for a spectrum of diseases (Wong, 2011).

1.2. Distinct Mechanisms of Apoptosis

Apoptotic mechanisms are mediated by molecular events that are energy-dependent cascades. Therefore it suffices to say that the mechanisms behind apoptosis are diverse and highly complex. There are two mechanisms of apoptosis: Caspase-dependent mechanisms and Caspase-independent mechanisms. Caspase-dependent mechanisms, as their name implies, are dependent on caspases and consist of extrinsic, intrinsic, execution (which is the end point of both and based on the activation of execution caspases -3, -6 and -7) and Perforine/Granzyme pathways (Ouyang et al., 2012). All of these pathways have their end-point in activation of execution caspases. The caspase-dependent pathways can be activated by certain drugs and ligands. There are known anti-cancer drugs, such as cisplatin and ligands, such as TNF-α (Tumor Necrosis Factor alpha) that can induce apoptosis in cells (Kolb et al., 2017). Caspase-independent mechanisms can be described as the mechanisms of apoptosis where there is no caspase activation. They consist of pro-apoptotic proteins that are released from the mitochondria in late-apoptosis phase and also the Granzyme A pathway that activates the apoptosis by inducing single-strand DNA breaks (Cao et al., 2007; Kaufmann, 2007).

In this study, the focus is on caspase-dependent mechanisms. Therefore, there will be no explanation of caspase-independent pathways as well as Perforine/Granzyme pathway because they are not related with this study.

1.2.1. Caspase-Dependent Mechanisms

The main caspase-dependent pathways are the extrinsic pathway and intrinsic pathway. All of the pathways induce the cleavage of caspase-3, which is the starting point of the terminal-execution pathway (Figure 1.1). This pathway results in fragmentation of DNA, degradation of cytoskeletal and nuclear proteins, mass protein cross-linking, expression of ligands for phagocytosis and formation of apoptotic bodies (Elmore, 2007).

Caspases are proteases and they are the members of cysteine protein family. They are present in most cell types and are mainly playing roles in apoptotic and inflammatory signaling pathways. Their name is an abbreviation of the words "***cy***steine-***asp***artic prote***ases***" or "***cy***steine-dependent ***asp***artate-directed prote***ases***". They are proenzymes and are in an inactive state in the cytoplasm. They are activated when they are cleaved

and they start to activate other caspases (McIlwain, Berger & Mak, 2013). When caspase activation starts, the cell undergoes an irreversible and rapid apoptosis. There are sixteen caspases identified to date and they are categorized into three classes: initiator apoptotic caspases (caspase-2, -8, -9 and -10), executioner (effector) apoptotic caspases (caspase-3, -6 and -7) and inflammatory caspases (caspase-1, -4 and -5). Caspases 11 through 16 are not playing major roles in apoptosis; they are tissue specific and some of them are not related with apoptosis within the current knowledge (Elmore, 2007; Ichim & Tait, 2016).

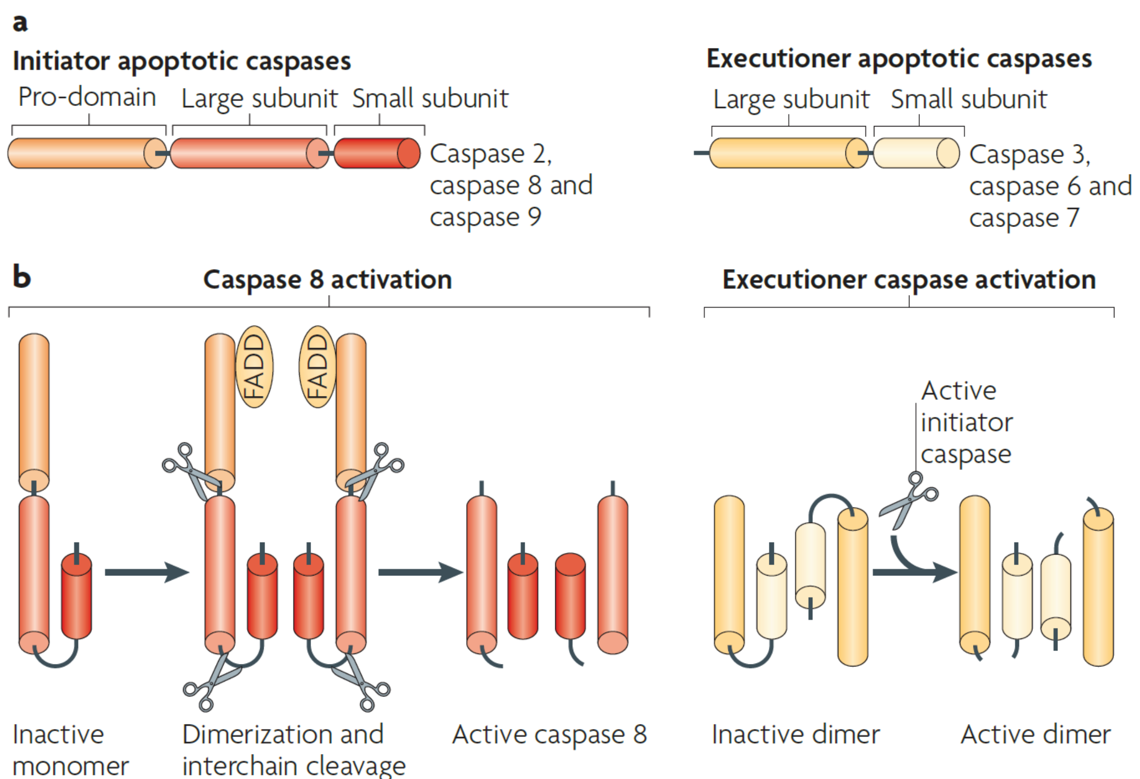


Figure 1.1. Representation of caspase activation. (a) Apoptotic caspases can be divided into two subgroups and the differences are shown in between these groups. (b) Initiator caspase activation is normally required for activation of executioner caspases. Initiator caspases have a lot less substrates (including self-cleavage, BH3-interacting domain death agonist (Bid) and executioner caspases) than executioner caspases (including cleaving of hundreds of different substrates and being responsible for the phenotypic changes during apoptosis). Dimerization and interdomain cleavage are required for activation and stabilization of mature caspase-8. Therefore, after death receptor ligation, dimers are formed by the recruitment of caspase-8 monomers through their pro-domains to the adaptor molecule FAS-associated death domain protein (FADD). The executioner caspases are activated. (Source: Tait & Green, 2010)

1.2.1.1. Extrinsic Pathway

There are six “death domain” containing receptors identified to date. Death domains, DD, are cytoplasmic 80 amino acid residues and transmit the “death” signals to intracellular signaling pathways. These are tumor necrosis factor receptor 1 (TNFR1), TNF receptor-related apoptosis-mediating protein (TRAMP), fatty acid synthase receptor (FasR), TNF-related apoptosis-inducing ligand receptor I and II (TRAIL-R1 and TRAIL-R2) and death receptor 6 (DR6) (Ashkenazi, 2008). The induction of apoptosis can be initiated by death receptors; transmembrane receptors like the tumor necrosis factor (TNF) receptor superfamily and thus, the transduced pathway is called an extrinsic signaling pathway (Park, 2011).

Fas and TNF receptors cluster at the cell surface and binding of trimeric Fas and TNF ligands to the corresponding receptors results in recruitment of adaptor proteins having death effector domains (DED): FADD (Fas-associated death domain protein) recruits FasR (Fas Receptor) upon FasL (Fas Ligand) binding; TRADD (TNF receptor-associated death domain) is recruited to the TNFR1 upon TNF-α binding. TRADD itself mediates recruitment of FADD and RIP (Receptor-interacting protein) and a death-inducing signaling complex (DISC) is formed after association of procascase-8 with FADD via dimerization of the death effector domain. The results of the formation of this complex are autocatalytic activation of procaspase-8 and induction of apoptotic execution pathway (Figure 1.2) (Park et al., 2007). However, the apoptotic execution pathway can be inhibited by c-FLIP (FLICE-inhibitory protein) by binding to the association of FADD/caspase-8 which inactivates them. c-FLIP can also suppress caspase-10 activation to inhibit the downstream apoptosis cascade (R Safa, 2013).

1.2.1.2. Intrinsic Pathway

The intrinsic pathway of apoptosis, also known as the mitochondrial pathway, is the most common mechanism of apoptosis. This pathway is activated by a variety of signals, such as developmental signals (hormones) and other stress-inducing stimuli (cytoskeletal disruption, DNA damage, accumulation of unfolded proteins, hypoxia). As a result of this activation, mitochondrial outer membrane permeabilization (MOMP) occurs in cells. This can be described as a loss of mitochondrial transmembrane potential

that is a result of an opening of the mitochondrial permeability transition (MPT) pore due to changes in the inner membrane of the mitochondria (Tait & Green, 2010). Some BCL family proteins are normally localized in the space between the outer and inner membranes of mitochondria but in the event of MPT formation, members that are pro-apoptotic are released from mitochondria. The pro-apoptotic members of the BCL family are as follows: Cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF, CAD and endonuclease G. These molecules can induce apoptosis by caspase-dependent or –independent manner. Caspase-dependent inducers are Cytochrome c, Smac/DIABLO and Omi/HtrA2. Cytochrome c, when released, binds and activates Apaf-1 and procaspase-9 and this formation is called an "apoptosome". Apoptosome formation leads to caspase-9 activation, thus leading to activation of the downstream executioner caspases, caspase-3 and caspase-7 (Figure 1.2). Smac/DIABLO and HtrA2/Omi induce apoptosis by inhibiting the inhibitors of apoptosis proteins (IAP) activity (Elmore, 2007; Inoue et al., 2009; Kaufmann, 2007).

The BCL-2 family of proteins plays very important roles in the control and regulation of intrinsic pathway events. The members are either pro-apoptotic or anti-apoptotic and they regulate the permeability of the mitochondrial membrane. These proteins are regulated by the p53, the tumor suppressor protein. Some of the anti-apoptotic members of this family are Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG. Some of the pro-apoptotic members of this family are Bcl-10, Bax, Bak, Bid, Bad and Blk (Taylor et al., 2008). The balancing act between pro-apoptotic and anti-apoptotic members of the BCL-2 family controls cell fate by controlling the release of Cytochrome c (Figure 1.2) (Jiang & Wang, 2004).

There is a cross-talk between the extrinsic and intrinsic pathways. Bid is cleaved by caspase-8 after the induction of apoptosis via the extrinsic pathway and thus, the mitochondria is damaged by truncated Bid. Truncated Bid migrates to the mitochondria where it induces the outer mitochondrial membrane permeabilization that is dependent on Bax and/or Bak. Thus Bid acts as a sentinel for protease-mediated death signals (Figure 1.2) (Billen, Shamas-Din & Andrews, 2008).

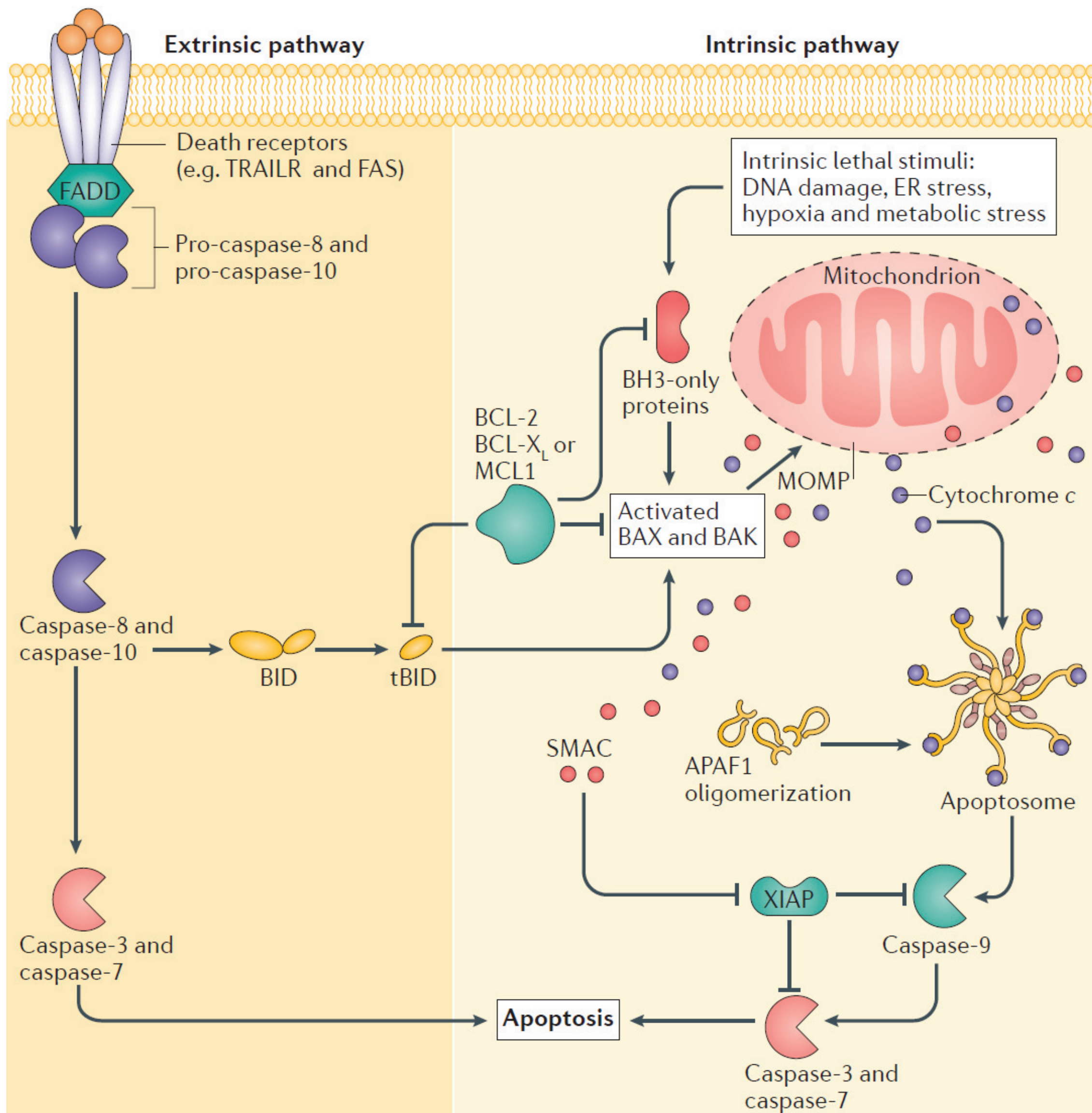


Figure 1.2. Extrinsic and intrinsic apoptotic signaling pathways. In the extrinsic apoptotic pathway, death receptors such as TRAILR and FAS can activate initiator caspases (caspase-8 and caspase-10) when binding to their ligand through dimerization that is mediated by adaptor proteins, such as FADD. The effector caspase-3 and caspase-7 are cleaved and activated by active caspase-8 and caspase-10, leading to apoptosis. The intrinsic (or mitochondrial) pathway of apoptosis requires MOMP. Anti-apoptotic BCL-2 family proteins counteract BH3-only protein activation that is engaged by cell stress which leads to BAX and BAK activity that triggers MOMP. Following MOMP, second mitochondria-derived activator of caspases (SMAC) and Cytochrome c, which are some of the mitochondrial intermembrane space proteins, are released into the cytoplasm. Cytochrome c interacts with APAF1, triggering apoptosome assembly, which activates caspase-9. Active caspase-9 then activates caspase-3 and caspase-7, leading to apoptosis. After release from mitochondria, SMAC mediates apoptosis by blocking the caspase inhibitor X-linked inhibitor of apoptosis protein (XIAP). (Source: Ichim & Tait, 2016)

1.3. Long Non-Coding RNAs

The story of so called “junk DNA” is one with a very surprising end. The genome size, based on C-value, is not an indicator of organism size or complexity. This phenomenon, called “C-value paradox” was partially solved after the discovery of the non-coding portion of the genome, which is much more abundant than the coding regions of eukaryotic genomes. Scientists called this huge non-coding portion “junk DNA” because it did not share similarities with coding sequences and contains lots of transposons, simple repeats and pseudogenes, which are estimated to constitute nearly 70% of the human genome (Eddy, 2012; Palazzo & Gregory, 2014). Today, it is understood that there is no paradox, instead much research needed to discover the roles and functions of these non-coding regions. Even though they were called “junk”, scientists studied these sequences as far back as the 1970s (Pennisi, 2012). The first speculations about these portions of the genome were related with rRNAs and tRNAs, which are non-coding RNAs themselves. Studies in the 2000s provided very promising results for the future of non-coding RNAs. It was found that nearly 90% of the human genome is transcribed at some point in development of an individual. This phenomenon is called “pervasive transcription” and may be related to alternative splicing and extensions of known protein coding genes (Encode Consortium, Carolina & Hill, 2013). Novel studies support non-coding gene transcription in intergenic regions that have a correlation with chromatin signatures, histone modifications or transcription factor binding. Today, the number of long non-coding RNAs that have been identified passes the twenty thousand mark and computationally predicted lncRNAs have passed the hundred thousand mark and these numbers are increasing every day (Volders et al., 2015).

An lncRNA, by definition, is a non-coding RNA that is larger than 200 nucleotides. Their lengths can go up to 100 kilobases (kb) and they are transcribed by RNA polymerase II and are poly-adenylated (Bhat et al., 2016). lncRNAs are mRNA-like transcripts but they lack stable open reading frames. They are generally tissue-specific and transcribed in lower amounts when compared to coding genes (Fatima et al., 2015). Studies suggest that lncRNAs play negative or positive roles in multiple levels of regulation in cells, such as gene expression regulation at the transcriptional and post-transcriptional levels in development, differentiation and human diseases (Nie et al., 2012).

1.3.1. Classification of Long Non-Coding RNAs

The current approach of scientists towards the classification of lncRNAs is to categorize them based on their genomic locations. Therefore, there are six groups of lncRNAs based on this categorization: Sense, Anti-sense, Bi-directional, Intronic, Intergenic and Enhancer (Figure 1.3) (Devaux et al., 2015). It has been shown that up to 70% of sense transcripts have anti-sense counterparts. There are sense-anti-sense (SAS) pairs that can overlap completely, partially or be nested. There are natural anti-sense transcript (NAT) lncRNAs that tend to locate around the promoter or terminator regions (end) of the sense transcripts. It was known that small ncRNAs such as snoRNAs and miRNAs are transcribed from introns (Nie et al., 2012). Recent studies show similar transcription of lncRNAs from annotated genes. Although few of them have been studied detailed, many of them were differently expressed in various conditions and in cancer (Zhao et al., 2015).

1.3.2. Functional Roles of Long Non-Coding RNAs

Our current understanding of the functional roles of lncRNAs is very limited. However, some lncRNAs such as *Xist*, *Tsix*, *HOTAIR*, *MALAT1* and *ANRIL* were functionally characterized and are known members of the lncRNA world. Even though the number of studies and identified lncRNAs are relatively low, the studies show a pattern about the roles of lncRNAs in regulation of cellular events. The differentially expressed lncRNAs are associated with disease states and developmental processes (Engreitz et al., 2013; Wilusz, 2017; Zhai et al., 2016).

lncRNAs play roles in many diverse cellular processes. One of them, and the most-studied one, is epigenetic regulation of allelic expression and some lncRNAs in this category play role in genomic imprinting and dosage compensation. An example would be the well-known event of X chromosome inactivation (XCI). During XCI, the X chromosome to be inactivated has a very active cluster of lncRNA loci, X-inactivation center (Xic), and from this loci, X (inactive)-specific transcript (*Xist*) that is 17 kb long is highly expressed. Then, *Xist* lncRNAs coat the X chromosome in which they are expressed to form a "*Xist*" cloud and act as scaffold to recruit the silencing factors. There

is another lncRNA, named *Tsix* that binds to *Xist* lncRNA and nullifies its effects on inactivated X chromosome (Chu et al., 2015; Engreitz et al., 2013).

lncRNAs also play roles in several regulatory pathways and several (if not all) cellular processes. They directly affect development of an organism, diseases and apoptosis of cells. Some of the known lncRNAs that play roles in cancer cell proliferation and apoptosis are *ANRIL*, *HOTAIR*, *MALAT1*, *PANDA* and *GAS5* (Fatima et al., 2015; Pickard & Williams, 2014; Schmitz, Grote & Herrmann, 2016; Zhai et al., 2016).

1.3.3. Action Mechanisms of Long Non-Coding RNAs

Current knowledge about lncRNA mechanisms of action is subject to change in the future. This is because there are many studies about this topic and novel findings will expand our knowledge about the mechanisms of action of lncRNAs.

LncRNAs can evict proteins from chromatin in order to ensure mRNA transcription. An example is the pancRNAs that prevent DNMT from methylating the DNA in their promoter region, thus they ensure mRNA transcription (Devaux et al., 2015). LncRNAs can stabilize looping and recruit transcriptional regulators or they may inhibit loop formation, meaning they can regulate the transcription process. An example is that they can interfere with RNA Polymerase II activity in both ways, promoting transcription or preventing it by promoting or preventing the recruitment of transcription factor complex (Chen, 2016). They may act as decoys for transcription factors or compete for transcription factor binding to the gene and they may even affect the cellular localization of transcription factors (Pickard & Williams, 2016). They may recruit proteins, such as chromatin-modifying complexes to specific target sites in the genome (Patil et al., 2016). They may act as scaffolds linking different proteins required for concerted action (Tsai et al., 2010). LncRNAs may bind and sequester proteins to prevent or attenuate their action (Li & Fox, 2016). They may bind to mRNAs and sequester miRNAs to prevent their binding to mRNAs (Ballantyne, McDonald & Baker, 2016). They may change the splicing pattern of an RNA by binding to a primary RNA transcript (Gonzalez et al., 2015). They may stabilize an mRNA by recruiting appropriate proteins, thereby preventing degradation of mRNAs (Figure 1.4) (Shibayama et al., 2014). LncRNAs may act as sponges for miRNAs and some other small non-coding RNAs (Paraskevopoulou & Hatzigeorgiou, 2016). They are shown to interfere with miRNA-

mediated mRNA destabilization by competing with the miRNAs or masking their binding sites (Jalali et al., 2013). Some lncRNAs have miRNA binding sites in their 3'-UTRs and these binding sites may serve as sponges to keep miRNAs away from their mRNA targets (Paraskevopoulou & Hatzigeorgiou, 2016). Also, lncRNAs may be host genes for small non-coding RNAs, like miRNAs (Guo et al., 2014).

Scaffolding, recruiting and tethering features that are mentioned before are associated with epigenetics. By *cis* acting, they mediate the recruitment of proteins for regulation of chromatin states. They may also achieve the same goal by *trans* acting (Kapusta et al., 2013).

1.3.4. Bridge between Long Non-Coding RNAs and Apoptosis

Studies showed that the expression rates of lncRNAs are subject to change in apoptosis conditions. They may be upregulated or downregulated during apoptosis and this information may give insight about their approach to apoptosis. They may act as pro-apoptotic or anti-apoptotic agents. In the literature, there are known pro-apoptotic and anti-apoptotic lncRNAs in human cells. These lncRNAs have distinct mechanism of action in regulation of apoptosis. LncRNA *PANDA* limits the expression of certain pro-apoptotic genes (Hung et al., 2011). LncRNA *LET* interacts with p53 to suppress the effects of pro-apoptotic proteins, prevents epithelial-to-mesenchymal transition and is downregulated in tumor cells (Liu et al., 2016). LncRNA *HOXA-AS2* (HOX Anti-sense lincRNA) positively regulates the proliferation of tumor cells by suppressing the transcription of certain pro-apoptotic genes (Wang et al., 2016). *LincRNA-p21* is a posttranscriptional inhibitor of translation (Yoon et al., 2012) and it enhances p53 tumor suppressor activity (Wu et al., 2014). LncRNA *GAS5* (growth arrest-specific transcript 5) induces apoptosis in cells by regulating several tumor-suppressor proteins. It is a small nucleolar ribonucleic acid (snoRNA) host gene and these snoRNAs also play role in induction of apoptosis. It also play role in miRNA sequestration (Mourtada-Maarabouni et al., 2009; Pickard, Mourtada-Maarabouni & Williams, 2013; Pickard & Williams, 2016; Pickard & Williams, 2014; Zhang et al., 2013). LncRNA *INXS* is a critical mediator of Bcl-XS induced apoptosis by leading the synthesis of Bcl-XS pro-apoptotic protein that is an apoptosis inducer (Deocesano-Pereira et al., 2014).

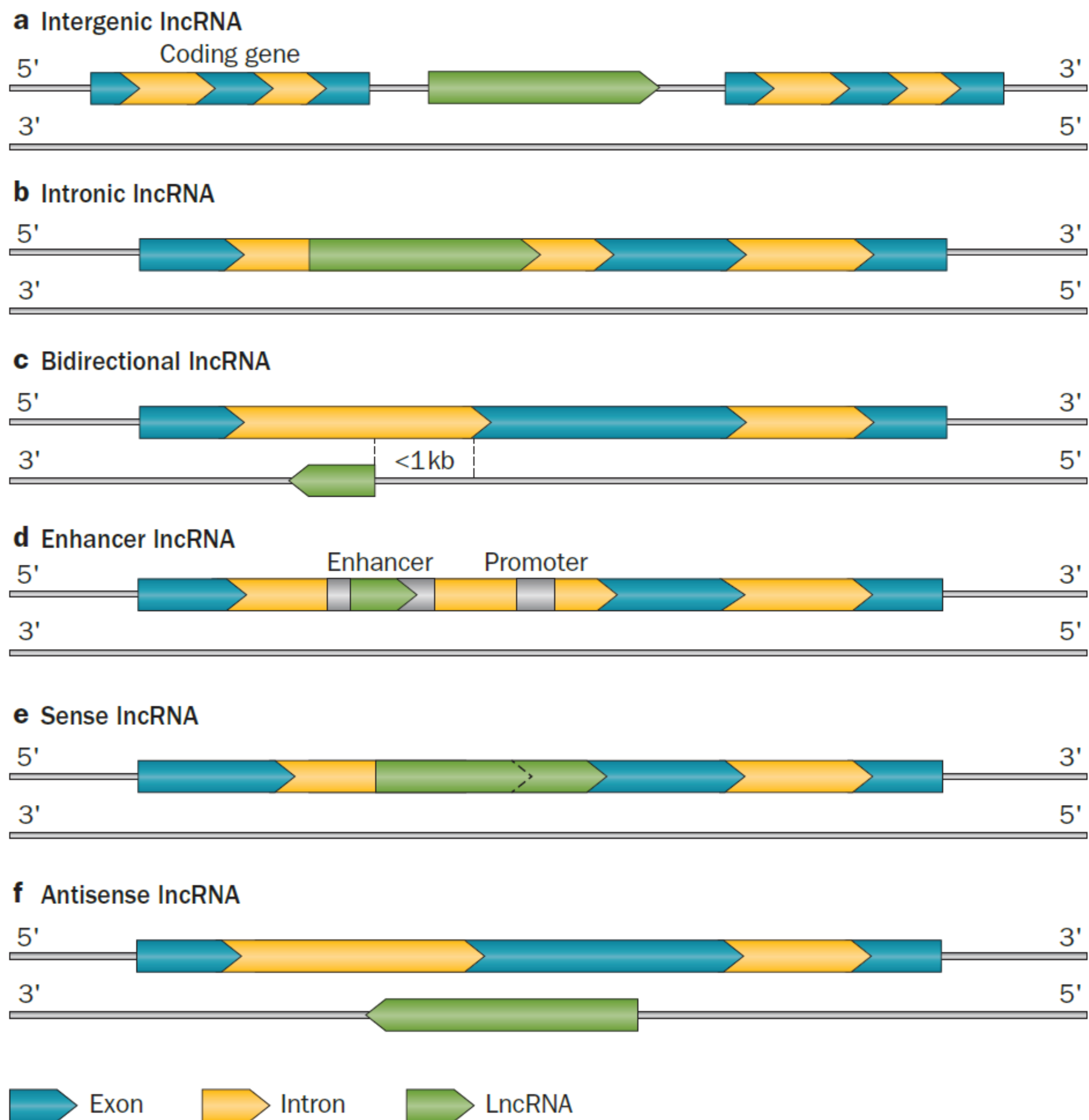


Figure 1.3. Classification of lncRNAs according to their genomic location. (a) Long intergenic non-coding RNAs (lincRNAs) are transcripts located between two protein-coding genes. (b) Intronic lncRNAs are located in an intron of a coding gene. (c) Bi-directional lncRNAs are located within 1 kb of promoters in the opposite direction from the protein-coding transcript. (d) Enhancer lncRNAs (elncRNAs) are located in enhancer regions. (e) Sense lncRNAs overlap one or several introns and exons and are transcribed from the sense strand of protein-coding genes. (f) Anti-sense lncRNAs overlap one or several introns and exons of the sense sequence and are transcribed from the anti-sense strand of protein-coding genes. (Source: Devaux et al., 2015)

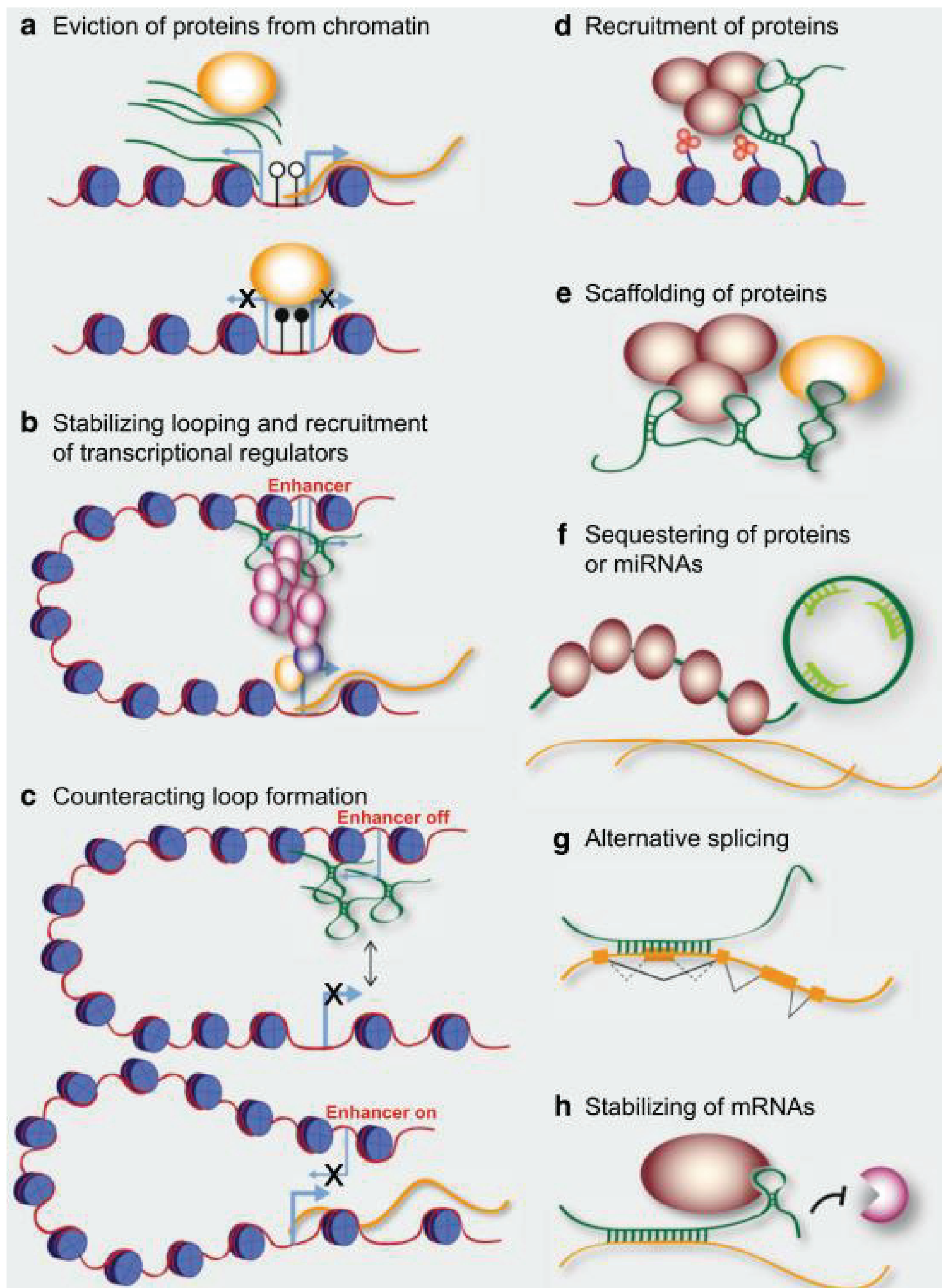


Figure 1.4. Mechanisms of action of lncRNAs. (a) LncRNA transcripts may evict proteins from chromatin. (b) LncRNAs may stabilize loop formation and transcription of the associated gene by recruiting transcriptional regulators. (c) ElncRNAs may interfere with enhancer-promoter contact, thus may inhibit transcription of the gene. (d) LncRNAs may recruit proteins to specific target sites. (e) LncRNAs may act as scaffolds, linking different proteins required for concerted action. (f) LncRNAs may bind and sequester proteins to prevent or attenuate their action. (g) LncRNAs may change the splicing pattern by binding to a primary RNA transcript. (h) LncRNAs may stabilize an mRNA by recruiting proteins, thus prevent degradation. (Source: Schmitz, Grote & Herrmann, 2016)

1.4. Information about GTF2A-1 Gene

TÜBİTAK project “113Z371” named “Identification of Long Non-coding RNAs that Regulate Apoptosis in Human” was aimed to find the lncRNA candidates that may regulate apoptosis in HeLa cervical cancer cells. The outcomes of this project were 20 candidates that are significantly upregulated or downregulated when apoptosis is induced in cells. All of the candidates were chosen based on their corresponding genes, which are related with apoptosis regulation and upregulated or downregulated significantly. One of these candidates was GTF2A-1 anti-sense lncRNA “CTD-2506P8.6-001” (ENSG00000273783) and is located on chromosome 14 (14:81.221.218-81.222.460). This candidate was chosen because it was known anti-sense lncRNA and its corresponding gene is a transcription factor.

General Transcription Factor IIA subunit 1 (GTF2A-1) is a component of the RNA polymerase II transcription machinery and plays an important role in activation of transcription. It mediates transcriptional activity when in a complex with TATA-Binding Protein (TBP). Basically, this protein is essential for transcription with RNA polymerase II (Høiby et al., 2007; Zhou et al., 2006).

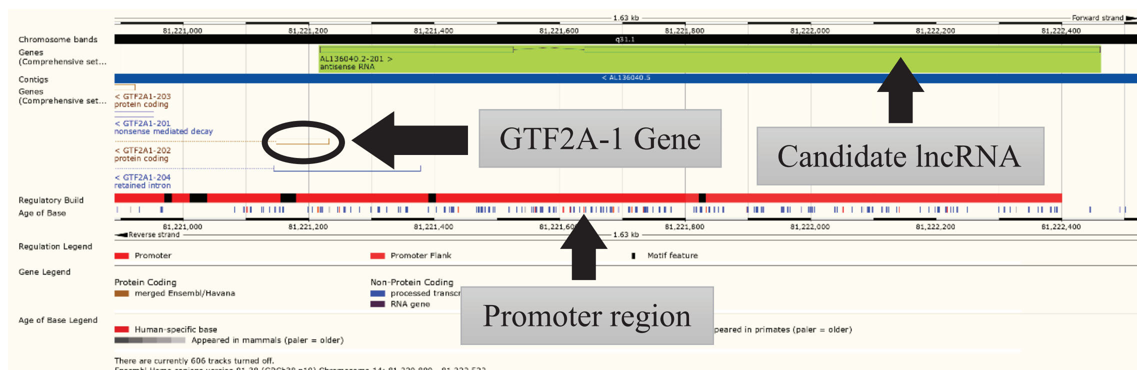


Figure 1.5. Candidate lncRNA, GTF2A-1 anti-sense. The figure shows the genomic location of the candidate lncRNA GTF2A-1 anti-sense lncRNA (highlighted with green) with its corresponding gene GTF2A-1 (Source: Ensembl Genome Browser, http://www.ensembl.org).

1.5. Aim of the Project

The aim of this project is to perform molecular characterization of the anti-sense lncRNA of the gene GTF2A-1 in apoptotic conditions via silencing this anti-sense lncRNA with target-specific GapmeR in HeLa cervical cancer cell line and then measuring differential gene expression by utilizing qRT-PCR and observing target protein levels and activations by performing western blot.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bioinformatics Analyses

The RNA-Seq data that is containing the information regarding HeLa cell apoptosis induction with different drugs was obtained from TÜBİTAK project "113Z371". The data was used in order to verify the candidates that were identified in previous work. Then, the candidate named GTF2A-1 anti-sense lncRNA was chosen after this verification step.

There is another RNA-Seq data that was obtained from TÜBİTAK project "113Z371", which is the RNA-Seq data that is containing the information regarding HeLa cell apoptosis induction by silencing the GTF2A-1 anti-sense lncRNA with LNA GapmeR. The data has been used to designate the candidates that are differentially expressed when the apoptosis is induced by silencing the GTF2A-1 anti-sense lncRNA with LNA GapmeR. This was done in order to determine the differentially expressed genes for verification of the data by qRT-PCR. The data were filtered based on fold changes (two-fold and more) and P value that is lower than 0.01. The candidates that fit these criteria were picked from the data and further analyzed.

Further analysis was performed using "PANTHER classification system" from www.pantherdb.com website. The upregulated and downregulated RNAs were run in this program and results were evaluated based on PANTHER's pathway analysis tool. The program assigned the upregulated and downregulated proteins into pathways and the analysis was continued based on this information. The pathways related with apoptosis were then selected and proteins in those pathways were further investigated in order to determine a resemblance between all the differentially expressed protein genes and one or more distinct apoptosis pathways (Mi et al., 2013; PANTHER, 2016).

Reactome (http://www.reactome.org) database was used to find the apoptosis-related protein-coding genes. The pathway analysis tool was used to run RNA-Seq data and apoptosis-related protein-coding genes were found and fetched from database (Fabregat et al., 2016).

2.2. Cell Culture and Transfection

HeLa cells (from DSMZ GmbH) were cultured in a mixture of RPMI 1640 (with L-Glutamine, Gibco), 10% inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco) in a humidified incubator (Thermo Scientific) with 5% CO_2 level at 37°C. The HeLa cells were seeded at two days interval and with a ratio of 1/3 or 1/4 (nearly 2 x 10^6 cells in each seeding). The remaining cells were destroyed.

LNA (Locked Nucleic Acid) long non-coding RNA GapmeRs are chimeric anti-sense oligonucleotides that have perfect sequence complementary to their target RNA. They bind to their target RNA in highly stable DNA:RNA heteroduplexes when they are introduced into cells and thus, they lead to RNase H mediated degradation of their target lncRNA. They are short (10-14 nucleotides long) and their center that is playing role in RNase H mediated degradation is flanked with Bridged Nucleic Acids (BNA) or other artificially modified ribonucleotide monomers (Fluiter et al., 2009).

The transfection procedure was performed in 24-well plates (Sarstedt) using 15.000 cells (because the confluency should be between 50-70%) and triplicates for every sample. In order to silence the target lncRNA and induce apoptosis, the LNA GapmeRs (Exiqon) were used. The GapmeR for the target gene was designed (sequence: 5'-AATGGACTTGGCGTGA-3') and ordered from the Exiqon Company, as well as negative GapmeR (sequence: 5'-AACACGTCTATACGC-3'), which is designed to have no effect on cells. Since FBS interferes with the FuGene transfection reagent (Promega) during the incubation of the transfection mixture, mixture was prepared by using only RPMI 1640 and cells were treated with antibiotics-free culture mixture. The transfection mixture was prepared with the following formula: 1.5 µl transfection reagent + x amount of GapmeR (40 nM) + y amount of RPMI = 100 µl of mixture. Then, this 100 µl of mixture was incubated for 15 minutes after vortexing, in dark. 15 minutes later, it was applied to appropriate wells that contain 400 µl of antibiotic-free culture mixture. The groups were as follows: control (no treatment), transfection reagent (only transfection reagent with no GapmeR because GapmeRs were added to the cells with transfection reagent), negative GapmeR (negative GapmeR used), target GapmeR (target GapmeR used), DMSO (only DMSO added) and cisplatin (cisplatin dissolved in DMSO added).

Cisplatin (SantaCruz) was freshly prepared in sterile DMSO as 83.2 mM stock in every drug screening experiment due to its chemical instability. Cisplatin concentration

of 80 μM was applied to cells for 16 hours because it is the optimal value. Due to toxic effect of DMSO and because cisplatin was dissolved in it, one more control was set as DMSO (0.1%) control.

2.3. Measurement of Apoptosis of Transfected Cells

Annexin-V (BD) and 7AAD (BD) were used in detection of apoptosis and all experiments were repeated 3 times in order to be scientifically accurate and acceptable for statistical analysis. Annexin-V was diluted 1:5 with PBS (1X) and 7AAD was diluted 1:10 with PBS (1X). The cells were harvested with Trypsin-EDTA (Gibco, 0.25%) and washed twice with ice-cold PBS (1X). After removal of PBS from last wash, cells were suspended in 50 μl Annexin binding buffer (BD). After that, 5 μl of Annexin-V and 7AAD were added into each Eppendorf. After 15 minutes of incubation in dark, cells were suspended with 150 μl PBS prior to analysis with Flow Cytometer (Muse). Cells with Annexin-V signal were considered to be at the early stage of apoptosis. Cells with both Annexin-V and 7AAD signal were considered to be at the late onset of apoptosis. Dead cells were only 7AAD positive and live cells were both Annexin-V and 7AAD negative.

2.4. Total Protein Purification from Transfected Cells and Western Blotting with Purified Proteins

Total protein extracts were prepared by using RIPA lysis buffer (Cell Signaling). After transfection procedure, the cells (nearly 30.000 because there is an expected doubling of cells in the 72h time period) were harvested with 100 μl Trypsin-EDTA (0.25%) and washed twice with 500 μl ice-cold PBS (1X). 2 μl Protease Inhibitor Cocktail (100X) (SantaCruz) was added to cells and immediately after the cells were lysed with RIPA (48 μl) and cell lysates were kept on ice for 45 minutes with vortexing them in every 10 minutes for 1 minute. Lysates were centrifuged for 10 minutes at 14.000 g at 4°C. Supernatants were aliquoted into two or three Eppendorf tubes and stored at -80°C.

In order to determine protein concentration, Bradford Assay was used. Standard curve was drawn with 40 μl of different BSA (bovine serum albumin) concentrations

ranging between 20 and 200 μg/ml in Bradford reagent [0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid] in order to relate protein concentration with absorbance (595 nm). Equation obtained from standard curve was used to estimate protein concentration from absorbance reads of samples. Protein samples were diluted in the rate of 1:10 with dH_2O whereas the blank was prepared by diluting the RIPA in the same rate. All of these dilutions were added into 1.5 ml of Bradford reagent that was placed in cuvettes. After incubation of 10 minutes in dark, optical density of each sample was measured immediately with a spectrophotometer.

Western blotting was carried out in order to show in which pathways the apoptosis is induced. All of the antibodies were ordered from Cell Signaling Technologies and caspase-3, -8 and -9, and β-Actin monoclonal antibodies were originated from mouse, whereas Bcl-2, BID, Cytochrome c, c-FLIP and p53 monoclonal antibodies were originated from rabbit.

Protein amount was fixed to 20 μg per well and protein samples with SDS loading dye [375 mM Tris-HCl, 9% SDS (w/v) 50% Glycerol (v/v), 0.03% Bromophenol blue (w/v) and 9% β-mercaptoethanol] was heated for 5 minutes at 90°C. Protein samples were run on 15% separating and 5% stacking gels [dH_2O, separating/stacking buffer, 40% Acrylamide (Sigma), 10% SDS (Applichem), TEMED (Sigma), 10% APS (Applichem)] vertically for two hours at 100V (mini-PROTEAN, Bio-Rad) in running buffer [25 mM Tris, 190 mM Glycine, 0.1% SDS (w/v)].

One of the gels was used to stain with Coomassie Blue solution [1 mg/ml Coomasie Blue (Sigma), 10% acetic acid (v/v), 30% methanol (v/v)] in order to check proper running and verify presence of proteins. Incubation of the gel with Coomassie Blue solution was held at room temperature for an hour followed by the incubation with Coomassie destaining solution [10% acetic acid (v/v) and 30% methanol (v/v)] for an hour in order to visualize protein bands on gel. On the other side, the other gel was run for blotting. The gel was placed between a Whatman paper (Bio-Rad) and methanol-activated PVDF membrane (Millipore). Proteins were transferred to PVDF membrane from gel at 30V overnight in transfer buffer [25 mM Tris, 190 mM Glycine and 20% methanol (v/v)] and in cold environment. Transfer was tested with Ponceau-S [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] staining of PVDF membrane for 15 minutes and destaining with ultrapure water for 30 minutes.

Membranes were blocked with blocking buffer (1X TBS, 0.05% Tween 20, 5% non-fat dry milk) for an hour at room temperature. Then, membranes were washed with

1X TBS-T 5 times for total of 30 minutes at room temperature. The mouse-originated caspase-3 and -9 monoclonal antibodies were diluted 1:2000 whereas β-Actin monoclonal antibody was diluted 1:5000 in blocking buffer. The rabbit-originated Cytochrome c monoclonal antibody was diluted 1:1000 in blocking buffer. After that, they were put together with membranes and incubated overnight at 4°C on orbital shaker to be shaken gently. After primary antibody incubation, membranes were washed with 1X TBS-T 5 times for total of 30 minutes and incubated with gentle shaking for an hour with HRP-conjugated anti-mouse or anti-rabbit secondary antibody that was diluted 1:20000 in blocking buffer. The wash step was repeated after incubation of membranes with secondary antibody has finished. The membranes were then prepared for visualization with the addition of 1000 μl Luminata Forte Western HRP Substrate (Millipore) enhanced chemiluminescent on membrane. The chemiluminescent was spread onto membrane and incubated for 2.5 minutes in dark. Visualization was performed by using Fusion SL (Vilber Lourmat) digital imaging device.

2.5. Total RNA Isolation and qRT-PCR

After transfection procedure, the cells were harvested with 100 μl Trypsin-EDTA (0.25%) and washed twice with 500 μl ice-cold PBS (1X). After the complete removal of PBS after the last wash step, 1 ml of TRIzol (Life Technologies) was used to dissolve each pellet and the protocol from manufacturer was followed.

Cell lysates were incubated for 5 minutes at room temperature to facilitate complete dissociation of the nucleoprotein complex. 200 μl of RNase-free chloroform (Sigma) was added per 1 ml of TRIzol for homogenization. Tubes were incubated for 2-3 minutes at room temperature after vigorously shaken by hand for 15 seconds. Tubes were centrifuged at 12,000 g for 15 minutes at 4°C to induce phase separation; the most gentle and careful approach was maintained when aqueous phase was pipetted out into a new Eppendorf tube by angling the tube at 45’ without disturbing the middle phase and the bottom phase. 0.5 μl glycogen (Roche) added to the aqueous phase before the next step in order to better precipitation of RNA. 500 μl of RNase-free 100% isopropanol (Sigma) was added into tubes per 1 ml of TRIzol for homogenization and the tubes were incubated at room temperature for 10 minutes. After that, the samples were centrifuged at 12,000 g for 10 minutes at 4°C and supernatant was removed. Pellets were washed with

1 mL of 75% RNase-free ethanol (Sigma) per 1 ml of TRIzol added in the initial homogenization. Tubes were centrifuged at 7500 g for 5 minutes at 4°C after a short vortex and supernatant was discarded. This step was performed 2 times in order to reduce alcohol contamination as much as possible. RNA pellets then were dried by keeping the lid of their tubes open for 10-15 minutes. The dry pellets were dissolved with DNase and RNase free water and incubated in 60°C for 15-20 minutes and then aliquoted and kept at -80°C.

RNA quality control and amount were measured by NanoDrop (Thermo Scientific). 1 µl from each RNA sample was run in NanoDrop and 260/230 and 260/280 ratios were obtained for each RNA as well as their amount as ng/µl. These values help the determination of the purity, quality and amount of the RNA.

Total RNAs from each sample were then used for the cDNA synthesis based on a procedure from Promega. For cDNA synthesis, the RNA amount was fixed among each sample. For that purpose, 500 ng RNA was used for each synthesis reaction. 500 ng RNA was mixed with $d(T)_{23}$ VN primers (Promega) (x amount of RNA sample + 2 µl of primers + y amount of dH_2O = 8 µl of reaction) and denatured for 5 minutes at 70°C and put on ice in order to increase yield. M-MuLV reaction mix (Promega) and M-MuLV (Promega) enzyme mix were added in these tubes (10 µl reaction mix + 2 µl enzyme mix + 8 µl from tube = 20 µl). Negative control was created by adding 10 µl of reaction mix and 2 µl dH_2O instead of enzyme mix to one of the tubes. The tubes were then incubated at 42°C for 1 hour. Then the enzymes were inactivated by incubating the tubes at 80°C for 5 minutes. Finally, contents of the tube were diluted by addition of 30 µl of dH_2O to each tube. Tubes were stored at -20°C.

For qRT-PCR, GoTaq qPCR master mix protocol from Promega Company was followed. qRT-PCR experiment volume was set to 10 µl and calculations were done based on this. The reaction mix for each gene was prepared based on procedure (4 µl of GoTaq qPCR master mix (Promega) + 0.4 µl of primers + 3.6 µl of dH_2O = 8 µl). Each well in 96-well PCR plate (Roche) was contain 8 µl of reaction mixture plus 2 µl of cDNA in total of 10 µl qRT-PCR. The primers used were ordered from IDT Company (exceptions are GAPDH from Alpha DNA and GTF2A-1 from QIAGEN). The primers are FGFR4 (forward: 5'-ACCTCCATCTCCGAGACC-3', reverse: 5'-CCTTTGGCATGGACCCT-3'), FOS (forward: 5'-GGAATGAAGTTGGCACTGGA-3', reverse: 5'-AGCCTCTCTTACTACCACTCAAC-3'), FOSL1 (forward: 5'-ACTCATGGTGTTGATGCTTGG-3', reverse: 5'-GGGCATGTTCCGAGACTT-3'),

GAPDH (forward: 5’-ACTCCTCCACCTTTGACGC-3’, reverse: 5’-GCTGTAGCCAAATTCGTTGTC-3’), GTF2A-1 (forward: 5’-CTTCCGGCCTCTGGCGCGTT-3’, reverse: 5’-ATTTGTCAGCGAGGCTTTGA-3’), GTF2A-1 anti-sense lncRNA (forward: 5’-GCGTTCCTATTCGGGAAGGT-3’, reverse: 5’-GCGAAGCTCACAGTCAAAGC-3’), INHBE (forward: 5’-GGACAGGTGAAAAGTGAGCA-3’, reverse: 5’-AGACTACAGCCAGGGAGTG-3’) and NFKB2 (forward: 5’-AACCGAACCTCAATGTCATCT-3’, reverse: 5’-CCATCCATGACAGCAAATCTC-3’. After the preparation of plate was finished, the plate was sealed with Microseal ‘B’ film (Bio-Rad) and placed in Roche LightCycler 480 qPCR machine. The setting for qRT-PCR was arranged in qPCR machine. There were 3 phases set: hot-start activation (95°C for 2 minutes), denaturation/annealing/extension (95°C for 15 seconds, 60°C for 1 minute) and high-resolution melting (between 60°C and 95°C for 10 minutes). The machine was run the samples and results were generated. The results were taken as reports and analyzed.

CHAPTER 3

RESULTS

3.1. Differential Expression Rate of GTF2A-1 Anti-Sense lncRNA in HeLa Cells After the Induction of Apoptosis with Cisplatin

Apoptosis was induced in HeLa cells by using cisplatin in 80 μM concentration. Control and DMSO (0.1%) groups had minimal amounts of apoptosis whereas cisplatin induced 55,3% apoptosis in HeLa cells (Figure 3.1).

Data from TÜBİTAK project "113Z371" has shown the differentially expressed lncRNAs when apoptosis is induced by different drugs in HeLa cells. Induction of apoptosis via cisplatin resulted in very significant upregulation of GTF2A-1 anti-sense lncRNA, which is a transcription factor-related lncRNA. Therefore, it was chosen as a candidate for further investigation. GTF2A-1 gene is also upregulated (Table 3.1).

3.2. Measurement of Apoptosis with Flow Cytometer After Silencing of GTF2A-1 Anti-Sense lncRNA with GapmeR

The induction of apoptosis is achieved by silencing the GTF2A-1 anti-sense lncRNA with target-specific GapmeR. The induction of apoptosis is 17,2% for Negative GapmeR treated cells whereas the induction of apoptosis is 46,9% for GTF2A-1 AS lncRNA specific GapmeR treated cells. The migration of cells from healthy state to early apoptosis and late apoptosis/dead state is very distinct (Figure 3.2).

Table 3.1. The upregulation rates of GTF2A-1 anti-sense lncRNA and GTF2A-1 gene from RNA-Seq data. The representation is based on logarithmic scale and direct multiplication value of said genes.

Gene name	Upregulation (log2 scale)	Upregulation (x times)
GTF2A-1	1.21	~2.3
GTF2A-1 anti-sense lncRNA	5.15	~35.5

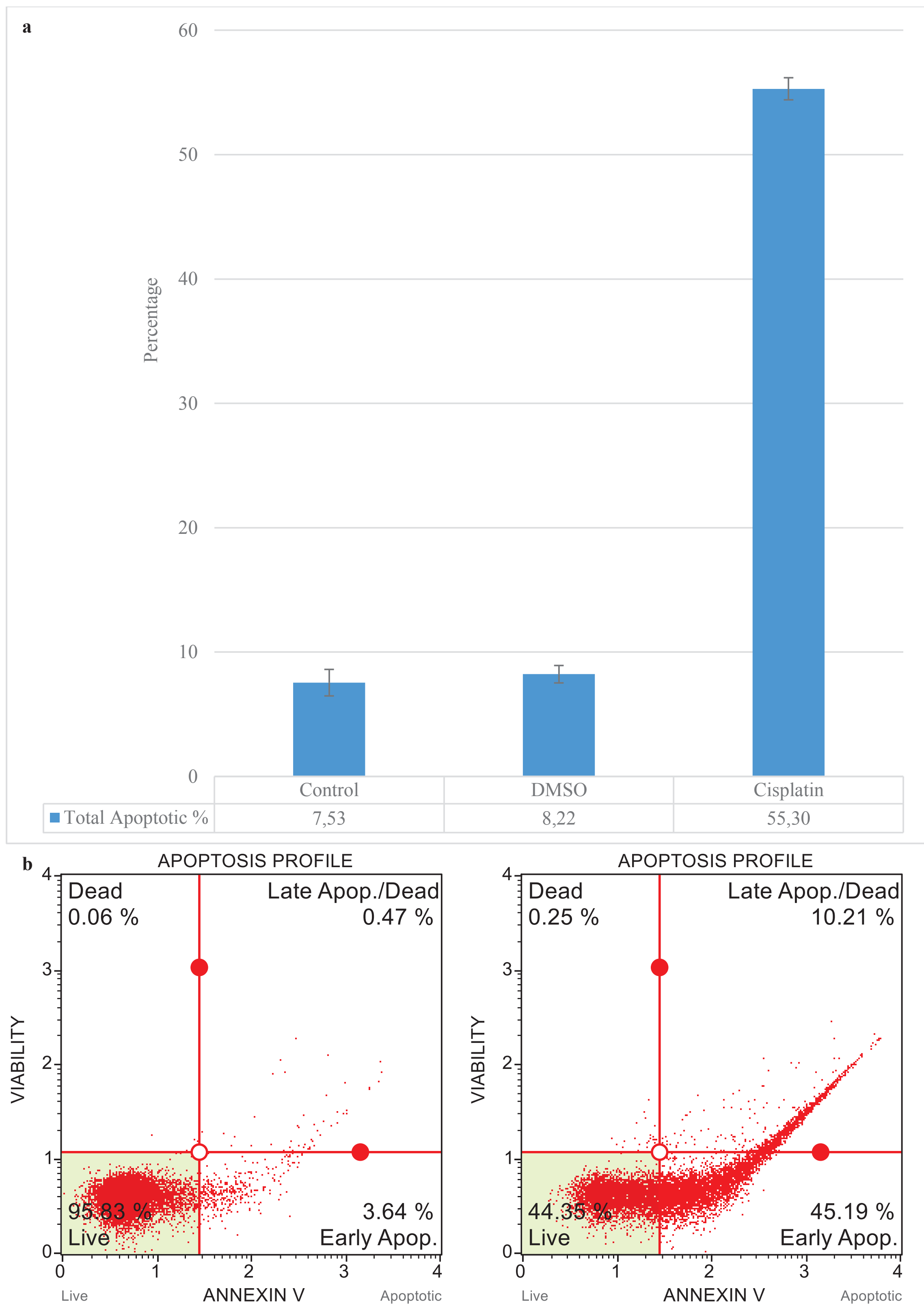


Figure 3.1. Induction of apoptosis via cisplatin. (a) Cisplatin has the concentration of 80 µM and DMSO has the volume of 0,1% in the medium. Student's t-test suggested that the change in apoptosis rates between DMSO and cisplatin is statistically significant ($p<0.05$). (b) The apoptosis profiles taken from flow cytometer show that the cells shift from live quadrant to apoptosis quadrants.

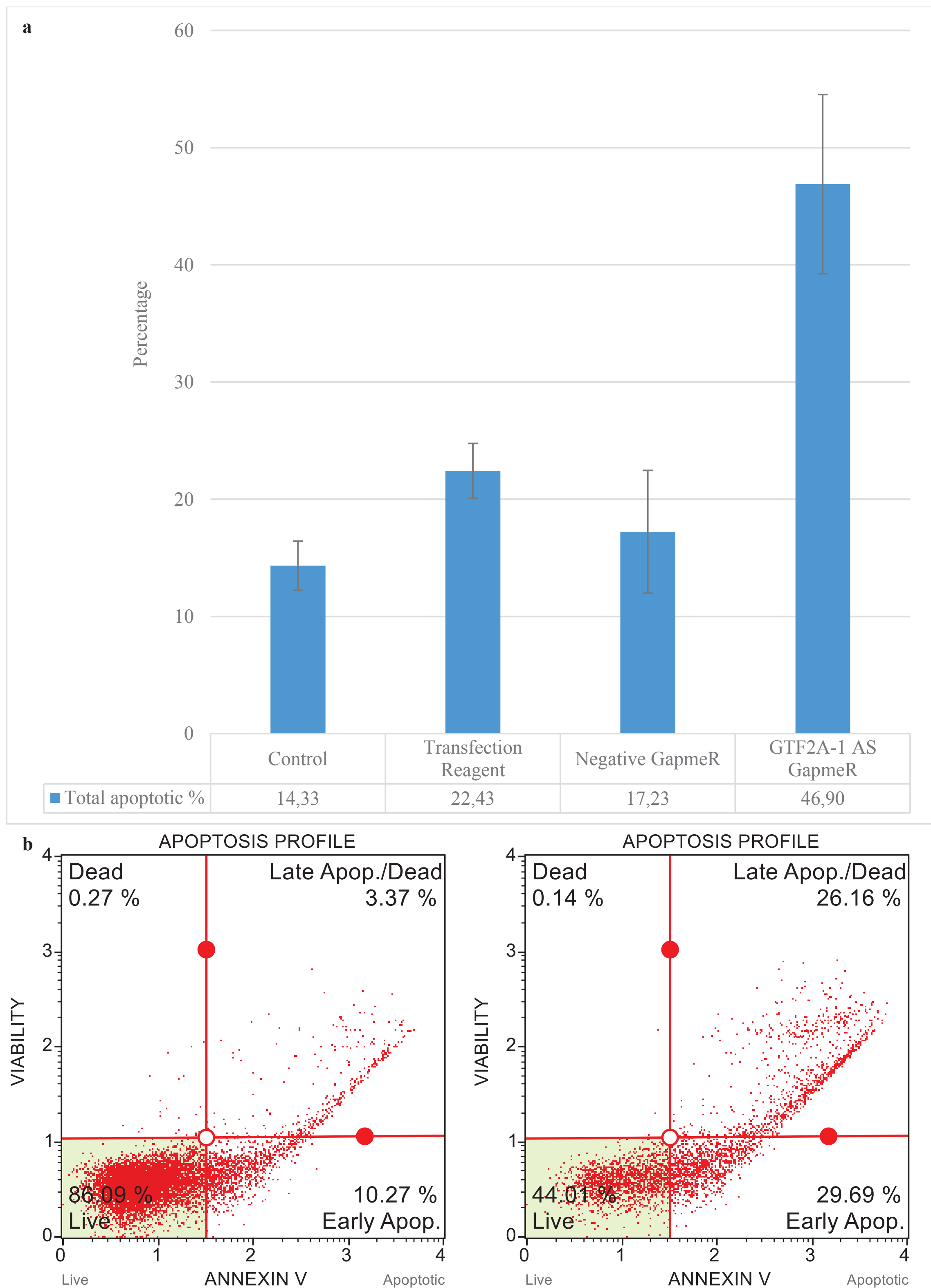
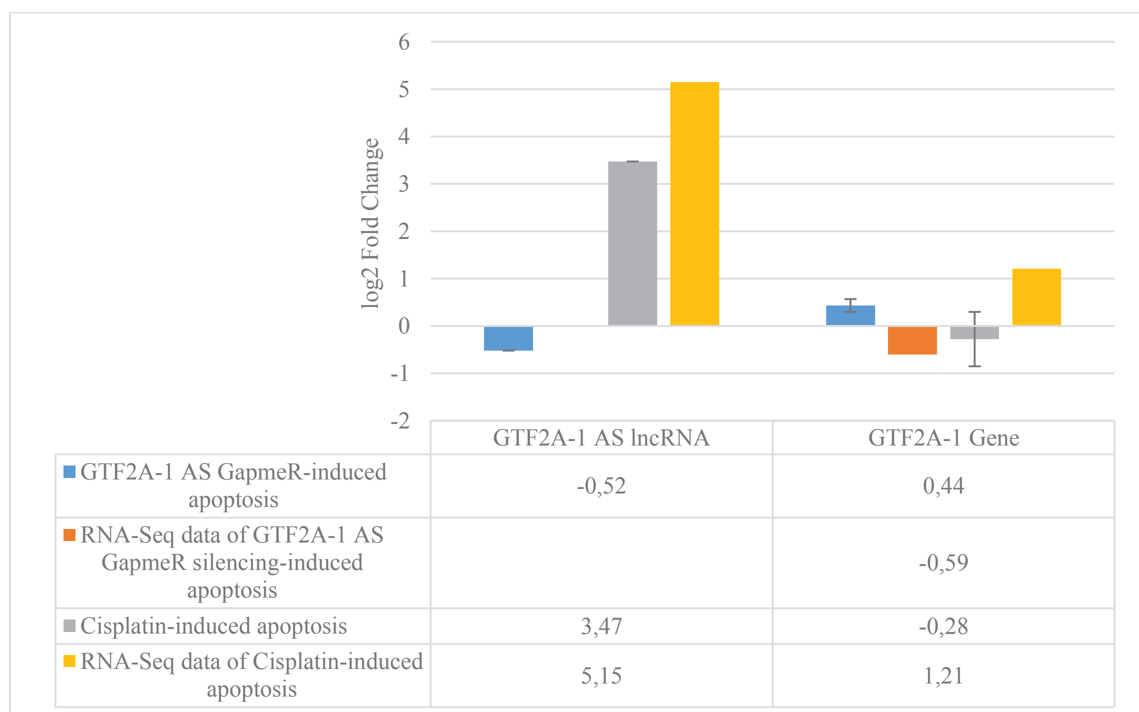


Figure 3.2. Induction of apoptosis via silencing the GTF2A-1 anti-sense lncRNA with target-specific GapmeR. (a) The GapmeR has the concentration of 40 nM in the medium. Student's t-test suggested that the change in apoptosis rates between Negative GapmeR and GTF2A-1 AS GapmeR is statistically significant ($p<0.05$). (b) The apoptosis profiles taken from flow cytometer show that the cells shift from live quadrant to apoptosis quadrants.

3.3. Differential Expression of GTF2A-1 Gene and GTF2A-1 Anti-Sense lncRNA via Cisplatin and GapmeR-Induced Apoptosis Measured by qRT-PCR

Total RNA was isolated from all the cells in the experiments where cisplatin induces apoptosis and where GTF2A-1 anti-sense lncRNA silencing with target-specific GapmeR induces apoptosis. These RNA were then transformed into cDNA to carry out qRT-PCR. In cisplatin induced apoptosis, GTF2A-1 anti-sense lncRNA is upregulated similarly like the previous work. In GapmeR induced apoptosis, since GapmeR leads to the degradation of target lncRNA, GTF2A-1 anti-sense lncRNA is downregulated as expected (Figure 3.3).



	GTF2A-1 AS lncRNA	GTF2A-1 Gene
GTF2A-1 AS GapmeR-induced apoptosis	-0,52	0,44
RNA-Seq data of GTF2A-1 AS GapmeR silencing-induced apoptosis		-0,59
Cisplatin-induced apoptosis	3,47	-0,28
RNA-Seq data of Cisplatin-induced apoptosis	5,15	1,21

Figure 3.3. qRT-PCR result for GTF2A-1 gene and GTF2A-1 anti-sense lncRNA and their comparison with the RNA-Seq data where the apoptosis is induced by cisplatin and GTF2A-1 anti-sense lncRNA silencing with GapmeR. The results for GTF2A-1 AS GapmeR group are relative values against normalized Negative GapmeR results whereas cisplatin results are the relative value against normalized DMSO results. The numbers for RNA-Seq data are the relative values against normalized control group results. There is a similar upregulation and downregulation pattern for these genes between qRT-PCR data and RNA-Seq data.

3.4. Bioinformatics Analyses to Determine Targets for Molecular Characterization of GTF2A-1 Anti-Sense lncRNA in Apoptosis

In order to characterize GTF2A-1 anti-sense lncRNA in the perspective of apoptosis, a series of bioinformatics analyses regarding the data from the TÜBİTAK project "113Z371" has been performed.

The RNA-Seq data was run in PANTHER database and the hits were analyzed based on their pathway. The apoptosis-related pathways were chosen and fetched from the database (Figure 3.4).

The RNA-Seq data was analyzed to find candidates that are upregulated or downregulated commonly in both instances of apoptosis. This was performed in order to validate the RNA-Seq data. In this analysis, the candidates for qRT-PCR were found as follows: FGFR4, FOS, FOSL1, INHBE and NFKB2 (Figure 3.5).

The RNA-Seq data was run in Reactome database to understand which protein-coding genes are upregulated or downregulated when apoptosis is induced. The protein-coding genes are as follows: BIRC3, DAPK3, GSN, LMNA, PLEC, PSMB10, SFN, TFDP2 and UNC5B (Table 3.2).

Literature was reviewed to find protein targets for western blotting and candidates were found as follows: Bcl-2, Bid, Cytochrome c, c-FLIP and p53.

Table 3.2. The upregulated and downregulated protein-coding genes that are related with apoptosis were found by using Reactome pathway tool.

Gene Name	BIRC3	DAPK3	GSN	LMNA	PLEC	PSMB10	SFN	TFDP2	UNC5B
log2 Fold Change	1,85	1,17	1,07	1,05	1,58	1,01	1,36	-1,22	-2,82

3.5. qRT-PCR of All the Candidates

The total results of qRT-PCR are surprising, because some of them are conflicting with the data from previous project. This experiment was done in order to confirm the RNA-Seq data but some results are opposite of what has been found (Figure 3.6).

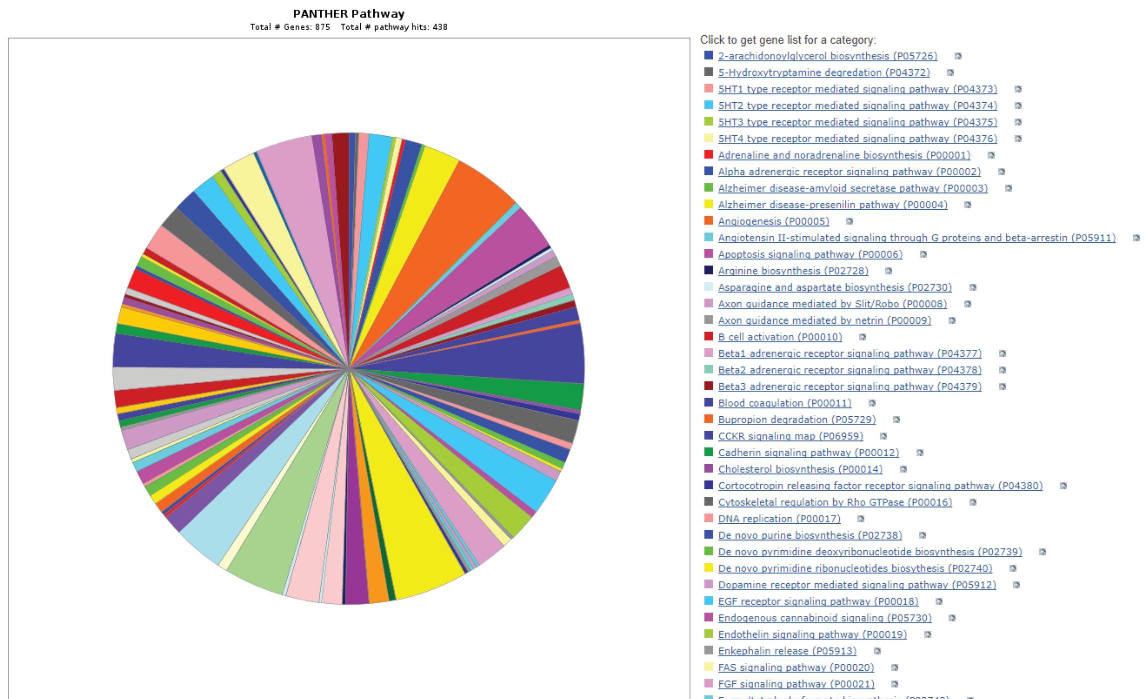
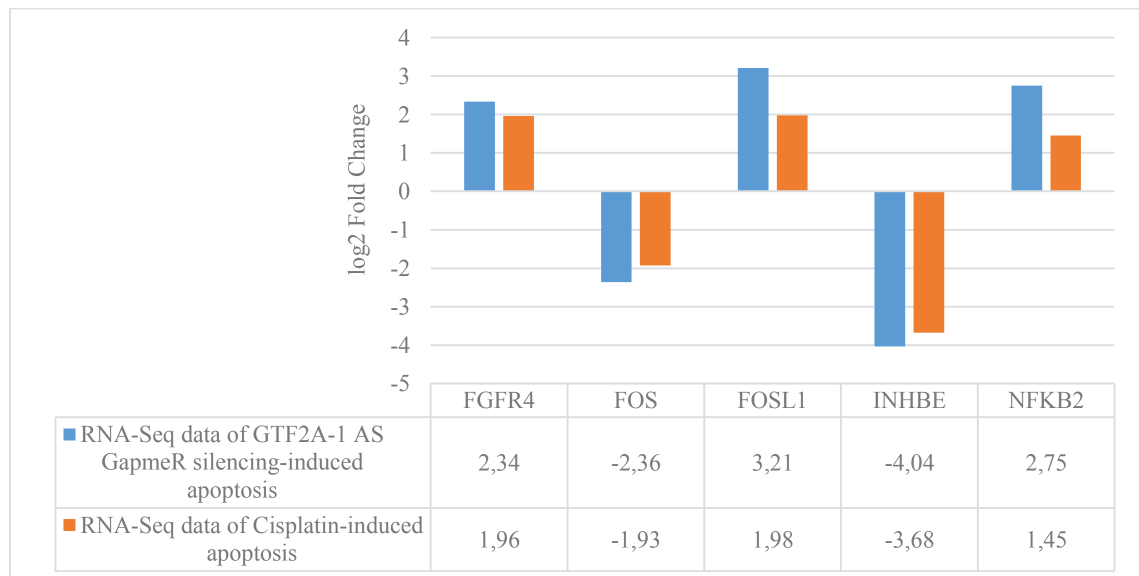


Figure 3.4. Analysis of RNA-Seq data in PANTHER database against pathways. There are almost 900 genes and almost half of them are present in the list of pathways. The list is long, however because of the limitations figure could not include all.



	FGFR4	FOS	FOSL1	INHBE	NFKB2
RNA-Seq data of GTF2A-1 AS GapmeR silencing-induced apoptosis	2,34	-2,36	3,21	-4,04	2,75
RNA-Seq data of Cisplatin-induced apoptosis	1,96	-1,93	1,98	-3,68	1,45

Figure 3.5. Analysis of RNA-Seq data for commonly upregulated and downregulated genes. The numbers for RNA-Seq data are the relative values against normalized control group results. Here are the targets for validation of RNA-Seq data.

	GTF2A-1 AS lncRNA	GTF2A-1 Gene	FGFR4	FOS	FOSL1	INHBE	NFKB2
GTF2A-1 AS GapmeR-induced apoptosis	-0,52	0,44	0,18	1,22	-1,23	0,28	1,26
RNA-Seq data of GTF2A-1 AS GapmeR silencing-induced apoptosis		-0,59	1,96	-1,93	1,98	-3,68	1,45
Cisplatin-induced apoptosis	3,47	-0,28	-0,25	-4,01	-0,76	1,09	2,55
RNA-Seq data of Cisplatin-induced apoptosis	5,15	1,21	2,34	-2,36	3,21	-4,04	2,75

Figure 3.6. qRT-PCR result for all of the chosen candidate genes and their comparison with the RNA-Seq data where the apoptosis is induced by cisplatin and GTF2A-1 anti-sense lncRNA silencing with GapmeR. The results for GTF2A-1 AS GapmeR group are relative values against normalized Negative GapmeR results whereas cisplatin results are the relative value against normalized DMSO results. The numbers for RNA-Seq data are the relative values against normalized control group results. Important differences are between the FOS gene results, FOSL1 gene results and INHBE gene results. FOS and FOSL1 genes give opposite results and INHBE gene gives no significant result when compared to previous data.

3.6. Western Blotting of All the Candidates

The western blotting was performed to investigate the effect of the silencing of target lncRNA on caspases and chosen proteins. The results showed that caspase-3 cleavage (activation) was undetectable (Figure 3.7) but there is caspase-9 cleavage in cells where apoptosis is induced (Figure 3.8). There is decrease in amount of Bcl-2, BID, Cytochrome c, c-FLIP and p53 in the sample where GTF2A-1 anti-sense lncRNA was silenced to induce apoptosis and in cisplatin-induced apoptosis, only the Cytochrome c amount is not changed and the others are decreased (Figure 3.9).

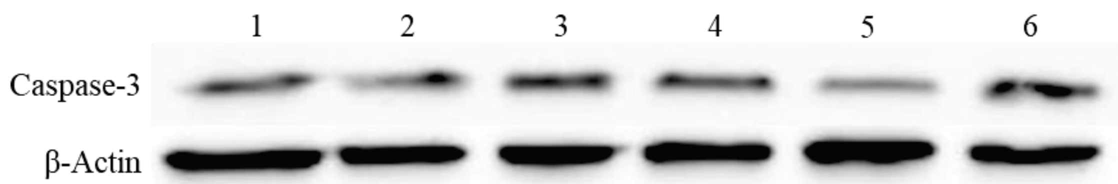


Figure 3.7. Detection of caspase-3 activation by western blotting. Result shows that there is decrease in caspase-3 amount in cisplatin-induced apoptosis but no activation can be seen. 1 is control, 2 is transfection reagent. 3 is negative GapmeR, 4 is GTF2A-1 anti-sense lncRNA, 5 is cisplatin (80 µM) and 6 is DMSO (0,1%).

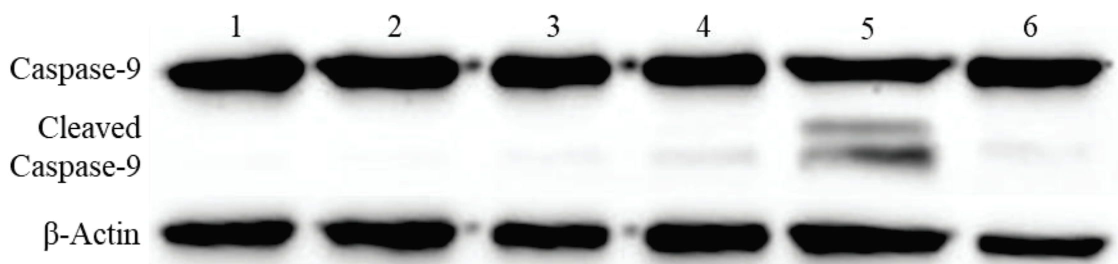


Figure 3.8. Detection of caspase-9 activation by western blotting. Result shows that there is decrease in caspase-3 amount in cisplatin-induced apoptosis but no activation can be seen. 1 is control, 2 is transfection reagent. 3 is negative GapmeR, 4 is GTF2A-1 anti-sense lncRNA, 5 is cisplatin (80 µM) and 6 is DMSO (0,1%).

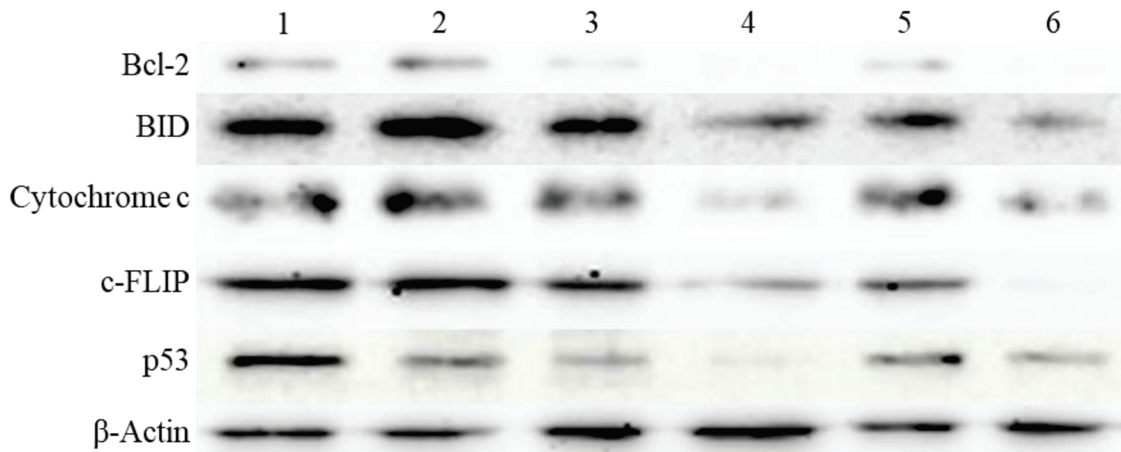


Figure 3.9. Detection of Bcl-2, BID, Cytochrome c, c-FLIP and p53 amount by western blotting. Results showed that the amount of proteins that are checked are decreased when GTF2A-1 anti-sense lncRNA is silenced and apoptosis is induced by cisplatin. Only difference is Cytochrome c amount, which remains same in cisplatin-induced apoptosis. 1 is control, 2 is transfection reagent. 3 is negative GapmeR, 4 is GTF2A-1 anti-sense lncRNA, 5 is DMSO (0,1%) and 6 is cisplatin (80 μM).

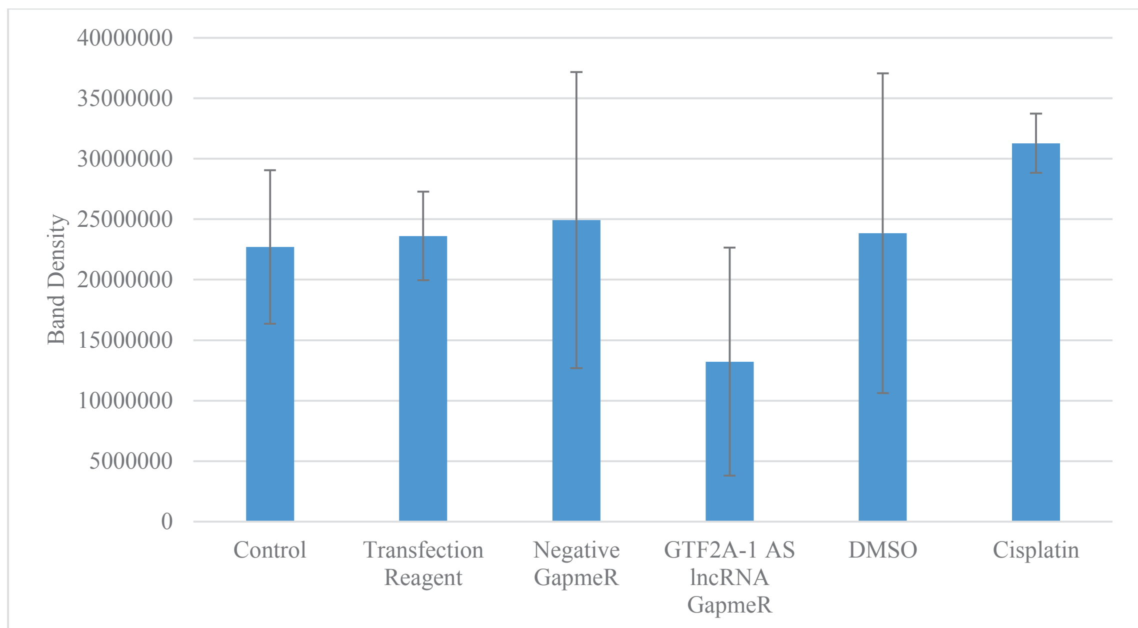


Figure 3.10. Measurements of average band densities for Cytochrome c bands. Results showed that the band densities are decreased in GTF2A-1 anti-sense lncRNA silencing-induced apoptosis when compared to Negative GapmeR treatment. DMSO (0,1%) and cisplatin (80 μM) were also checked. The results were statistically significant ($p<0.05$).

CHAPTER 4

DISCUSSION

The upregulation rate of GTF2A-1 anti-sense lncRNA is 5.15 times in log2 scale in the RNA-Seq data; it is upregulated nearly 35.5 times where apoptosis is induced by cisplatin in HeLa cells. The upregulation rate is very significant and decided to be investigated further. Therefore, GTF2A-1 anti-sense lncRNA is chosen as a candidate to induce apoptosis in HeLa cells by silencing it. Whether GTF2A-1 is an anti-apoptotic protein or pro-apoptotic protein is not known, but it is only logical to silence the anti-sense lncRNA of it and check whether it is pro-apoptotic or anti-apoptotic. Therefore, it is thought that, based on the results, GTF2A-1 should be pro-apoptotic. Because when its anti-sense lncRNA gets silenced, it is not under the effects of it and thus it can regulate its processes. Since the silencing of the anti-sense lncRNA of GTF2A-1 induces apoptosis, mentioned conclusion can be done about GTF2A-1.

The apoptosis rate is significantly increased in HeLa cells where they are transfected with GTF2A-1 anti-sense lncRNA GapmeR compared to all other groups. Only the single non-coding RNA is getting silenced and there is a significant amount of apoptosis induction observed. This result shows that the candidate is related with apoptosis. Since apoptosis is increased when GTF2A-1 anti-sense lncRNA is silenced with target-specific GapmeR, the conclusion would be that it is anti-apoptotic.

The apoptosis is induced by cisplatin and GTF2A-1 anti-sense lncRNA silencing with GapmeR and the knockdown of GTF2A-1 anti-sense lncRNA is achieved at 45%. GTF2A-1 gene is also downregulated. However, in cisplatin induced apoptosis, the values are different but the upregulation pattern is similar. Therefore, these results are validating the RNA-Seq data from drug treatments as well as RNA-Seq data.

The bioinformatics analyses are performed by using the RNA-Seq data. The data was analyzed in order to find candidate genes to validate the RNA-Seq data via qRT-PCR method first and in order to find candidate proteins to characterize the GTF2A-1 anti-sense lncRNA in perspective of apoptosis via western blotting. The targets were all refined thoroughly and chosen among the best candidates.

The genes for RNA-Seq data validation were chosen based on their relation with apoptosis regulation. The chosen candidates are FGFR4, FOS, FOSL1, INHBE and NFKB2. FGFR4 (fibroblast growth factor receptor 4) is overexpressed in different cancer types and associated with decreased overall survival durations. When silenced, proliferation, survival and invasiveness of cells are decreased and apoptosis is induced (Li et al., 2013; Zaid et al., 2013). FOS is a pro-apoptotic agent that represses the anti-apoptotic molecule c-FLIP. c-Fos protein binds the c-FLIP promoter, represses its transcriptional activity, and reduces c-FLIP mRNA and protein levels (Zhang et al., 2007). FOSL1 (FOS-related antigen 1, FRA-1) is involved in proliferation as well as cell melanoma cell migration. FOSL1 acts as an oncogenic agent and reprogrammed melanocytes in transformation (Maurus et al., 2017). INHBE (Inhibin Beta E Subunit, Activin Beta-E Chain) plays role in inhibition of the secretion of follitropin by pituitary gland and other regulate cell proliferation, immune response and apoptosis. Inhibin group of proteins work completely opposite of activing group of proteins and found mostly in liver cells (Vejda et al., 2003). NFKB2 encodes the p100/p52 protein, which is a known critical mediator of the NFκB signaling pathways that are canonical and non-canonical. Aberrant NFKB2 signaling interferes with the TCR-mediated proliferation of T cells as well as causes humoral immune deficiency (Lindsley et al., 2014).

The protein-coding genes in the RNA-Seq data that are upregulated or downregulated and related with apoptosis pathways are BIRC3, DAPK3, GSN, LMNA, PLEC, PSMB10, SFN, TFDP2 and UNC5B. BIRC3 is an inhibitor of apoptosis that effects the terminal segment of apoptosis which is leading to evasion from (Wang et al., 2016). DAPK3 (death-associated protein kinase 3) has an inverse correlation with pAKT, which is a known apoptosis inhibitor. Overexpression of DAPK3 reverts the proliferative function of AKT and ectopic expression of AKT inhibits DAPK3 function and induces proliferation in tumor cells (Das et al., 2016). GSN (Gelsolin) is an actin-severing protein that protects cells from excitotoxic cell death. GSN has anti-apoptotic properties that correlate to its dynamic actions on the cytoskeleton (Harms et al., 2004). LMNA (lamin A/C) is a basic component of the nuclear lamina. Mutations in LMNA induces apoptosis in cells. Both extrinsic and intrinsic pathways are activated in LMNA mutation (Lu et al., 2010). PLEC (plectin) is a major cross-linking protein of the three main cytoplasmic filament systems. Active caspase-8 is translocated to plectin and cleaving it at the center of the protein. Plectin is required for the microfilament system reorganization during apoptosis (Stegh et al., 2000). PSMB10 (proteasome subunit beta 10) is a subunit of

proteasome complex. In non-lysosomal protein degradation, multi-subunit proteasome complex is the principal mediator. It also plays role in the function of transcription factor NFκB. It is required for generation of subunits p50 and p52 NFκB. Proteasome complex protects cells from TNF-α induced apoptosis by catalyzing the degradation of phosphorylated IκBα (Hayashi & Faustman, 2000; Wójcik, 2002). SFN (stratifin, 14-3-3 sigma) is an oncogene differentially expressed related to cell proliferation. Suppression of SFN expression significantly reduces cell proliferation. SFN facilitates lung tumor development and progression (Shiba-Ishii et al., 2015). TFDP2 (transcription factor DP2) functions as binding partner for E2F transcription factors. E2F/DP activity is tightly regulated because this complex plays role in so many essential roles in vital cellular functions. Active E2F1/DP promotes apoptosis in both extrinsic and intrinsic apoptotic pathways, as well as caspase-independent apoptosis. DP proteins possess their biological functions when they bind to E2F proteins (Hitchens & Robbins, 2003). UNC5B (unc-5 netrin receptor B) is a tumor suppressor, and induces apoptosis when its cognate ligand netrins is absent. UNC5B is a direct transcriptional target of p53 (He et al., 2011).

Antibodies chosen for western blotting are caspase-3, caspase-8, caspase-9, Bcl-2, Bid, Cytochrome c, c-FLIP and p53. Caspases are cleaved (activated) during apoptosis if the apoptotic process is caspase-dependent. Caspase-8 is activated in extrinsic pathway and caspase-9 is activated in intrinsic pathway whereas caspase-3 is activated to finalize apoptosis (McIlwain et al., 2013). Bcl-2 (B-cell lymphoma-2 gene) inhibits apoptosis by controlling the permeability of mitochondrial membrane. Inhibits caspase activity by preventing Cytochrome c or by binding to the apoptosis-activating factor (APAF-1) and functions with caspases in a feedback loop system (Cory & Adams, 2002). Bid (BH-3 interacting-domain death agonist) can be cleaved by caspase-8 during apoptosis and truncated Bids (tBid) are formed. These tBids are then migrate to mitochondria in order to induce MOMP, which causes the pro-apoptotic Cytochrome c proteins to be released from mitochondria and induce apoptosis. Bid is also playing role in cross-talk between extrinsic and intrinsic pathways of apoptosis (Billen et al., 2008). Cytochrome c is found only in mitochondria and it is released from mitochondria when there is an internal stress. When Cytochrome c is released after MOMP, it binds to and activates Apaf-1 and pro-caspase-9 and to form an apoptosome. This leads to caspase-9 activation and this leads to the executioner caspase activation (Jiang & Wang, 2004). c-FLIP (FLICE-inhibitory protein) binds the caspase-8 and/or caspase-10 and forming apoptosis inhibitory complex (AIC) with them and this complex prevents the DISC formation (R Safa, 2013). p53 is

the master regulator of tumor suppression. It leads to integrate multiple stress signals into diverse series of anti-proliferative responses. p53 activates apoptosis through transcription-dependent and –independent mechanisms that act in harmony (Fridman & Lowe, 2003).

The results of qRT-PCR were unreliable because the standard deviations of average values were too high. This is because, for some genes, one of the three biological replicates show upregulation and other two shows downregulation. The melting curves of some genes were yield unreliable results. These problems might be caused by primers, which were pre-designed and the improper application of qRT-PCR procedure. Therefore, the results of qRT-PCR were regarded as unreliable. The results of qRT-PCR have shown that the RNA-Seq data should be the data that is relied on for conclusions and therefore, the approach based on this data is a correct step to the molecular characterization of the target lncRNA. The similarities and differences are on par with each other in manner of sheer numbers and therefore, the conclusion that is RNA-Seq is the reliable one among the two data can be made. This conclusion was done based on the comparison of methods. qRT-PCR is limited to several genes and depending on optimizations for each primer. However, in RNA-Seq technique, the approach is to quantify all the RNAs in the sample and then compare two groups based on the amount of RNA (Costa et al., 2013).

The results of western blotting are showing that the Bcl-2, BID, Caspase-3, Caspase-9, Cytochrome c, c-FLIP and p53 amount is lowered and Caspase-9 is cleaved in the proteins that are extracted from the cells that are transfected with the GapmeR designed to silence GTF2A-1 anti-sense lncRNA. All results except Cytochrome c are similar with proteins that are extracted from the cells that underwent cisplatin-induced apoptosis. The decrease in the amount of anti-apoptotic proteins and the activation of pro-apoptotic proteins showed that there is clearly an apoptosis induction similar to cisplatin-induced apoptosis. More proteins and RNAs should be checked before making any solid conclusions about the act of mechanism of GTF2A-1 anti-sense lncRNA and pathways regulated by this lncRNA.

In HeLa cells, the p53 protein have reduced stability and found in very low amounts because of the HPV-encoded E6 protein activity. The studies show that the p53 amount in HeLa cells is increased after cisplatin treatment and reaches its maximum after 6 hours and actively induces the apoptosis. Since they have reduced stability, their degradation is accelerated. By treating the HeLa cells with cisplatin, the cellular level of

E6 protein is decreased. The p53 amount is decreased after 15 hours has passed since cisplatin treatment (Wesierska-Gadek et al., 2002). After the apoptosis induced in HeLa cells via transfection of GTF2A-1 anti-sense lncRNA specific GapmeR, the p53 amount in HeLa cells were decreased compared to control groups. This result is incompatible with the other results in the literature. There is a discrepancy between the results and the literature, therefore the reasons behind the decrease in amount of p53 in apoptosis induced by GTF2A-1 anti-sense lncRNA silencing with target-specific GapmeR should be further studied.

There is one result that is interesting, which is the decrease in the amount of Cytochrome c; because up until now, there is nothing found about the decrease in the amount of Cytochrome c related with apoptosis in literature. The decrease in amount is supported with the measurement of band densities of total of five different western blots. After the necessary calculations, such as student's t-test were performed, it has been found out that the band densities were decreased nearly 50% in GTF2A-1 anti-sense lncRNA silencing-induced apoptosis when compared to all other control and treatment groups. There should be more analyses performed to conclude this unfitting result, whether it is correct or wrong, and based on the conclusion, there should be increased focus on studying the relation between the amount of Cytochrome c and lncRNA silencing-induced apoptosis.

CHAPTER 5

CONCLUSION

In this study, the molecular characterization of GTF2A-1 anti-sense lncRNA in apoptosis was performed by using data analysis, transfection, qRT-PCR and western blot.

All of the methods used were dependent to each other. Apoptosis was induced by several anti-cancer drugs and ligands in HeLa cells and then the candidate was chosen based on differentially expressed lncRNAs that are analyzed by RNA-Seq. The GapmeR probe was designed specifically for this candidate anti-sense lncRNA afterwards. The GapmeR probe was transfected to HeLa cells and apoptosis rate was observed by flow cytometer. The apoptosis was induced by silencing of this candidate lncRNA. RNA-Seq was performed to see differentially expressed genes when apoptosis was induced by silencing of the candidate. Based on these results, the candidate genes and proteins were chosen in order to characterize this candidate lncRNA. qRT-PCR and western blotting were performed to validate the data and characterize the candidate lncRNA.

Based on all the data, there is a definite induction of apoptosis by silencing of this candidate anti-sense lncRNA by target-specific GapmeR probe. The way how apoptosis induced resembles the apoptosis induced by cisplatin treatment. RNA-Seq data showed that the same apoptosis-related genes are upregulated and downregulated with similar amounts and same apoptosis-related proteins are decreased and activated.

There is one intriguing result, which is the decrease in amount of Cytochrome c where the apoptosis is induced by silencing of GTF2A-1 anti-sense lncRNA. In literature, there is no relation between Cytochrome c amount decrease and apoptosis.

As a conclusion, the candidate that is chosen, GTF2A-1 anti-sense lncRNA, is directly regulating the apoptosis in HeLa cells.

For future studies, more apoptotic genes and proteins should be checked in order to fully understand the role of GTF2A-1 anti-sense lncRNA in apoptosis regulation; the overexpression of the GTF2A-1 anti-sense lncRNA in HeLa cells should be performed in order to further validate these results; the silencing and overexpression of GTF2A-1 anti-sense lncRNA should be performed in different cell lines; Cytochrome c dilemma should be investigated further and the reasons behind its decrease in amount should be found.

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