INVESTIGATION OF THE EFFECT OF DR5-AS LONG NON-CODING RNA ON CELL PROLIFERATION

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ABSTRACT

INVESTIGATION OF THE EFFECT OF DR5-AS LONG NON-CODING RNA ON CELL PROLIFERATION

Cell proliferation is the process of increasing cell number in a multicellular organism. In literature, there are numerous proteins and non-coding RNAs reported as regulators of cell proliferation, yet, many of others are waiting to be explored. Unravelling the mechanism behind the regulation of cell proliferation is crucial to develop new strategies for fighting numerous diseases such as cancer, immune diseases, or neurodegenerative diseases. Long non-coding RNAs (lncRNAs) are known to regulate various cellular processes. To determine which ones are related to cell proliferation and apoptosis in HeLa cells, a transcriptomics study was performed under cisplatin, doxorubicin, TNF-α and Anti-Fas treatments. DR5-AS is a novel lncRNA transcript selected from this transcriptomics study as a promising regulatory lncRNA candidate due to its overlap with DR5 protein-coding gene which is known to regulate apoptosis and proliferation. Several phenotypic characterization methods were performed to understand the function of DR5-AS lncRNA. These studies showed that DR5-AS knockdown causes a significant decrease in cell proliferation, an alteration in the normal HeLa cell morphology, a shift through S and G_2/M phases in cell cycle profile, and significant accumulation of cells in the metaphase phase. A second transcriptomics study was performed with DR5-AS knockdown HeLa cells to uncover which pathways are responsible for these changes. The results suggest that DR5-AS lncRNA regulates expression of numerous key proteins in cell cycle regulation. This observation was confirmed by several qPCR experiments. In conclusion, this study provides the first evidence that DR5-AS lncRNA modulates cell cycle and proliferation in HeLa cells.

Keywords: Cell Cycle, Proliferation, Long Non-coding RNA, DR5-AS, Transcriptomics, Antisense

ÖZET

DR5-AS UZUN KODLAMAYAN RNA'SININ HÜCRE PROLİFERASYONU ÜZERINE OLAN ETKISININ ARAŞTIRILMASI

Hücre proliferasyonu, çok hücreli canlılarda hücre sayısının artması işlemidir. Literatürde hücre proliferasyonunun düzenleyicileri olarak tanımlanan birçok protein ve kodlamayan RNA bilinmektedir ancak birçoğu da keşfedilmeyi beklemektedir. Hücre proliferasyonun düzenlenmesinin altındaki mekanizmanın araştırılması kanser, bağışıklık hastalıkları veya nörodejeneratif hastalıklarla mücadelede yeni stratejilerin geliştirilmesi için oldukça önemlidir. Uzun kodlamayan RNA'ların (ukmRNA) hücre içindeki birçok aktiviteyi düzenlediği bilinmektedir; ve hangilerinin HeLa hücrelerinde proliferasyonu ve apoptozu düzenlediğini belirlemek için sisplatin, doksorubisin, TNF-α ve Anti-Fas muamelesi ile bir transkriptomik çalışma yapılmıştır. DR5-AS bu veriden yeni bir ukmRNA transkripti olarak elde edilmiş, proliferasyon ve apoptozu düzenlediği bilinen DR5 geni ile çalışmasından dolayı da umut vadeden bir düzenleyici ukmRNA adayıdır. DR5-AS ukmRNA'sının işlevini anlamak için çeşitli fenotipik karakterizasyon yöntemleri uygulanmıştır. Bu veriler DR5-AS susturulmasının hücre proliferasyonunda anlamlı bir azalmaya, normal HeLa hücre morfolojisinde bir değişikliğe, hücre döngüsü profilinde S ve G_2/M fazına doğru bir kaymaya ve hücre populasyonunda metafaz fazında takılmış hücrelerin birikmesine neden olduğunu göstermiştir. Bu değişikliklerden sorumlu olan sinyal yolaklarını bulmak için DR5-AS susturulmuş HeLa hücreleri ile ikinci bir transkriptomik çalışma yapılmıştır. Bunun sonucunda DR5-AS ukmRNA'sının hücre döngüsünde anahtar rol oynayan bazı proteinlerin ekspresyonunu düzenlediğini göstermiştir. Bu veriler aynı zamanda qPCR ile doğrulanmıştır. Özetle, bu çalışma DR5-AS ukmRNA'sının HeLa hücrelerinde hücre döngüsü ve proliferasyonu düzenlediğine dair ilk kanıtları sunmaktadır.

Anahtar Kelimeler: Hücre Döngüsü, Proliferasyon, Uzun Kodlamayan RNA, DR5-AS, Transkriptomik, Antisens

Junk DNA matters.

TABLE OF CONTENTS

LIST OF FIGURES

Figures

Page

LIST OF TABLES

Table **Page**

CHAPTER 1

INTRODUCTION

The actual meaning of cell growth is an expansion in physical size and mass. In a normal cell, water forms up to 70% of the cell mass. The remaining 30% is made up of main macromolecules, which are proteins, nucleic acids, polysaccharides and lipids besides the small molecules and ions (Sinclair 1922). Synthesis of these macromolecules is under strict control of several biological, chemical, and physical factors. Understanding the mechanism underlying the regulation of biosynthesis of macromolecules helps to uncover the rationale behind the signaling pathways that control cell growth and survival which are related to a variety of disorders such as cancer and immune diseases (Vander Heiden et al. 2001).

1.1. Cell Proliferation

Cell proliferation is a fundamental phenomenon that involves an increase in cell number in multicellular organisms (Heath 2008). It is required for tissue and organ growth, embryonic development, immune response, wound healing, and many other physiological processes. Proliferation is maintained by the cyclic behavior of the cells, and it can be measured by numbers of cells that are cycling within a given time. Homeostasis is attained by the balance of cell division and cell death rates and dividing cells are dominant over the dead cells in proliferating cells (Duronio and Xiong 2013).

Cell cycle is the process of cell division through the life cycle of actively proliferating cells. It is a highly complex and strictly regulated process because the main goal of the cell cycle is ensuring accurate replication of chromosomes and their precise segregation into two daughter cells (Gary S. Stein and Arthur B. Pardee 2004; Bizard and Hickson 2018). The distortion of the cell cycle regulation process is one of the leading causes of cancer (Leal-Esteban and Fajas 2020; Kohrman and Matus 2017).

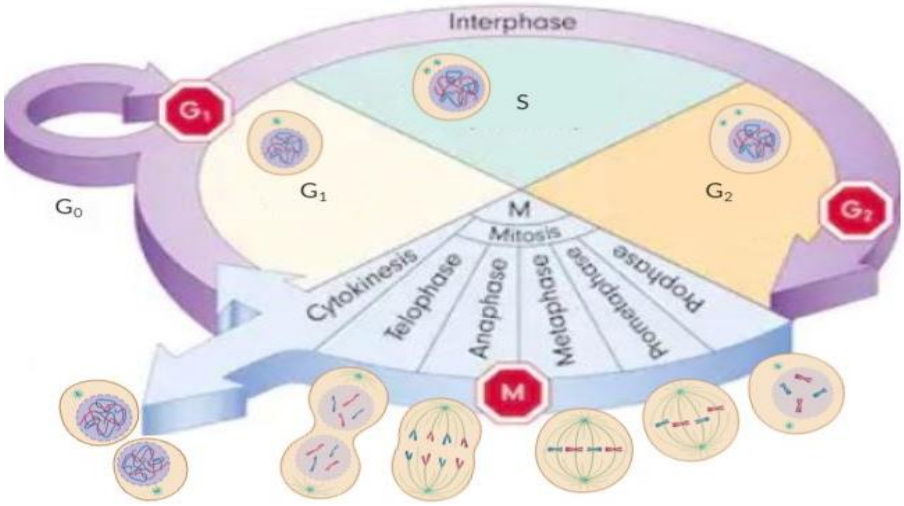

Figure 1.1. Schematic representation of cell cycle stages. The cell cycle process consists of two main stages as interphase and mitotic phase. Interphase stage is further divided into three subphases as G_1, S, and G_2. The mitotic phase consists of two main stages as mitosis and cytokinesis. Mitosis is further divided into prophase, prometaphase, metaphase, anaphase, and telophase phases. (Created with BioRender.com)

To complete the cell cycle, a cell goes through several critical events that result in two daughter cells. This process requires replication of DNA and some organelles followed by the allocation of cytoplasmic content at the end of the cell division (Peters 1999). In eukaryotic cells, the cell cycle occurs in two stages, which are interphase and mitotic phase. The interphase stage is further divided into three phases as G_1 (gap 1), S (synthesis), and G_2 (gap 2) (Okayama 2011; Ravi, Lal, and Begum 2018). G_1 phase is the growth phase for cells. It is the time between the end of the previous mitotic phase and the initiation of DNA replication. In the presence of enough hormones and nutrients, cells start replicating centrosome and producing proteins like histones as well as other molecules required for DNA replication. Nutrient availability and other factors determine the duration of G_1 phase. In G_1 phase, the cellular environment and other conditions are monitored to ensure the cell is ready to proceed into S phase. If the requirements are not met for some time, cells pause the cycle at G_1 phase and enter a snooze phase called G_0 which can last several days, even years. Cell cycle duration depends on the organism, cell type, and cellular environment. In a cell that completes the cell cycle in about 24 hours, G_1 phase takes about 10 hours which makes it the most extended phase (Duronio and Xiong 2013). If all the required conditions are met, cells proceed to S phase, which is the

DNA replication stage. S phase is highly regulated because the accuracy of DNA synthesis is very crucial. DNA becomes unguarded against mutagens during the replication process, which can lead to severe DNA damage and cell death. Thus, DNA synthesis must take place rapidly enough to protect unpaired bases from mutagenesis and long enough to ensure high fidelity. The duration of S phase entirely depends on the amount of DNA to be replicated. Therefore, it is also cell type-dependent; however, it usually takes about 8 to 9 hours. Histone synthesis and cellular growth starting at G_1 stage continue in the S phase (Mehta et al. 2013). After the completion of DNA replication, cells proceed into G_2 phase, which is the last substage of interphase before entering mitosis starting with the condensation of chromatins into chromosomes. Some cancer cells and embryonic cells may skip the G_2 phase and directly enter mitosis after completing S phase. Therefore, the G_2/M transition is a fascinating research topic to gain insight into cancer progression. In normal cells, G_2 phase includes rapid protein synthesis required for mitosis and continuation of cell growth. It serves as the final check point for the cell to ensure that centrosome and entire DNA are successfully replicated. Completion of G_2 phase takes usually 3 to 4 hours (Wendell, Wilson, and Jordan 1993).

Once the cell completes all the preparatory steps, it proceeds into the mitotic phase. The mitotic phase consists of two parts: mitosis, which refers to the division of nucleus, and cytokinesis, which means cytoplasmic division (Björklund 2019). In mitosis, the cell goes through 5 stages that are prophase, prometaphase, metaphase, anaphase, and telophase. In the prophase stage, chromatins condense into chromosomes and become visible. Sister chromatids are connected from the centromere region and became X-shaped structures. Kinetochore is formed around centromere. In addition, centrioles start positioning at the opposite poles of the cell. By that time, cytoskeleton disassembles, and the mitotic spindle begins to form. Kinetochore microtubules start to elongate from the centrioles through the kinetochores (Wendell, Wilson, and Jordan 1993). These processes are followed by the second stage of mitosis, prometaphase. In prometaphase, the nuclear membrane disappears due to the nuclear lamin phosphorylation, which gives spindle fibers direct access to sister chromatids. Spindle fiber attached microtubules started to position back and forth until all the mitotic chromosomes are aligned, which is the beginning of metaphase (Tin Su 2001). In metaphase, kinetochore microtubules are connected to the kinetochore, and sister chromatid pairs are aligned at the equator region of the cell. At this point, the cell needs to check if all the chromosomes are aligned correctly, and all kinetochores are adequately connected to the microtubules. After this

checkpoint, the cell is ready to proceed to the third phase, anaphase (Duronio and Xiong 2013). Anaphase is the division phase. Sister chromatids become disconnected from their centrosome regions at the same time, then spindle fibers that connect kinetochores to the centrosomes became shorter and broader. As a result, sister chromatids are pulled through their attached centrosomes which results in localization of equal amount of genetic material in the opposite poles (Nakajima et al. 2007; Mehta et al. 2013; Nasmyth 1999; De Gramont and Cohen-Fix 2005). Then, cells proceed into the telophase stage, which is the final phase of the mitosis. Around the separated chromosomes, a nuclear membrane started to form to create a distinction between genetic material and cytoplasm. Chromosomes start to uncoil and return to the chromatin state. After the completion of mitosis, cytokinesis occurs, which states for the physical division of two cells. A protein filament structure located underneath the plasma membrane is formed around the equatorial region of the cell called contractile ring. This contractile ring starts to shrivel, which forces the plasma membrane to pinch inwards. This shrinkage continues until the two cells are separated entirely and have their own plasma membranes (Potapova et al. 2006).

1.2. Mechanism and Regulation of Cell Proliferation

Cell proliferation is a dynamic process, and the cyclic production and degradation of specific macromolecules are required for the proper progression of the cell cycle. Cyclins are the main regulatory elements of the cell cycle. They were discovered in 1982 by R. Timoty Hunt as a family of proteins whose concentrations fluctuate through the phases of the cell cycle in sea urchins. Interestingly, “cyclin” name comes from Hunt’s cycling hobby, and as a coincidence, these proteins are actually cycling during the cell cycle (Evans et al. 1983). Cyclins activate specific kinases called cyclin-dependent kinases (CDKs) by physically attaching to them, and they also regulate a set of transcription factors that are required for the following cell cycle stage (Bonke et al. 2013). In somatic cells, cell proliferation is regulated by Myc transcription factor, which is encoded by c-myc gene. Myc and Max form a heterodimeric structure and promote cell growth by activating ribosomal RNA production and downregulating Bcl-2 to suppress apoptosis. Myc-Max complex is able to recruit histone acetyltransferases (HATs) to enhance transcription elongation, but it is also known to suppress transcription of several

genes such as p21 which is a CDK inhibitor and has a negative effect on the cell cycle progression. C-myc gene acts as an oncogene in various cancers due to its constitutive activation. In this way, cancer cells proliferate rapidly and can repress apoptosis (Bouchard, Staller, and Eilers 1998; Carabet, Rennie, and Cherkasov 2019).

Cell growth and cell cycle are regulated by a variety of internal and external factors. Exceeding a cell size threshold or a cellular response to growth hormone can induce cell cycle progression externally; however, the cell needs to check numerous events before committing and during the progression of cell division. All these mechanisms are regulated by CDKs and cell cycle checkpoints (Heath 2008; Lin and Alan Diehl 2004).

1.2.1. Cyclin-Dependent Kinases

CDKs are serine-threonine kinases that regulate the progression of particular phases of the cell cycle. CDKs are activated by specific cyclins or phosphorylation at particular sites. Following cyclin binding or phosphorylation, CDKs change conformation and become capable of recognizing their substrates. When they are activated, they induce several downstream events by phosphorylating their target substrates (Diaz-Moralli et al. 2013).

Different pairs of cyclins and CDKs function in each stage of the cell cycle. CDK4, CDK6, and CDK2 are responsible for the G_1 phase. CDK4 and CDK2 interact with cyclin D1, cyclin D2, and cyclin D3 to regulate entry into G_1. CDK2 and cyclin E interaction is essential for G_1 to S progression. Cyclin A and CDK2 interaction have a crucial role during S phase. However, during G_2 to M transition, cyclin A interacts with CDK1. Throughout the M phase, CDK1 interacts with cyclin B (Ding et al. 2020; Schafer 1998). These complicated events are under strict regulation. Over the course of cell cycle progression, CDK levels remain stable; however, a particular set of cyclin levels are modulated dynamically. The level of cyclins is regulated both transcriptionally and post-transcriptionally. For example, the same CDKs function in G_1 and G_1/S, but cells synthesize G_1 and G_1/S cyclins at different times along the G_1 phase. Furthermore, regulation of cyclin degradation is as crucial as the cyclin production regulation. Cyclins are degraded by ubiquitin pathway in proteasome dependent manner in distinct times in the cell cycle (Glotzer, Murray, and Kirschner 1991). When a particular type of cyclin is

degraded, the CDK that is in interaction with that cyclin becomes inactive. Cell cycle arrest occurs in the case of complications in cyclin degradation. Cyclins themselves have no enzymatic activity, but as pairs with CDKs, they are responsible for post-translational modifications of a specific group of downstream protein substrates. Proper phosphorylation of these substrate proteins in correct times is essential for cell cycle progression (Coutts and Weston 2016; Duronio and Xiong 2013; Vermeulen, Van Bockstaele, and Berneman 2003).

The activity of CDKs is also regulated by cyclin-dependent kinase inhibitors (CDKIs). These proteins deactivate CDK activity either by forming stable complexes with CDKs to prevent cyclin binding or inactive cyclin-CDK complex (Hochegger, Takeda, and Hunt 2008). There are two well-studied families of CDKI proteins, which are INK4 family and Cip/Kip family. INK4 family proteins consist of p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) proteins. Their primary role is inactivating G_1 CDKs, which are CDK4 and CDK6. They bind CDK4 and CDK2 and prevent their interaction with cyclin D (Schafer 1998; Terzi, Izmirli, and Gogebakan 2016). Cip/Kip family proteins consist of p21 (Cip1, Waf1, CDKN1A), p27 (Cip2) and p57 (Kip2). These proteins inactivate the CDK1-cyclin B complex in G_1 phase. p21 has a role in also S phase. It inhibits PCNA, which results in inhibition of DNA synthesis. The expression of p21 is under the regulation of tumor suppressor p53 protein. p21 gene has a binding site for p53. Thus, when p53 is activated, transcription of increases which leads to cell cycle arrest. p15 and p27 are regulated by growth factors like TGFβ (Lawless et al. 2010).

Intracellular localization of certain proteins must also be regulated to ensure accurate progression of the cell cycle. Cyclin B is responsible for the nuclear exclusion signal. Through the prophase, it is continuously exported from the nucleus by active transport. The intracellular trafficking of cell cycle-related proteins is regulated by 14-3-3 group of proteins. In the interphase stage, Cdc25 which activates CDKs is ensured to stay in the cytoplasm by 14-3-3 group of proteins. These proteins sequester CDK1-cyclin B complexes as well (Pennington et al. 2018).

CDK activity is essential for S phase progression and entry into mitosis. Moreover, loss of CDK activity is required for mitotic exit and re-entry into interphase. In G_1, pRB is phosphorylated, which results in histone deacetylase protein (HDAC) degradation. Following this event, E2F-1 and Dp-1 transcription factors are released and activate the transcription of cyclin A, cyclin E and cdc25 whose activity is required for S phase progression. pRb is kept hyperphosphorylated until the end of the cell cycle by the

CDK2-cyclin E complex. CDK2-cyclin E phosphorylates p27 which is its inhibitor and leads to its degradation by a proteasome-dependent pathway in G_1/S. Histone H1 is another substrate of CDK2-cyclin E complex whose activity is crucial for chromosome condensation. CDK1-cyclin B also targets Histone H1. DNA polymerase alpha primase is activated by cyclin A-dependent kinase, which leads to DNA replication initiation. Nuclear laminins and microtubules are also a target for CDKs which have a role in both mitosis and cytokinesis processes (Potapova et al. 2006).

1.2.2. Checkpoint Regulation

Three main checkpoints regulate cell cycle progression in G_1, G_2 and M stages. G_1 checkpoint is also called restriction point, and cell decides to commit to divide irreversibly at this time. It controls all the conditions such as cell size and essential enzymes before replicating DNA and entering to cell division process. If a cell decides not to proceed, it can enter quiescence (G_0) and rest there (Matellán and Monje-Casas 2020). An internal or external signal is required for re-entering the cell cycle from quiescence. At G_2 checkpoint, cell controls if it is ready to enter mitosis. Cell must ensure that all DNA is replicated and there is no DNA damage. If DNA is damaged, cell pauses the cycle and tries to repair it to complete the cell cycle. If the damage is overwhelming, cell commits apoptosis under the regulation of tumor suppressor proteins. Following of entering mitosis and transition into metaphase, the cell needs a final check for controlling whether the chromatids are appropriately attached to the mitotic spindle. Anaphase is an irreversible step in mitosis. If the kinetochores are not connected to the mitotic spindle by at least two microtubules belonging with opposite centromeres, cells do not progress to the anaphase (Coutts and Weston 2016).

Cyclin 3 regulates the cell size checkpoint in G_1 while cdc25 is responsible for monitoring the cell size and nutritional conditions in G_2 checkpoint. Controlling cell size can vary in different cell types; however, their logic is similar. One way to ensure cell is large enough to commit division is checking the translational rate. Several regulatory proteins act as translational sizers and cln3, and cdc25 are two of them. Another method for monitoring cell size is to determine cell geometry. In yeast, it is determined by Pom1 protein. It inhibits the Cdr1-Cdr2-Wee1-Cdc20, and as the cell grows, Pom1, which is located on the tips of the cell can no longer inhibit this complex which leads to cell cycle

progression. In animal cells, cell size is measured by surface to volume ratio. Production of plasma membrane components are monitored like cholesterol, and the level of these molecules determine the level of cell growth (Ding et al. 2020).

The interphase stage is very critical time to determine DNA damage and arrest the cell cycle for giving cell time for repairing. DNA lesions may be sourced intrinsically, ranging reactive oxygen species to shortened telomeres, oncogene activity, or replicative errors. On the other hand, various extrinsic sources cause DNA damage such as X-rays from sunlight and chemical mutagens. DNA damage checkpoints must keep the CDKs deactivated through the DNA repairing process. Checkpoint kinases (Chk1 and Chk2) are activated in response to DNA damage. Intrinsic or extrinsic single-stranded DNA breaks (ssDNA breaks) activate ataxia-telangiectasia, mutated and Rad3 related (ATR) protein which activates Chk1. Double-stranded DNA breaks (dsDNA breaks) are recognized by Mre11-Rad50-NbsI complex (MRN complex), which leads to the activation of ataxia-telangiectasia mutated (ATM) protein followed by Chk2 activation. Both of these events lead to cell cycle arrest prior to DNA repair or apoptosis. Chk2 activation by ATR leads to the activation of p53 and in the meantime Chk1 is degraded. p53 activation and Chk1 degradation result in a commitment to apoptosis. Chk1 activation leads to cdc2 phosphorylation on Y15 to maintain it inactivated. Chk1 also phosphorylates cdc25 which is a phosphatase that removes Y15 phosphate from cdc2 to repress its activity. As a result, cell cannot proceed to mitosis. After the completion of DNA repair, Chk1 is deactivated by type 1 phosphatases which lead removal of Y15 phosphorylation from cdc20 to continue cell cycle (Nasmyth 1999; Hochegger, Takeda, and Hunt 2008; Swaffer et al. 2016).

Transcription factor mediating DNA damage checkpoints also have a crucial role in higher organisms, especially in G_1 phase. When DNA damage is recognized by MRN complex, p53 is activated and promotes the transcription of p21, which is a G_1 CDK inhibitor. As a result, cell cycle stalls at G_1 prior to DNA replication to give time to the cell for repairing the damaged DNA. Moreover, p53 may have inhibitory effects for transcription of several proteins which results in elongation in G_2 arrest in response to DNA damage after replication. As a matter of fact, the leading role of p53 is regulating apoptosis, but in the evolutionary time, it gained the function of regulating cell cycle as well (Schafer 1998).

Mitotic spindle consists of microtubules and helper motor proteins that are located in centrosome and kinetochore ends and also between microtubules. After the cell ensures

all chromosomes are aligned properly and all sister chromatids are correctly attached to the mitotic spindle, it can proceed into the anaphase which is driven by a large complex of E3 ubiquitin ligase called as Anaphase Promoting Complex or Cyclosome (APC/C). APC/C activity is required Cdc20 and Cdh1 proteins. APC/C ubiquitinates mitotic cyclins and securin, which leads to their degradation. Once securin is degraded, separase disentangles and free to cleave cohesin which is the protein that holds sister chromatids together. After that, the cell is ready to proceed into anaphase (Peters 1999). However, before APC/C completes its function, the cell needs to scan detached chromosomes. In this step, kinetochores have a crucial role; if there are any unsatisfied kinetochores which means remaining kinetochores that lack attachment from both sides and have tension, give a signal. Actually, this signaling event lasts until the last kinetochore is adequately attached to the spindle and have tension. At that point, the signal is terminated, and anaphase begins with the activation of APC/C (Peters 2002). Errors in the mitotic spindle checkpoint may have destructive effects on the cell and tissue homeostasis. The daughter cells may lose or gain chromosomes which leads to aneuploidy. These chromosomal imbalances usually trigger apoptosis, and the organism can get rid of problematic cells, but sometimes due to mutations in some tumor suppressor genes or epigenetic dysregulations, these problematic cells can remain unnoticed and leads to uncontrolled growth. Uncontrolled proliferation is one of the hallmarks of cancer formation and progression (Kohrman and Matus 2017). Consequently, unravelling the mechanism underlying the control of the cell cycle is vital for progression in the cancer researches.

Besides these key regulator proteins such as cyclins, CDKs, p53 and pRB, there is also an epigenetic layer of regulation that controls cell cycle progression by non-coding RNAs. These non-coding RNAs can regulate these essential proteins and many others transcriptionally or post-transcriptionally, they can act as a scaffold for protein complexes, or they can compete for other non-coding RNA interactions to positively or negatively regulate the protein of interest and the cell cycle consequently (Kitagawa et al. 2013).

1.3. Long Non-Coding RNAs

RNA is a molecule that is produced as a first step of any phenotype. According to the central dogma of molecular biology, the information can only flow through from DNA

to RNA, which was thought as an intermediate molecule, then is translated into protein that creates phenotype (Jarroux, Morillon, and Pinskaya 2017; Hüttenhofer, Schattner, and Polacek 2005). As a result, scientists thought that a large portion of the human genome must be coding. However, completion of human genome project revealed that most of the genome is transcribed, but only 1% of the DNA contains instructions for producing proteins (Collins, Morgan, and Patrinos 2003; Eddy 2012). Consequently, a large portion of the genome was described as "junk DNA". With the help of the developments in deep sequencing technology, we know that more than 100,000 transcripts are produced in mammalians, but only approximately 20,000 of them are translated into protein (Fernandes et al. 2019; Ponting, Oliver, and Reik 2009). These non-coding transcripts were thought as transcriptional noise; however, our knowledge about the transcriptome and central dogma of molecular biology has been revised. We now know that this dark matter of our genome has remarkable functions in the cell (Jarroux, Morillon, and Pinskaya 2017).

Non-coding RNAs (ncRNAs) can be described as RNA molecules that do not contain a functional or longer than 100 amino acids open reading frame (ORF). Long non-coding RNAs (lncRNAs) are ncRNA molecules that are longer than 200 nucleotides (nt). They are primarily transcribed by RNA Polymerase II, might have 5' cap structure and may have polyA tail, may translocate into the cytosol. Some lncRNAs can be translated into small peptides (Hüttenhofer, Schattner, and Polacek 2005; Qian et al. 2019; Brannan et al. 1990; J. Li and Liu 2019). lncRNAs exist in a wide range of organisms ranging from viruses and prokaryotes to plants and animals. However, they are evolutionary less conserved compared to mRNAs (Brannan et al. 1990). Poor conservation of lncRNAs make harder to ascertain their functionality, however, they may have species-specific functions and characteristics. Despite their low abundance in the cell, it is evident that they have roles in a wide range of cellular processes such as cell proliferation, cell cycle, cell migration, invasion, and others. They may act as oncogenes and tumor suppressors or may have roles in drug resistance in cancers (J. yan Wang, Lu, and Chen 2019).

1.3.1. Classification of Long Non-Coding RNAs

Long non-coding RNAs are classified based on their genomic location and context with respect to protein-coding genes as sense, antisense, bidirectional, intronic and intergenic lncRNAs (Hombach and Kretz 2016; Chen, Feng, and Wang 2018; Esteller 2011). Sense lncRNAs are located in the same direction with a protein-coding gene (PCG) on the same strand, and they overlap with the PCG (Fang and Fullwood 2016; Jarroux, Morillon, and Pinskaya 2017). Antisense lncRNAs are located on the opposite strand of a PCG and overlaps partially or completely with PCG. Antisense lncRNAs are also called as natural antisense transcripts (NATs) and may regulate the sense PCG in *cis* or other PCGs in *trans* (Fang and Fullwood 2016; Chen, Feng, and Wang 2018; Jarroux, Morillon, and Pinskaya 2017). Bidirectional lncRNAs are located in the opposite strand on the PCG similar to antisense lncRNAs; but without an overlap with the PCG, and their transcription initiation sites must be less than 1000 bp away (Meng et al. 2017). Intronic lncRNAs are derived from intronic sequence of another PCG. They may be a byproduct of pre-mRNA processing of template PCG or separately transcribed (Matsui and Corey 2017). Intergenic lncRNAs are located between two different PCGs and do not share any common sequence with them (Esteller 2011). Long intergenic RNAs (lincRNAs) are the most conserved class of lncRNAs, and they showed over 70% of sequence similarity with mouse lincRNAs. If the 80% of PCG sequence similarity between mouse and the human genome is considered, the comparable conservation of lincRNAs indicates a high probability of having functions in cellular processes (Ma, Bajic, and Zhang 2013).

1.3.2. Functional Roles of Long Non-Coding RNAs

Long non-coding RNAs have a prominent role in epigenetic regulation of gene expression. Until now, many lncRNA functions have been well characterized, including *H19*, *Xist*, *MALAT1*, *HOTAIR*, and *ANRIL* (Alahari, Eastlack, and Alahari 2016). Several lncRNAs have been biologically associated with human disorders including cancer. Differential expressions of particular lncRNAs in different types of cancer provide a clue about their function in proliferation, migration/invasion, apoptosis and drug resistance (X. Li and Li 2018; Jarroux, Morillon, and Pinskaya 2017).

H19 is the first lncRNA that is identified as a functional lncRNA in 1989. It was

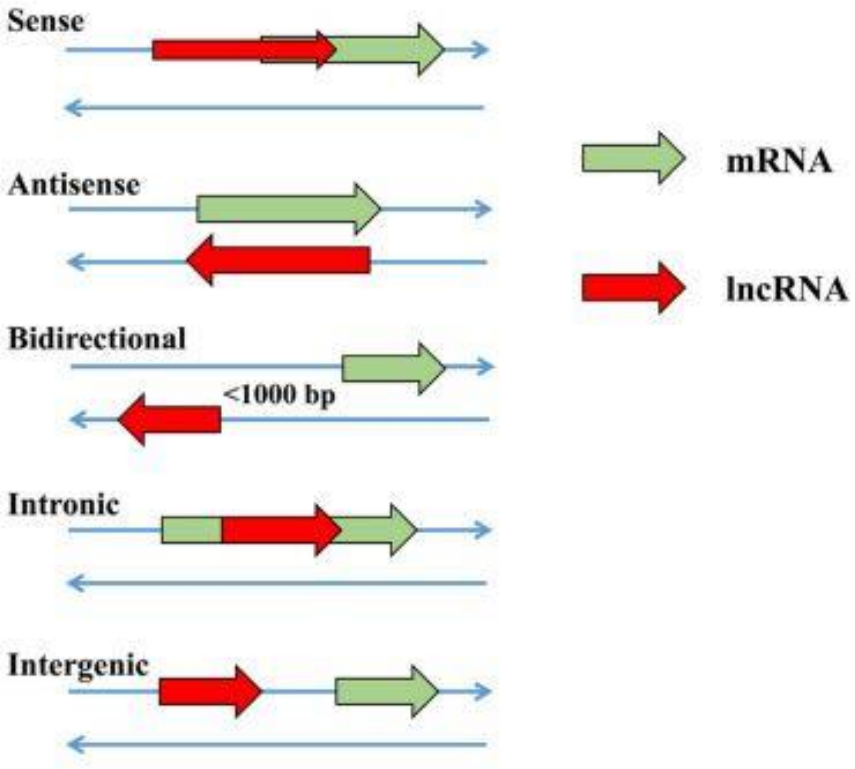

Figure 1.2. Classification of lncRNAs in terms of their genomic location and context with respect to protein-coding genes (PCGs). Sense lncRNAs and antisense lncRNAs overlaps with a PCG and located on the same strand and opposite strand, respectively. Bidirectional lncRNAs located within a range of 1000bp of a PCG and on the opposite strand. Intronic lncRNAs are derived from introns of PCGs. Intergenic lncRNAs are located between two PCGs. (Source: Meng et al. 2017)

discovered in mouse first and considered as mRNA because it is transcribed by RNA Pol II, it is processed and contains poor ORFs. However, transgenic mouse studies have revealed that it is responsible for overgrowth, without a protein product, and it is located in an strictly controlled imprinted region (Brannan et al. 1990). The idea of RNA functioning as an RNA molecule on its own was new and speculative; however, after a short time Xist was discovered, which is the most well-known lncRNA today. Xist is expressed in a monoallelic manner in cells that contain more than one X chromosome and responsible for epigenetic silencing of one random X chromosome for compensation. Xist lncRNA physically covers the randomly selected X chromosome and help to recruit methyl transferases for transcriptionally inactivate that chromosome (Jarroux, Morillon, and Pinskaya 2017). MALAT1, HOTAIR, and ANRIL are other well-characterized lncRNAs that have a role in cell proliferation, cell cycle, epithelial to mesenchymal

transition (ECM), migration and apoptosis (Alahari, Eastlack, and Alahari 2016; Congrains et al. 2013).

1.3.3. Long Non-Coding RNAs: Act of Mechanism

There appears to be a relationship between cellular location of lncRNAs and their molecular functions. They can be divided into two main class of nuclear and cytoplasmic lncRNAs (K. C. Wang and Chang 2011).

Nuclear lncRNAs can regulate their target genes in a *cis*- or *trans*-acting manner. *Cis*-acting lncRNAs target their neighboring genes while *trans*-acting ones have a function on distant loci. Despite their actions on different proximity of genes, their working principle is similar. They regulate transcription through altering chromatin structure by recruiting chromatin-modifying enzymes. They can act on a distinct locus or a whole chromosome, for example, *Xist.* In addition, they can recruit or remove transcription factors or transcriptional machinery components from the promoter region. Some lncRNAs can even act as an enhancer and stimulate target genes transcription. Besides, lncRNAs can regulate co-transcriptional and post-transcriptional events such as splicing by directly interacting with pre-mRNA or interfere with splicing machinery. In addition to these functions, nuclear lncRNAs can alter the chromosomal architecture by forming or disrupting chromosomal loops by interacting with DNA itself. Other lncRNAs can regulate the formation of subcellular structures such as nuclear speckles, paraspeckles, or Polycomb bodies (Neguembor, Jothi, and Gabellini 2014; Grossi et al. 2020; López-Urrutia et al. 2019; K. C. Wang and Chang 2011).

Cytoplasmic lncRNAs are produced and processed in the nucleus and transported into the cytoplasm. Some lncRNAs help translocation other proteins from the nucleus to the cytosol as well. In the cytoplasm, they regulate translation in a variety of ways. An exciting class of lncRNAs sequester miRNAs and form sponges. By that way, they can compete mRNAs for targeting miRNAs; thus, they can regulate miRNA targeting mRNA's degradation or translational availability. This class of lncRNAs is also categorized as competing endogenous RNAs (ceRNAs). ceRNAs can regulate other non-coding and/or lncRNAs as well (Salmena et al. 2011; Tang et al. 2019). Cytoplasmic lncRNAs can also regulate the translation rate of some mRNAs directly by interfering with polysome complexes by loading themselves into ribosomes through their internal

ribosome entry sites (IRES). By this way, they can promote or repress translation. Some lncRNAs can be even translated into small functional peptides by IRES recognition (Carlevaro-Fita et al. 2016; Zeng, Fukunaga, and Hamada 2018). Last but not least, one class of cytoplasmic lncRNAs can promote or suppress a particular mRNA's stability through regulating the mRNA decay mechanism by interfering with RNA methylation readers such as YTHFD2 and writer proteins such as METTL3 (Yan et al. 2019; Rashid, Shah, and Shan 2016).

1.3.4. Experimental and Bioinformatic Approaches to Study lncRNA Function

lncRNAs possess comparable characteristics of mRNAs due to their splicing, 5'-capping and polyadenylation although they are not translated. Since the discovery of the first lncRNA, a variety of tools and approaches have been developed and standardized. All these methods can be collected and categorized under the following order; identification of a lncRNA as a functional molecule, determination of proteins that are interacting with this lncRNA, discovery of macromolecule that interact with lncRNA of interest and resolving the functional role of this interaction in cellular events (McDonel and Guttman 2019).

The most basic idea behind whether a lncRNA is functional is to examine whether its depletion generates a cellular phenotype. There are several ways to disrupt the function of a lncRNA. By CRISPR-Cas9, the promoter region of interested lncRNA can be deleted to suppress its transcription. This approach is quite convenient for lincRNAs and bidirectional RNAs; however, in the case of antisense lncRNAs, overlapping sense lncRNAs, and intergenic lncRNAs, deletion will also affect the related protein-coding gene. Therefore, the phenotypic outcome could be an off-target effect (Goyal et al. 2017; Zhen and Li 2019; Zare et al. 2018). Besides sequence deletion, CRISPR technology can be used to insert a polyadenylation site after the transcription start site (TSS) to generate a premature transcript. This approach can be used when the promoter region should remain intact, and the early poly-A signal can be inserted close enough to TSS to disrupt the RNA function (McDonel and Guttman 2019). Besides deletion and insertion, the CRISPR-dCas9 approach can be used to suppress lncRNA loci transcriptionally.

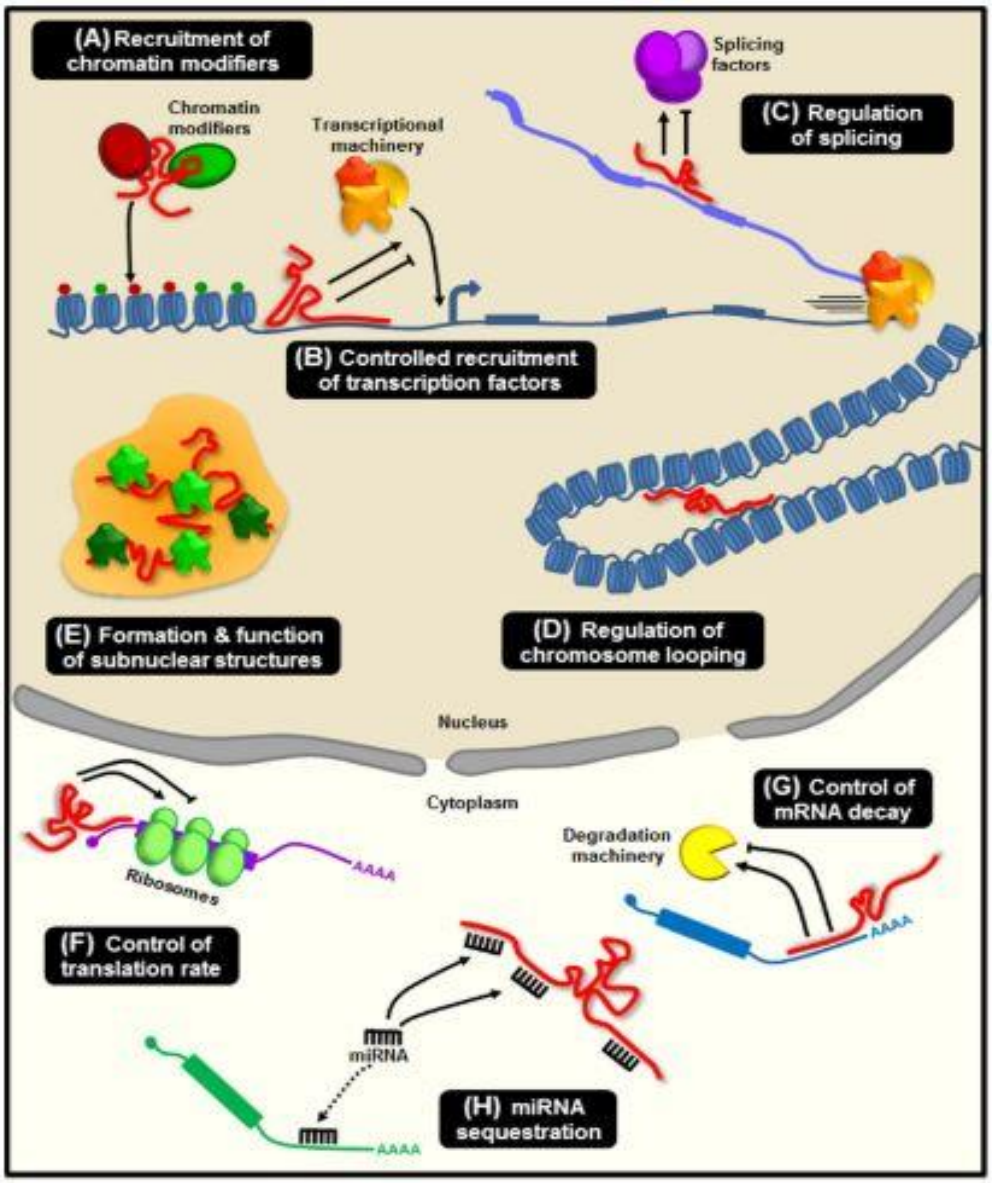

Figure 1.3. Schematic representation of the lncRNA mechanism of action. (a) LncRNAs shown in red can recruit and scaffold chromatin-modifying enzymes. (b) LncRNAs can regulate transcription factor recruitment and critical components of the transcription machinery. (c) LncRNAs can interact directly with mRNAs and regulate splicing. (d) LncRNAs can interact with DNA and form higher-order chromatin structures. (e) LncRNAs can regulate the formation and action of subnuclear structures. (f) LncRNAs regulate translation rate by interacting with polysomes. (g) LncRNAs can protect mRNA from decay or promote its degradation. (h) LncRNAs can sponge miRNAs and act as ceRNAs. (Source: Neguembor, Jothi, and Gabellini 2014)

CRISPR-dCas9 has deactivated Cas9; thus, it has no ability to cut DNA. By using this method, chromatin-modifying enzymes can be attached to the CRISPR-dCas9 complex, and with the help of creating site-directed heterochromatinization, lncRNA can be silenced (Gjaltema and Schulz 2018; Awwad 2019). In another approach, an already known protein interaction site of an lncRNA can be mutated and then rescued to uncover the mechanism behind the lncRNA-protein interaction. To rescue the mutated binding site, we can take advantage of RNA aptamer sequences that can bind the protein of interest with the help of a specific tag recognized by the aptamer sequence (Pintacuda et al. 2017).

The most direct and widely used method in lncRNA function studies is to induce degradation of mature lncRNA post-transcriptionally through RNA interference (RNAi) (Behlke 2016). In this method, siRNA targets lncRNA, and by RNAi-induced silencing complex (RISC), targeted lncRNA is cleaved, followed by its degradation by exosome and XRN1 from 3' and 5' ends respectively (Orban and Izaurralde 2005). However, this mechanism works well for cytoplasmic lncRNAs because RISC components exist mainly in the cytoplasm and rough ER. In order to degrade nuclear lncRNAs, RNase-H should be activated. RNase-H is the enzyme that recognizes RNA-DNA hybrids and degrades it in a non-sequence-specific manner. To target a lncRNA and digest it by RNase-H, locked nucleic acids containing GapmeR molecules are used. These antisense oligos contain LNA-DNA-LNA structure and recognize the lncRNA of interest while converting it as a prey for RNase H. By this method; nuclear lncRNAs can be silenced successfully (Lennox and Behlke 2016; Fluiter et al. 2009).

Once the functionality of a lncRNA has been demonstrated, the next step involves revealing the molecular mechanism of its function. To this end, macromolecules that interact with the lncRNA of interest may be identified by two reciprocal methods. Either the lncRNA can be tagged with specially designed probes, and then the protein and DNA that is interacting with are precipitated by RNA immunoprecipitation or RNA binding proteins of interest are tagged, and RNAs precipitated by protein immunoprecipitation. In the first method, identity of the related protein is determined by mass spectrometry. However, this approach is quite challenging due to the low abundance of lncRNAs in the cell, which leads to meager amount of precipitated protein. On the other hand, the protein immunoprecipitation method could be useful for identifying all lncRNAs that are interacting with a specific protein of interest by isolating precipitating RNAs and then sequencing them. By using this method, it is not possible to find the target protein by

starting from lncRNA, but the target lncRNAs can be found interacting with specific RNA-binding protein (Selth, Gilbert, and Svejstrup 2009; Ramanathan, Porter, and Khavari 2019). A new method is developed called in-cell protein-RNA interaction (incPRINT) that is based on the MS2-MS2CP system with luciferase tag. MS2 tagged lncRNA of interest is co-transfected with flag tagged RNA binding protein library into MS2CP luciferase-expressing cell. In the luciferase assay, even the lncRNA expression level is quite low, the lncRNA-protein interaction can be determined, and the target protein can be identified with ELISA. This method is highly promising and not much labor intensive but failed to answer if lncRNA and protein are indirectly interacting (Graindorge et al. 2019).

Once the lncRNA-protein interaction is identified, the next question is about the functional outcome of this interaction. In this step, targeted mutagenesis can be applied to disrupt lncRNA-protein interaction, and the phenotype is observed. However, this method is not applicable if the binding site cannot be defined clearly, for example, Xist. In this case, the mutagenesis can be applied to the RNA-binding protein. The most important issue to consider here is the confirmation of the outcome of this interaction by a rescue experiment. To conclude, there is a large variety of different approaches and methods developed to study lncRNA function. The path that will be followed must be chosen wisely (McDonel and Guttman 2019).

Besides these experimental approaches, computational approaches have an important application in studying lncRNA biology. Unlike microarray, deep sequencing technology allows identifying a vast number of novel lncRNA transcripts, including alternative splicing isoforms. There are five stages in a standardized computational pipeline for lncRNA annotations: (1) pre-processing the raw reads by removing adapter sequences via adapter trimming tools such as cutadapt, (2) quality control of the reads by FastQC, (3) alignment of the reads to the reference genome by an alignment algorithm like STAR or Bowtie, (4) read counting of gene features by HTSeq, (5) and as the last step, differential gene expression (DE) analysis by edgeR or DESeq (Arrigoni et al. 2016). To successfully analyze lncRNAs by RNA-Seq, cDNA must be prepared by using random primers instead of oligo DT because not all the lncRNAs are polyadenylated. Besides, for the read alignment, the comprehensive gene annotation file must be used instead of polyA feature annotation file to be able to annotate non-poly-adenylated transcripts as well (Frankish et al. 2019).

1.4. Long Non-Coding RNAs in Cell Proliferation

The key regulatory proteins of cell cycle, which are cyclins and CDKs, are subject to regulation by lncRNAs in human. For example, promoter-associated non-coding RNA (pncRNA) which is located upstream of the *CCND1* gene, negatively regulates cyclin D1 in HeLa cells (Yoneda et al. 2020). Following DNA damage, this lncRNA is activated, followed by recruiting TLS. TLS-ncRNA$_{CCND1}$ prevents p300 binding, which results in transcriptional repression of the *CCND1* gene. As a result, cell cycle arrest at G_1 stage occurs. (X. Wang et al. 2008). A well-studied lncRNA *MALAT-1* has a role in mRNA splicing in human. Overexpression of *MALAT-1* was shown in various cancer types, and it promotes cancer cell proliferation. Removal of *MALAT-1* results in the activation of several cell cycle regulators such as p53, p16, p21, and p27 and leads to the cell cycle arrest at G_1. In addition, it is known that *MALAT-1* plays a role in splicing of B-Myb, which is required for G_2/M transition indicating *MALAT-1* functions in several phases of cell cycle. It is regulated by cell cycle stage-specific regulator proteins and also regulates others (Sun, Hao, and Prasanth 2018). lncRNAs regulate CDK inhibitors. *INK4* locus has a crucial role in cell cycle regulation. This locus contains p15, p16, and ARF proteins and responsible for p53 and pRB degradation. It was shown that the antisense lncRNA of p15, *ANRIL*, represses its own locus by recruiting PRC2 complex, which leads to activation of cyclin D and CDK4/6 and cell cycle progressing. However, excessive RAS signaling suppresses *ANRIL,* which transcriptionally activates *INK4* locus resulting in the expression of p15 and p16. These proteins inhibit cyclin D and CDK4/6, which leads to cell cycle arrest or senescence (Kong et al. 2018; Congrains et al. 2013). Another lncRNA, *lncRNA-p21* is regulated by p53 and physically interacts with hnRNP-K to recruit it to target genes. It was also shown that *lncRNA-p21* functions with Rck/p54 RNA helicase, which results in repression of target mRNA's translation. Interestingly, this lncRNA can regulate transcription in the nucleus and translation in cytoplasm (Kitagawa et al. 2013). Besides these lncRNAs, there are a number of lncRNAs that were shown as regulatory players in cell proliferation such as *PANDA, gadd7, H19 lncRNA, p53-induced eRNA,* and *SRA.* PANDA stands for p21-associated ncRNA DNA damage-activated. PANDA is activated by p53 in case of DNA damage and suppresses FAS and BIK to repress apoptosis. It is also known that PANDA lncRNA stabilizes p53; thus, they are working in a positive feedback loop (Kotake et al. 2016). Gadd7 lncRNA regulates Cdk6

post-transcriptionally by causing its degradation (Jeong et al. 2016). Although lncRNA H19 is the first functional lncRNA to be identified, not all of functions of it are identified. One of its functions is processing into a miRNA, which causes pRB degradation and suppression of S phase entry (Kitagawa et al. 2013). Other lncRNAs that have a role in cell proliferation are still waiting to be unraveled.

In this laboratory, previous unpublished data from TUBİTAK project "113Z371" identified several lncRNAs that have a role in apoptosis and/or cell proliferation. In that project, HeLa cells were treated with cisplatin, doxorubicin, TNF-α, and Anti-FAS drugs and ligands. Then differentially expressed non-coding RNAs were determined by deep sequencing and confirmed by qPCR. As an outcome of that project, several lncRNAs are identified as candidate regulators of apoptosis and cell fate. In Dr. Ahmadov's previous unpublished data, four antisense lncRNAs were selected as candidates, including DR5-AS.

1.5. DR5-AS Long Non-Coding RNA Gene

DR5-AS was one of the candidate lncRNAs among a number of differentially expressed lncRNAs that is a drug inducible antisense lncRNA under apoptotic conditions (TUBİTAK Project 113Z371) (Ahmadov 2015). This lncRNA was quite an interesting candidate because it overlaps with DR5, which is a p53-dependent TRAIL-induced death receptor that regulates cell proliferation and apoptosis.

AC107959.2 gene (DR5-AS) is a novel transcript and annotated as lncRNA in the latest human reference genome release GRCh38.p13. It is located on chromosome 8p21.3, and it is 2,636 bp long. Due to its 807 bp overlap with DR5 gene, it was named as Death Receptor 5-Antisense. DR5 is a surface receptor activated by TRAIL and can induce apoptosis by interacting with caspase cascade (Ke et al. 2018). It is regulated by p53 and ATF3 and activated in response to DNA damage. DR5 has a dual role in survival; it can both promote cell proliferation and apoptosis by interacting with different sets of proteins. Moreover, intracellular localization of DR5 also has regulatory roles. It can interact with importin β1 and be translocated in nucleus, and in there, it interacts with miRNA processing machinery and inhibit let-7 maturation, which leads to upregulation of LIN28B and HMGA2 promoting cell growth (Mert and Sanlioglu 2016). A long non-coding RNA that overlaps this protein-coding gene is quite likely to have function.

DR5-AS has three exons and only one transcript variant. It is a novel transcript, without any functional information in the literature. There are three questions to be asked to tackle functionality of a novel non-coding transcript: (1) is it processed? (2) Is it conserved? (3) Is it associated with any phenotype? Our data reveals that it is conserved among primates with high sequence coverage with bonobo, chimpanzee, gorilla and orangutan. Furthermore, previous transcriptomic data has revealed that it is differentially expressed in different cancer tissues (Gibb et al. 2011). Besides, according to the International Cancer Consortium Data Portal, in different cancer tissues, this locus carries several numbers of mutations. All these little pieces of information suggest that DR5-AS might have a cellular function.

In Dr. Sweef's previous unpublished data, it was shown that DR5-AS transcript is processed. Its 5' and 3' ends were confirmed by RACE experiment, which reveals it has 5' cap yet no poly-A tail. DR5-AS was also shown to be localized in nucleus (Sweef 2020).

1.6. Aim

This study aims to unravel the function of DR5-AS lncRNA by using a reverse genetics approach in which the molecular and phenotypic effect(s) of GapmeR-mediated gene silencing will be examined by RNA sequencing and cellular studies.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culture, Transfection, and Imaging

HeLa cells were obtained from DSMZ GmbH (Gibco). The culture conditions were humidified air containing 5% CO_2 at 37°C in RPMI 1640 (with L-Glutamine, Gibco) medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin-streptomycin (Gibco). The cells were subcultured every two days, with 2.0 – 2.5 x 10^6 cells in 75 cm^2 cell culture flask.

Cisplatin treatment on HeLa cells was performed by seeding 0.3 x 10^6 cells on a 6 well-plate (Sarstedt) and growing overnight. Time- and dose- kinetic experiments with cisplatin were performed to acquire 50% apoptosis rate in HeLa cells that were performed in previous studies (TUBITAK Project 113Z371). Cisplatin (SantaCruz) was dissolved in DMSO to obtain 83.2 mM fresh stock on account of its chemical instability. Following experiments were set to 80 μM for 16 hours. DMSO, solvent of cisplatin, was used for negative control in 0.1 % concentration.

Silencing experiments were performed in 6 well-plates (Sarstedt). One day before the experiment, 7.5 x 10^4 cells per plate were seeded and allowed to be grown overnight in antibiotic-free FBS containing medium. The next day, transfection mixture was prepared by following these steps; firstly 40 nM of DR5-AS GapmeR (Qiagen) probes were dissolved in RPMI 1640 (with L-Glutamine, Gibco) without containing FBS or antibiotics followed by addition of 4.5 μL of FuGENE HD Transfection Reagent (Promega) then vortexed for 3-5 seconds. The total reaction mixture was 150 μL. Then the transfection mixture was incubated on room temperature for 15 minutes. By that time, the media of cells were replaced with fresh RPMI 1640 (with L-Glutamine, Gibco) containing FBS. When the incubation was completed, the tubes were spun shortly and added into the media dropwise to ensure even distribution. The total volume of the well was 2 mL. These transfected cells were incubated for 24 to 72 hours in the first trial, and subsequent experiments were set to 72 hours. Negative control antisense LNA GapmeR was an oligo without any target. FuGENE HD Transfection Reagent (Promega) was used

as a second negative control due to its possible toxic effects. For proliferation measurement, 40 nM of LNA GapmeR (Qiagen) was used with 0.15 µL of FuGENE HD Transfection Reagent (Promega) mixed in RPMI 1640 (with L-Glutamine, Gibco) up to 5 µL of volume per well in 96 well-plate and the same procedure that was mentioned above was followed. The total working volume for 96 well-plate was 100 µL.

For overexpression experiment, the sequence of DR5-AS lncRNA gene was synthesized commercially (Epoch) and cloned into pcDNA3.1(+) mammalian expression vector (Invitrogen). pcDNA3.1-DR5-AS construct was generated by performing double digestion of pcDNA3.1 with NheI and XhoI restriction enzymes and ligation with DR5-AS lncRNA gene. For optimization, 500 ng to 2500 ng of plasmid DNA were transfected. The same transfection procedure as GapmeR transfection was followed. After transfection, the transfected cells were incubated for 1h followed by a fresh RPMI 1640 (with L-Glutamine, Gibco) containing FBS replacement due to toxicity of plasmid DNA transfection. After that, the cells were incubated for 24 to 72 hours. The subsequent experiments were set to 1500 ng for 48 hours. As positive control, green fluorescence protein (GFP) containing vector was used. After transfection, cells were imaged with fluorescence microscope to confirm GFP expression, as well as transfection. For 96 well plate, 5 ng of plasmid DNA was used for transfection. Vector was dissolved into RPMI 1640 (with L-Glutamine, Gibco) without containing FBS or antibiotics followed by 0.15 µL of FuGENE HD Transfection Reagent (Promega). The total reaction mixture was 5 µL, and the total volume of the well was 100 µL.

For co-transfection, 40 nM GapmeR was transfected first and media was replaced with fresh RPMI 1640 (with L-Glutamine, Gibco) containing FBS after 1h, 2h, 4h, and 8h following transfection. Media replacement set to 8h for GapmeR. After GapmeR transfection and 8h incubation, media was removed, transfection mixture containing 1500 ng of pcDNA3.1-DR5-AS plasmid was added on cells, incubated for 1h and then the media was replaced with fresh RPMI 1640 (with L-Glutamine, Gibco) containing FBS. The final transfected cells were incubated for 48h.

Live cell imaging was performed with staining the cells by NucRed Dead 647 ReadyProbes Reagent (Thermo Fisher Scientific). NucRed Dead 647 ReadyProbes Reagent were added directly to the culture media as 2 drops per mL then incubated in the dark for 15 minutes-1hour. Then cells were imaged with Cy5 filter at far-red color at 642/661 excitation/emission. For brightfield live cell imaging, cells were imaged by using brightfield microscopy with 10X objective lens.

2.2. Measurement of Apoptosis

The apoptosis rates were detected by staining cells with Annexin V and 7AAD (BD) then analyzed by the MUSE cell analyzer (Millipore). Annexin V and 7AAD (BD) were diluted 1:10 with PBS. For harvesting the cells, Trypsin-EDTA (Gibco, 0.25%) was used and then washed with ice-cold PBS twice. After complete removal of PBS, cells were suspended into 50 μL of annexin binding buffer (BD). 10 μL of Annexin V and 10 μL of 7AAD were added into the cell suspension then incubated for 15 minutes in the dark at room temperature. The stained cells were diluted into 200 μL of PBS just before the analysis. Viable cells were considered as both Annexin V- and 7AAD-negative. Annexin V-positive and 7AAD-negative cells represent early apoptotic stage while Annexin V- and 7AAD-positive population represents late apoptotic stage. 7AAD positive cells were considered as dead.

2.3. Measurement of Proliferation

The proliferation was measured by WST-1 staining. Following various treatments of transfection conditions, 10 μL of cell proliferation reagent WST-1 (Sigma) was added directly to the cell media then incubated at 37°C and 5% CO_2 for 2-3 hours. In pursuit of the incubation, the absorbances of samples were measured at 450 nm wavelength. RPMI 1640 (with L-Glutamine, Gibco) was used as blank.

2.4. Cell Cycle Analysis

Cell cycle analysis was performed by ethanol fixation, Propidium Iodide (PI) staining, and flow cytometry analysis. Firstly, cells were harvested with 100 μL of Tyripsin-EDTA (0.25%) (Gibco) into 15 mL centrifuge tubes followed by centrifugation for 5 minutes at 1000 RPM. The supernatant was removed without disturbing the pellet, and the samples were placed on ice. In this step, pellets were dissolved with 1 mL of ice-cold PBS (1X) followed by 4 mL of ice-cold RNase-free ethanol addition. Ethanol was added slowly while vortexing. After that, the fixed samples were centrifuged at 1200

RPM for 10 minutes at 4°C. The supernatant was removed, and the pellet was dissolved in 5 mL of ice-cold PBS (1X). Next, the samples were re-centrifuged at 1200 RPM for 10 minutes at 4°C, and the supernatant was removed. In the next step, the pellet was dissolved in 0.1 % Triton X-100 PBS (1X) followed by 100 µL of RNase A (200 µg/mL) addition. These samples were incubated for 30 min at 37°C, and then 100 µL of PI (Thermo) was added following a second incubation for 15 minutes at room temperature. Lastly, the samples were analyzed by flow cytometry (FacsCanto, BD).

2.5. RNA Extraction and Deep Sequencing

Cells were harvested with Trypsin-EDTA (Gibco, 0.25%). The growth media of the cells were also saved to have dead cells as well. Harvested cells were washed with ice-cold PBS twice, and after complete removal of PBS, cells were lysed with 1 mL cold TRIzol (Invitrogen). After complete dissolving of pellets, the lysates were incubated at room temperature for 5 minutes to ensure of complete dissociation of nucleoprotein complexes. Then 0.2 µL of chloroform (Sigma) was added for 1 mL of TRIzol followed by vigorous shake for 15 seconds. After 2-3 minutes of incubation at room temperature, samples were centrifuged at 12,000 x g at 4°C for 15 minutes. At this point, the sample was separated into three phases, the upper aqueous phase was collected carefully by angling the tube 45° without disturbing the middle and bottom phase. After that, 0.5 mL of 100% RNase free isopropanol (Sigma) and 1 µL of RNase free glycogen (Sigma) for 1 mL TRIzol used were added then incubated at -20°C for 2 hours followed by centrifugation for 10 minutes at 12,000 x g at 4°C. The supernatant was completely removed carefully, and the pellet was washed with 1 mL of 75% ice-cold ethanol twice by centrifuging at 7,500 x g at 4°C for 5 minutes for each wash. Then the supernatant was removed completely, and pellets were air-dried then dissolved into 20-50 µL of DNase and RNase free water. RNA concentrations were measured by using NanoDrop Spectrophotometer (Thermo Fisher Scientific). RNA quality was checked by 260/280 and 260/260 absorbance ratios, and 1 % agarose gel run in TBE buffer (Tris-borate-EDTA buffer, 1M Tris base, 1M Boric acid, and 0.02M EDTA) for 25 minutes at 100V. Gel was visualized by using AlphaImager (Model IS-2200, AlphaImager High Performance Gel Documentation, and Image Analysis System).

Three replicates of DR5-AS GapmeR treated cells, and their negative GapmeR treated negative controls were sent to Fasteris SA (Switzerland) for deep sequencing by using HiSeq 2500 Illumina Platform.

2.6. Bioinformatic Analyses

The identification of differential expressed mRNAs was performed by Fasteris SA (Switzerland). First, adapter of the reads was removed by Trimmomatic tool then the reads were mapped to human GRCh37 reference genome by using Bowtie, Tophat, and Samtools (Langmead 2011). The mapped reads were count by Cufflinks program, which also normalizes the reads by RPKM (reads per kilobase per million) and determines the differentially expressed genes. The data was provided including gene names and Ensembl gene IDs with their respective loG_2 Fold Change values.

Pathway enrichment analysis was performed by using Reactome database, which is a cross-reference of NCBI, Ensembl, UniProt, KEGG, ChEBI, PubMed and GO databases (Jassal et al. 2020). The output of pathway enrichment analysis was visualized by using R programming language (Ihaka and Gentleman 1996) and ggplot2 software (Wickham 2006). The most significant 30 pathways were determined in a positively or negatively regulated manner. From this list of pathways, the ones that are parallel to the phenotype were determined, and candidate genes were selected for further confirmation of RNA-Seq data.

2.7. cDNA Synthesis and Quantitative PCR

For cDNA synthesis, RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was used. Two μg of RNA, 1 μL of Random Hexamer (Thermo Fisher Scientific) primer and RNase and DNase free water up to 12 μL were mixed first. Then 4 μL of 5X Reaction Buffer, 1 μL of RiboLock RNase Inhibitor, 2 μL of 10 mM dNTP mix and 1 μL of RevertAid M-MuLV RT (Thermo Fisher Scientific) were added and centrifuged briefly. The mixture was first incubated at 25°C for 5 minutes, then 42°C for 60 minutes followed by 70°C incubation for terminating the reaction for 5 minutes. Prior to qPCR, all cDNA samples were 1/2 diluted. For qPCR, 6.25 μL of GoTaq Master Mix

(Promega), 1 µL of cDNA, 4.75 µL of DNase and RNase free water and 0.5 µL of forward and reverse primer mix of DR5-AS were mixed for each well of 96 well reaction plate. Standard two-step PCR amplification was applied by LightCycler 96 (Roche) as; at 95°C for 2 minutes as initial denaturation, 45 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute following a melting step. Primer sequences are presented in Table 2.1.

Table 2.1 The list of primer sequences that were used in this study

Gene Name	***Forward 5'-3'***	***Reverse 5'-3'***
DR5-AS	Qiagen Cat. No. PH15855A-200	
DR5	CAGGTGTGATTCAGGTGAAGTGG	CCCCACTGTGCTTTGTACCTG
ANAPC4	ATAGACTCTTGGTCCAGCTGCC	TGCATGGTACGGGTGGGAATAG
ANAPC2	CAGTGACGACGAGAGCGACT	AGGCCCAGTCACCACAAACA
HMGA2	ATAAGCAAGAGTGGGCGGGT	TGAATGCCCGACGTCACAAG
CENPP	CATCCTGCAGACAGGGAGACAG	CTGTGTGACCTGGAGCTGATCTT
JUN	CTGTTGACAGCGGCGGAAAG	CACTTGTCTCCGGTCCTCCC
GADD45B	CGACATCAACATCGTGCGGG	AGACAATGCAGGTCTCGGGC
P21	CTGTGATGCGCTAATGGCGG	CCTCCAGTGGTGTCTCGGTG

CHAPTER 3

RESULTS

3.1. DR5-AS as a Functional lncRNA Candidate

DR5-AS lncRNA is a novel transcript. Phenotypic association and conservation analyses were performed to estimate the possibility of its functionality. The phenotypic association was performed by using several public transcriptomic data. As a result, DR5-AS was found to be differentially expressed in different cancer tissues and the loci that DR5-AS lncRNA is found carries mutations in different cancer patients (Figure 3.1.) (Gibb et al. 2011).

In multiple sequence alignment (MSA) analysis, 27 primate species were used and 4 of them have high-quality assembly according to EPO pipeline (Herrero et al. 2016). 14 primate species show low coverage and 8 of them show no similarity (Figure 3.1). DR5-AS was not conserved in further distant mammals and other species.

3.2. DR5-AS Knock-down Modulates Cell Morphology

DR5-AS GapmeR targets DR5-AS and then leads to its degradation. Negative GapmeRs that do not target any transcript were used as a negative control for the effect of oligo transfection. For overexpression, specifically constructed vector containing DR5-AS gene in pcDNA3.1 plasmid was used. As a negative control, empty pcDNA3.1 vector was used. In co-transfection, DR5-AS GapmeRs and pcDNA3.1-DR5-AS vector were transfected together to rescue the phenotype. The qPCR results show the relative abundance of DR5-AS lncRNA and DR5 gene in corresponding treatments as a confirmation for transient transfection in HeLa cells. DR5 expression was not affected by either DR5-AS silencing or DR5-AS overexpression (Figure 3.2).

As a result of DR5-AS silencing, the morphology of HeLa cells was altered. The healthy HeLa cell population consists of mostly angular, slightly filamentous-shaped cells with few round ones. However, silencing of DR5-AS leads to an increase of round cells

in HeLa cell population. When they are examined more in-depth, the metaphase plates can be seen clearly (Figure 3.3). The number of these round cells that are in metaphase stage is 15% higher in DR5-AS silenced population in comparison to negative control and DR5-AS overexpression. In co-transfection, the number of mitotic cells in metaphase is 5% which is less than DR5-AS knockdown and higher than negative control and DR5-AS overexpression (Figure 3.4).

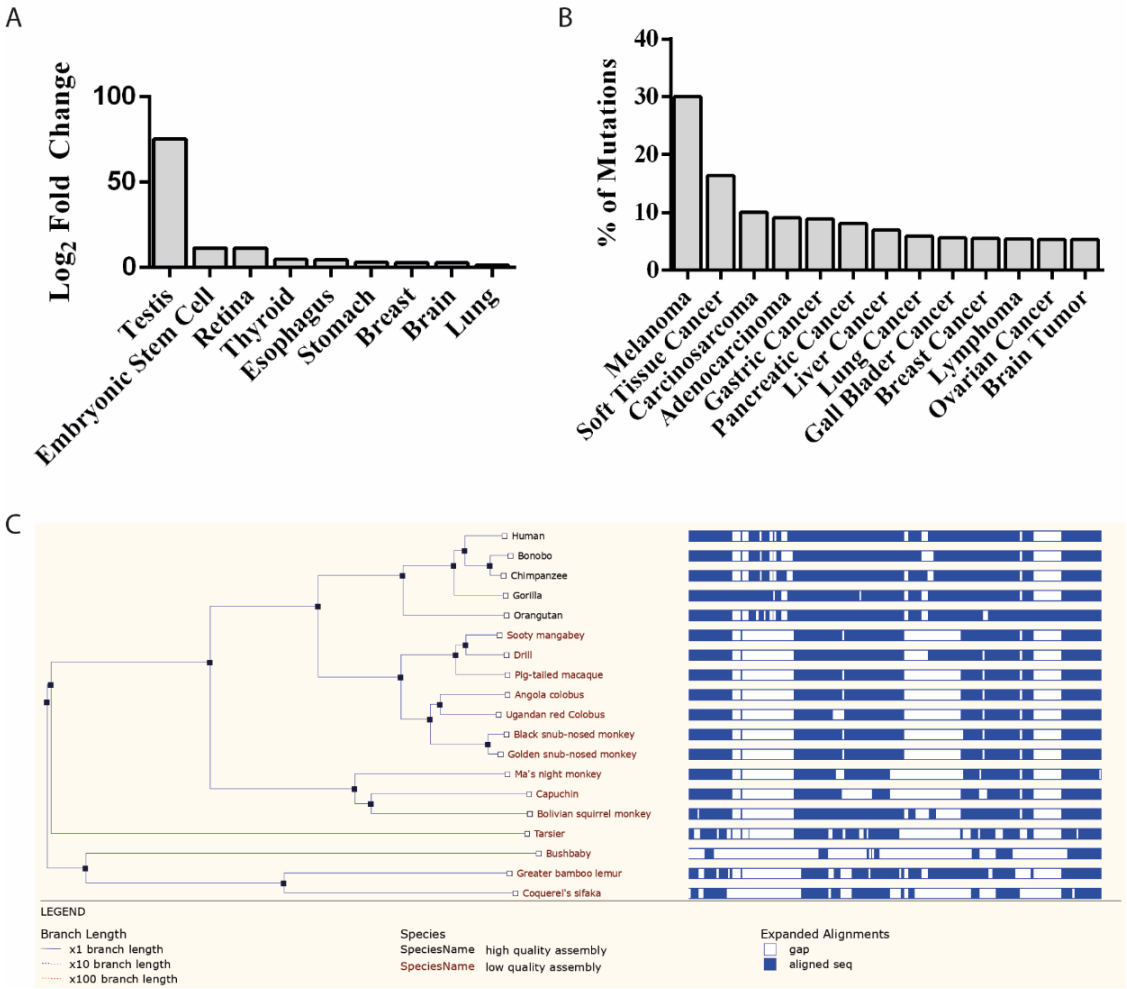

Figure 3.1. Phenotypic association and MSA analysis of DR5-AS lncRNA. (A) Log_2 fold change of DR5-AS in different cancer tissues. (B) % of mutations DR5-AS loci carries in different cancer patients. (C) MSA analysis of DR5-AS by 27 primates EPO-Low-Coverage.

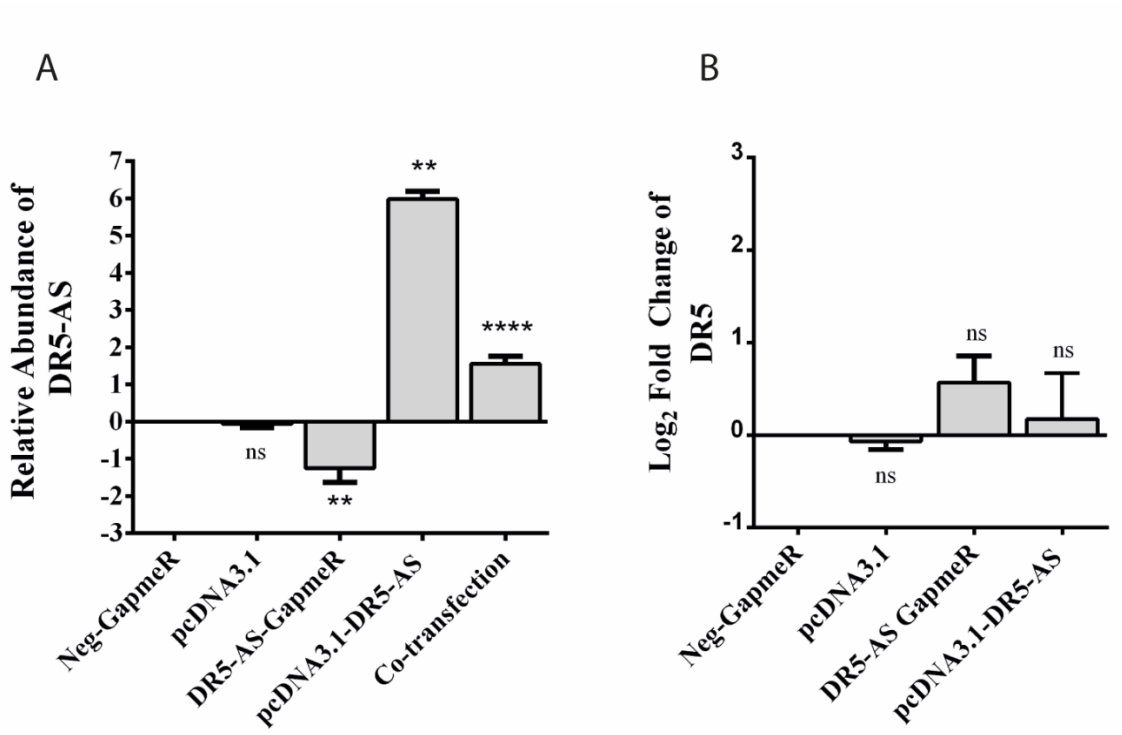

Figure 3.2. qPCR results of DR5-AS lncRNA and DR5 gene under different conditions. (A) DR5-AS GapmeR was used for silencing DR5-AS, and Neg-GapmeR was used as a negative control. For overexpression, pcDNA3.1-DR5-AS construct was used, and pcDNA3.1 was used as empty vector negative control. In co-transfection, DR5-AS GapmeR and pcDNA3.1-DR5-AS were transfected consecutively. (B) DR5 relative expression was measured by qPCR in response to DR5-AS knockdown and overexpression. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, P>0.05. p<0.01 (**), p<0.001 (***), p>0.0001 (****).

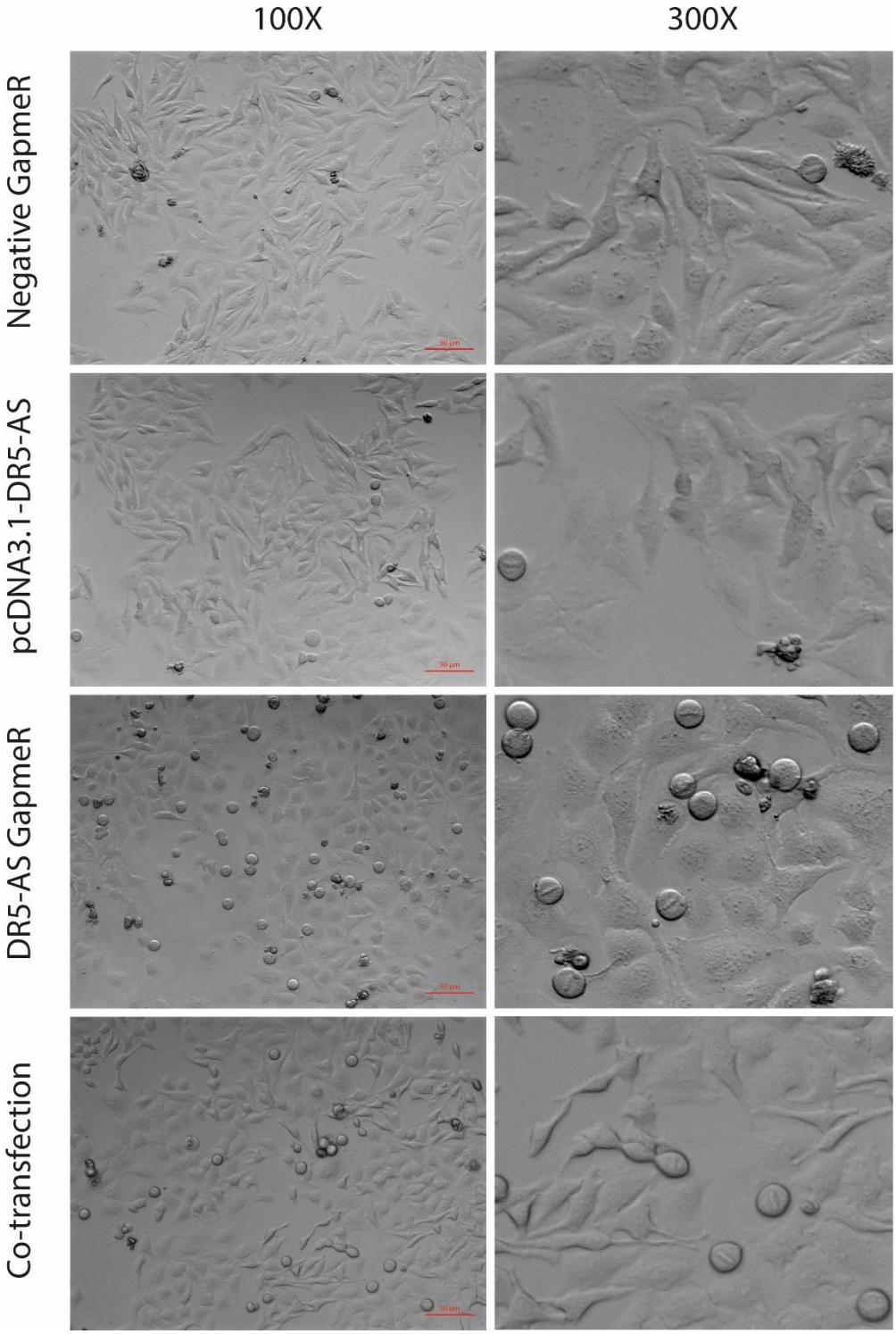

Figure 3.3. Brightfield microscopy analysis of HeLa cell morphology under treatment of Negative GapmeR, overexpression vector, DR5-AS GapmeR, and co-transfection. The images were taken under 10X objective lenses and magnified 3X by Adobe Illustrator software.

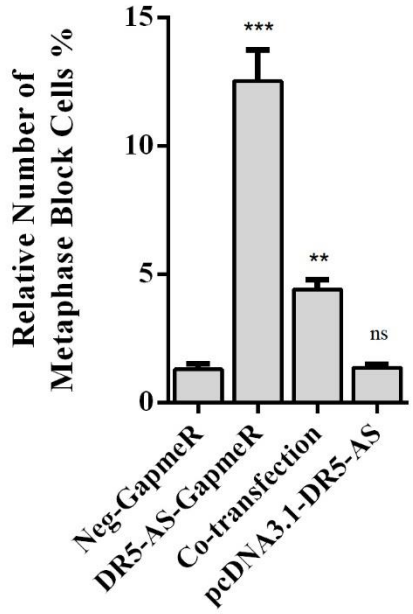

Figure 3.4. Relative percentage of metaphasic cells. The cells that contain metaphase plates were counted manually in ImageJ software then normalized with cells that have normal HeLa morphology. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

HeLa cells remain attached to the cell culture dish in a healthy culture, and detached cells are usually considered as dead cells. Live cell imaging was performed to ensure that they are alive by using NucRed Dead 647 ReadyProbes Reagent that stains DNA. Dead cells give fluorescent signal due to the penetration of the dye into the nucleus, which is clearly seen in lethal dose (5 %) of DMSO that was used as a positive control. However, there is no signal in Negative GapmeR, and DR5-AS GapmeR transfected HeLa cells, which means the population in those samples is alive (Figure 3.5).

3.3. Apoptosis Measurement

Because DR5-AS is a cisplatin- inducible lncRNA, it is possible that this lncRNA might modulate apoptosis. To test this hypothesis, apoptosis and proliferation rates of DR5-AS silenced, overexpressed, and co-transfected HeLa cells were measured.

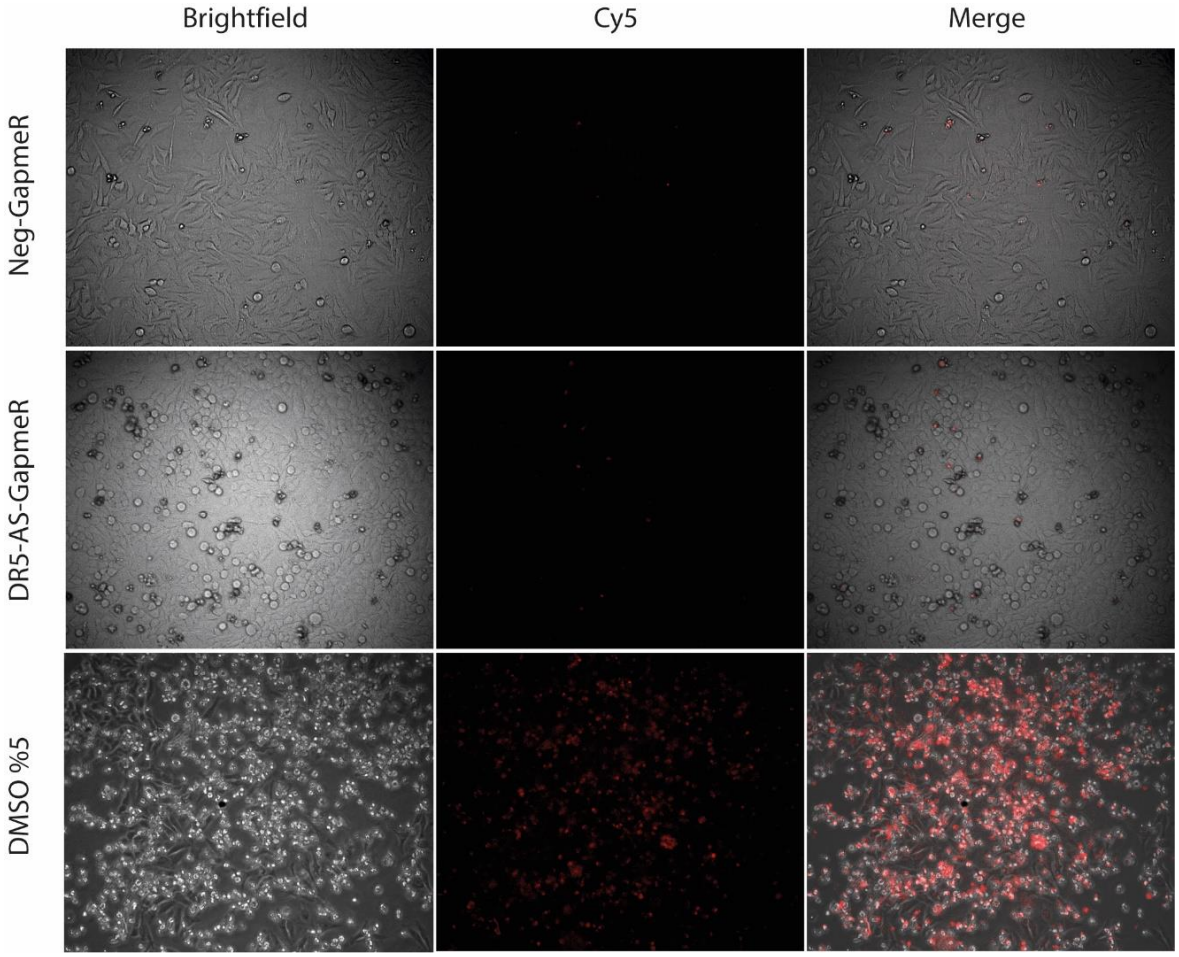

Figure 3.5. Live cell imaging of DR5-AS silencing in HeLa cells with NucRed Dead 647 ReadyProbes Reagent. Dead cells have signal in far red due to the penetration of the reagent to the nucleus. Cy5 images were taken at 642/661 excitation/emission. Brightfield and Cy5 images were merged in Adobe Photoshop software.

DR5-AS silencing, overexpression, and co-transfection have no remarkable effect on the apoptosis rate. Vector transfections caused a slight increase in apoptosis rate, yet the overall rate of early apoptotic cells is not higher than 4% (Figure 3.6).

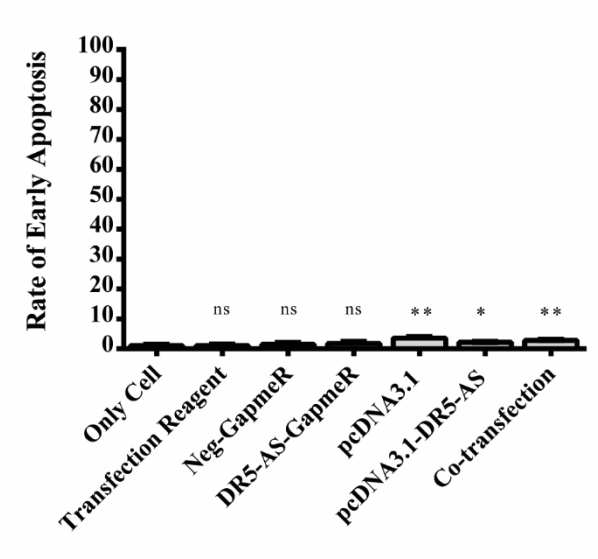

Figure 3.6. Apoptosis and proliferation rate measurement of DR5-AS knockdown, DR5-AS overexpression, and co-transfection. Flow cytometry analysis was performed via Annexin V and 7AAD staining. Annexin V positive and 7AAD negative cells were considered as early apoptotic cells. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, $P>0.05$. $P<0.01$ (**), $P<0.001$ (***), $P>0.0001$ (****).

3.4. Deep Sequencing and Bioinformatic Analysis

RNA-Seq analysis was performed to reveal the mechanism behind the phenotypic changes due to DR5-AS deficiency in HeLa cells. The obtained differentially expressed (DE) gene list was first filtered subject to fold change (two-fold and higher) and q value ($q<0.05$). As a result of this filtering, the analysis identified 2215 protein-coding genes that are differentially expressed in case of DR5-AS knockdown. These 2215 DE genes were subjected to pathway enrichment analysis by using Reactome Pathway Database. The database found 1635 affected pathways in human. These pathways were sorted based on entities ratio, which measures the set of DEs over the genes that are found in a particular pathway, and the first 30 pathways were taken into account (Figure 3.7).

The data analysis reveals the negative effect on cell cycle and mitotic cell cycle pathways and positive effect on cellular responses to stress and external stimuli of DR5-AS lncRNA deficiency. To confirm RNA-Seq data, cell cycle and proliferation related candidate genes were selected. LoG_2 fold change values of these candidates were presented in Table 3.1.

Table 3.1 The list of selected candidate genes provided with their loG_2 fold change values in DR5-AS silenced RNA-Seq data

Gene Name	***Log_2 Fold Change in RNA-Seq data***
ANAPC2	-2.39116
ANAPC4	-3.13217
HMGA2	4.22259
CENPP	-4.58038
JUN	-1.64037
GADD45B	1.86403
P21	1.16312

3.5. Cell Proliferation Measurement and Cell Cycle Analysis

RNA-Seq data suggest that DR5-AS knock-down may regulate proliferation through affecting cell cycle. To test this hypothesis, cell proliferation rate was measured, and cell cycle analysis was performed. Negative GapmeR caused 30% decrease in cell proliferation; however, silencing of DR5-AS leads to a more significant decrease as 80%. DR5-AS overexpression resulted in a 10% increase in proliferation in contrary to DR5-AS knockdown. Decrease in proliferation rate due to DR5-AS knockdown was rescued by co-transfection and increased as much as Negative GapmeR control (Figure 3.8).

Silencing of DR5-AS in HeLa cells results in shifting through S and G_2/M phase in cell cycle profile, which states DR5-AS knockdown causes cell cycle arrest at S and G_2/M phase. There is a 20% decrease in the G_1 and 10% increase in the S population in DR5-AS deficient cells. G_2/M population increased 10% in DR5-AS knockdown, which explains the cells that are stuck at metaphase stage (Figure 3.9).

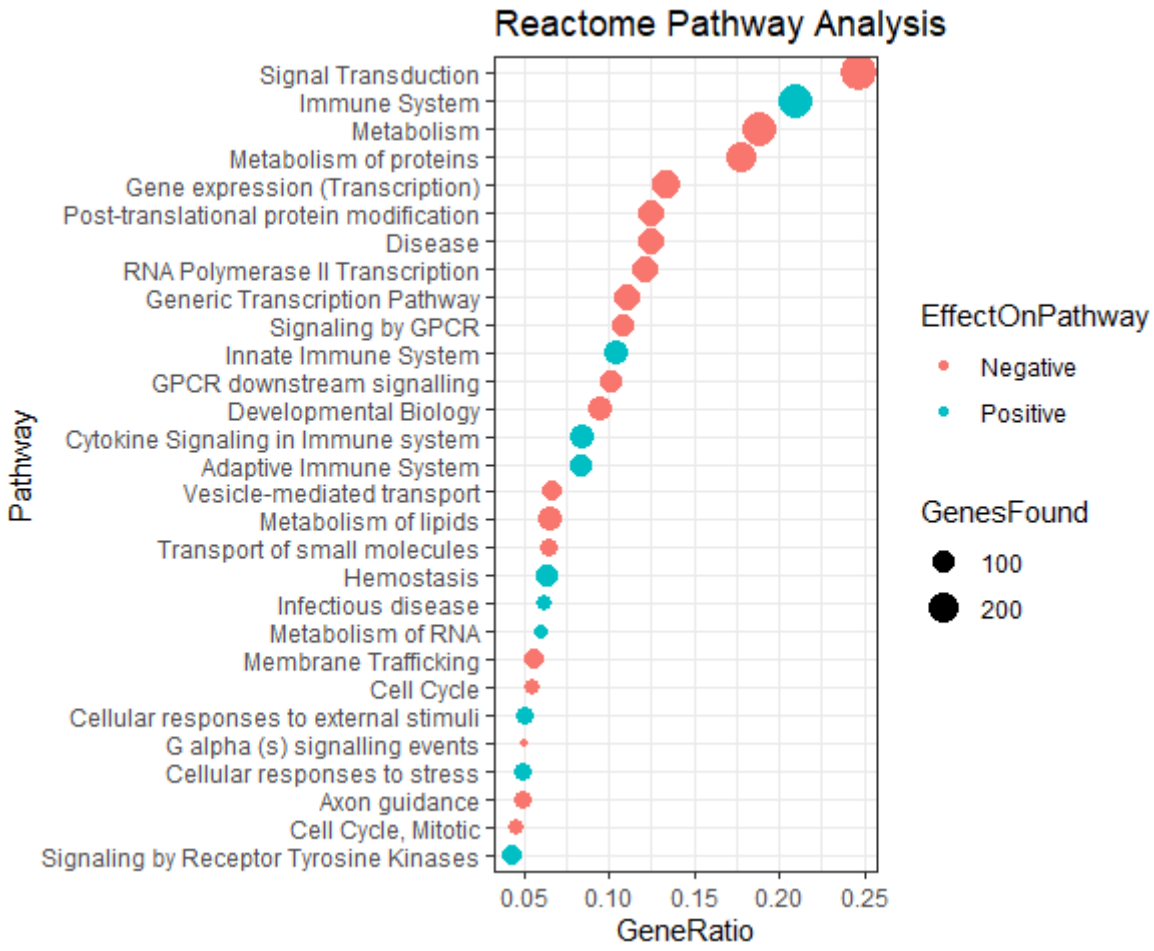

Figure 3.7. Pathway enrichment analysis of DR5-AS knockdown RNA-Seq data by using Reactome Pathway Database. EffectOnPathway stands for the overall fold changes of the entities found in that particular pathway. Negative means the genes in that pathway are downregulated and positive means the genes in that pathway are upregulated. GenesFound stands for the total number of entities in a particular pathway. GeneRatio is the number calculated by entities found in a pathway over the total number of genes in that pathway. 1079 of 2215 genes are not found in this database. The scatter plot was generated by using ggplot2 package in R environment.

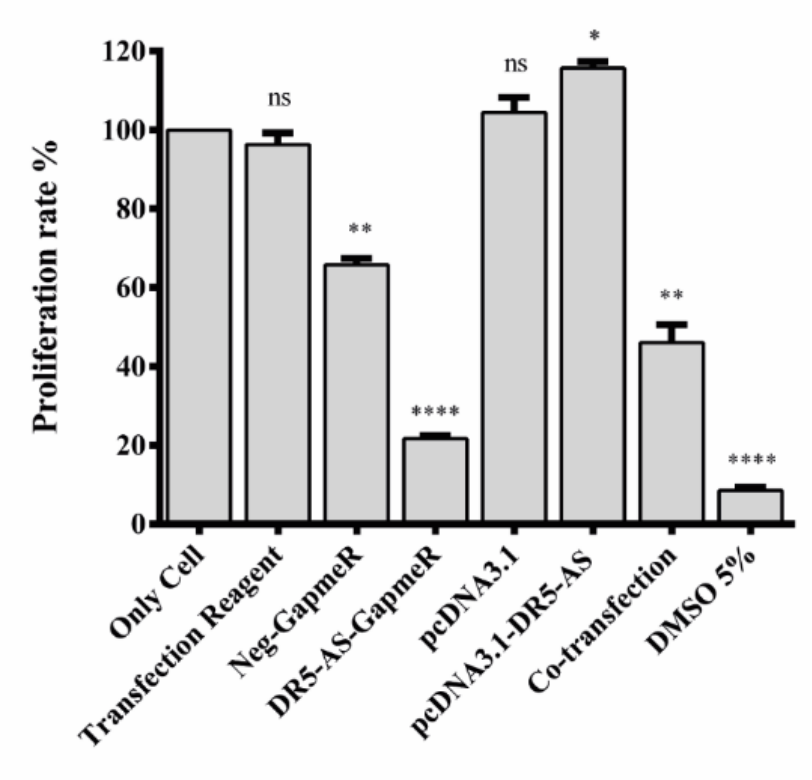

Figure 3.8. Spectrophotometric analysis of proliferation rate by WST-1 staining at 450 nm. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

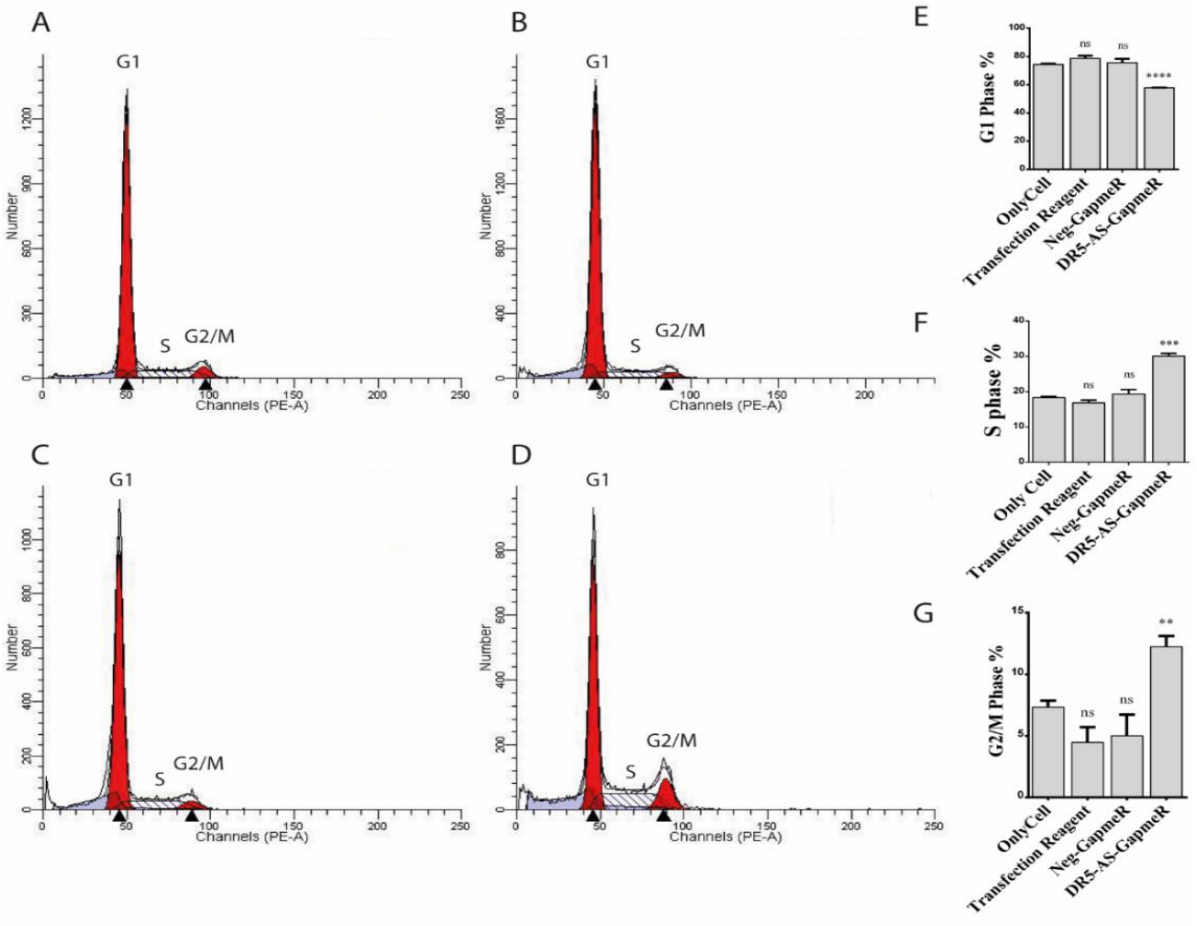

Figure 3.9. Cell cycle analysis of DR5-AS knockdown in HeLa cells. PI staining was performed, followed by flow cytometry analysis. (A) Only Cell (B) Transfection Reagent (C) Negative GapmeR (D) DR5-AS GapmeR (E), (F), and (G) are G_1, S, and G_2/M phases, respectively. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, P>0.05. P<0.01 (**), P<0.001 (***), P>0.0001 (****).

3.6. Quantitative PCR of Cell Cycle Related Genes

Quantitative PCR of candidate genes that are related to cell cycle and proliferation was performed to confirm RNAseq analysis. ANAPC4, ANAPC2, HMGA2, and CENPP genes were downregulated significantly while JUN, GADD45B, and p21 were upregulated in the event of DR5-AS knockdown. The overall fold change trend of differentially expressed genes is parallel with RNAseq data except for HMGA2. In RNAseq data, HMGA2 is upregulated, while it is significantly downregulated in qPCR results (Figure 3.10).

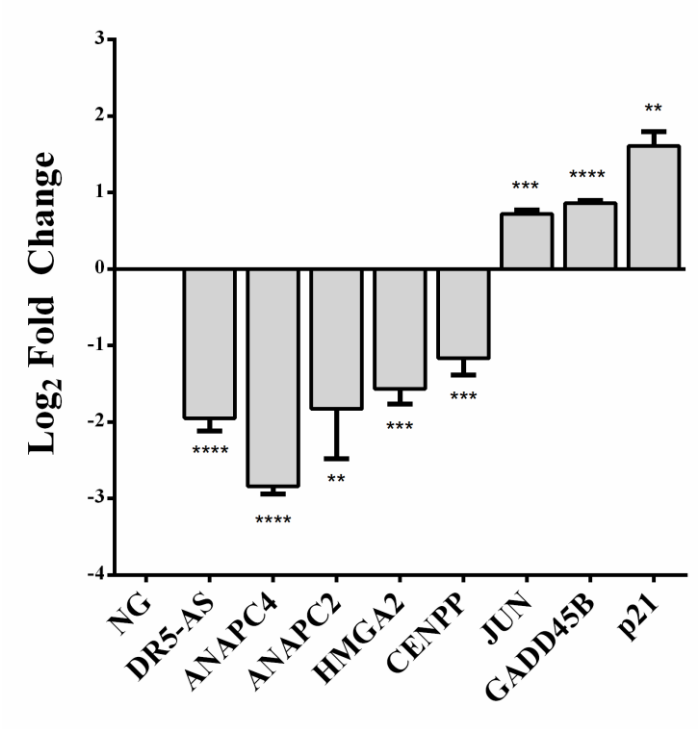

Figure 3.10. Quantitative PCR results of cell cycle-related candidate genes. Log_2 fold change in response to DR5-AS knockdown. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, $P>0.05$. $P<0.01$ (**), $P<0.001$ (***), $P>0.0001$ (****).

Relative expressions of cell cycle-related candidate genes were also measured in response to DR5-AS overexpression and co-transfection. ANAPC4, GADD455, JUN, and p21 did not show significant differential expression. HMGA2 expression was not affected by DR5-AS overexpression; however, DR5-AS co-transfection caused significant upregulation of HMGA2. CENPP significantly upregulated when DR5-AS is overexpressed, while co-transfection did not affect its expression (Figure 3.11).

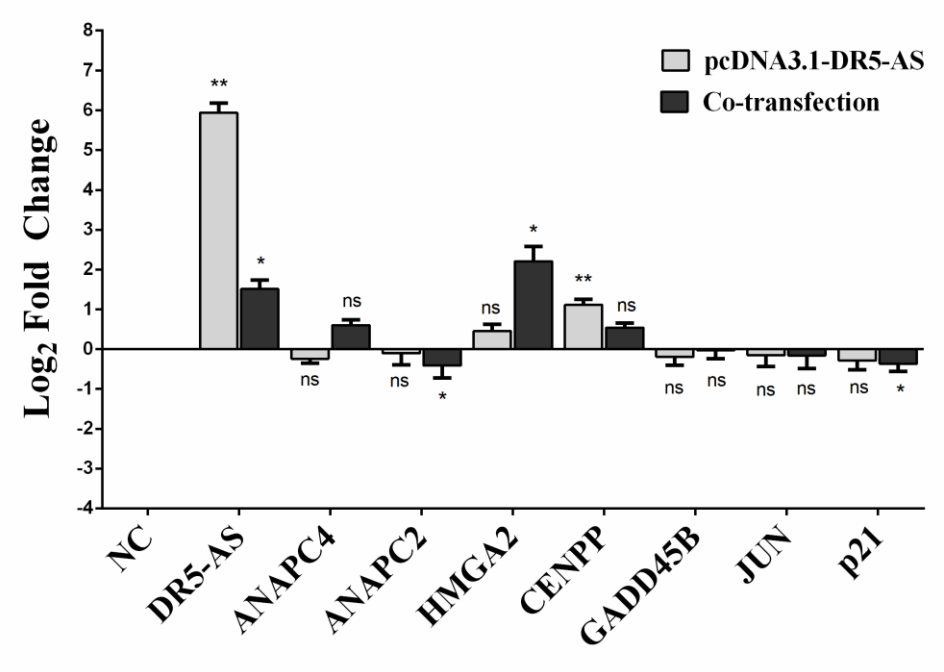

Figure 3.11. Quantitative PCR results of cell cycle-related candidate genes in overexpression of DR5-AS and rescue events. Experiments were performed in triplicates. Statistical analyses were performed by using Student's t-test, P>0.05. p<0.01 (**), p<0.001 (***), p>0.0001 (****).

CHAPTER 4

DISCUSSION

DR5-AS was initially selected due to its antisense characteristics and overlapping of DR5 gene in response to cisplatin, doxorubicin, TNF-α, and anti-FAS ligand treatments in HeLa cells. It was a promising functional candidate lncRNA because it was differentially expressed in several cancer tissues, different cancer patients carry mutations in its locus, and it was conserved among several primate species (Figure 3.1). It was hypothesized that DR5-AS lncRNA might regulate DR5 *in cis* to regulate cell proliferation. However, the qPCR results showed that DR5 expression levels do not depend on DR5-AS expression levels because neither DR5-AS knockdown nor DR5-AS overexpression affected DR5 abundance in HeLa cells (Figure 3.2B). This qPCR results also showed that knockdown, overexpression, and co-transfection of DR5-AS lncRNA was performed successfully (Figure 3.2A).

Microscope image in Figure 3.3 reveals the morphological change that occurred in HeLa cells in response to DR5-AS knockdown. They were initially suspected as dead cells because the majority of the DR5-AS knockdown population assumed a round shape and seemed detached. However, staining cells with NucRed Dead 647 ReadyProbes showed that they are not dead because the dye did not penetrate through the cell membrane (Figure 3.5). Furthermore, when those HeLa cells having different morphology were examined closer, the reason for this change could be understood. The HeLa cells seem stuck in mitosis, especially metaphase stage for some reason (Figure 3.3). To confirm the difference in mitotic HeLa cell number between control groups and DR5-AS knockdown statistically, the three replicates of Negative GapmeR, DR5-AS GapmeR, pcDNA3.1-DR5-AS treated and co-transfected HeLa cell images were analyzed by ImageJ. The cells were counted with Cell Counter plugin; then, the results were normalized with negative control as percentage. Figure 3.4 shows the significance of the cells that contain metaphase plate in DR5-AS knockdown sample compared to negative control and DR5-AS overexpression. Furthermore, this change in phenotype was rescued by co-transfection, which means treating the cells with DR5-AS GapmeR and pcDNA3.1-DR5-AS overexpression plasmid at the same time. In Figure 3.3, the microscope image shows the decrease in metaphasic cells in co-transfection compared to

DR5-AS knockdown. This rescue was also confirmed statistically, as can be seen in Figure 3.4. The number of mitotic cells was significantly reduced in co-transfected HeLa cells. This data provides the proof that the morphological change of HeLa cells is due to DR5-AS knockdown, not for any other off-target effect of transfection.

The next step was further phenotypic characterization by measuring apoptosis rate of HeLa cells in response to DR5-AS knockdown, DR5-AS overexpression, and co-transfection (Figure 3.6). The data states that apoptosis is not triggered by silencing or overexpression of DR5-AS in HeLa cells. To understand the mechanism behind the morphological change upon DR5-AS deficiency, a transcriptomics approach was performed with DR5-AS knock-down HeLa cells. Cells were treated with negative GapmeR and DR5-AS GapmeR followed by RNA-Seq experiment. In RNA-Seq sample preparation process, cDNA libraries were constructed by using oligo(dT) primers to have polyA + transcripts only. Analysis of this transcriptomic data showed that 2215 genes were differentially expressed in response to DR5-AS knockdown in HeLa cells. These differentially expressed genes were further subjected to pathway enrichment analysis to understand which molecular pathways are responsible for the morphological change caused by DR5-AS knockdown. The pathway enrichment analysis was performed by using Reactome Pathway Database. Reactome found 1635 affected pathways by this set of differentially expressed genes. These pathways were sorted based on entities found, and first 30 pathway was shown by the scatter plot in Figure 3.7. In the figure, pink circles represent the pathways that are suppressed while the blue circles represent the activated pathways. The size of circles shows the number of differentially expressed genes in the particular pathway, and the total 30 pathways were ordered based on GeneRatio, which is calculated by the number of differentially expressed genes from transcriptomic data over the total number of genes in that pathway. As can be seen from this figure, Cell Cycle and Mitotic Cell Cycle pathways were negatively affected while Cellular Response to Stress and Cellular Response to External Stimuli pathways were affected positively. From this point, proliferation rate was measured, and cell cycle analysis was performed. As can be seen from Figure 3.8, DR5-AS knock-down caused 80% decrease in proliferation rate. This decrease in proliferation rate was not seen in DR5-AS overexpression and could be rescued by co-transfection. The explanation for the decrease in proliferation rate without triggering apoptosis was considered as cell cycle arrest. To test this idea, DR5-AS knockdown HeLa cells were subjected to PI staining and then analyzed in flow cytometry to show cell cycle stages. PI staining showed that DR5-AS

silencing leads to a significant shift through S and G_2/M phase in cell cycle profile. This shift can also be seen from the graph that shows the percentage of cells in different cell cycle stages. In response to DR5-AS knockdown, cell population in G_1 stage were reduced significantly while the population in S and G_2/M stages are increased significantly (Figure 3.9). The reason why cells both shift to S and G_2/M in response to DR5-AS knockdown might be the fact that they are not initially synchronized.

All these phenotypic characterization experiments revealed that DR5-AS lncRNA has particular function in the cell cycle, especially in the mitotic phase. To confirm RNA-Seq data and understand the mechanism behind this regulation better, differentially expressed genes that are associated with proliferation and cell cycle such as ANAPC2, ANAPC4, HMGA2, CENPP, JUN, GADD45B and p21 were selected from RNA-Seq data. ANAPC4 and ANAPC2 genes were selected because they are one of the critical molecules that act as subunits of the anaphase promoting complex/cyclosome (APC/C), which is required for metaphase to anaphase transition (Kernan, Bonacci, and Emanuele 2018). In RNAseq data, ANAPC2 and ANAPC4 were downregulated 2.4 and 3.1 fold respectively, and in qPCR, their loG_2 fold change values are quite similar. The absence of ANAPC2 and ANAPC4 can explain the failure of progressing into anaphase, as ANAPC2 codes for the catalytic subunit of APC/C and ANAPC4 codes the scaffold subunit (McLean et al. 2011). When their mRNAs are not abundant, the complex could not be recruited and functioned properly. Impairment of APC/C leads to cell cycle arrest at metaphase stage because the cell needs to ubiquitinylate securin followed by the release of separin and degradation of cohesin to become able to separate sister chromatids (Ross and Cohen-Fix 2002). Besides the APC/C subunits, CENPP is another crucial regulator of the mitotic progression because it is required for proper kinetochore function at the centromere region. It is significantly downregulated in both RNA-Seq data and qPCR while being upregulated upon the DR5-AS overexpression. CENPP expression is directly proportional to DR5-AS level, which means DR5-AS could somehow regulate CENPP expression. p21 is one of the essential cell cycle regulators that acts as a CDK inhibitor (Schafer 1998). p21 upregulation was expected in response to DR5-AS knockdown because silencing of DR5-AS causes cell cycle arrest. DR5-AS overexpression and co-transfection did not affect the expression level of p21. GADD45B is known as upregulated in response to stressful growth arrest events and exposure to DNA damaging agents (Diaz-Moralli et al. 2013). In this case, DR5-AS knockdown leads to the upregulation of GADD45B as expected, while overexpression and co-transfection of

DR5-AS did not affect GADD45B expression at all. HMGA2 is a DNA binding protein that binds DNA and thus alters the DNA topology. It is also an oncogene that promotes growth in cancer cells (Sánchez et al. 2014). In RNA-Seq data, HMGA2 is surprisingly upregulated as 4.2-fold. That is why it was selected as another candidate; however, qPCR results showed that HMGA2 expression level actually decreases around 2-fold in response to DR5-AS knockdown, which makes more sense. Sometimes RNA-Seq can give opposite or nonsense results; that is why it is imperative to confirm it before completely trusting it. JUN was selected as another candidate because it is the subunit of the transcription factor AP-1, which is the major regulator of the DR5 gene (Mert and Sanlioglu 2016). JUN is upregulated when DR5-AS is silenced; in this scenario, DR5 upregulation was also expected, but this is not the case. DR5-AS overexpression and co-transfection did not affect JUN expression level.

CHAPTER 5

CONCLUSION

In this study, we discovered a lncRNA that plays a role in cell proliferation, especially in mitosis, by performing several phenotypic characterization experiments and with the help of sophisticated deep sequencing technology. DR5-AS deficiency causes cell cycle arrest in the metaphase stage in HeLa cells by directly or indirectly regulating the expression levels of key proteins in cell cycle progression like ANAPC2, ANAPC4, GADD45B, JUN, $HMAG_2$, CENPP, and p21.

DR5-AS was initially selected as a drug inducible antisense lncRNA that might have a function in cell proliferation. This data shows that it regulates cell proliferation, however, independently of the DR5 gene. To understand its mechanism of action further, several other experiments must be performed. From this point, the protein(s) that are interacting with DR5-AS should be investigated. There is no available experimental design that studies DR5-AS-RNA binding protein or DR5-AS-DNA interaction. As a future direction, these experiments can be performed. Once the molecule DR5-AS interacts is revealed, the exact function of this lncRNA can be understood better. After all, it is a possible candidate for prognostic or diagnostic markers; however, a more in-depth investigation is required, especially in different cancer cell lines and by *in vivo* studies. It may also have a role in chemotherapy resistance, so as another future direction, the effect of this lncRNA in drug metabolism can be investigated.

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