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Haloperidol Paradoxical Behavior Induced Cell Proliferation Through Pkb/Akt And Abolished By Mir-Let-7c

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Haloperidol is a typical antipsychotic drug; it is widely used for people with various psychiatric conditions such as schizophrenia, bipolar disorder, major depressive disorder, and dementia. Despite its effectiveness in controlling delusions, hallucinations, agitation, and other disruptive behavioral symptoms in these psychiatric conditions, it has adverse side effects such as extrapyramidal manifestation and endocrinologic and metabolic changes. Initial evidence shows that haloperidol has antitumor properties in several cancer types by inhibiting cell proliferation and inducing apoptosis. However, other reports indicate that haloperidol promotes cell proliferation in other cells, raising concerns about its association with an increased risk of cancer which suggests haloperidol has a paradoxical behavior in different cell types. Most haloperidol studies focused on cancer cells, not normal cells. However, a study showed that haloperidol protects lung endothelial cells from injury and another study proved that haloperidol stimulated the cellular transformation of human endometrial epithelial cells (HECCs) into human endometrial carcinoma cells (HECCs). Nevertheless, the role of haloperidol on the immortalized non-

tumorigenic epithelial cell line, human bronchial epithelium BEAS-2B, remains unknown. Also, the paradoxical effect of haloperidol in cancer and non-cancer cells and on Protein Kinase B protein kinase B (PKB/AKT) and its downstream regulatory proteins is puzzling. PKB/AKT promotes cell proliferation, survival, and metabolism, including tumorigenesis, by phosphorylating its downstream target proteins. Additionally, studies have proven that Several miRNAs were observed to be differentially expressed by antipsychotic drug treatment. However, limited studies directly investigate microRNAs' role in the haloperidol paradoxical effect. Therefore, it is essential to study the effects of haloperidol on PKB/AKT and its downstream regulatory proteins and explore the role of microRNAs targeting PKB/Akt messenger ribonucleic acid (mRNA) in regulating haloperidol's paradoxical behavior, focusing on miRNA, hsa-Let-7c, known for its roles as a cycle regulator, tumor suppressor, as well as its involvement in regulating neuronal differentiation, neural subtype specification, and synapse formation in humans.

This research proposes two aims to address these complexities. Aim 1: this study focuses on understanding haloperidol's paradoxical (i.e., contradictory) behavior relative to cell proliferation in non-cancerous cellular environments. The hypothesis is that haloperidol promotes cell proliferation and survival, increasing cell cycle progression gene expression in BEAS-2B cells. Aim 2: this study deciphers whether microRNAs (miRNAs) prevent haloperidol's paradoxical behavior that induces cell proliferation through the PKB/AKT signaling pathway in BEAS-2B cells since the miRNAs are suitable biomarkers and can be utilized for determining the paradoxical behavior of PBK/AKT in different cellular environments. The hypothesis is that overexpression of miRNA (i.e., hsa-let-7c

DNA construct, which targets PKB/AKT mRNA) would reduce PKB/AKT-induced expression by haloperidol in BEAS-2B cells, reducing cell proliferation and promoting apoptosis. To achieve the study aims and fulfill the study hypotheses, BEAS-2B cells were treated with 3.5 µM of haloperidol for 24 and 48 hours, and they were treated with 3.5 µM of haloperidol and transfected with hsa-let-7c- GFP-DNA construct for 24 and 48 hours. Also, untreated control experiments were included. After each interval, the cells were examined under an inverted microscope; protein and RNA were isolated; cDNA was synthesized; semi-qPCR and Western blot were performed. Li-COR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE) using Image Studio Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE) was used for visualization and quantification. Haloperidol increases BEAS-2B cell proliferation without hsa-let-7c expression, suggesting it promotes cell cycle progression. Conversely, overexpression of miRNA, hsa-let-7c, inhibits haloperidol-induced cell proliferation and PBK/AKT, including the downstream genes regulated by PBK/AKT. Conclusion: haloperidol-induced PBK/AKT expression to promote BEAS-2B cell proliferation and survival. However, overexpression of hsa-let-7c inhibits haloperidol-induced PBK/AKT and its downstream target genes. This study validates the proliferating paradoxical effect of haloperidol. It demonstrates that miRNA, hsa-let-7c, regulates PBK/AKT mRNA, reducing its protein expression in BEAS-2B cells and suggesting hsa-let-7c is a suitable biomarker for solving haloperidol paradoxical behaviors involving cell proliferation, survival, and apoptosis.

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LIST OF SYMBOLS/ABBREVIATIONS

µg	Micrograms
µL	Microliters
µM	Micromolar
3'UTR	3'-untranslated region
5'UTR	5'-untranslated region
AChE	Acetyl Cholinesterase
ACTB	Actin Beta
AKT	AK Strain Transforming
APAF-1	Activation of Apoptosome 1
APP	Amyloid Precursor Protein
ATCC®	American Type Culture Collection
BAK	Bcl-2 Homologous Antagonist Killer
BAX	BCL2-Associated X Protein
BCL	B-cell leukemia/lymphoma
BCL-2	B-Cell Lymphoma 2
BCL-XL	B-Cell Lymphoma Extra Large
BEAS-2B	Bronchial Epithelium transformed with Ad12-SV40 2B
Bid	BH3 interacting domain death agonist
C. elegans	Caenorhabditis elegans

CASP	Cysteine-Aspartic Proteases
CASP3	Cysteine-Aspartic Proteases 3
CASP9	Cysteine-Aspartic Proteases 9
CCN	Cyclin
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CO2	Carbon Dioxide
DEPC	Diethyl Pyrocarbonate
DFF45	DNA Fragmentation Factor 45
DISC	Death-induced signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DRD2	Dopamine D2 receptors
DT	Deoxythymidine
E2F	E2 factor
ECL	Enhanced Luminol-Based Chemiluminescent
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition

EMT	Epithelial-Mesenchymal Transition
ER	Endoplasmic Reticulum
ERK	Extracellular-signal-related kinase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	FGF receptor
GLY	Glycine
GPX	Glutathione Peroxidase
GPXs	Glutathione Peroxidases
GSH	Glutathione
GTP	Guanosine triphosphate
H	Hour
H2O	Water
HAL	Haloperidol
HD	Huntington Disease
HepG2	Hepatocarcinoma
HER	Hormone epidermal growth factor receptor
HMF	Hydroxy methyl furfural
HRP	Horseradish Peroxidase
IAP	Inhibitor of Apoptosis Protein
IARC	International Agency for Research on Cancer

IFGR	Insulin-like growth factor receptor
IL-3	Interleukin 3
kg	Kilogram
M	Molar
MAPK	Mitogen-activated protein kinase
MCF-3	Human Caucasian Breast Adenocarcinoma Cell Line
MDM2	Mouse Double Minute 2 Homolog
MEK	Mitogen-activated protein kinase
MgCl2	Magnesium Chloride
min	Minutes
miRNAs	MicroRNAs
mL	Milliliters
mM	Millimolar
MOMP	Mitochondrial Outer Membrane Permeabilization
MPT	Mitochondrial Membrane Permeability Transition
mRNA	Messenger Ribonucleic Acid
mtDNA	Mitochondrial Deoxyribonucleic Acid
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	Nanograms
NICE	National Institute for Health and Care Excellence
NIH	National Institute of Health
O	Oxygen
OD	Optical Density

OH	Hydroxyl Radical
p53	Tumor protein 53
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PBS	Phosphate-buffer saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease
PDK1	Phosphoinositide-dependent protein kinase-1
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit
PIK3R	Phosphoinositide-3-kinase regulatory
PIP	Phosphatidylinositol-1, 4, 5-triphosphate
PKB	Protein Kinase B
PMSF	Phenyl methane sulfonyl fluoride
PP2A	Protein phosphatase 2 activators
pre-miRNA	Precursor miRNA
pri-miRNA	Primary micro-RNA
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma tumor suppressor
RH	Random Hexamer
RIPA	Radio Immunoprecipitation Assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid

RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SNFA	Swedish National Food Administration
SOD	Superoxide Dismutase
SPR1	Arabidopsis Thaliana SPIRAL1
SPTBN2	Spectrin Beta Chain, Brain 2
STAT	Signal transducer and activator of transcription
TF	Transcription Factors
TP53	Tumor Protein 53
UTR	Untranslated Region
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

Background

Haloperidol (HAL) is a typical antipsychotic medication discovered over five decades (Granger, 1999). It is used worldwide in the treatment of mental health issues, in pediatric patients with autism, development of mental disorder, and aggressive behaviors, in adult patients with schizophrenia and bipolar disorder. It is used in the treatment of Alzheimer's disease and elderly patients with disruptive behavioral symptoms associated with dementia (Hutchings, 2013). Furthermore, because it relieves agitated delirium, nausea, and vomiting, it is frequently used in the palliative care of cancer patients (Zaporowska-Stachowiak et al., 2020).

The American Psychiatric Association recommends haloperidol as a drug of choice in the treatment of adult patients with schizophrenia despite the discovery of new medication (Lehman, 2004). Furthermore, haloperidol is considered by the World Health Organization (WHO) Model list as a necessary Medicine to treat psychotic disorders (WHO, 2015) that are required to manage agitation, hallucinations, delusions, and other symptoms of disruptive behavior.

The National Institute for Health and Care Excellence (NICE) recommends haloperidol for the treatment of patients with alcohol-related psychosis (NICE 2015), and Food and Drug Administration (FDA)-approved or off-label clinical applications of

haloperidol in clinical settings for conditions like Tourette syndrome, hyperactivity, acute mania, and intractable hiccups (Bixby, 1970; Wijemanne, 2013; Zaporowska-Stachowiak, 2020). Haloperidol is one of the fewest medications that have been used for over 50 years after being discovered. Usually, efficacious, and safer newer drugs will develop into obsolete older drugs. However, haloperidol continues to be prescribed as a medication for many patients, possibly because of its low cost and its effectiveness in comparison to other treatments (Apiquián et al., 2003).

Side Effects of Haloperidol

Patients receiving haloperidol show heterogeneity in drug response and various adverse effects, such as impairment of extrapyramidal nerve tracts and tardive dyskinesia (Malhotra, 2004; Schäfer, 2001 Thakur, 2014). The symptoms of extrapyramidal and movement disorders, including parkinsonian symptoms such as rigidity, bradykinesia, and tremor, are developed in 40%–76% of chronically treated patients with haloperidol (Beresford & Ward, 1987). In addition, metabolic changes such as weight gain, hyperlipidemia, insulin resistance, diabetes, and polydipsia have been occurring more frequently. Another common side effect reported is cardiotoxicity. Moreover, in long-term follow-up, haloperidol was linked to higher overall mortality when compared to 66 other antipsychotics (Lao et al., 2020).

Potential Therapeutic and Molecular Target of Haloperidol

Haloperidol, a member of the butyrophenone series, is a potent neuroleptic drug with a wide range of clinical applications (Janssen, 1965). Haloperidol's mechanism of action and pathways involved are complex and not fully understood. It is primarily metabolized by CYP3A4, with potential genetic and ethnic variations in metabolism

(Kudo, 1999). Haloperidol exerts its mechanism of action through the blockage of dopamine D2 receptors (DRD2) in the basal ganglia, with secondary effects in the thalamus and cortex (Holcomb, 1996). In addition to its high affinity to dopamine D2, it binds to sigma receptors and blocks activities for noradrenergic, cholinergic, glutamatergic, and histaminergic receptors (Bowen, 1990, Fjalland, 2009, Ohta, M. 1976**)**. Haloperidol's affinity to sigma receptors is so significant that even just one orally administered dose that increases the plasma level of haloperidol in nanomolar concentration is sufficient to cause immediate binding to sigma receptors (Stone et al., 2006). Despite the inhibitory effect of haloperidol on the dopamine and other receptors occurring immediately, its antipsychotic full effect requires long-term (several weeks) treatment (Meltzer, 1991), implying that there may be chronic term changes mediated through regulation of downstream intracellular signaling pathways, which includes changes in gene expression (Fasulo et al., 2003). Previous studies have shown that the dopamine D2 receptor belongs to the G protein-coupled receptor (GPCR) family. Dopamine D2 receptor mediates their actions through both G protein-dependent and independent (β-arrestin 2-dependent) signaling pathways; dopamine D2 receptor has been associated with insulin signaling, which is mediated by phosphatidyl inositol 3-kinase (PI3K), AKT Serine/Threonine Kinase (AKT) and Glycogen Synthase Kinase 3 (GSK-3) signaling pathway (Beaulieu, 2007, Dwyer et al., 2008 and Kim, 2005). Many studies have identified the role of miRNAs, in the pathogenesis of schizophrenia (Caputo, 2015 and Wright, 2013), and the reports also indicate that haloperidol can influence the host epigenome and potentially impact drug response among the users (Swathy, 2017). These findings highlight the complex interplay between haloperidol and miRNAs and underscore the need for further research in this area.

Paradoxical Effects of Haloperidol

Several clinical studies of patients under haloperidol treatment observed a reduction in the risk of cancer (Wei et al., 2006; Wiklund et al., 2010). Haloperidol inhibits cell proliferation and induces apoptosis in human melanoma, glioblastoma, pancreatic, and colon cancer (Wei et al., 2006; Papadopoulos, 2020). It reported that haloperidol induces apoptosis, decreases Bcl-2 and BclxL expression in various cell types, including pre-neuronal, neuroblastoma, and glioblastoma cells (Wei, 2006; Papadopoulos, 2020), and inhibits the PI3K-Akt signaling pathway in pheochromocytoma (PC12) and neuronal cell cultures (Dai et al., 2007).

In contrast, several studies have reported recently that haloperidol is a subtle agent for initiating and promoting breast, prostate, colorectal, liver, ovarian, and endometrial cancers (Chiang et al., 2022). Researchers at Washington University School of Medicine in St. Louis have found that haloperidol and other antipsychotic medication, are associated with a significant increase in the risk of breast cancer (Rahman et al., 2022). Another recent study has raised concerns about the potential link between haloperidol, medication, and an increased risk of breast cancer (Hope, 2023). Research conducted in vitro has demonstrated that haloperidol activates nuclear factor kappa B (NF-B) and its downstream signaling target, thereby stimulating the cellular transformation of human endometrial epithelial cells (HECCs) into human endometrial carcinoma cells (HECCs) and facilitating their proliferation, migration, and invasion (Chiang et al., 2022).

The variability in haloperidol therapeutic response and induced adverse effects among the users have been studied, and it has been proposed that the potential role of polymorphic genes is responsible for paradoxical responses (Malhotra et al., 2004; Schäfer

et al., 2001). However, these suggestions provide limited success in predicting the drug's paradoxical effects, which implies that there might be another mechanism to account for haloperidol's paradoxical reactions (Ivanov et al., 2012).

In this study, we have been interested in the potential of microRNAs (miRNAs) impact on haloperidol response. MiRNAs are implicated in regulating the expression of various genes involved in drug metabolism, transport, and transduction pathways (Li et al., 2016). Certain genes must be expressed for the drugs to work, and variations in the expression level of these genes can impact the drug response by either over- or underexpressing miRNA. In cancer cells, miRNAs have been found to modulate drug response, with their dysregulation contributing to drug resistance (Wu et al., 2009). The potential of miRNAs as biomarkers for personalized medicine has also been highlighted, given their role in drug response variability (Latini, 2019).

Research has demonstrated that the use of antipsychotic medications was found to differentially express several miRNAs. Interestingly, certain patterns of miRNA expression resembled previously documented miRNA observations on the pathogenesis of schizophrenia and other psychological disorders (Caputo et al., 2015; Swathy and Banerjee, 2017 b).

Most importantly, the expression of miRNA let -7c, in addition to its roles as a cycle regulator and tumor suppressor (Roush and Slack, 2008) in the brain, has been shown to regulate neuronal differentiation, neural subtype specification, and synapse formation in humans and it has been associated to many neurological and psychological disorders (Kim et al., 2014; McGowan et al., 2018). However, none of these studies directly investigate the role of hsa let-7c in haloperidol response.

Most of the studies on the haloperidol effect were on postmortem brain tissue peripheral blood, or cancer cell lines (Backhouse, 1982; Bloomfield, 2018; Korpi, 1984; Kornhuber, 1999). The patient might be on multiple medications with different disease conditions in a clinical setup. Significant alterations in miRNA profiles occur when a healthy tissue or cell becomes diseased, and these changes may impact the expression of drug target genes (Hesse, 2014; Lu et al., 2005). As a result, it is challenging to understand the effects of haloperidol, how it affects the expression of different miRNAs and the genes that they target, and how different expressions of miRNAs affect the genes that haloperidol regulates.

To resolve this change, we aimed to identify the role of miRNAs (hsa-let-7c) on the genes that were targeted by haloperidol treatment under an in vitro condition using the BEAS-2B cell line, derived from normal human bronchial epithelial cells.

The Aims of the Study

This study aims to decipher whether the expression of miRNA, hsa-Let-7c, will regulate the expression of AKT, a regulator involved in cell survival, apoptosis, and cell proliferation in BEAS-2B cells when exposed to haloperidol, a stimulant of AKT. The effects of hsa-Let-7c on AKT expression would explain a part of haloperidol's paradoxical effects on cell lines. Three aims were proposed for the study to understand the impact of hsa-Let-7c on haloperidol-induced AKT expression in BEAS-2B cells.

Aim 1: Investigation of Haloperidol's Paradoxical (i.e., contradictory) Effects on Cell Proliferation, Growth and Survival in Non-Cancerous BEAS-2B Cells. Objective 1 evaluated the effect of haloperidol treatment on the PKB/AKT signaling pathway and its downstream proteins involved in cell cycle progression and survival in BEAS-2B cells.

Objective 2 was to examine the effect of haloperidol on protein-involved regulation of protein synthesis, potentially facilitating increased cellular protein production and growth. Hypothesis: Haloperidol promotes cell proliferation through activation of the PKB/AKT pathway, promoting cell survival and cell cycle progression and modulation of protein involved in the regulation of protein synthesis.

Aim 2: Investigation of miRNA Differential Expression in Haloperidol-Treated BEAS-2B Cells. The objective was to compare the differential expressions of tumor suppressor miRNAs, specifically miR-15a and miR-has-let-7c, in response to haloperidol treatment in BEAS-2B cells. Hypothesis: Haloperidol treatment downregulates the expression of miR-15a and miR-has-let-7c, contributing to cell proliferation observed on treated BEAS-2B.

Aim 3: Examination of miRNA let-7c's Regulatory Effect on Haloperidol Response Objective: To investigate the effect of miRNA, hsa-let-7c on the protein's expression targeted by haloperidol treatment in BEAS-2B cells. Hypothesis: Overexpression of miRNA (i.e., hsa-let-7c DNA construct) counteracts haloperidol-induced effects, affecting the PI3K/AKT pathway and altering proteins expression associated with cell proliferation and survival in BEAS-2B.

Experimental Approach: To achieve the study aims and fulfill the study hypotheses, BEAS-2B cells were treated with 3.5 µM of haloperidol for 24 and 48 hours, and they were treated with 3.5 µM of haloperidol and transfected with hsa-let-7c DNA construct for 24 and 48 hours. Also, untreated control experiments were included. After each interval, the cells were examined under an inverted microscope; protein and RNA were isolated; cDNA was synthesized; semi-qPCR and Western blot were performed. The

LI-COR Image Studio 5. 2 Software (LI-COR, Lincoln, NE) was used for visualization and quantification and statistical analysis was conducted to compare results between experimental groups.

This study aims to provide a comprehensive understanding of the molecular mechanisms underlying haloperidol-induced cell proliferation, survival, and the modulation of cell cycle progression, and translation proteins as well as the potential regulatory role of miRNA let-7c in modulating haloperidol impacts on BEAS-2B cells (Figure 1). These findings will provide valuable insights into the complex effects of haloperidol and its potential implications for cellular processes.

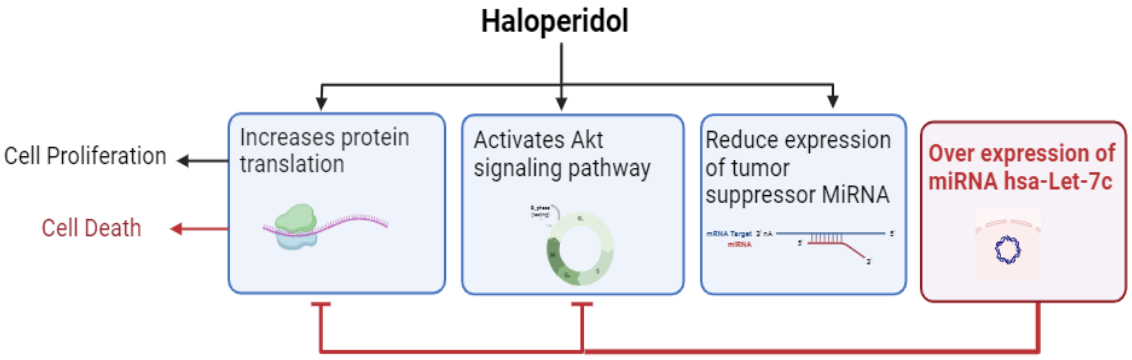


Figure 1: Graphical Abstract of the Effects of Haloperidol on Cell Proliferation and the Role of miR-has-Let-7c

The Graphical abstract provides a visual summary of the effects of haloperidol on cell proliferation and the role of miR-has-Let-7c (i.e., construct) in modulating these effects. Created by an online tool available at BioRender.com.

CHAPTER 2

LITERARY REVIEW

Several mechanisms are known to be involved in haloperidol activity and its variability in the response. Haloperidol treatment has a complex effect on gene expression, as evidenced by studies that link it to changes in the expression of genes related to neurotransmission, neural plasticity, oxidative stress, signal transduction, ionic homeostasis, and metabolism (Feher, 2005; Narayan, 2007; Thomas, 2003). The gene expression process is a complex of various steps that primarily occur through gene-specific transcription, but it's important to note that various post-transcriptional events also play a significant role in shaping cell-specific expression patterns, ultimately influencing cellular function (Corbett, 2018). This complexity underscores a wide array of molecular interactions and potential targets for haloperidol within the cellular machinery. Gaining insights into these multifaceted effects is paramount for unraveling haloperidol's mechanisms and comprehending its paradoxical behavior across diverse cellular environments. MiRNAs, as significant regulators of gene expression (Garzon et al., 2009; Klein et al., 2001), add another layer of complexity to the understanding of haloperidol's actions. Their role in influencing drug-related genes, as well as those associated with cell proliferation, cell cycle, and apoptosis, provides a plausible mechanism for the drug's diverse responses (Klein et al., 2001). Unraveling the interplay between haloperidol, miRNAs, and various cellular processes is crucial for comprehending the drug's intricate mechanisms and addressing its paradoxical effects in different cellular environments.

Transcriptional Regulation of Specific Genes

A cell controls gene activity by transcriptional regulation, where the information encoded in DNA is converted into RNA. This process is tightly regulated and involves a complex interplay of various factors to ensure that genes are expressed in the right amount, at the right time, and in the right cell type (Cramer, 2019).

Transcriptional Regulation of Survival-Related Genes

Phosphoinositide-3-kinase Regulatory (PIK3R)

A component (subunit) of the enzyme phosphatidylinositol 3-kinase (PI3K) is encoded by phosphoinositide-3-kinase regulatory (PIK3R). The subunit's main job is to control the enzyme's activity (Engelman et al., 2006). PI3K is a kinase, which means that it phosphorylates other proteins by adding a group of oxygen and phosphorus atoms, or a phosphate group, to them. Certain signaling molecules are phosphorylated by PI3K, which sets off a chain of subsequent processes. PI3K signaling is essential for many cellular functions, including cell migration, growth and division (proliferation), the synthesis of new proteins, material transport within cells, and cell survival (Engelman, Luo et al., 2006).

According to their structural features and substrate selectivity, PI3Ks have been categorized into three groups, class I, II, and III (Fruman et al., 1998). Class I enzymes are directly triggered by cell surface receptors. Class I PI3Ks are further broken down into class IA enzymes, which are triggered by tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs), and some oncogenes like the small G protein Ras, and class IB enzymes, which are solely governed by GPCRs (Liu et al., 2009). Regulatory subunit p85 and catalytic subunit p110 form heterodimers that makeup class IA PI3Ks (Engelman, Luo et al., 2006). The regulatory component mediates the location, activation, and binding of

the enzyme to receptors. Three genes in mammals, PIK3R1, PIK3R2, and PIK3R3, are responsible for producing the regulatory subunits p85 α (and its splicing variants p55 α and p50 α), p85 β, and p55 γ, respectively (Engelman, Luo et al., 2006).

PI3K is normally inactivated as the result of the interaction between p58 and p110 (Jean et al., 2014). By interacting with the tyrosine phosphate motifs on active receptors, the p85 subunit of PI3K can recruit to the membrane in response to a range of growth hormones and signal transduction molecules, including insulin, vascular protein I (Ang1), VEGF, human growth factor (HGF), and fibroblast growth factor (FGF). Class I PI3Ks convert phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) which is a second messenger that powers numerous downstream signaling cascades regulating cellular functions (Fruman et al., 1998).

AKT is moved from the cytoplasm to the cell membrane by the action of PIP3, which is produced following PI3K activation. AKT was activated when threonine and serine were phosphorylated by PDK1 and PDK2, respectively (Stokoe et al., 1997). The nuclear transcription factor B (NF-B), mTOR, Bad, Caspase 9, cyclin D1, and other downstream signal molecules were all targeted by the activated AKT after it had been moved from the cell membrane to the cytoplasm or nucleus Johnson (Ge et al., 2008). Also, class I PI3K activation significantly boosts the amounts of cyclin D, which are necessary to activate CDK 4 or CDK 6 in the G1 phase of the cell cycle. The retinoblastoma protein (RB) is phosphorylated by this CDK, permitting the release of the associated E2F, which is required for G1 progression (Sherr et al., 1999).

AKT Serine/Threonine Kinase (AKT)/ Protein Kinase B (PKB)

Protein kinase B (PKB), also known as Akt, is the collective of a set of three serine/threonine-specific protein kinases that participate in important and diverse cell roles in response to hormones, growth factors, cytokines, and neurotrophic factors (Dummler and Hemmings, 2007). The AKT is known for regulating metabolism, lipid synthesis, protein synthesis, growth, angiogenesis, and survival of most cell types (Manning et al., 2017). AKT dysfunction can lead to diverse pathological consequences, including developmental and overgrowth syndromes, cancer, cardiovascular disease, insulin resistance and type 2 diabetes, inflammatory and autoimmune disorders, and neurological disorders (Manning et al., 2017). In mammalian genomes, there are three Akt /PKB isoforms: Akt1 (PKBa), Akt2 (PKBb), and Akt 3(PKBg) (Cantley, 2004–2005). All tissues express one or more Akt isoforms, with Akt1 being the most widely expressed, Akt2 being enriched insulin-responsive metabolic tissues, and Akt3 in the brain (Dummler and Hemmings, 2007).

The Akt is a major downstream target of the phosphoinositide 3-kinase (PI3K). Activation of PI3K by extracellular stimuli results in activation of Akt. The phosphorylation of PI3K results in the lipid second messenger 3,4,5-P3 (PIP3), which binds to Akt and causes Akt to be recruited to the membrane. It also binds to phosphoinositide-dependent kinase 1 (PDK1), which phosphorylates Akt in the kinase domain (Thr 308 in Akt). PDK2 is necessary to phosphorylate Akt's carboxyl-terminal regulatory domain (Ser 473 in Akt) for Akt to be fully activated.

The fully activated Akt moves to the cytoplasm and nucleus, where it phosphorylates, activates, or inhibits many downstream molecular targets to regulate the

cell functions (Kitagishi et al., 2012). Akt promotes cell survival by inhibition of proapoptotic Bcl2 family proteins, including BAD (Datta, 1997). Akt phosphorylates the transcription factors that promote the expression of cell death genes including Forkhead family transcription factors—phosphorylated Foxo exports to the cytoplasm where they are degraded by the ubiquitin-proteasome pathway (Greer, 2008). IκB kinase α (IKKα) can be phosphorylated and activated by AKT, leading to IκB degradation and NF-κB nuclear translocation which promotes the expression of caspase inhibitors have been identified although XIAP (X-linked mammalian inhibitor of apoptosis protein) and pro-survival Bcl-xL (Bui, 2001; Manning, 2007). AKT indirectly suppresses p53-mediated apoptosis through phosphorylation of MDM2 and this promotes translocation of MDM2 to the nucleus, where it inhibits p53 function (Manning, 2007).

B-cell lymphoma-2 proteins (BCL-2)

B-cell lymphoma-2 (Bcl-2), encoded in humans by the BCL2 gene, is a founding member of the Bcl-2 family that regulates programmed cell death. Some members of the family (such as BCL-2 and BCL-XL) inhibit apoptosis, whereas others such as (BAX and BAK) promote cell death (Tsujimoto, 1998). Anti-apoptotic Bcl-2 was classified as an oncogene involved in human follicular lymphoma of B cell origin (Tsujimoto et al., 1985). Bcl-2 was shown to prevent apoptosis induced by various inducements, including heat shock, and chemotherapeutic drugs (Tsujimoto, 1998). The apoptotic stimuli activate cysteine proteases, called caspases, which cleave essential cellular proteins, leading to apoptosis (Tsujimoto, 1998). Bcl-2 prevents cell death by preventing the activation of caspases. Bcl-2 prevents the activation of caspase by two independent mechanisms (Tsujimoto, 1998). Bcl-2 is localized in the mitochondrial membrane, the endoplasmic

reticulum membrane, and the nuclear envelope. Bcl-2 in the mitochondria prevents the release of apoptogenic factors, such as cytochrome c and apoptosis-inducing factor (AIF) from the mitochondrial inter-membrane space into the cytoplasm, subsequent caspase activation and apoptosis (Ts, 1998; Liu, 1998; Liu et al., 1996). Another mechanism by which Bcl-2 prevents the activation of caspases is through its ability to sequester pro-caspases and prevent their activation (Tsujimoto, 1998). In addition to the role of BCL-2 in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins.BCL-2 regulates mitochondrial dynamics and is involved in the regulation of mitochondrial fusion and fission (Kataoka, 2022). The maintenance of the mitochondrial dynamic and bioenergetics is important to cells. Studies demonstrate that Overexpression of BCL2 contributes to many cancer antiapoptotic effects and BCL-2 proteins are fundamental in controlling carcinogenesis and treatment (Lucantoni et al., 2021).

Caspase 3 (CASP3)

The protein encoded by this gene is a cysteine-aspartic acid protease (Caspase-3) that is known for its enzymatic function at the end of the intrinsic apoptotic cascade to mediate the cleavage of specific target proteins. Two classes of caspases are involved in the process of apoptosis, initiator (2, 8, 9, and 10) and executioner caspases (caspase 3, 6, and 7) (Ponder et al., 2019). Apoptotic stimuli trigger the activation of the initiator caspase-8 or caspase-9, which then cleaves and thereby activates the effector caspases-3 which ultimately leads to cleaved proteins within the cell as cell cycle proteins, and DNase proteins, leading to apoptosis. (Galluzzi et al., 2016). However, accumulating evidence indicates that caspase-3 also plays a role in regulating the growth and homeostatic

maintenance of both normal and malignant cells and tissues in multicellular organisms (Ebrahim et al., 2020).

Studies have shown that many anticancer therapies can suppress cell growth by activating caspase (Liu et al., 2015). However, a recent study shows that caspase-3 can endorse carcinogenesis after cellular exposure to chemicals and radiation (Liu et al., 2015) as well as regulate colon cancer cell migration, invasion, and metastasis (Zhou et al., 2018).

Transcriptional Regulation of Cell Cycle -Related Genes

Cyclin Dependent Kinase 4 (CDK4)

The protein encoded by this gene is a member of the Ser/Thr protein kinase family. This protein is important for cell cycle G1 phase progression when the cell prepares to initiate DNA synthesis (Baker et al., 2022). In many cells, CDK4 is associated and activated by members of the cyclin-cyclin-dependent kinase CYCLIN D family (D1, D2, and D3) and mediates cell progression. The retinoblastoma (RB) protein is hyperphosphorylated in response to CDK4/CYCLIN D activation. By interacting with and sequestering several cellular proteins, the retinoblastoma (RB) protein regulates the cell cycle. When RB protein is phosphorylated, the transcription factor E2F protein factors are released (Sherr et al., 1999). The transcription factor E2F is a crucial binding protein that positively activates the transcription of genes whose products are necessary for the progression of S-phase (Classon et al., 2002). At least two mechanisms exist for inhibiting a fully active CDK/CYCLIN complex through two families of cyclin kinase inhibitors (CKIs), the INK4 and CIP/KIP families of proteins can negatively regulate CYCLIN/CDK complexes, or regulatory kinases can phosphorylate the CDK subunit at inhibitory sites (Gil et al., 2006). CYCLIN D expression is stimulated by growth factors, and if these

factors are removed, CYCLIN D levels drop immediately regardless of the stage of the cell cycle (Malumbres & Barbacid, 2009). Activation of CDK4 represents the pushing force of tumorigenesis in several cancer types. Small molecule inhibitors of CDK4 have been used in the treatment of hormone receptor-positive breast cancers and are in clinical trials for other cancer types (Fassl et al., 2022).

Retinoblastoma (RB)

The retinoblastoma protein (RB) is the product of the RB1 gene which is a tumor-suppressor gene involved in hereditary and sporadic retinoblastoma pathogenesis, and it was the first tumor suppressor to be discovered (Sherr et al., 2002). The RB protein has a negative regulatory effect on cell cycle proliferation and tumor progression. Activation of CYCLIN/CDK complexes contributes to hyperphosphorylation of the retinoblastoma (RB) protein. Hyperphosphorylation of the retinoblastoma (RB) results in a release of Rb from its binding proteins such as the transcription factor E2F-1, which activates the transcription of genes whose products are required for S-phase progression (Classon et al., 2002). Hypo-phosphorylated RB protein inhibits gene transcription by directly binding to the transactivation domain of E2F and by binding to the promoter of these genes as a complex with E2F (Sherr et al., 1999). Also, RB represses gene transcription by remodeling chromatin structure through interaction with proteins such as hBRM, BRG1, HDAC1, and SUV39H1, which are involved in nucleosome remodeling, histone acetylation/deacetylation and methylation, respectively (Giacinti et al., 2006). Loss of RB functions may induce cell cycle deregulation and so lead to a malignant phenotype (Giacinti et al., 2006).

Cyclin-dependent Kinase Inhibitor (CDKN1A)

This gene encodes a potent cyclin-dependent kinase inhibitor p21/WAF1/CIP1/CDKN1A and was the first discovered transcriptional target of p53 (El-Deiry et al., 1994). It is one of the essential proteins that regulate the cell cycle. P21 plays a role in several processes, including the cellular response to DNA damage, cell growth, invasion, metastasis, apoptosis, and senescence. It has been demonstrated that P21, depending on cellular location and posttranslational modifications, can either have an oncogenic or tumor-suppressive effect (Kreis, et al., 2019). It was shown that p21 induces tumor growth suppression through wild-type p53 activity (El-Deiry W.S., et al., 1993). P53 binds to domains in the p21/CDNKN1A and activates its transcription (El-Deiry et al., 1994). The tumor suppressor p21 controls Rb phosphorylation. The hypophosphorylated Rb can be changed to hyperphosphorylation Rb through the activation of kinase activity of CYCLIN/CDK complexes. These kinases can be inhibited by p21 (Sherr et al., 1995). Thus, p21 prevents phosphorylation of retinoblastoma proteins and stimulates Rb-E2F complex formation. The release of E2F transcription factors is important for S-phase progression (Engeland et al., 2022). Also, there is an immediate interaction between p21 and the proliferating cell nuclear antigen (PCNA) which inhibits the PCNA function in DNA replication but not in DNA repair (Xiong et al., 1993). Through regulating the function of cyclin-dependent kinase enzymes (CDKs) or interacting with proliferating cell nuclear antigen (PCNA) by p21 that employs a significant regulatory effect on both G1/S and G2/M checkpoints (Engeland et al., 2022). Although p21 is considered a tumor suppressor, it can also exhibit oncogenic properties in certain conditions. The location of p21 and the status of the p53 protein determine the contentious aspects of p21. Therefore,

depending on whether it is found in the cytoplasm or the nucleus, p21 may either be an oncogenic protein or a tumor suppressor (Kreis et al., 2019). Mechanisms (such as phosphorylation) that enable p21 to change location from the nucleus to the cytoplasm, where it can suppress apoptosis, are the basis of these actions (Zhou et al., 2001), Another example is offered by p53-deficient cell model systems where it has been demonstrated that a certain group of cells may have the capacity to avoid senescence following the protracted expression of p21, resuming proliferation at the expense of DNA replication stress (Ćmielová & Řezáčová, 2011). Studies on cancer cells revealed that p21 is phosphorylated by AKT in the nucleus, where it then translocates to the cytoplasm where it inhibits apoptosis (Li et al., 2002). Additionally, it was discovered that cytoplasmic p21 inhibits caspase 3 and the apoptotic kinases ASK1 and JNK by binding to and inhibiting them (Asada et al., 1999). In agreement with these results, increased cytoplasmic p21 was also associated with poor survival in breast cancer patients and with cisplatin resistance in human ovarian cancer (Abukhdeir, 2008 and Xia et al., 2011).

Post-transcriptional Regulation of Gene Expression

While much of this differential gene expression is achieved at the level of gene-specific transcription, numerous post-transcriptional events also contribute to the cell-specific expression patterns, which determine the function (Dassi, 2017). Following the mRNA's transcription but before translation, post-transcriptional regulation takes place. This control can take place at the mRNA processing level, during cytoplasmic transit from the nucleus, or during binding to ribosomes. The mRNA's stability in the cytoplasm is another type of post-transcriptional control. Proteins known as RNA-binding proteins (RBPs) can bind to untranslated regions (UTRs). Depending on which RBPs bind to these

areas, the stability of an RNA molecule may be increased or decreased. RNA molecules can also be bound by microRNAs, or miRNAs which play an important role in mRNA's stability.

Mitochondrial Ribosomal RNA (rRNA) Methyltransferase (RNMTL1)

Mitochondrial Ribosomal RNA (mt-rRNA) Methyltransferase (RNMTL1) is a member of the RNA methyltransferase family that may be responsible for 2′-O-ribose modifications and participate in methylation of the 16 S rRNA core of the large mitochondrial ribosome (mitoribosomes) subunit (Lee et al., 2013 and Lopez Sanchez, et al., 2020). Mammalian cells contain a mitochondrial genome (mitochondrial DNA; mtDNA) to encode 13 mRNAs for oxidative phosphorylation proteins that are translated on mitochondrial ribosomes using a set of 22 tRNAs (Lopez, 2020). The mitochondrial small and large ribosomal subunits contain 12 S and 16 S rRNAs encoded by mtDNA. All organisms' ribosomal RNA (rRNA) is subject to post-transcriptional modifications that broaden the range of its functionalities and composition and can control the structure, stability, and translation efficiency of the mRNAs encoded by the mitochondrial genome. (Decatur & Fournier, 2002).

Mitoribosome biogenesis and/or mitochondrial translation are frequently inhibited when mt-rRNA modifiers such as RNMTL1 are inactivated (Garone et al., 2017). Patients with deficiencies in oxidative phosphorylation have been found to have defects in mitochondrial translation as a result of issues with post-transcriptional mitochondrial (mt) RNA. The most commonly affected tissues are the heart and central nervous system; leukoencephalopathy or hypertrophic cardiomyopathy are the most common presentations

in these patients. Developmental delay, ataxia, seizures, spasticity, and peripheral neuropathy are examples of neurological symptoms (Garone et al., 2017).

MicroRNAs (miRNAs)

MiRNAs are small non-coding RNAs (miRNAs) that have an average length of 22 nucleotides. MiRNAs are synthesized from DNA sequences into precursor miRNAs (pre-miRNAs) and processed into mature miRNAs (Ha et al., 2014). RNA polymerase II/III transcripts are processed post- or co-transcriptionally to start the production of miRNAs (Ha et al., 2014). About half of all miRNAs that have been found to date are intragenic, mainly derived from protein-coding genes' introns and a few exons (Ha et al., 2014). The remaining miRNAs are intergenic and transcribed independently of a host gene, these miRNAs regulate their transcription, by their promoter. On rare occasions, a cluster of miRNAs can be produced by transcription. miRNAs are viewed as a family in this situation (Tanzer et al., 2004).

Most of the time, miRNAs block the expression and mRNA deadenylation and decapping of target mRNAs by binding to their 3′ untranslated region (3′ UTR) (Ha et al., 2014 and Huntzinger & Izaurralde, 2011). But it's also been discovered that miRNAs interreact with the 5′untranslated region (5′ UTR), coding sequences, and gene promoters (Broughton et al., 2016). The binding of miRNAs to 5′ UTR and coding regions have silencing effects on gene expression (Forman et al., 2008). However, miRNA interaction with promoter region has been reported to induce transcription (Dharap et al., 2013). MiRNAs have a role in several biological processes and are essential for optimal development (Fu et al., 2013). A variety of human disorders are linked to miRNAs' irregular expression such as lung cancer, gastric cancer, and breast cancer (Tufekci et al.,

2014). MiRNAs discharge extracellular fluids. Extracellular miRNAs are signaling molecules that facilitate cell-cell contact, and they have been reported as potential biomarkers for several illnesses (Huang et al., 2017).

microRNA let-7c (let-7c)

miRNA hsa-let-7 was first identified in Caenorhabditis elegans, where it regulates stem cell division and differentiation. miRNA hsa-let-7 and its family members have since been discovered to have crucial roles in tumor suppression. Several mechanisms explain how miRNA let-7c functions as a cancer suppressor, including preventing the expression of early cancer progression (Park et al., 2007), inhibiting the migration and invasion of human non-small cell lung cancer and colorectal cancer (Han et al., 2012) and inducing cell apoptosis and cell cycle irregularity in human hepatocellular carcinoma cells (Zhao et al., 2014). Let-7c expression is decreased in breast cancer patients' tissues and serum, (Li et al., 2015). Higher levels of let-7c expression have been linked to a better clinical prognosis for patients with estrogen receptor-positive breast cancer (Sun et al., 2016).

microRNA 15a (MIR15A)

Recently, miR-15a-5p has been linked to a variety of cancers. MiR-15a serves as a tumor suppressor in lung cancer, melanoma chronic myeloid leukemia (CML), and endometrial cancer (Wang et al., 2021) by limiting cell proliferation and causing cell death (Wu et al., 2019, Handa et al., 2019, Veronese et al., 2015). According to a recent study, miR-15a expression was downregulated in colon tumor tissues and cell lines by targeting the G1/S-specific cyclin-D1 (CCND1), miR-15a-5p prevented colon cancer from proliferating, migrating, and invading the body (Li et al., 2021).

Translational Regulation of Gene Expression

Eukaryotic Translation Initiation Factor 2-alpha Kinase 1, EIF2α Kinase Heme-Regulated Inhibitor (EIF2AK1/HRI)

Eukaryotic translation initiation factor 2-alpha kinase 1 (EIF2AK1), also known as EIF2α kinase heme-regulated inhibitor (HRI) is a kinase enzyme that phosphorylates EIF2α in response to various cell stressors, resulting in attenuation of mRNA translation and inhibits protein synthesis. Although EIF2AK1 (HRI) is a kinase that can be inactivated by hemin and is activated by heme deficiency, EIF2AK1 is best known for its role in coupling heme availability to globin synthesis in early erythroid cells (Chen, 2014). A study found that EIF2AK1 (HRI) is downregulated in most ovarian cancers compared with normal ovarian tissues (Hwang et al., 2000). It has been shown that pharmacologic activation of HRI exhibits anti-cancer activity against a variety of tumors, including multiple myeloma, plasma cell neoplasm, and specific subtypes of breast cancer (Burwick et al., 2017). Bortezomib is an example of a chemotherapy drug used clinically that induces eIF2α phosphorylation and has also been found to increase apoptosis and defeat drug resistance in cancer cells (Chauhan et al., 2011). Also, in neuronal cells, EIF2AK1 (HRI) has been found to mediate the translation of GluN2B, a subunit for the N-methyl-D-aspartate (NMDA) receptor and mediated translation of β-site APP cleaving enzyme-1 (BACE1) may have a role in memory association (Storey, 2011). The study has shown that Inhibition of HRI in mice impairs memory recovery (Ramos-Fernandez et al., 2016).

Haloperidol and AKT/PKB Insulin Signaling Pathway

Haloperidol (HAL1; 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol) belongs to the butyrophenones class. Haloperidol may generate ROS by more

than one mechanism. Studies have shown that haloperidol exerts oxidative stress through its metabolites. Haloperidol is known to be extensively metabolized with only about 1% of the administered dose excreted unchanged in urine (Forsman et al., 1977). Haloperidol metabolized ed via N-dealkylation by cytochrome P450 in the liver with the preferential involvement of CYP3A4 and CYP2D6 with a small contribution of CYP1A2 (Forsman et al., 1977). The metabolic pathway leads to the formation of HPP+ (the 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl] is structurally similar with MPP+ (1-methyl-4-phenylpyridinium), dopaminergic neurotoxic. MPP+ is a toxic metabolite of N-methyl-4-phenyl-l,2,3,6-tetrahydropyridine (MPTP), a contaminant produced during the manufacture of the synthetic opioid MPPP (1-methyl-4-phenyl-4-propionoxypiperidine). MPP+ is ultimately responsible for an irreversible neurodegenerative condition like Parkinson's disease (Rollema et al.,1994). Many studies have reported several side effects among patients treated with haloperidol and a positive relationship between the severity of these side effects and peripheral blood levels of HPP+ Ulrich et al., 1998). Studies have verified that HPP+ is highly toxic and increases oxidative stress-inducing plasma membrane damage (Murata, 2007). It has been known that Haloperidol applied its clinical effect by antagonizing dopamine D2 receptors. The effect of haloperidol, a D2 receptor antagonist, on AKT pathway activity has also been investigated. However, findings on the relationship between Haloperidol and the AKT pathway are contradictory. Activation of D2Rs facilitates the formation of the (PKB or Akt) complex along with the phosphatase PP2A, resulting in dephosphorylation of Akt (inactivation), followed by dephosphorylation (activation) of GSK3 (Beaulieu et al., 2007). Studies have shown that haloperidol, an antagonist to dopamine D2 receptors, can activate the AKT pathway. In the rat frontal

cortex, haloperidol up-regulated Ser473 Akt phosphorylation (Roh et al., 2007) and decreased activity of phosphatase 2A (PP2A) (Kim et al., 2008). In the striatal neurons of mice, haloperidol was able to significantly increase activation of the Akt-mTORC1 pathway (Bowling et al., 2014). Haloperidol increases the phosphorylation level of Akt and GSK-3β in SH-SY5Y neuroblastoma cells (Deslauriers et al., 2014) and increases the levels of phospho-Ser9-GSK-3β in mice (Emamian et al., 2004). On the contrary, studies reported a significant decrease in the phospho-Akt (Ser473) in the brain cortex of rats treated with haloperidol (Ukai, 2004 and Ibarra-Lecue et al., 2020) which is consistent with another study that has been linked haloperidol with inhibition of pro-survival Akt signaling and induces cytotoxicity in pheochromocytoma (PC12) and neuronal cell cultures (Dai et al., 2007). It has also been revealed that treatment of haloperidol suppresses the phosphorylation of Akt Thr308 and Ser473, as well as GSK-3 beta in rat cortical neuronal cell cultures (Takaki et al., 2018). These contradictory results made it necessary for this investigation to examine the impact of haloperidol on the AKT/PKB insulin signaling pathway, evaluate a low haloperidol dose, and consider a constrained number of time points. Additionally, it was necessary to investigate the regulators of the AKT/PKB insulin signaling pathway in response to haloperidol to see if this route would be involved in the paradoxical response to haloperidol.

CHAPTER 3

DESIGN OF THE STUDY

Cell Culture of BEAS-2B Cells

BEAS-2B cells were purchased from the American Type Culture Collection (ATCC® CRL-9609, Manassas, VA). The cell was cultured in 75 cm^2 Corning™ Cell Culture Treated Flasks (VWR International, Radnor, PA) with a 5% CO2 humidified incubator. The media used for the culture was Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 1% GlutaMAX, and 1% Penicillin/Streptomycin (Life Technologies, Carlsbad, CA). At 80% confluence, the cells were trypsinized using Gibco™ Trypsin-EDTA (0.05%) (Life Technologies, Carlsbad, CA), and cells were seeded in twenty-one Corning™ Tissue-Culture Treated Culture Dishes (60 x 15 mm) (VWR International, Radnor, PA). The cells were treated with drugs, and the controls (untreated cells).

Treatments of Cells

BEAS-2B cells were seeded in the tissue-culture dishes for 24 hours. Dishes were divided into three groups. Each group had five dishes that were treated in the following order: Two culture dishes were untreated controls that were incubated for 24 h and 48 h. Five culture dishes were treated with 3.5µM of haloperidol. After the treatments cells were incubated in a 5% CO2 humidified incubator for 24 h, 48 h, and 72 h, untreated cells were

incubated for 24 h, 48 h. The tissue-culture dishes were used in proteins, and RNA was isolated from cells seeded in the tissue-culture dishes.

Cell Proliferation

Rapid BEAS-2B cell proliferation in each tissue-culture dish was visualized and validated under the Eclipse Ti-E Inverted Nikon Microscope, (Nikon Instruments Inc., Melville, NY).

Proteins Isolation

The extraction of protein from a set of seeded BEAS-2B cells was conducted by using the Radio-Immunoprecipitation Assay (RIPA) reagent (Cell Signaling, Danvers, MA). According to the manufacturer's instructions. 1X RIPA lysis buffer supplemented with 1 mM phenyl-methane-sulfonyl fluoride (PMSF), protease inhibitor (1:100), and protease I phosphate inhibitor (1:100) (Cell Signaling, Beverly, MA), were added to the cells in each tissue-culture dish then Cells in each tissue-culture dish were scraped off and transfer the contain into an individual 1.5 mL microcentrifuge tube. Following 45 minutes of ice treatment, the lysed cells in the 1.5 mL microcentrifuge tubes were centrifuged for 20 minutes at 14,000 revolutions per minute (rpm) in a Precision, Winchester, VA, 4°C microcentrifuge. After 20 min of centrifugation, the aqueous proteins were removed from the individual 1.5 mL microcentrifuge tube and moved into the new individual 1.5 mL microcentrifuge tube stored at -80°C.

Total RNA Isolation

The isolation of total RNA from a set of seeded BEAS-2B cells was performed by using TRIzol™ reagent (Thermofisher™, Waltham, MA). According to the manufacturer's

instruction, 1 mL TRIzol™ reagent (Thermofisher™, Waltham, MA) was added to the cells in each tissue-culture dish to lyse the cells. The lysed cells were transferred into new individual 1.5 mL microcentrifuge tubes and gently mixed by using the vortex then 200 mL of chloroform at room temperature was added to each lysed TRIzol-cell mixture and incubated at room temperature for 5 min then centrifuged at 14,000 rpm in a 4°C microcentrifuge (Precision, Winchester, VA) for 20 min to separate the sample into chloroform and aqueous layer. The aqueous solution layer of individual TRIzol-cell mixture was removed and transferred into a new individual 1.5 mL microcentrifuge tube then 500 mL isopropanol was added to each aqueous solution layer and incubated at room temperature for 10 min to precipitate the RNA. The incubated tubes were centrifuged at 14,000 rpm in a 4°C microcentrifuge (Precision, Winchester, VA) for 20 min to participate RNA pallet to the bottom of the microcentrifuge tubes and the aqueous solution-isopropanol mixture was separated at the top of the microcentrifuge tubes. The aqueous solution was removed from the pallet. The pallet of the individual RNA sample was rinsed and centrifuged twice. The pellet contained an RNA sample in each 1.5 mL microcentrifuge tube and was air-dried at room temperature. Each RNA sample was dissolved in Nuclease-Free Water (Thermofisher™, Waltham, MA) then the RNA samples were stored at -80°C. The integrity of each sample of extracted total RNA was determined based on the expression of the ribosomal RNA bands—28S, 18S, and 5S by using a 1% TAE agarose gel stained with ethidium bromide and visualized inside Li-COR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE) using Image Studio Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE).

Optical Density 280 (OD_{280}) Reading

Optical density (OD_{280}) was used to determine the amount of the extracted proteins, and Spectronic BioMate 3 UV-V (Thermofisher™, Waltham, MA) was used to confirm the OD280 of each experimental sample protein. For the OD_{280} measurement of the extracted proteins, 5 microliters (µl) of the extracted proteins were taken from each experimental protein sample and added to 495 µl of Ultrapure™ DNase/RNase-Free Distilled Water (Thermofisher™, Waltham, MA) in individual 1.5 mL microcentrifuge tube, and each protein-distilled water mixture was mixed by tapping several times. Each protein-distilled water mixture was individually transferred into an optical density reading cuvette and the experimental protein sample OD_{280} was measured and recorded. Each experimental protein sample was aliquoted into several individual 0.6 mL microcentrifuge tubes and stored as working stock at -80°C.

Optical density (OD_{260}) Reading

Optical density (OD_{260}) was used to determine the amount of each extracted total RNA, and Spectronic BioMate 3 UV-Vis (Thermofisher™, Waltham, MA) was used to confirm the OD_{260} of the individual total RNA sample. For the (OD_{260} measurement of each extracted total RNA, 5 µl of the individual extracted total RNA was added to 495 µl of UltraPure™ DNase/RNase-Free Distilled Water (Thermofisher™, Waltham, MA) in an individual 1.5 mL microcentrifuge tube and mixed. Individual diluted RNA was individually transferred into an OD reading cuvette and was measured and recorded. Each total RNA sample was stored at -80°C.

Transient Transfection of Pre-hsa-let-7c-GFP DNA Construct into BEAS-2B Cells

To culture the BEAS-2B cells (ATCC, Manassas, VA), cells were seeded in ten of 60 x15 mm cell cultured plates (a total of 8 plates were used for the transient transfection and two plates as control), using DMEM media supplemented with, L-glutamine, 1% GlutaMAX, 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). The BEAS-2B cells were incubated for 24 hours at 37 °C in an incubator with 5% CO2. For the experimental sets, transient transfection was carried out using the pre-hsa-let-7c-GFP DNA construct; 5.0 µg of this DNA construct was treated with lipofectamine reagent (Life Technologies, Grand Island, NY) following the manufacturer's procedure. Transfection experiments of 24 h. and 48 h. were performed then 3.5µM of Haloperidol was added to transfected BEAS-2B cells for 24 h. and 48 h. Proteins were isolated from controls, transient transfected, and haloperidol-treated and transfected BEAS-2B cells at intervals of (24 h and 48 h). BEAS-2B cells were lysed using mammalian protein extraction Radio-Immunoprecipitation Assay (RIPA) reagent, RIPA (1X) lysis buffer supplemented with a phenylmethanesulfonyl fluoride (PMSF), and protease I phosphate inhibitor (1:100), protease (1:100) (Cell Signaling, Beverly, MA), according to the manufacturer's instructions.

Protein Gel Electrophoresis and Western Blot

The experimental extracted protein samples were electrophoresed in 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels using the BIORAD Mini-PROTEAN Tetra System (BIORAD, Hercules, CA) according to the BIORAD protocol. A total of 100 micrograms (µg) of the individual extracted protein sample was loaded into the individual well in 10% SDS-PAGE gel and a marker, Precision

Plus Protein™ WesternC™ Blotting Standard (BIO-RAD, Hercules, CA), was also loaded into an individual well. The 10% SDS-PAGE gel containing the experimental protein samples was run according to the BIORAD protocol. Multiple 10% SDS-PAGE gels were run, and the 10% SDS-PAGE gels were used for Coomassie blue dye staining and Western blotting experiments.

For Western Blotting: Using the Trans-Blot® TurboTM Transfer System (BIORAD, Hercules, CA) following BIORAD protocol, the proteins electrophoresed in a 10% SDS-PAGE gel were transferred into a nitrocellulose membrane. The proteins on the nitrocellulose membranes were probed based on the primary rabbit or mouse IgG, HRP-linked antibodies and the secondary Anti-rabbit or anti-mouse IgG, HRP-linked antibody. ACTB, BCl-xL, P21, CDK4, p-PI3K-p85/p55, RNMTL1, E1F2AK1, p-Rb-S780, and CASP3, proteins were probed with polyclonal rabbit or mouse IgG HRP-linked antibodies and secondary Anti-Rabbit or Anti-mouse IgG HRP-linked antibodies (Cell Signaling, Danvers, MA), according to Cell Singling's protocol. Proteins were probed with a primary polyclonal rabbit AKT 1/2/3 IgG, HRP-linked antibodies, and a secondary Goat Anti-Rabbit IgG H&L (HRP) antibody (Abcam, Cambridge, MA), according to Abcam protocol.

For the detection and visualization of the proteins on the antibody-treated nitrocellulose membranes, the antibody-treated nitrocellulose membranes were treated with Clarity™ and Clarity Max™ Western (ECL) Blotting Substrates (BIO-RAD, Hercules, CA), according to BIO-RAD's protocol. The Clarity™ and Clarity Max™ Western (ECL) Blotting Substrates (BIO-RAD, Hercules, CA) were used to generate a chemiluminescent detection mechanism that detects and makes protein bands visible inside

a chemiluminescence imaging system. The substrate-treated nitrocellulose membranes were imaged in the Li-COR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE) under chemiluminescence imaging, and the protein bands were detected, and using trimmed intensity signal in Image Studio Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE), the expression of the protein bands was quantified. Five percent of the pixels with the highest and lowest pixel intensities are removed from the total pixel intensity signals in Image Studio Lite Version 5.2's trimmed intensity signal.

Complementary DNA (cDNA) Synthesis

The individual extracted total RNA was used to synthesize complementary DNA (cDNA) using SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. A single 0.2 mL microcentrifuge tube was used to hold 5 micrograms (µg) of extracted total RNA for cDNA synthesis. The total RNA was then treated with DNAse and DNAse I 10X Buffer and incubated for 1 hour at 37 °C in a BIO-RAD C1000 TouchTM Thermal Cycler (Hercules, CA). Following a 1-hour DNase I treatment, the DNase reaction of each total RNA sample was inactivated following the manufacturer's protocol. The cDNA inside the C1000 TouchTM Thermal Cycler (BIO-RAD, Hercules, CA) was synthesized using both oligo (dT) and random hexamer primers in the SuperScriptTM III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol.

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Polymerase chain reaction (PCR) was used to investigate the RNA expression in haloperidol-treated and untreated BEAS-2B cells both, miRNAs, hsa-let-7c, and hsa-miR-

15a primer sets were used, and to create the hsa-let-7c overexpression construct. The sequences of the primer sets are:

- hsa-let-7c primer set (Forward Primer: 5'-GTTGTATGGTTTAGAGTTACAC-3'
- and Reverse Primer: 5'-GCTCCAAGGAAAGCTAGAAGGTT-3').
- miR-15a (Forward Primer: 5'-CCTTGGAGTAAAGTAGCAGCACA-3' and Reverse Primer: 5'-ACAATATGGCCTGCACCTTTTCA-3 ').

For the PCR: A total volume of the PCR reaction mixture was 15.0 µl (contains 2.5 µl of cDNA (~12.5 ng), 7.5 µl of HotStar Taq Master Mix (Qiagen, Valencia, CA), 1.0 µl (100 ng) of forward primer, 1.0 µl (100 ng) of reverse primer (MilliporeSigma, Burlington, MA), and 3 µl of nuclease-free H_2O (Thermo Fisher Scientific, Waltham, MA)). The PCR reactions were placed and run inside a C1000 Touch™ Thermal Cycler (BIORAD, Hercules, CA). The PCR condition is as follows: 95°C for 15 min, 94°C for 45 sec, 60°C or 55°C for 30 sec, 72°C for 60 sec, 45 cycles, and 72°C for 10 minutes.

For gel electrophoresis: A 5.0 µl of the amplified PCR products of the individual experimental sample was mixed with DNA loading dye and electrophoresed on a 2.5% agarose gel stained with ethidium bromide. Each stained agarose gel was placed inside the Li-COR Odyssey® Fc imager (LI-COR Biosciences, Lincoln, NE) to visualize the amplified DNA bands and to capture the gel image photos. The amplified DNA bands were quantified in Image Studio Lite Version 5.2 (LI-COR Biosciences, Lincoln, NE) using a trimmed intensity signal. The trimmed intensity signal in Image Studio Lite Version 5.2 excludes five percent of pixels with the highest and lowest pixel intensity from the total pixel intensity signals.

Quantification and Statistical Analysis

In this study, the graphs were generated using Microsoft Excel for clear visualization of experimental data. The quantitative analysis of Western blots and semi-quantitative PCR results was conducted using Licor Image Studio software 5.2. This involved measuring the density of the bands multiple times to ensure the reliability and consistency of the measurements. One-way ANOVA was conducted for multiple comparisons between different experimental groups Subsequently, Tukey's tests were utilized as post hoc analysis to further comparison between specific groups. The data presented as mean ± standard error of the mean (SEM) and statistical significance, where *$p < 0.05$**$p < 0.001$, ***$p < 0.0001$. This quantification and statistical analysis strategy ensures the reliability and validity of the experimental results, allowing for meaningful interpretations and conclusions.

CHAPTER 4

RESULTS AND DISCUSSION

Haloperidol Promotes Survival and Proliferation of BEAS-2B Cells

Haloperidol Increases Cell Proliferation

In this study, to determine the effect of haloperidol on cell proliferation we assess the impact of haloperidol on cell viability BEAS-2B cell. The BEAS-2B cell line was established originally via the immortalization of a human non-tumorigenic lung epithelial cell line using an adenovirus12-SV40 hybrid virus (Reddel et al.,1988). We observed that the cells were exposed to haloperidol (3.5 µM) for 24 h. showed no morphological features of cells undergoing program cell death but lost their epithelial morphology in favor of smaller elongated and needle-shaped cells. Also, in comparison to the control, cells that were treated with haloperidol for 24 hr. showed no increase in cell proliferation based on cell confluence compared to 24 h control, suggesting the cells may likely have been in a quiescence state (Figure 2 [A, B]). Haloperidol-treated cells for 48 h. started to become disorganized and the changes in cell shape became evident. Also, at 48 h the confluence of haloperidol-treated cells increases compared to 48 h. control (Figure 2 [C D]), suggesting proliferation and averting quiescence.

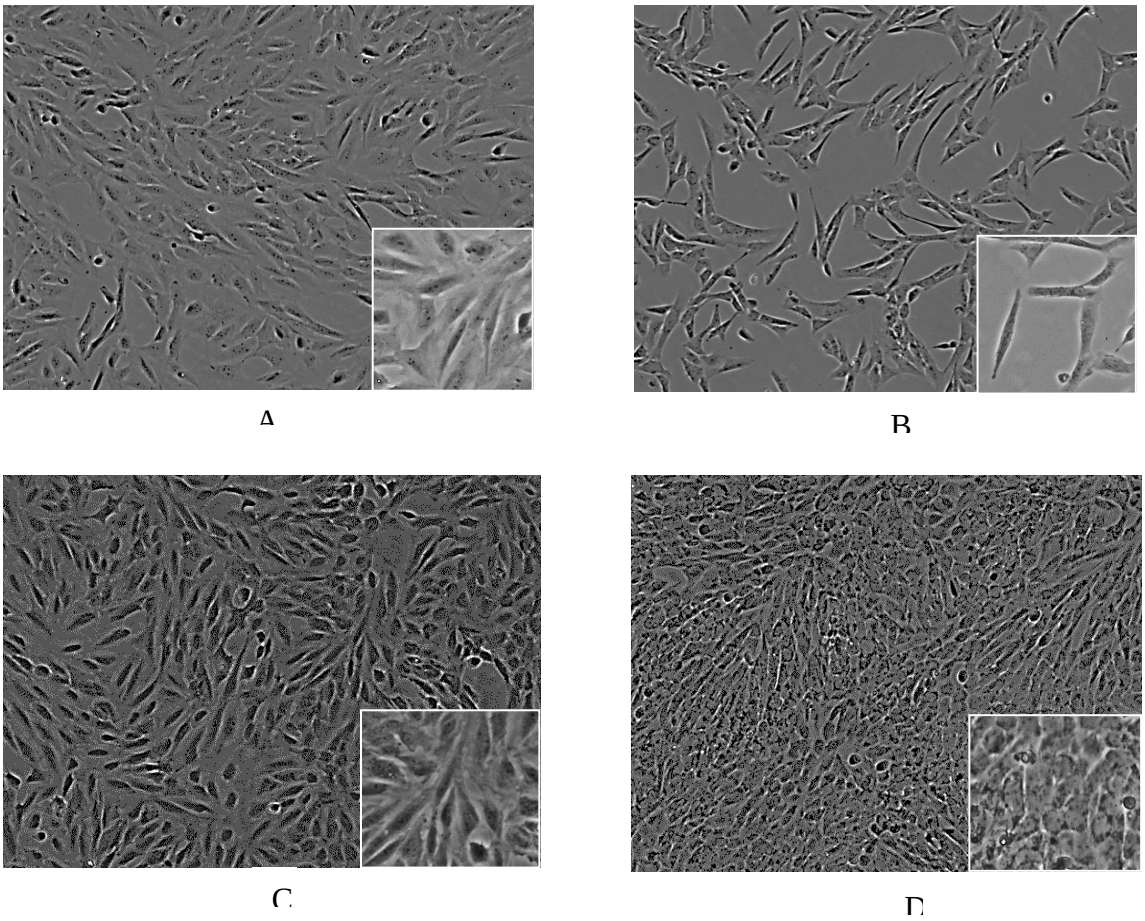


Figure 2: BEAS-2B Cells Proliferation Differences Between Controls and Haloperidol-treated Cells

The A, B, C, and D, in the figure delineate the labeling of the experimental samples in the following (A) Control 24 h (untreated); (B) Control 48 h (untreated); (C) Haloperidol treated (HAL: 3.5 µM) 24 h; and (D) Haloperidol treated (HAL: 3.5 µM) 48 h. An inverted microscope at the 10X phase contrast was used to observe the cells.

Haloperidol promotes BEAS-2B cell Survival through activation of AKT and Bcl-xL

To characterize the effects of haloperidol on the survival pathway Western blots analysis has been used to determine the expression of Akt and Bcl-xL and examine potential pathways that contribute to cell survival (Figure 3A). Akt is an important factor for cell survival as an activator of many factors involved in cell survival. Akt increases the transcription of anti-apoptotic genes in the nucleus while suppressing transcription factors that support the expression of genes linked to cell death. Bcl-xL is anti-apoptotic and a survival pathway regulator that prevents the release of mitochondrial contents such as cytochrome c, which leads to caspase activation and ultimately, programmed cell death (Michels et al., 2013). In this study, following exposure to (3.5 µM) haloperidol, the expression of Akt was upregulated at 24 h and significantly at 48, and 72 h., and Bcl-xL was activated at 24, 48, and significantly at 72 h. when compared to the untreated controls (Figure 3B). The results show that haloperidol promotes survival and proliferation in BEAS-2B cells by increasing the expression of Akt and the anti-apoptotic Bcl-xL proteins.

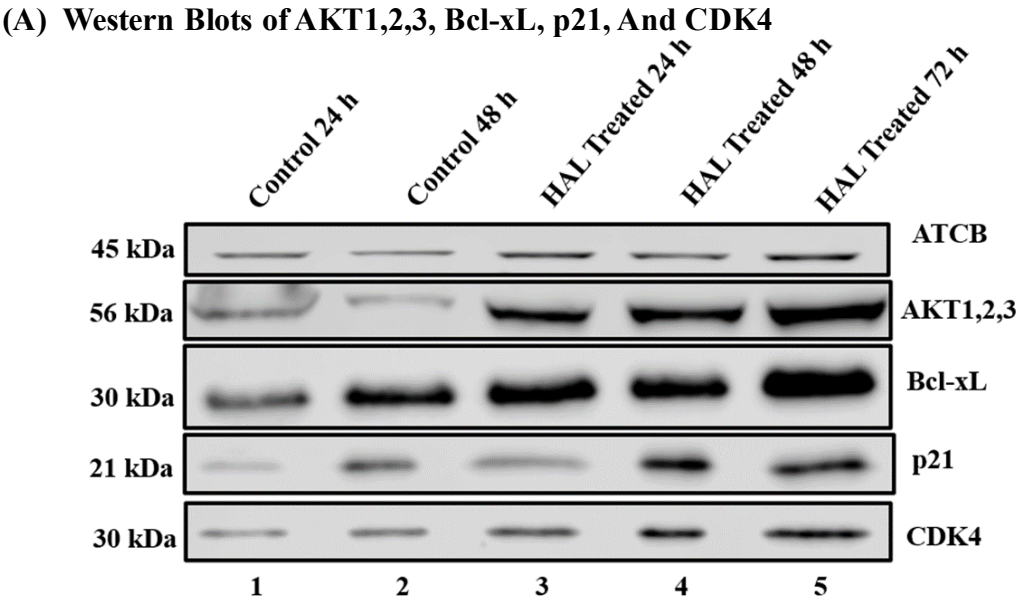
Haloperidol Induces BEAS-2B Cell Proliferation via Cell Cycle Protein Regulation

To investigate the molecular mechanism involved in the haloperidol-induced proliferation of BEAS-2B cells, the expression levels of cell cycle proteins were examined specifically AKT, cyclin-dependent kinase inhibitor (P21), and cyclin-dependent kinase 4 (CDK4). Western blot analysis was performed to determine the level of protein in BEAS-2B cells treated with (3.5 µM) haloperidol compared to untreated controls as shown in Figure 3A. Haloperidol treatment resulted in an increase in the expression of Akt at 24, 48,

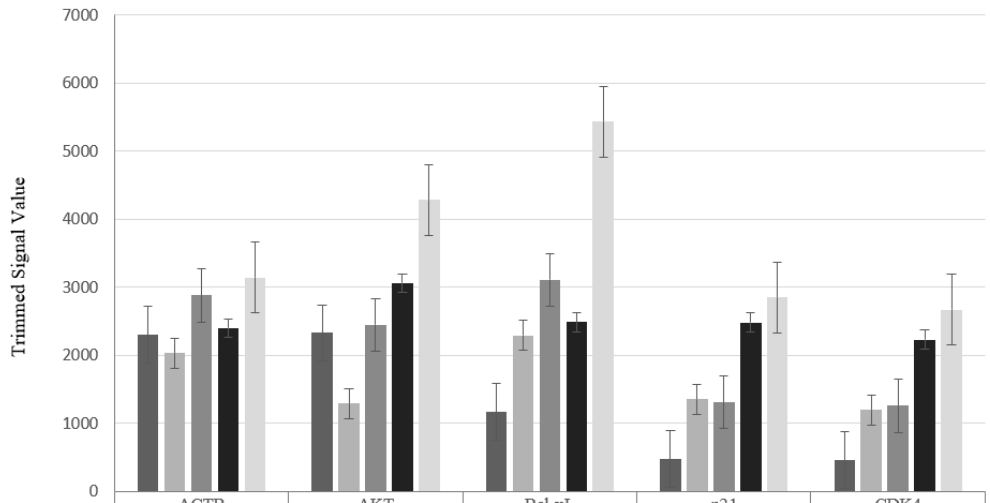
and 72 h. (Figure 3B). AKT is serine/threonine kinase, a key signaling molecule involved in promoting cell survival and proliferation. AKT exerts its effects by phosphorylating and regulating the activity of downstream target proteins involved in cell cycle progression (Li et al., 2002).

Also, the expression of CDK4 was upregulated following haloperidol (3.5 µM) treatment at 24, 48, and 72 h (Figure 3B). CDK4 is a critical regulator of the G1/S phase transition in the cell cycle. This upregulation suggests enhanced cell cycle progression and proliferation in response to haloperidol exposure. Furthermore, we observed an increase in the expression of P21/CDKN1, a cyclin-dependent kinase inhibitor, at 24, 48, and 72 h. following (3.5 µM) haloperidol treatment in comparison to the control (Figure 3B). P21/CDKN1 is known to inhibit cell cycle progression. Interestingly, P21/CDKN1 expression increased in a time-dependent manner following haloperidol treatment. Studies on cancer cells show that AKT phosphorylates p21 in the nucleus, after which it moves to the cytoplasm where it inhibits the apoptotic kinases JNK and ASK1, thereby preventing apoptosis (Asada et al., 1999; Li et al., 2002). Studies also reported that high levels of p21 were connected to poor survival rates in patients with breast cancer and cisplatin resistance in ovarian cancer in humans (Abukhdeir, 2008; Xia et al., 2011).

Overall, our findings indicate that haloperidol modulates the expression of Akt, P21/CDKN1, and CDK4, suggesting its involvement in the regulation of cell survival and cell cycle progression in BEAS-2B cells. These results provide an understanding of the molecular mechanisms underlying haloperidol's paradoxical effects and its impact on cellular homeostasis.



(B) ACTB, AKT1,2,3, Bcl-xL, p21, and CDK4 Protein Expression



	ACTB	AKT	Bcl-xL	p21	CDK4
Control 24h	2300	2330	1170	479	464
Control 48h	2030	1290	2290	1360	1200
HAL. Treated 24h	2880	2450	3110	1310	1260
HAL. Treated 48 h	2400	3060	2490	2480	2230
HAL. Treated 72 h	3140	4280	5430	2850	2670

Figure 3: **Western Blot Results of ACTB, AKT1,2,3, Bcl-xL, p21, and CDK4**

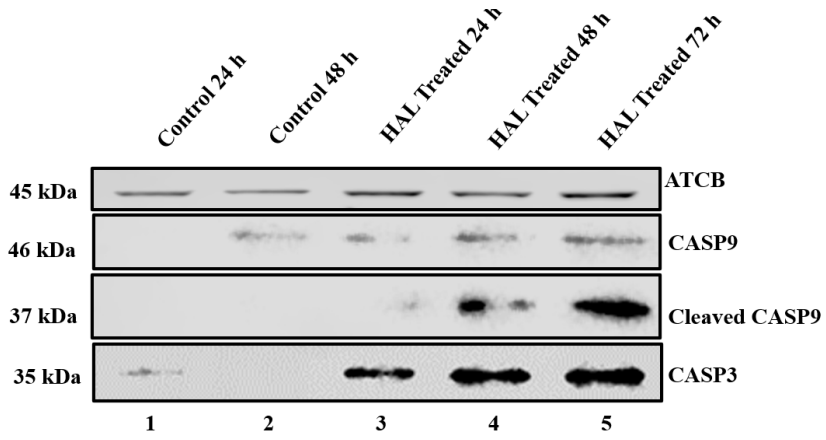
(Figure 3A), and bar graphs and a numerical table delineate the expression ACTB, AKT1,2,3, Bcl-xL, p21, and CDK4, protein in the experimental samples (Figure 3B).

Haloperidol Upregulation Expression of Caspase 3 and Caspase 9 Protein in BEAS-2B Cell

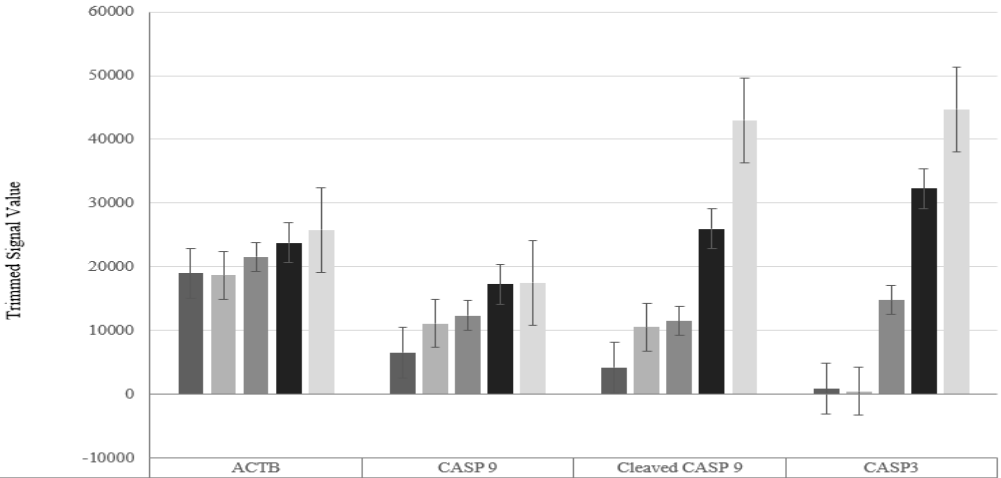
To investigate the molecular mechanism of haloperidol involved in cell survival and proliferation, Western blots analysis was conducted focusing on the expression levels of caspase 3 and caspase 9. BEAS-2B cells were treated with 3.5 μM haloperidol for 24, 48, and 72 h., and caspase protein levels were compared to untreated controls as shown in Figure 4A. The Western blot analysis revealed an increase in the expression of both caspase 9 and caspase 3 proteins in BEAS-2B cells treated with 3.5 μM haloperidol and the increase was significant at 72 h compared to untreated controls. (Figure 4B). These results revealed interesting findings, despite the activation of proliferation pathways indicated by the significant increase in the AKT expression we also noted an upregulation of caspase 3, caspase 9, and cleaved caspase 9 proteins expression following haloperidol treatment. One possible explanation for the increase in caspase 3 and caspase 9 protein expression could be a compensatory response to haloperidol-induced cellular stress. Treatment with haloperidol may initiate signaling pathways that activate caspases as a feedback mechanism to control cell division and preserve homeostasis within the cell. Another possible explanation from recent understanding is that caspases are associated with non-apoptotic pathways. While caspases 9 and 3 are classically associated with apoptosis, emerging evidence suggests their involvement in non-apoptotic processes, including cell proliferation and differentiation (Dick, 2015; Lamkanfi, 2007; Ohsawa, 2010). The upregulation of caspase 3 and caspase 9 in response to haloperidol treatment may reflect their roles in promoting cell proliferation or modulating cellular signaling pathways. The

upregulation of caspase 3 and caspase 9 alongside the protein responsible for cell proliferation following haloperidol treatment presents a paradoxical situation that requires further investigation.

(A) Western Blots of CASP9, Cleaved CASP9 And CASP3



(B) ACTB, CASP 9, Cleaved CASP 9, and CASP 3 Protein Expression



	ACTB	CASP 9	Cleaved CASP 9	CASP3
Control 24 h	19000	6590	4230	886
Control 48 h"	18700	11100	10600	476
HAL Treated 24 h	21600	12400	11500	14800
HAL Treated 48 h	23800	17300	26000	32300
HAL Treated 72 h	25800	17500	43000	44700

Figure 4: **Western Blot Results of Casp3, Casp9, and Cleaved Casp9**

(Figure 4A), and bar graphs and a numerical table delineate the expression of Casp3, Casp9, and cleaved Casp9 protein in the experimental samples (Figure 4B).

Haloperidol Down-Regulates hsa-let-7c and MiR-15a Expression

Several miRNAs have been reported to be dysregulated and associated with multiple diseases (Caputo et al., 2015). In schizophrenia, let-7c and miR-15a have been reported to be upregulated (Gururajan et al., 2016 and Miller et al., 2012). Therefore, if we assume that elevated miRNAs are accurate predictors of diseases, then the expression of let-7c and miR-15a must be regulated throughout haloperidol that is used in schizophrenia treatment. Also, hsa-let-7c and miR-15a are categorized as tumor suppressors, because they reduce cancer aggressiveness by regulating the cell cycle, and cell signaling (Gururajan et al., 2016).

In this study, the expression levels of miR-15a and let-7c in BEAS-2B cell lines were examined by semi-quantitative real-time PCR. Figure 5A illustrates a contrast agarose gel photo of PCR amplified hsa-let-7c and miR-15 cDNA products analyzed on a 2.5% TAE agarose gel stained with ethidium bromide and a bar chart based on the semi-quantification expression of PCR-amplified hsa-Let-7c and miR-15 a cDNA product (Figure 5B). The result shows the expression of let-7c and miR-15a were decreased in haloperidol-treated BEAS-2B cells at 24 h., 48 h., and 72 h. compared to untreated control. The data suggested that haloperidol down-regulates the expressions of let-7c and miR-15a which could contribute to haloperidol proliferative effect. Understanding the differential expression of specific miRNAs in response to haloperidol treatment can provide valuable comprehension of the molecular mechanisms underlying haloperidol-induced effects on cell proliferation.

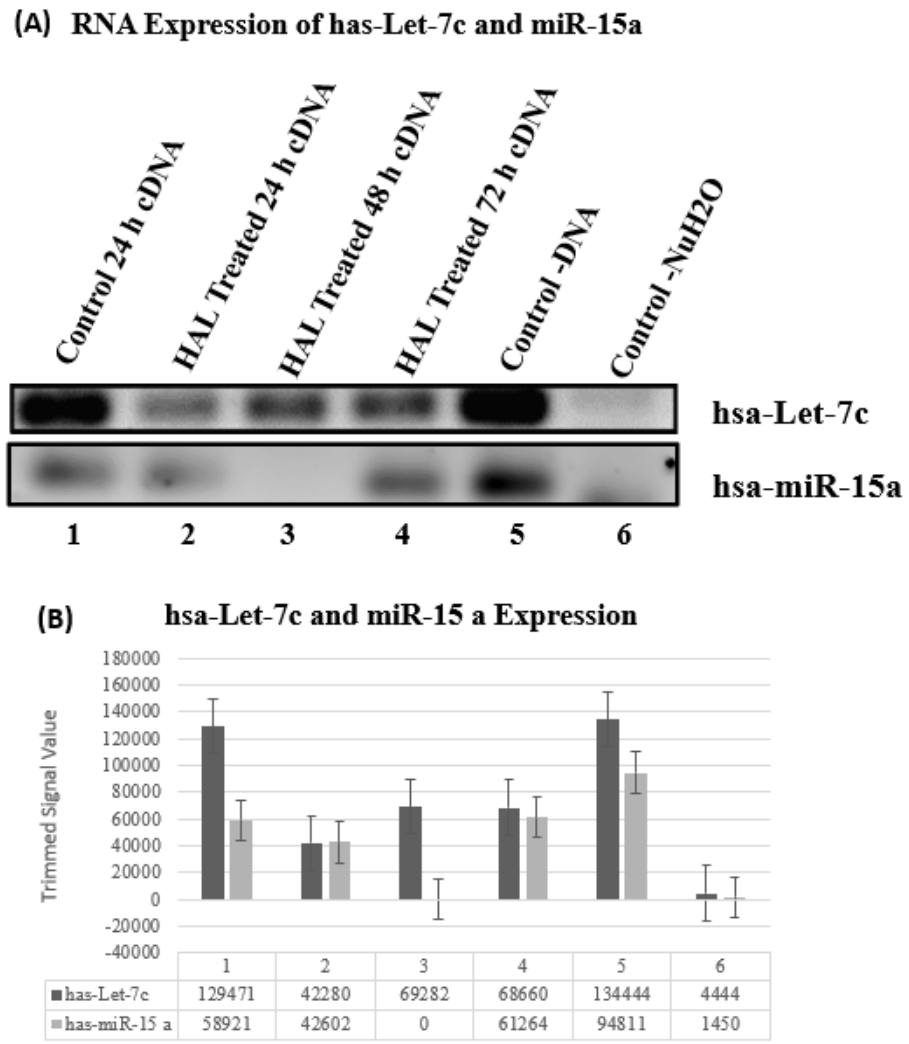


Figure 5: Semi-quantification PCR Results of miRNA Has-let-7c-5p and Has-miR-15a

(Figure 5A), and bar graphs and a numerical table delineate the expression of miRNA has-let-7c-5p and has-miR-15a in the experimental samples (Figure 5B).

Upregulation of has-let-7c Reverses Haloperidol Proliferative Effect

MicroRNAs (miRNAs) are crucial epigenetic markers that have a significant impact on medication responsiveness. MiRNAs play a role in controlling the expression of several genes involved in the drug mechanism of action and genes involved in regulating cell cycles. They can bind to the 3' untranslated region (3' UTR) of target messenger RNAs (mRNAs) and inhibit their translation or induce mRNA degradation. Studies have revealed that let-7c regulates the expression of 27 genes in the PI3K-AKT signaling pathway, which has been reported to be dysfunctional in many psychological disorders (Gururajan et al., 2016). For this reason, hsa-let-7c was chosen to investigate its role in regulating haloperidol-related genes that are involved in its proliferative effect.

Prediction of Potential Targets of has-let-7 in the Regulation of Haloperidol Target Genes Involved in the PKB/AKT Pathway and Cell Cycle Progression

To detect potential targets of hsa-let-7c involved in the regulation of haloperidol target genes within the PKB/AKT pathway and cell cycle progression, a search was conducted using the miRNA Database (miRDB) to retrieve hsa-let-7c p predicted target genes. The miRDB is an online database for miRNA target prediction and functional annotations. A bioinformatics tool called MirTarget was used to predict all targets in the miRDB. MirTarget was created by examining thousands of miRNA-target interactions from high-quantity sequencing experiments (Yuhao Chen, 2020). The following are the potential target genes of let-7c that are predicted to be involved in the regulation of the AKT pathway and cell cycle proteins:

- AKT: The gene encoding the AKT, serine/threonine kinase plays a central role in the AKT pathway by promoting cell survival and growth.
- PIK3CA: The gene encoding the p110α catalytic subunit of phosphoinositide 3-kinase (PI3K), which is upstream of AKT in the signaling pathway.
- PIK3R: The gene encoding the regulatory subunit of PI3K, which regulates PI3K activity.
- Cyclin D1: The gene encoding the Cyclin D1, cell cycle protein that regulates the G1/S transition.
- CDK4: The gene encoding cyclin-dependent kinases that form complexes with cyclin D1 and play a role in cell cycle progression.
- P21 (CDKN1A): The gene encoding cyclin-dependent kinase inhibitor that regulates cell cycle arrest.
- RB1 (Retinoblastoma): The retinoblastoma transcriptional corepressor gene plays a role in cell cycle progression.
- E2F transcription factors: E2F transcription factors play a role in regulating the expression of genes required for DNA replication and cell cycle progression.

By integrating this research finding of haloperidol's effects on the PKB/AKT and cell cycle proteins, and the prediction targets of let-7c from miRDatabase, we determined the potential genes regulated by let-7c within the AKT pathway and cell cycle progression that involves the haloperidol mechanism of action as shown in Figure 6. However, to validate these interactions and understand the functional effects of hsa-let-7c on

haloperidol-target within the AKT pathway and cell cycle progression further research experiments were conducted to investigate how hsa-let-7c influences the expression of the target genes identified, such as AKT, PIK3R, CDK4, P21, RB1 regulated by haloperidol.

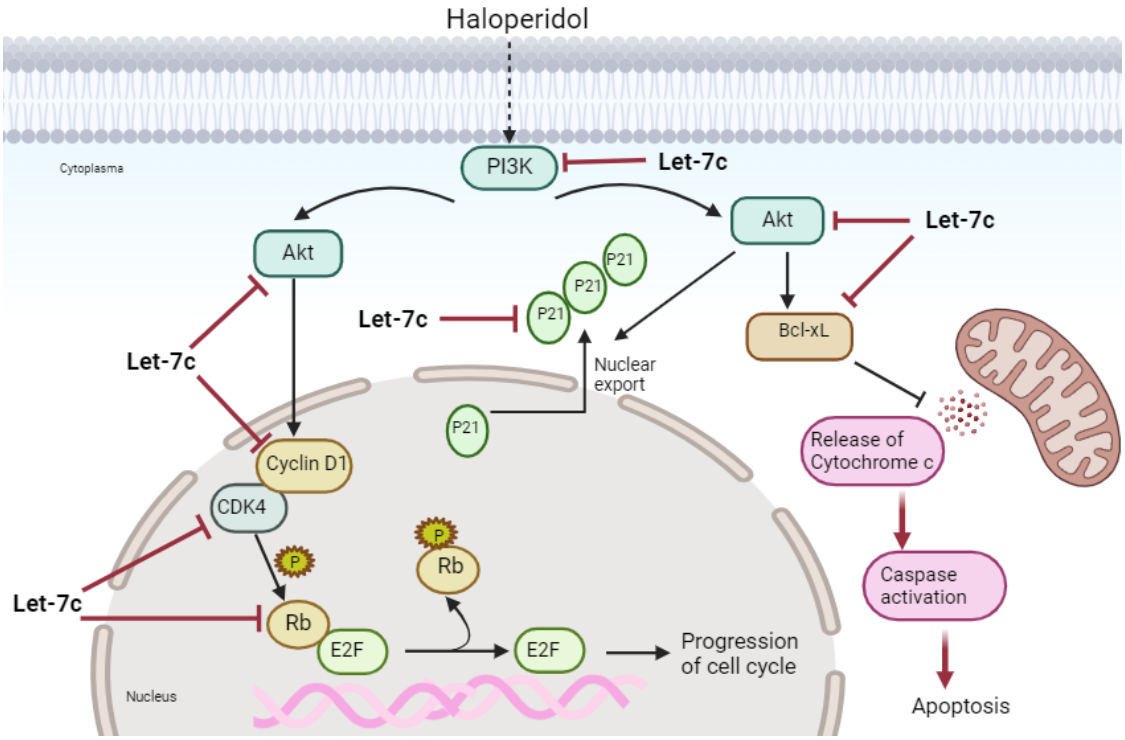


Figure 6: Regulatory Interactions Between miRNA- hsa-let-7 and the Predicted Target Genes Associated with the PKB/AKT Pathway and Cell Signaling Progression Modulated by Haloperidol.

This figure is made by an online tool at BioRender.com.

Overexpression of hsa-let-7c Inhibits the Expression of Haloperidol-Activated Proliferative Proteins p-PI3K and AKT

To investigate the role of hsa-let-7c in regulating the expression of proliferative proteins activated by haloperidol. We investigated the effect of haloperidol on the protein expression of phosphorylated PI3K p85/p55 and AKT in BEAS-2B cells, along with the influence of hsa-let-7c on the expression of these proteins and modulates the haloperidol proliferation effect.

The protein expression levels of p-PI3K-p55, p-PI3K-p85, and AKT were measured using Western blot analysis. BEAS-2B cells were treated with 3.5 µM of haloperidol for 24 h. and 48 h. Additionally, miRNA let-7c was transfected into another group of BEAS-2B cells. After 24 h. of the transfection, 3.5 µM of haloperidol was added to the medium, and the cells were incubated for another 24 and 48 h. then the cells were collected, protein extracted, and a Western blot assay was performed to detect the expression levels of AKT and PIK3 proteins in the different experimental conditions.

We observed that the expressions of p-PI3K-p55 protein were significantly increased in haloperidol-treated BEAS-2B cells for 24 h. and 48 h. compared to the respective control cells (Figures 8A, and 8B). While the protein expression of the p-PI3K-p85 regulatory subunit increases at 24 h. but not at 48 h in haloperidol-treated cells in comparison to 24 h control cells (Figures 8A, and 8B). Also, the expression of AKT protein was significantly induced in haloperidol-treated cells compared to the respective control cells (Figures 9A, and 9B). Furthermore, the overexpression of hsa-let-7c in BEAS-2B haloperidol-treated cells reduced the expression level of AKT after 24 and 48 h. compared

to haloperidol-treated BEAS-2B cells at 24 and 48 h. (Figures 9A, and 9B). This suggests that hsa-let-7c may act as a negative regulator of AKT expression that reverses the proliferation effect of haloperidol. Also, overexpression of hsa-let-7c in haloperidol-treated BEAS-2B reduces the expression of p-PI3K regulatory p55 subunit significantly at 24 and 72 h. (Figures 8A, and 8B). Interestingly, overexpression of has-let-7c in haloperidol-treated BEAS-2B does not affect the expression level of the p-PI3K-p85 regulatory subunit in comparison to haloperidol-treated cells (Figures 8A, and 8B). This implies that hsa-let-7c may specifically target the p55 subunit of PI3K, potentially modulating the activity of the PI3K-AKT pathway.

These results suggested that haloperidol promoted cell proliferation through the PI3K-AKT pathway via phosphorylation of regulatory subunits p55 of the phosphatidylinositol 3-kinase (PI3K) enzyme and increased Akt protein expression. Phosphorylation of p55, a regulatory subunit of PI3K, modulates PI3K activity by binding to the catalytic subunit (p110) of PI3K, enhancing PI3K activity and leading to increased production of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which serves as a second messenger in the PI3K signaling pathway. PIP3 recruits and activates AKT (protein kinase B) which regulates numerous downstream effectors involved in cell survival, proliferation, metabolism, and growth (Fruman, 1998 and Jean, 2014). Overall, these results provide an understanding of the molecular mechanism of haloperidol proliferation and highlight the potential regulatory role of hsa-let-7c in modulating the expression of proliferative proteins, specifically AKT and the p-PI3K-p55 subunit and counteract the haloperidol proliferation effect on BEAS-2B.

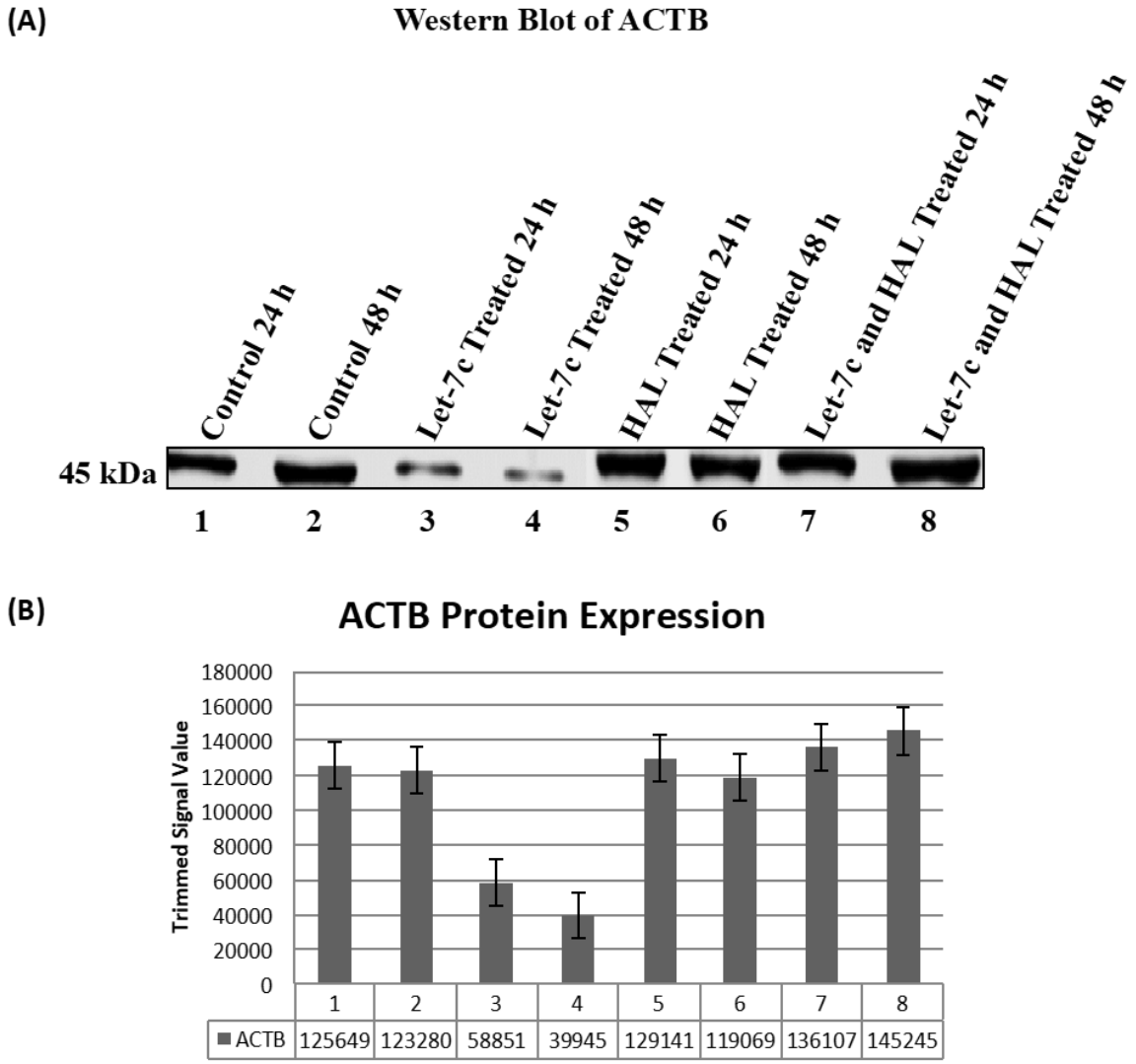


Figure 7: Western Blot Results of ACTB

(Figure 7A), a bar graph, and a numerical table delineate the expression of ACTB protein in the experimental samples (Figure 7B)

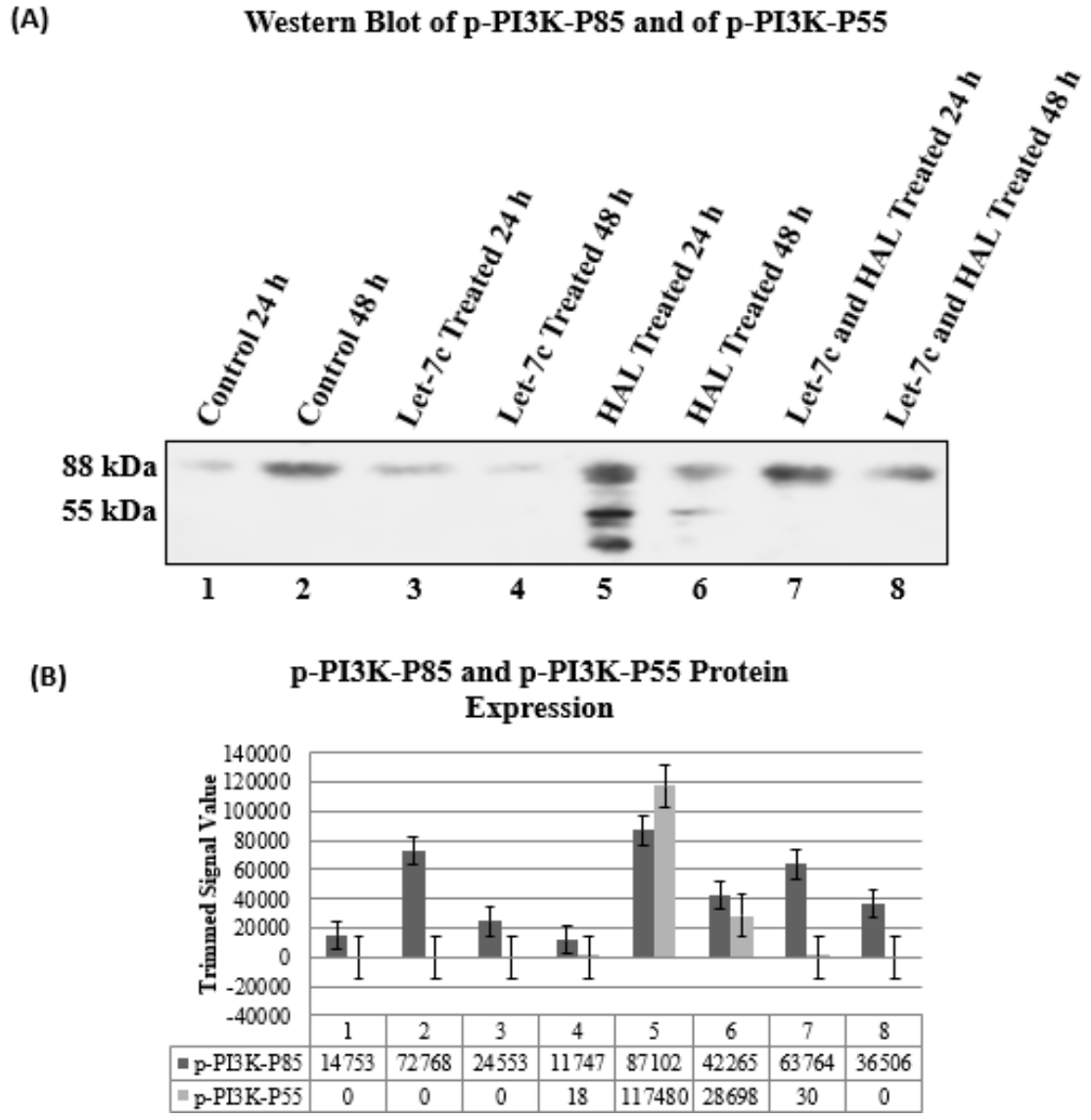


Figure 8: Western Blot Results of p-PI3K-P85 and p-PI3K-P55

(Figure 8A), and a bar graph and a numerical table delineate the expression of p-PI3K-P85 and p-PI3K-P55 protein in the experimental samples (Figure 8B).

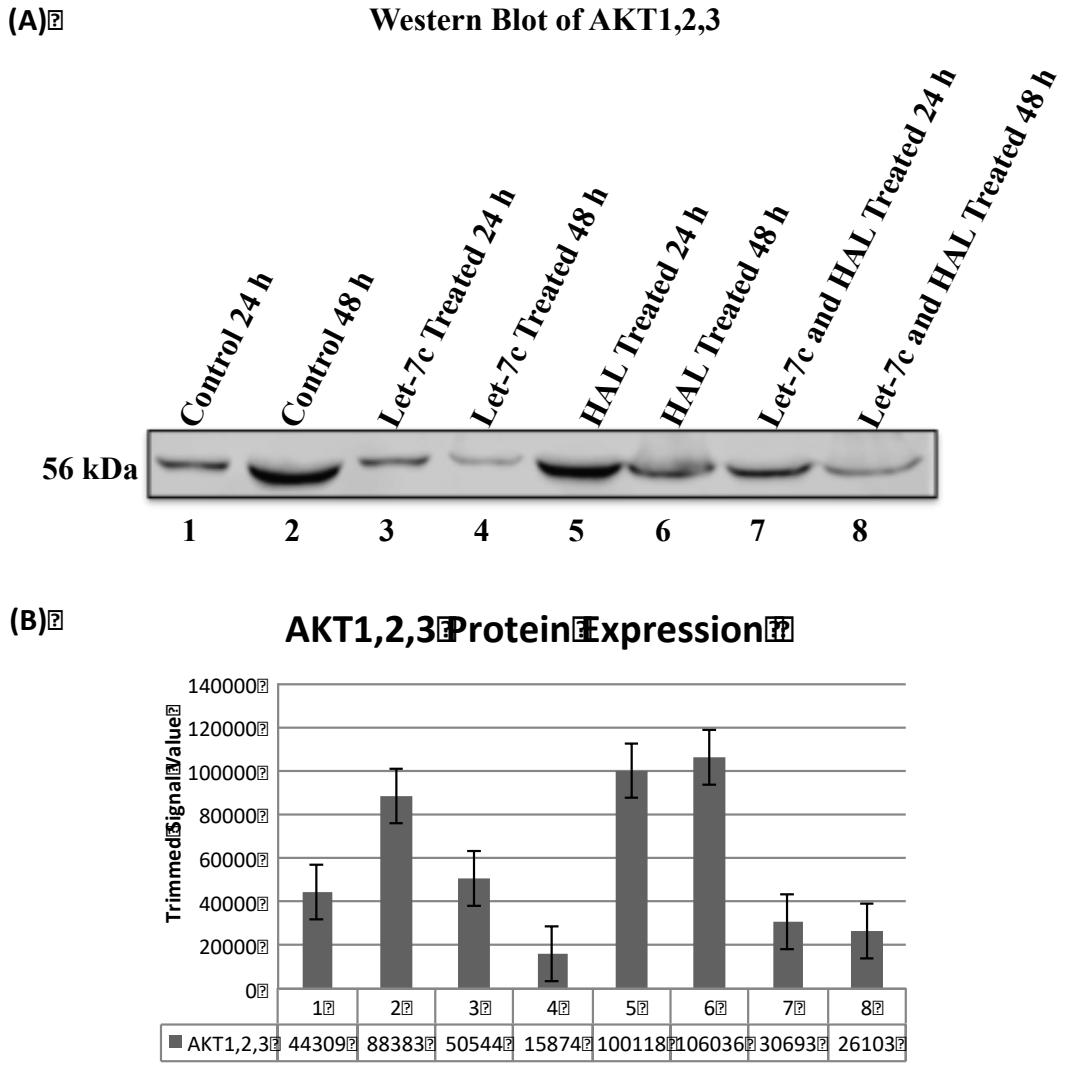


Figure 9: Western Blot Results of AKT1,2,3

(Figure 9A), and a bar graph and a numerical table delineate the expression of AKT1,2,3 protein in the experimental samples (Figure 9B).

Overexpression of hsa-let-7c Suppresses the Expression of Haloperidol-Induced Cell Cycle Regulatory Proteins P21, CDK4, and p-RB

Since cell cycle regulation plays an important role in the induction of cell proliferation. In this study, we investigated the role of hsa-let-7c in regulating the expression of cell cycle proteins induced by haloperidol. Specifically, we investigated the effect of haloperidol on the expression of cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor P21 (CDKN1A), and phosphorylated Retinoblastoma (p-RB) proteins along with the influence of hsa-let-7c on the expression of these proteins and modulates the haloperidol-induced proliferation effect.

Using Western blot analysis, the protein expression levels of CDK4, P21 (CDKN1A), and phosphorylated RB proteins were determined. BEAS-2B cells were treated with 3.5 μM of haloperidol for 24 h. and 48 h. In another group set of BEAS-2B, miRNA let-7c was transfected cells. After 24 h. of the transfection, 3.5 μM of haloperidol was added to the medium, and the cells were incubated for another 24 and 48 h., protein was extracted, and a Western blots assay was performed.

The results showed that the expression of CDK4 protein was significantly increased in haloperidol-treated BEAS-2B cells at 24 and 48h. compared to the respective control cells. However, the overexpression of hsa-let-7c in BEAS-2B cells led to a reduction in the expression level of CDK4 at 48 h. compared to control cells and overexpression of hsa-let-7c in haloperidol-treated BEAS-2B cells led to a significant reduction in the expression level of CDK4 at 24 and 48 hours compared to the haloperidol-treated cells (Figures 10A and B).

Also, the results of Western blots analysis show that phosphorylated RB protein was induced by haloperidol at 24 and 48 h. The phosphorylated activation of RB was attenuated by the overexpression of hsa-let-7c in haloperidol-treated BEAS-2B cells at 24 and 48 h. compared to the haloperidol-treated cells (Figures 11A and B).

This study also observed an increase in p21(CDKN1A), in the haloperidol-treated cells at 24 and 48 h. Overexpression of has-let-7c in haloperidol-treated BEAS-2B cells decreased the expression of the p21 protein significantly at 24 and 48 h. compared to the haloperidol-treated cells (Figures 12A and B). P21 is a negative regulator of the cell cycle, which could regulate the G1 checkpoint of the cell cycle through binding to CDK/cyclin complexes (Starostina and Kipreos, 2012). However, Akt-dependent cytoplasmic localization of p21 occurs in a variety of cells where it promotes tumorigenesis by inhibiting proteins essential for apoptosis (Asada, 1999; Zhou, 2001).In this study, along with the increased expression of Akt, the expression of CDK4, phosphorylated RB (p-RB), and p21 in response to haloperidol treatment significantly increased suggesting a regulatory network involving the Akt pathway and downstream effectors in modulating cell cycle progression. Protein kinase B/ AKT mediated phosphorylation of CDK4 can have a significant effect on cell cycle progression. Phosphorylation of CDK4 by AKT enhances its activity, leading to increased phosphorylation of the retinoblastoma protein (RB). Phosphorylation of RB weakens its interaction with E2F, leading to the release of E2F transcription factors and subsequent cell cycle progression from G1 to S phase (Sherr et al., 1999).

Therefore, the observed increase in CDK4, phosphorylated RB (p-RB), and p21 expression, along with Akt activation, in response to haloperidol treatment suggests a potential role for Akt in promoting cell cycle progression induced by haloperidol. Additionally, the repression of haloperidol-induced CDK4, phosphorylated RB (p-RB), and expression by hsa-let-7c upregulation may involve modulation of the Akt signaling pathway, indicating a multifaceted interaction between hsa-let-7c, the Akt pathway, and cell cycle regulation which could reverse the haloperidol proliferation effect.

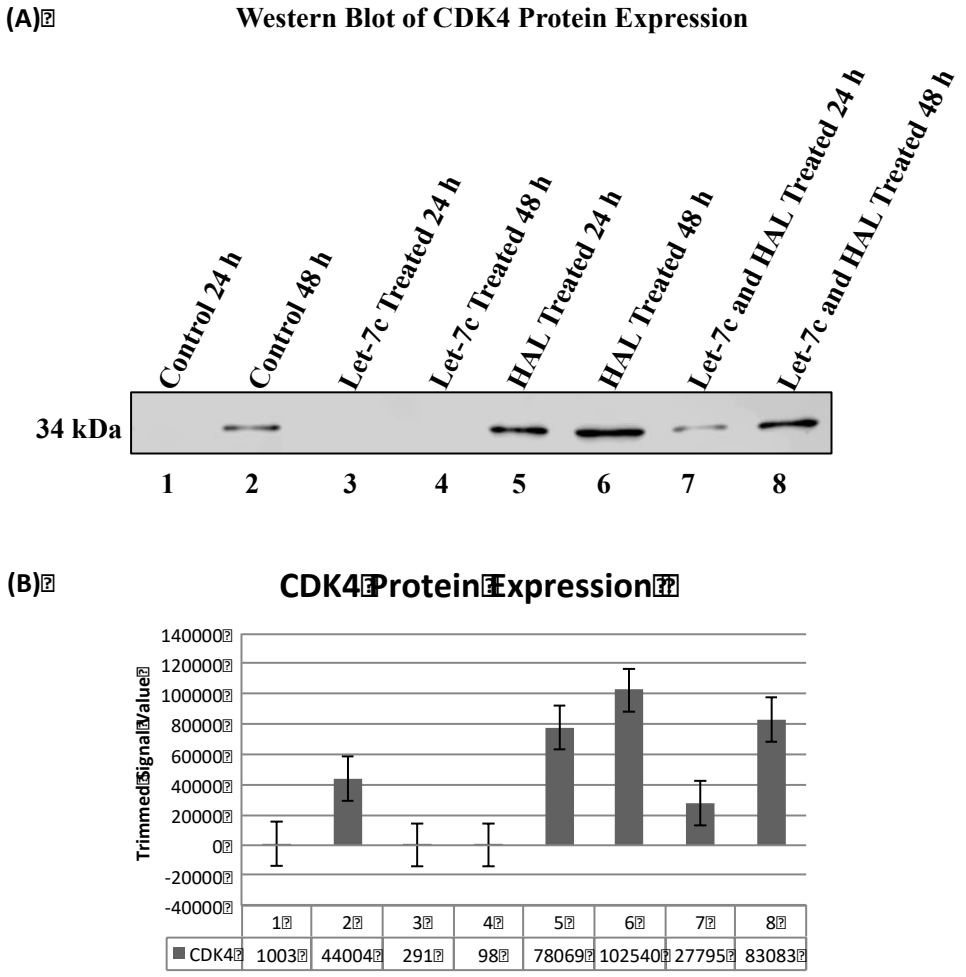


Figure 10: Western Blot Results of CDK4

(Figure 10A), a bar graph, and a numerical table delineate the expression of CDK4 protein in the experimental samples (Figure 10B).

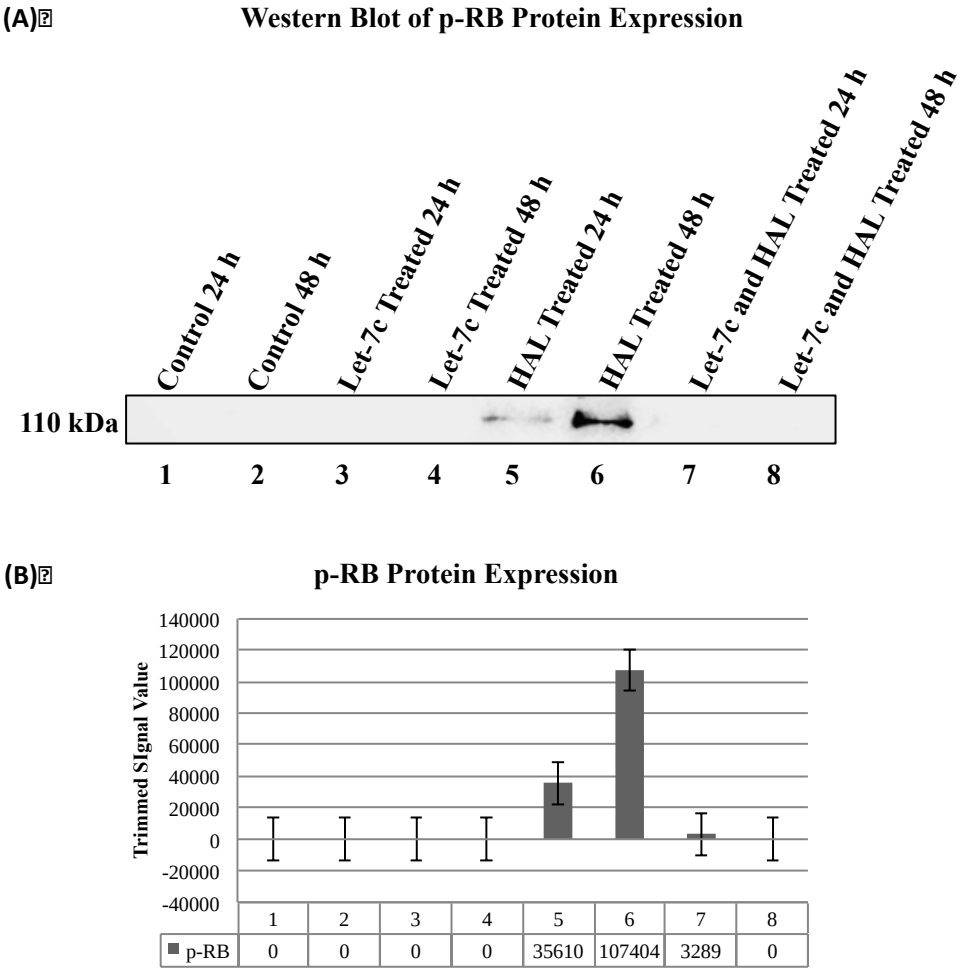


Figure 11: Western Blot Results of p-RB

(Figure 11A), a bar graph, and a numerical table delineate the expression of p-RB protein in the experimental samples (Figure 11B).

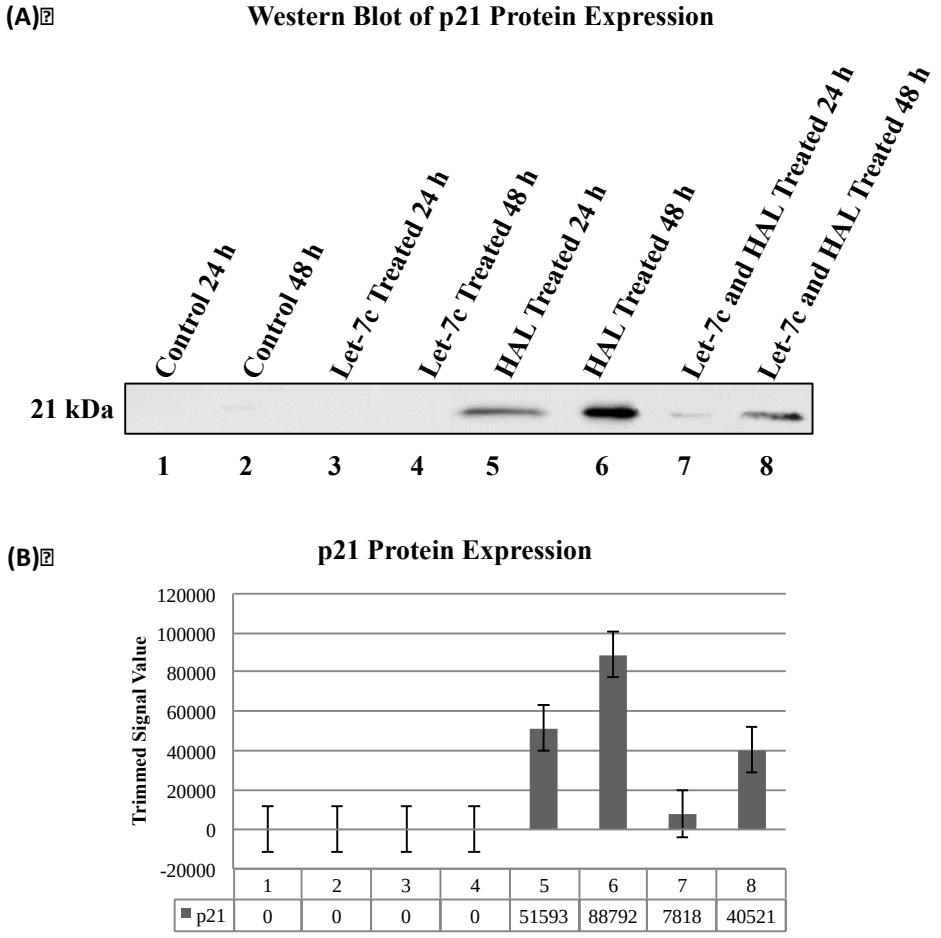


Figure 12: Western Blot Results of p21

(Figure 12A), a bar graph, and a numerical table delineate the expression of p21 protein in the experimental samples (Figure 12B).

Overexpression of hsa-let-7c Inhibits the Expression of Haloperidol-Activated Caspase-3

In this study, we investigated the impact of hsa-let-7c on the expression of caspase-3 in BEAS-2B cells treated with haloperidol. Caspase-3 is usually known as an activator of cell apoptosis pathways because of its proteolytic activity in cells responding to specific extrinsic or intrinsic factors which ultimately lead to cell death. However recent evidence suggests it may also have non-apoptotic functions associated with cell growth, differentiation, and intercellular communication through cytokine release and NF-κB activation in both normal and malignant cells (Dick, 2015; Lamkanfi, 2007; Ohsawa, 2010). Also, it has been suggested that an increased expression of the caspase-3 protein has been observed and linked to the activation of the Retinoblastoma (RB) protein and induced cell cycle progression (Eskandari et al., 2022). Western blot analysis was used to assess the expression of the Caspase-3 protein in response to haloperidol treatment. BEAS-2B cells were treated with 3.5 µM of haloperidol for 24 h. and 48 h. Also, a different group set of BEAS-2B cells was transfected with miRNA let-7c. miRNA let-7c. After 24 h. of the transfection, 3.5 µM of haloperidol was added, and the cells were incubated for another 24 and 48 h. then protein was extracted, and a Western blots assay was performed to detect the expression levels of Caspase-3 protein. The results showed that haloperidol increases the activation of caspase-3 at 24 and 48 hours after treatment in comparison to 24 and 48-hour controls. However, when hsa-let-7c was overexpressed in BEAS-2B cells, the expression level of caspase-3 decreased significantly in haloperidol-treated BEAS-2B cells compared to the haloperidol-treated cells (Figure 13A and B).

These results suggest that the haloperidol-induced proliferation in BEAS-2B cells might be a result of increased caspase-3 activity, which is reversible by hsa-let-7c overexpression. This suggests that hsa-let-7c has a regulatory role in modulating caspase-3 activity and, subsequently, the effects of haloperidol treatment on cell proliferation in BEAS-2B cells.

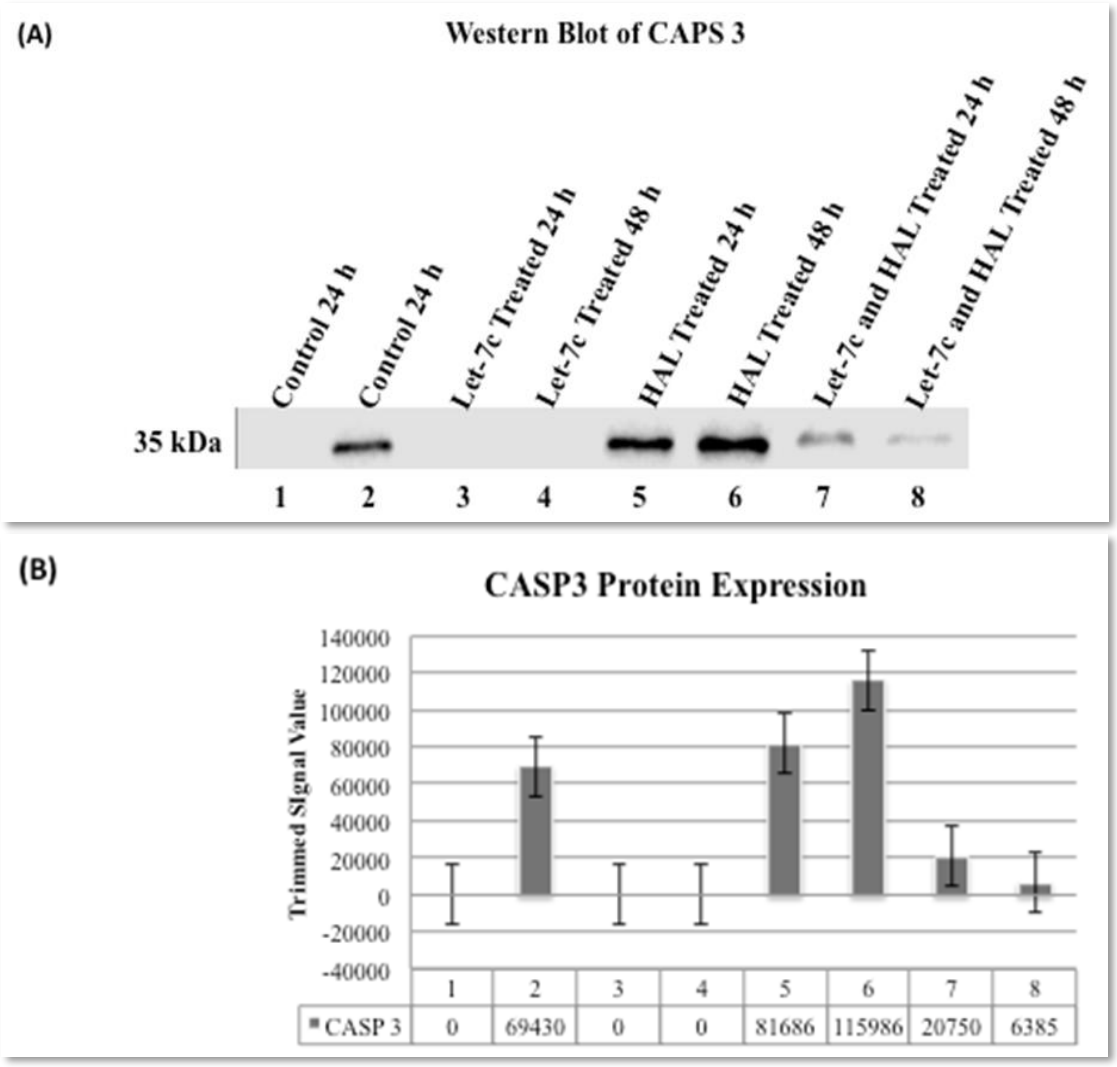


Figure 13: Western Blot Results of CASP3

(Figure 13A), a bar graph, and a numerical table delineate the expression of CASP 3 protein in the experimental samples (Figure 13B).

Overexpression of hsa-let-7c Increased EIF2AK1 Expression and Counteracted the Haloperidol's Inhibition Effect

In this study. We investigated the effect of haloperidol on cell proliferation mediated through the regulation of the protein translation process, specifically by regulation of Eukaryotic translation initiation factor 2-alpha kinase 1 (EIF2AK1) protein expression. Also, we investigated the impact of hsa-let-7c on the expression of EIF2AK1 protein in BEAS-2B cells treated with haloperidol over points of time. Regulation of protein translation in cells occurs mostly at the initiation step through the phosphorylation of components of the translational process (Holcik, 2005; Sonenberg, 2009). Phosphorylation of the alpha (α) sub-unit of eukaryotic initiation factor 2 (eIF2α) is one of the well-studied mechanisms in regulating the overall rate of protein synthesis in eukaryote cells (de Haro, 1996; Holcik, 2005). Eukaryotic translation initiation factor 2-alpha kinase 1 (EIF2AK1), is a kinase enzyme that phosphorylates EIF2α in response to various cell stimuli, such as oxidative stress, resulting in attenuation of mRNA translation and inhibits protein synthesis (Chen, 2014; de Haro, 1996). Western blot analysis was performed to assess the expression of EIF2AK1 in BEAS-2B cells treated with haloperidol for 24 and 48 hours and to determine the impact of hsa-let-7c overexpression on EIF2AK1 expression in haloperidol-treated BEAS-2B cells.

The result indicates that haloperidol leads to a significant decrease in the expression of EIF2AK1 in BEAS-2B cells at 24h. and 48h. in comparison to the corresponding control. Figure 16 shows a proposal schematic representation, illustrating the regulatory

interactions between Eukaryotic translation initiation factor 2-alpha kinase 1 (EIF2AK1) and EIF2α in response to haloperidol. The reduction in the expression of EIF2AK1 by haloperidol in BEAS-2B cells was reversed and increased significantly by overexpression of has-let-7c in haloperidol-treated BEAS-2B cells for 24 h. and 48h (Figure 15A and B). These findings suggest that haloperidol causes an increase in protein synthesis due to reduced EIF2AK1 expression and overexpression of hsa-let-7c in BEAS-2B cells increased EIF2AK1 expression and counteracted the effect of haloperidol, which led to reduction in protein synthesis and, ultimately, reduced cell proliferation.

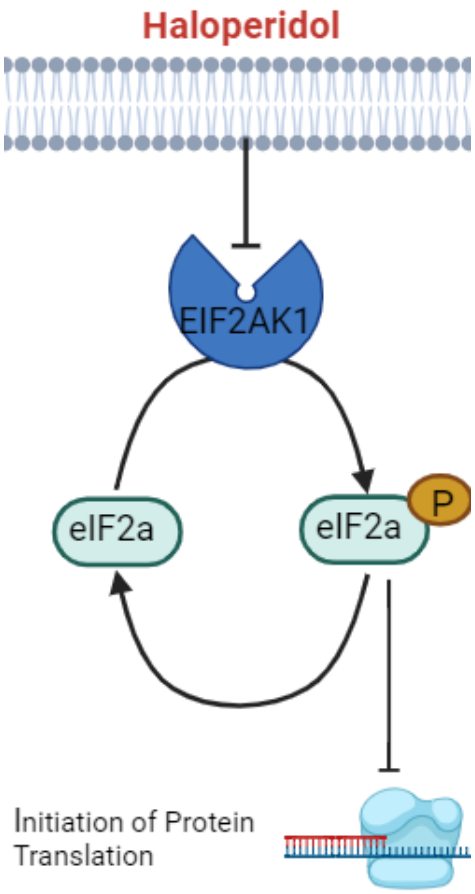
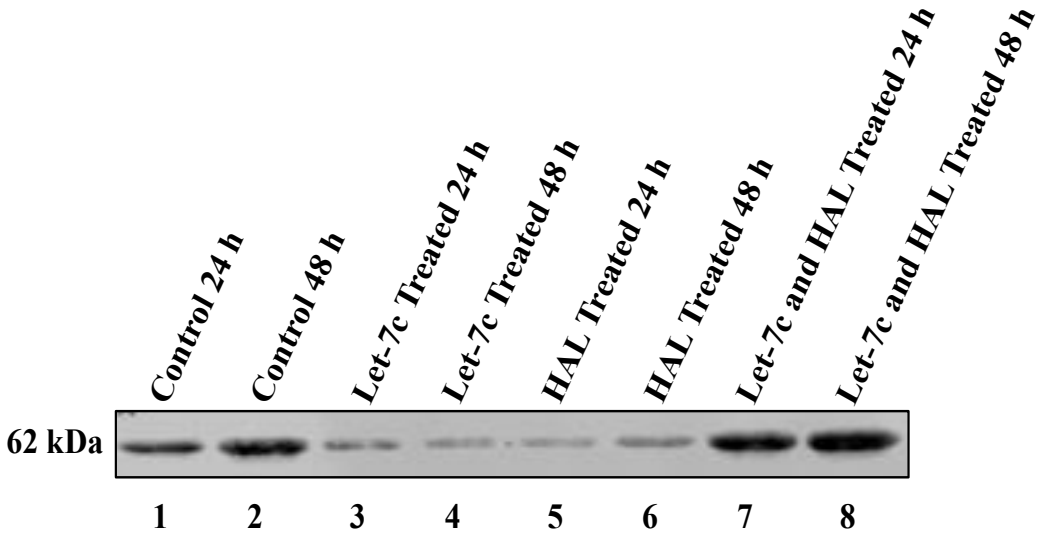


Figure 14: Schematic Representation Illustrating the Regulatory Interactions between Eukaryotic Translation Initiation Factor 2-alpha Kinase 1 (EIF2AK1) and EIF2α in Response to Haloperidol

Leading to the inhibition of EIF2AK1 and inhibiting the phosphorylation of EIF2α and subsequent initiation of mRNA translation, ultimately resulting in increased protein synthesis. This figure is made by an online tool at BioRender.com.

(A)



(B)

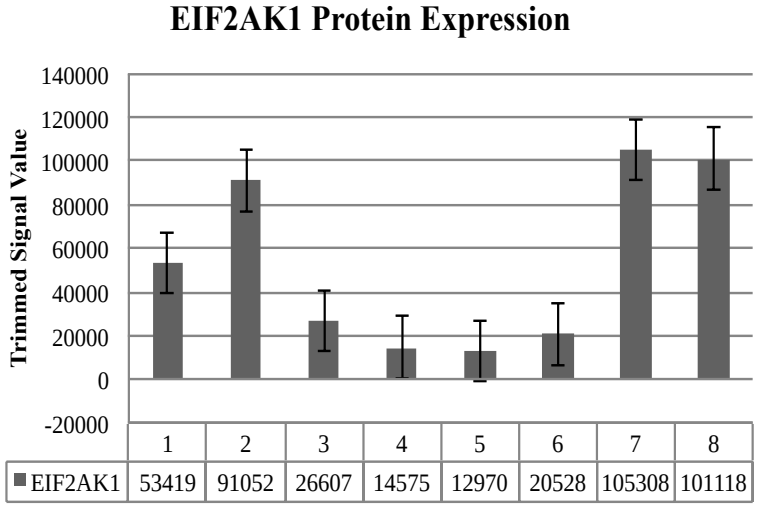


Figure 15: Western Blot Results of EIF2AK1

(Figure 15A), a bar graph, and a numerical table delineate the expression of EIF2AK1 protein in the experimental samples (Figure 15B)

Upregulation of hsa-let-7c Attenuates Haloperidol-Induced Mitochondria Dysfunction through Modulating of RNMTL1

Haloperidol has been associated with mitochondrial dysfunction in some studies (Murata,2007; Hu, 2021). So, in this study, we evaluated the impact of haloperidol on RNMTL1, a member of the RNA methyltransferase family, which is responsible for the 2'-O-ribose modifications of the large mitochondrial ribosomal subunit's 16 S rRNA core, which is crucial for mitochondrial protein translation (Lee et al., 2013 and Lopez Sanchez et al., 2020). Mitochondria, specialized mitochondrial ribosomes (mitoribosomes) are responsible for the synthesis of 13 oxidative phosphorylation proteins encoded by the mitochondrial genome (Lopez, 2020). Mitoribosomal RNA undergoes post-translational modification, this form of post-transcription regulation of mitochondrial gene expression affects mitoribosome biogenesis and function which ultimately impacts cell homeostasis (Decatur & Fournier, 2002). Also, in this work, we examined the impact of hsa-let-7c on the expression of RNMTL1 protein in BEAS-2B cells treated with haloperidol over points of time.

The result of a Western blots analysis showed that haloperidol decreased RNMTL1 expression at 24 and 48 h. in comparison to control. However, overexpression of hsa-let-7c significantly increased the expression of RNMTL1 in BEAS-2B untreated cells at 48 h. Also, overexpression of hsa-let-7c in haloperidol-treated BEAS-2B cells reversed haloperidol effects and increased RNMTL1 expression at 24 and 48 h. in comparison to haloperidol treated BEAS-2B (Figure 16A and B). These results suggest that haloperidol might influence mitochondrial translation by controlling RNMTL1's post-transcriptional

processing of mitochondrial RNA and that haloperidol's effects might be reversed by overexpressing hsa-let-7c expression in BEAS-2B cells. This result suggests that haloperidol might impair mitochondrial translation by modulating RNMTL1. Impaired mitochondrial translation can lead to dysfunctional electron transport chain complexes, resulting in increased production of reactive oxygen species (ROS). Overproduction of ROS can lead to oxidative damage to lipids, proteins, and DNA in cells, resulting in genomic instability, oncogenic mutations, and tumor progression (Sullivan,2014). Moreover, other studies have proven that in cancer cells impaired mitochondria translation may disrupt the apoptotic signaling pathway leading to apoptosis resistance (Cavdar, 2001; Lopez, 2015) and a decline in mitochondria energy metabolism which might lead to the development of cancer (Warburg, 1956). On the other hand, the overexpression of hsa-let-7c showed an opposite effect on RNMTL1 expression in untreated cells. Overexpression of hsa-let-7c reverses the downregulation of RNMTL1 caused by haloperidol treatment. This indicates that hsa-let-7c may have a protective role against haloperidol-induced mitochondrial dysfunction.

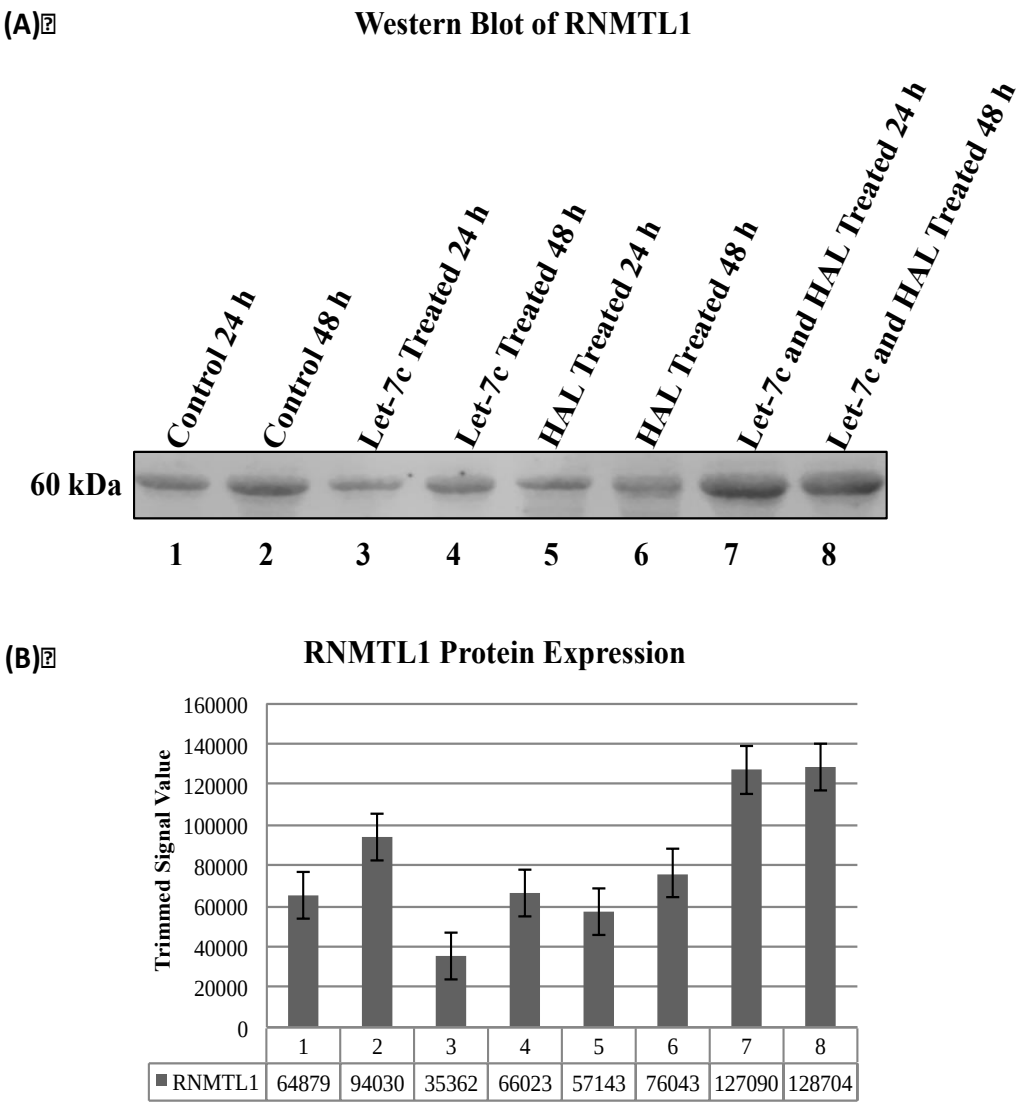
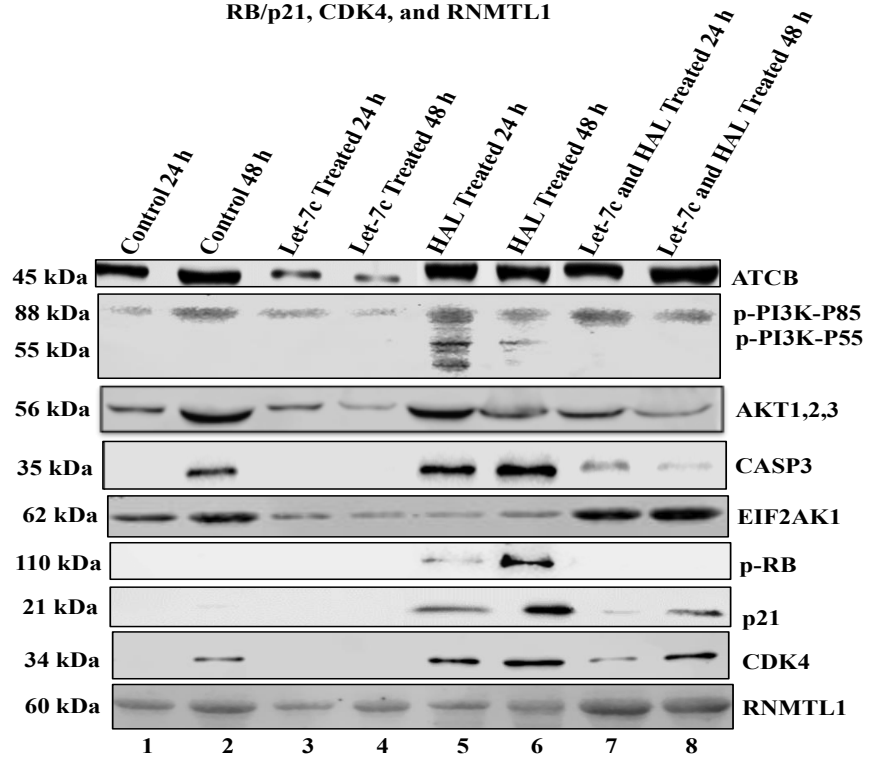


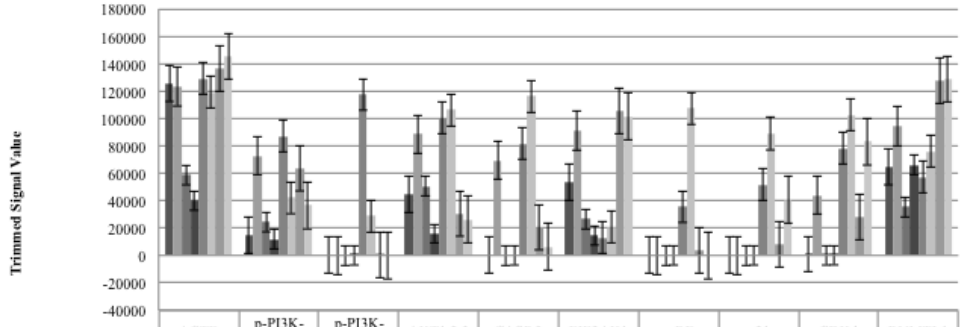
Figure 16: Western Blot Results of RNMTL1

(Figure 16A), a bar graph, and a numerical table delineate the expression of RNMTL1 protein in the experimental samples (Figure 16B).

(A) Western Blots of p-PI3K-P85, p-PI3K-P55, AKT1,2,3, CASP3, EIF2AK1, p-RB/p21, CDK4, and RNMTL1



(B) ACTB, p-PI3K-P85, p-PI3K-P55, AKT1,2,3, EIF2AK1, p-RB, p21, CDK4, p21, and RNMTL1 Protein Expression



	ACTB	p-PI3K-P85	p-PI3K-P55	AKT1,2,3	CASP 3	EIF2AK1	p-RB	p21	CDK4	RNMTL1
Control 24 h	125649	14753	0	44309	0	53419	0	0	1003	64879
Control 48 h	123280	72768	0	88383	69430	91052	0	0	44004	94030
Let-7c Treated 24 h	58851	24553	0	50544	0	26607	0	0	291	35362
Let-7c Treated 48 h	39945	11747	18	15874	0	14575	0	0	98	66023
HAL Treated 24 h	129141	87102	117480	100118	81686	12970	35610	51593	78069	57143
HAL Treated 48 h	119069	42265	28698	106036	115986	20528	107404	88792	102540	76043
Let-7c and HAL Treated 24 h	136107	63764	30	30693	20750	105308	3289	7818	27795	127090
Let-7c and HAL Treated 48 h	145245	36506	0	26103	6385	101118	0	40521	83083	128704

Figure 17: Western Blot Results of ACTB, p-PI3K-P85, p-PI3K-P55, AKT1,2,3, EIF2AK1, p-RB, p21, CDK4, p21, and RNMTL1

(Figure 17A), and bar graphs and a numerical table delineate the expression of ACTB, p-PI3K-P85, p-PI3K-P55, AKT1,2,3, EIF2AK1, p-RB, p21, CDK4, p21, and RNMTL1 protein in the experimental samples (Figure 17B).

Analysis of Variance (ANOVA)

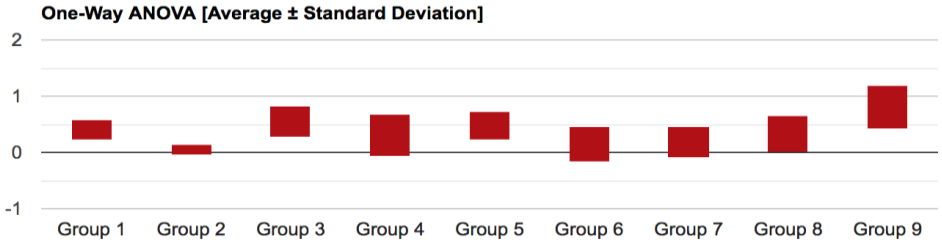
Analysis of Variance Results
F-statistic value = 5.00526
P-value = 0.00008 ≃ 0.0001

Data Summary

Groups	N	Mean	Std. Dev.	Std. Error
Group 1: p-PI3K-P85	8	0.396	0.1819	0.0643
Group 2: p-PI3K-P55	8	0.0471	0.0915	0.0324
Group 3: AKT1,2,3	8	0.5496	0.2915	0.103
Group 4: CASP3	8	0.2958	0.3763	0.1331
Group 5: EIF2AK1	8	0.4654	0.2546	0.09
Group 6: p-RB	8	0.1475	0.3197	0.113
Group 7: p21	8	0.1852	0.2732	0.0966
Group 8: CDK4	8	0.3274	0.3265	0.1154
Group 9: RNMTL1	8	0.8042	0.3832	0.1355

ANOVA Summary

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Stat	P-Value
	DF	SS	MS		
Between Groups	8	3.3953	0.4244	5.0053	0.0001
Within Groups	63	5.342	0.0848		
Total:	71	8.7374			



Post-Hoc Tukey's Honestly Significant Difference (HSD) Test

Post-Hoc Tukey's HSD Test Results

Group	Group	Pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD inferfence
Group B: p-PI3K-P55	Group C: AKT1,2,3	B:C	4.8805	0.0260943	* p<0.05
Group D: CASP3	Group I: RNMTL1	D:I	4.9385	0.0232345	* p<0.05
Group H: CDK4	Group I: RNMTL1	H:I	4.6318	0.0423334	* p<0.05
Group B: p-PI3K-P55	Group I: RNMTL1	B:I	7.3536	0.0010053	** p<0.01
Group F: p-RB	Group I: RNMTL1	F:I	6.3785	0.0010053	** p<0.01
Group G: p21	Group I: RNMTL1	G:I	6.0125	0.0021999	** p<0.01

Figure 18: Statistical Analysis, One-way ANOVA Conducted for Multiple Comparisons Between Different Experimental Groups

(Figure 18A). Tukey's tests were utilized as post hoc analysis to further compare between specific groups (Figure 18B). The data presented as mean ± standard error of the mean (SEM) and statistical significance, where *p < 0.05**p < 0.001, ***p < 0.0001.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

We hypothesized that overexpression of the miRNA (hsa-let-7c-5p targets PKB/Akt mRNA) would prevent PKB/Akt-induced expression by haloperidol in BEAS-2B cells, which would prevent cell proliferation and cause cell death. The goal of this work is to provide an understanding of the potential regulatory role of miRNA let-7c on the molecular mechanisms underlying the effects of haloperidol on BEAS-2B cell proliferation, and survival, as well as modulation of cell cycle progression and translation proteins. The findings show that the haloperidol proliferative effect on BEAS-2B cells is induced by PKB/Akt activation. PKB/Akt promoted the proliferative effects of haloperidol on BEAS-2B cells by activation of the expression of the antiapoptotic protein BcL-xL, and cell cycle progression proteins as cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor P21 (CDKN1A) and p-RB (phosphorylated Retinoblastoma) proteins. Additionally, this study demonstrated that haloperidol increased protein synthesis by inhibition of Eukaryotic translation initiation factor 2-alpha kinase 1 (EIF2AK1) and induced mitochondria dysfunction by downregulating the expression of RNA methyltransferase (RNMTL1) which is a critical protein for mitochondrial protein translation.

Furthermore, the study demonstrates the role of hsa-let-7c in regulating haloperidol's paradoxical behavior. Overexpression of hsa-let-7c inhibits haloperidol-induced cell proliferation through the modulation of target genes regulating the protein

kinase B (PKB/AKT) signaling pathway. Overexpression of hsa-let-7c in haloperidol-treated BEAS-2B cells resulted in a decrease in the expression level of cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor P21 (CDKN1A) and p-RB (phosphorylated Retinoblastoma) proteins, in comparison to the haloperidol-treated cells. Also, overexpressing has-let-7c in haloperidol-treated BEAS-2B increased and reversed the reduction in EIF2AK1 expression caused by haloperidol in BEAS-2B cells. This finding suggests that haloperidol increases protein synthesis by decreasing EIF2AK1 expression, and that haloperidol's effect is counteracted by overexpressing hsa-let-7c in BEAS-2B cells, which increases EIF2AK1 expression and attenuated haloperidol-inducing protein translation. Moreover, miRNA hsa-let-7c plays a protective role against haloperidol-induced mitochondrial dysfunction. Overexpression of hsa-let-7c in haloperidol-treated BEAS-2B cells reversed the effects of haloperidol and increased RNMTL1 expression, a protein needed for the mitochondrial protein translation process.

The finding of this study demonstrated the paradoxical proliferative effect of haloperidol in non-cancerous BEAS-2B cells. The regulatory effect of hsa-let-7c in controlling proliferation through the regulation of PKB/Akt and its downstream pathway and counteracting the proliferative effect of haloperidol is also demonstrated. The study revealed that understanding the differential expression of specific miRNAs in response to haloperidol treatment can provide a valuable understanding of the molecular mechanisms underlying haloperidol-induced effects on cell proliferation. This study also established the possibility of miRNAs as suitable biomarkers for addressing the issues of drugs with paradoxical behavior involving cell proliferation and apoptosis to predict the drug response among users.

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