MICRORNA-MEDIATED SENSITIZATION OF LUNG CANCER CELLS TO SELECTED CHEMOTHERAPEUTICS AND ZFN- MEDIATED EXPRESSION OF GFP IN HUMAN DERMAL FIBROBLAST

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DECLARATION

I, NNAEMEKA DARLINGTON NDODO, hereby declare that I carried out the investigations in this thesis by myself under the mentorship of Professor J. A.Nok, Professor S. S Adebisi and Dr. B. Danborno. That whatever citations made of other Investigators and other sources of information were properly acknowledged and referenced. That, no part of this Thesis has been submitted, or accepted elsewhere for a higher degree or diploma.

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Signature Date

CERTIFICATION

This is to certify that, this Thesis titled, "MicroRNA-Mediated Sensitization of Lung Cancers to Chemotherapeutics and ZFN- Mediated Expression of GFP in Human Dermal Fibroblast", is an original work carried out by Ndodo Nnaemeka Darlington, with REG. NO. PhD/MED/2773/2009/2010, under our guidance and supervision. That this investigation meets the requirements and regulation governing the award of the degree of Doctor of Philosophy (PhD) in Human Anatomy of the Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is totally dedicated to the Almighty God for His kindness and mercies towards me. And to my mother for ensuring I took studies seriously.

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LIST OF ABBREVIATIONS

3’ UTR	3’ untranslated region
5α-DHT	5α-dihydrotestosterone
AGO	Agonaute
AMO	Anti-miRNA oligonucleotide
ATCC	American Type Culture Collection
Bp	Basepair
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
Ct	Quantification cycle
DF	Dharmafect
dNTP	Deoxynucleotide triphosphate
dsRBD	Double-stranded RNA-binding domain
FFPE	Formalin-fixed, paraffin-embedded tissue
GO	Gene ontology
IP3	Inositol 1,4,5-triphosphate
Kb	Kilobases
LB	Luria–Bertani
M7G 7-	Methylguanosine
miRISC	MicroRNA induced silencing complex
miRNA	MicroRNA
miR	MicroRNA
mRNA	Messenger RNA
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NSCLC	Non-small cells lung cancer.
Nt	nucleotide
PCR	Polymerase Chain Reaction
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real time quantitative PCR
SCLC	Small Cell Lung Cancer
SRB	Sulphurhodamine Blue
TCA	Tri acetic acid.
TMA	Tissue microarray
XTT	3-[1-(phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4-methoxy-6-nitro benzene sulfonic acid hydrate)
ZFN	Zinc Finger Nucleases

ABSTRACT

Lung cancer and diabetes continue to be the leading cause of death globally. Earlier in the 20th century lung cancer was a rare malignancy. At the moment, it is occurring in epidemic proportions worldwide. It has become the most common cause of death from malignancy globally. Despite the use of surgery, chemotherapy and radiation in the treatment of lung cancer, the survival rate for patients remains extremely poor. Misregulation of genes that control cell-cycle and cell-fate determination often contributes to cancer. miRNAs are class of short noncoding RNAs that function as a regulator of gene expression via targeting mRNA for degradation or translational inhibition. They regulate expression of genes involved in tumorigenic processes, such as inflammation, cell cycle regulation, stress response, diferentiation, apoptosis, and invasion, and they have been found to play key roles in many cancers.This work was designed to look at the expression profile of two known oncogenic microRNAs, miR-21 and miR-155 in lung cancer, and to see whether re-introduction or inhibition of these would affect progression or aid sensitivities of the lung cancer cells to major Chemotherapeutics used in the management of lung cancer. It also examined zinc finger nucleases mediated expression of GFP in dermal fibroblast, targeting the AAVS1 locus, using *galk* recombineering scheme in SW102 strains. This work involved cell culture of H358 and A549 lung adenocarcinoma lines, as well as normal lung cells FC 7333 3KT lines (Broncho-aveolar cells). It compared the miRNA expression profile of two known microRNAs (miR-21 and miR-155) in cancer and wild type lung cell lines using real time qPCR (Qiagen miScript PCR system), and then treated with three known chemotherapeutics used in the management of Lung cancer namely Cisplatin, Etoposide and Paclitaxel and concluded by inhibiting over-expressed miRNA by transfecting the cancer cells with microRNA inhibitors called Antagomirs and then measured the level of proliferation with Sulforhodamine-B assay(SRB assay) which is essentially a survival assay. Concerning recombineering workflow, using *galk* selection scheme, that relies on homology directed repair (via zinc finger nucleases, ZFN) to effect gene modification or genome editing in SW102 strain of E.*coli*. *galk* plasmid was successfully amplified using iProof High Fidelity DNA Polymerase kit (BioRad) with primers with homology to the *galk* gene. The amplified galk gene was then used to transform competent heat-shocked SW102 cell using electroporation. The *galk* and GFP was then used to co-transform competent cells. Data from this work showed that miR-21 was over-expressed in all the cell lines used relative to the normal lung cell lines which is consistent with the role of miR -21 as an oncogenic microRNA, (oncomiR) while miR-155 was found to be down-regulated relative to the normal lung cells, suggesting that miR-155 could be acting as a tumour-suppressor contrary to the view that miR-155 is a universal oncomir. Furthermore, inhibition of miR-21 function in H358 lines using 50nM Ambion antimiR microRNA inhibitor led to decreased proliferation of H358 cells compared 50nM AntimiR-155 treated group. Inhibition of miR-21 also led to dose dependent apoptosis in H358 compared to negative control group. Downregulation of miR-21 was able to sensitize lung cancer cells to chemotherapeutics (Etoposide) used in this study. The IC_{50} for Etoposide following 72h incubation was 15.8µM, while after 96h incubation it was 0.1µM. Furthermore, the IC_{50} for Cisplatin after 72h incubation was given as 10µM and 5.45µM

after 96h of incubation in Cisplatin. For Paclitaxel the IC50 after 72h incubation was 0.7µM and 3.0µM after 96h of incubation in Paclitaxel. Result of the recombineering workflow showed that GFP was successfully expressed in the dermal fibroblast targeting the AAVS1 locus, the safe harbor for transgene expression in chromosome -19 of the dermal fibroblast genome using Zinc finger nucleases. Hence this work demonstrated that miR-21 at 50nM can sensitize lung cancer cells to Chemotherapeutics (Etoposide) and that miR-155, a known oncogenic miRNA seem to be acting as tumour-suppressor in lung cancer, which promise to be of immense therapeutic importance. Furthermore, that data from this study indicated that ZFN could target GFP expression into the AAVS1 site of human dermal fibroblast.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

MicroRNAs are commonly believed to be class of small, non-coding RNAs that post-transcriptionally control the translation and stability of mRNAs. MicroRNAs (miRNAs) are known to hybridize to the 3' untranslated region of target mRNAs causing repression at post-transcriptional level. Genes encoding miRNA molecules are found either inserted in introns as polycistronic clusters or in isolated regions of the genome (Jerome *et al.*, 2007). Through forward screening genetics the first microRNA was discovered. Even though it is known that miRNAs are dysregulated in various diseases, clear, causal evidence of their role in cancer has only recently come to light (Kasinski and Slack, 2011).

According to Stenvang and colleagues,(Stenvang *et al.*, 2012) manipulation of miRNA activity in vivo is of high interest due to the aberrant expression and implication of miRNAs in the pathogenesis of human diseases. The use of antimiR oligonucleotides to target disease-associated miRNAs is the most widely used approach to probe their functions in vivo and shows great promise in the development of novel miRNA-based therapeutics. They further stated that, an increasing number of studies have reported successful therapeutic miRNA silencing in a variety of animal disease models using antimiR oligonucleotides.

Kasinski and Slack noted that the ability of a miRNA to regulate a gene stems from a 6-nucleotide seed region in the miRNA that typically interacts with the 3′ untranslated region of an mRNA, so as to prevent translation or induce degradation of the mRNA. Due to their small which contributes to their pleiotropic role in targeting a large number of critical

protein-coding genes, misregulation of a single miRNA can give rise to multiple adverse cellular effects (Kasinski and Slack, 2011). Aberrant miRNA levels have been implicated in promoting and maintaining various disease states including cancer. In some cases, overexpression of single miRNA is sufficient to drive tumorigenesis, although its silencing leads to tumor regression (Kasinski and Slack, 2011). They further observed, that based on these findings, and on-going human tumor miRNA profiling studies that have confirmed altered levels of some key miRNAs, it is becoming increasingly attractive to target these master gene-regulators in vivo with cancer therapeutics (Medina *et al.*, 2010a).

It is equally important to know that several strains of mice lacking or overexpressing cancer-associated miRNAs have been developed and characterized. These include germline transgenic or knockout mice for: miR.155; miR.21; miR.17~92 and its paralogues; miR.15 and miR.16; miR.146; and miR.29. Additional mouse models are the LIN.28 overexpressing strain (for evaluation of the in vivo loss of mature let.7) and the multiple conditional DICER knockout models (Kasinski *et al.*, 2011).

This work would focus on two important microRNAs miR-21 and miR-155 known to be differentially expressed in cancers.

The insertion of exogenous genetic information into the genome of target cells is broadly used in biology. Gene insertion is traditionally achieved via virus-mediated or spontaneous integration of transfected DNA followed by a selection for cells carrying the new DNA. In cell-based therapy, lack of control over the transgene integration site can result in adverse events due to insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2002). Uncontrolled transgene integration results in unwanted phenotypic heterogeneity due to the varying permissivity of integration sites for transgene expression (position-effect variation) (Kwaks and Otte, 2006). In these situations, Cost and co-workers noted that it would be

advantageous to target DNA insertion to a specific, desirable site in the genome (Orlando *et al.*, 2010).

According to Porteus and colleagues,(Porteus, 2010), Homologous recombination is the most precise way to manipulate the genome. It has been used extensively in bacteria, yeast, murine embryonic stem cells, and a few other specialized cell lines, but it has not been available in other genetic systems such as mammalian somatic cells. However, the creation of a gene-specific DNA double-strand break can stimulate homologous recombination by several-thousand fold in mammalian somatic cells. These double-strand breaks can be created in mammalian genomes by zinc finger nucleases (ZFNs), artificial proteins in which a zinc finger DNA-binding domain is fused to a nonspecific nuclease domain.

Zinc finger nucleases (ZFNs) have been used successfully to create genome-specific double-strand breaks and thereby stimulate gene targeting by several thousand fold. ZFNs are chimeric proteins composed of a specific DNA-binding domain linked to a non-specific DNA-cleavage domain. By changing key residues in the recognition helix of the specific DNA-binding domain, one can alter the ZFN binding specificity and thereby change the sequence to which a ZFN pair is being targeted. For these and other reasons, ZFNs are being pursued as reagents for genome modification, including use in gene therapy (Pruett-Miller *et al.*, 2009).

Targeted gene transfer is typically performed by transfection of a selectable marker gene flanked by a substantial amount of DNA homologous to the target locus. Spontaneous double stranded breaks (DSBs) are formed at the target locus, likely from stalled DNA replication forks. While normally repaired correctly by homology-directed repair (HDR) templated by the sister chromosome, HDR can instead use the homologous donor DNA to heal the break. When additional DNA sequence is inserted between the two regions of

homology in the donor plasmid, the cellular DNA repair machinery unwittingly copies this genetic information into the chromosome. As this homology-based targeting relies on the capture of very rare DSBs within the region of donor homology, extensive homology to the target locus is needed to obtain targeted integration at a useful frequency (Porteus, 2010).

The initial idea that Zinc finger nucleases, ZFNs could stimulate gene targeting in mammalian somatic cells came from the work of Porteus and colleagues (Porteus and Carroll, 2005a). In these experiments, recognition sites for known zinc finger DNA-binding domains were inserted into a green fluorescent protein (GFP) reporter gene, which was integrated as a single copy into the genome of the human embryonic kidney cell line. In this system, gene targeting is measured by the correction of an integrated mutant GFP target gene by a transfected donor plasmid and the resultant conversion of GFP-negative cells into GFP-positive cells. In addition, this work showed that targeting with ZFN could be induced simultaneously at both the site of the break and at a distance of 400 bp from the break, demonstrating that a single pair of ZFNs can stimulate targeting in a relatively large region surrounding a DSB (Porteus and Carroll, 2005a).

Urnov and workers reported that designed ZFNs can cleave an endogenous human gene in cultured cells and lead to targeted gene replacement in up to 20% of the cells (Urnov *et al.*, 2005). The target was the gene for interleukin (IL) - 2Rγ, a cytokine receptor that is required for T-cell development and the establishment of a functional immune system. However, before ZFN methods become widely used several challenges must be scaled: first, applicability of the ZFN approach needs to be broadened; second, the method for delivering ZFNs and repair substrate to cells requires optimization; and third, the understanding of the process of homologous recombination itself needs to be enhanced. Efforts to broaden the applicability of the approach will require not only the design of

ZFNs targeting a greater variety of gene targets but also the determination of the conditions for performing gene targeting in different cell types. Until now, ZFNs have been primarily applied to transformed mammalian cell lines that are relatively resistant to apoptotic stimuli. An important advance, therefore, will be to develop ways of using ZFNs in primary cells (like the human dermal fibroblast that this research will address) known to be more sensitive to DNA damage.

1.2 AIMS AND OBJECTIVES OF THE STUDY

The main aim of this study is to sensitize lung cancer cells to chemotherapeutics using two microRNAs (miR-21 and miR-155) by determining the expression profile of these microRNAs via Real time PCR and then inhibiting or over-expressing them using microRNA Inhibitors or Mimics.

The second aim of this study is to express fluorescent proteins in human fibroblast targeting the AAVS1 Locus at chromosome 19 using Zinc finger Nucleases. These fluorescent proteins will be used to precisely monitor blood sugar levels in diabetic patients, which will in turn stimulate insulin release from biosensor to be implanted into the skin. The biosensor would be built by Massachusset Institute of Technology.

1.2.1 OBJECTIVES OF STUDY

The objectives of the study are to:

I. profile microRNA expression levels in wild type “WT” (FC 7333 3KT) versus “Cancer” (H358) Lung cell lines.

II. inhibit microRNAs that would be upregulated in the Lung cancer cells.

III. find IC_{50} for the chemotherapeutics used Cisplatin, Etoposide, and Paclitaxel.

IV. monitor transfection efficiency of the AntimiR microRNA inhibitors (Ambion) using Renilla Luciferase assay.

V. monitor apoptosis and cell survival using Sulforhodamine blue (SRB) assay following microRNA inhibition.

VI. use Zinc Finger Nucleases (ZFN), to express Green Fluorescent Protein (GFP), in the human dermal fibroblast at the AAVS1 locus using *galK* recombineering Scheme in SW102 strain.

1.3 STATEMENT OF PROBLEM

Lung cancer is the most common cause of cancer-related deaths around the world, whereas non-small cell lung cancer (NSCLC) represents the most frequent type of lung cancer. NSCLC is comprised of squamous cell carcinoma, adeno-carcinoma, and large cell undifferentiated carcinoma.

Recent studies from genomic and proteomic approaches have changed current view on cancer. From a disease previously perceived as manifested in the alteration of merely several genes, cancer is infact genetically very complex. Tumor cells frequently harbor an array of mutated genes, and each tumor mass can comprise hundreds of cancer cells with distinct cancer genotype.

Resistance to chemotherapeutics is known to be a serious obstacle to effective cancer therapy. Clinically relevant levels of resistance can emerge quickly after treatment. Beside intrinsic resistance, acquired or gradually developing resistance has been observed in tumors under therapy. Several mechanisms underlying resistance have been described, like decreased exposure to the drug, e.g. via reduced drug accumulation/drug-target interaction

or increased detoxification response, diminished cell-cycle effects, reduced apoptotic responses, or increased DNA-repair

Autoimmune destruction of pancreatic beta cells causes insulin-dependent diabetes mellitus or Type I diabetes. As a consequence of partial or complete loss of beta cells, little or no insulin is secreted by the pancreas. Most cells, with the exception of brain cells, require insulin for the uptake of glucose. Inadequate insulin production causes reduced glucose uptake and elevated blood glucose levels. Both reduced glucose uptake and high blood glucose levels are associated with a number of very serious health problems. In fact, without proper treatment, diabetes can be fatal. One conventional treatment for diabetes involves periodic administration of injectable exogenous insulin. This method has extended the life expectancy of millions of people with the disease.

However, blood glucose levels must be carefully monitored to ensure that the individual receives an appropriate amount of insulin. Too much insulin can cause blood glucose levels to drop to dangerously low levels. Too little insulin will result in elevated blood glucose levels. Even with careful monitoring of blood glucose levels, control of diet, and insulin injections, the health of the vast majority of individuals with diabetes is adversely impacted in some way. Hence, a need for efficient insulin delivery method that obviates these highlighted challenges.

1.4 JUSTIFICATION/SIGNIFICANCE OF STUDY

Gene targeting provides an important research tool for probing the complex interplay between the genome, the physiologic processes and the environment for manipulating the genome (Porteus and Carroll, 2005b). In gene targeting, an exogenously introduced DNA fragment replaces an endogenous segment of DNA by homologous recombination. This

process was reported in yeast more than 25 years ago and variations on this technique are now commonly used for evaluating gene function in that organism.

ZFN-mediated targeting holds promise of providing useful experimental tool for manipulating the mammalian genome for many of the potential applications. Given further development, the ZFN strategy may be applied in the treatment of human genetic diseases as well as in other disease like diabetes which this present work will be using ZFN to mediate the expression of fluorescent proteins that would be used by a biosensor to regulate blood sugar by triggering off precise release of insulin into blood stream, thereby obviating the use of manual insulin injection in diabetics.

The second aspect of this work will elucidate therapeutically important link between microRNA expressions, the prognosis of lung cancer, by demonstrating that anti-miR (microRNA inhibitor) molecules also sensitize lung cancer cells to chemotherapeutics. Studies show that up-regulation of miR-21 and miR-155 was frequently found in lung cancer samples. Again, it is hoped that antisense-mediated down-regulation of these micoRNAs could significantly inhibit migration and invasion and reverse the chemo- or radio-resistance of human lung cancer cells partially by targeting the tumor suppressor genes. This work will reveal novel roles of miR- 21 and miR-155 in the development of lung cancer and targeting miR-21and miR-155 will be a novel strategy for the treatment of lung cancer. The study will also help explain how microRNA becomes elevated or decreased in lung cancers and demonstrate that inhibition of miR-21 may have important therapeutic potential as a means to sensitize lung cancer cells.

1.5 SCOPE OF STUDY

The work was designed to address two thematic areas -Targeted gene therapy towards using microRNAs to sensitizing lung cancer cell to chemotherapy, which took place at Yale University, Department of Molecular, Cellular and Developmental Biology, USA, as well as ZFN-mediated targeted expression of GFP into AAVS1 site of dermal fibroblast aimed at targeting diabetes, which took place at Harvard Stem Cell Institute, Harvard University, USA.

At Yale Unversity, this work involved cell culture of H358 lung adenocarcinoma line, and A549 lung cancer cell lines, as well as normal lung cells FC 7333 3KT lines. It compared the miRNA expression profile of two known microRNAs (miR-21 and miR-155) in cancer and wild type lung cell lines using real time qPCR and then treated with 3 known chemotherapeutics used in the management of lung cancer namely Cisplatin, Etoposide and Paclitaxel and concluded by inhibiting over-expressed miRNA by transfecting the cancer cells with microRNA inhibitors called Antagomirs and then measured the level of proliferation with Sulforhodamine-B assay(SRB assay) which is essentially a survival assay.

This work was a part of collaboration between Harvard University, MIT and Boston Children Hospital aimed at producing engineered fluorescent proteins that would monitor blood sugar levels. Three different fluorescent proteins would be produced in total in the entire project, namely Green Fluorescent Protein, GFP, Red Fluorescent Protein, RFP and Yellow Fluorescent Protein, YFP which would change colour at Low blood sugar and normal blood sugar levels respectively. The green fluorescent protein would recognize high blood sugar which would be detected by Biosensor and mediate precise release of

insulin to control sugar metabolism in diabetics. Here, this work would focus on the building of the GFP and expressing it to target the AAVS1 site in chromosome -19 of the human dermal fibroblast guided by ZFN. This will be a proof of principle that would provide a platform subsequently for replacements with the other two fluorescent proteins, red fluorescent protein and yellow fluorescent protein (RFP and YFP).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MicroRNAs

2.1.1 RNAi and microRNA

RNA interference (RNAi) refers to the powerful phenomenon that double-stranded RNA (dsRNA) induces silencing of mRNAs that share sequence complimentarity with the dsRNA. Thus dsRNA is trigger and silencing of the mRNA is the consequence. The dsRNA trigger can be long (a few hundreds of nucleotides) or short (21 nucleotides)(Fire *et al.*, 1998).

The phenomenon of RNAi according to Hannon, (Hannon, 2002) was first discovered in the nematode worm *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA), which resulted in sequence-specific gene silencing (Fire *et al.*, 1998). Following on from the studies of Guo and Kemphues, who had found that sense RNA was as effective as antisense RNA for suppressing gene expression in worms, Fire and coworker (Fire *et al.*, 1998) attempted the use of antisense RNA as an approach to inhibit gene expression. Their goal was to test the synergy of sense and antisense RNAs, and they eventually found that the dsRNA mixture was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs alone (Hannon, 2002).

In order to function the long dsRNA must be primarily processed in cells into 21-nucleotide (nt) short/small RNA by an enzyme termed Dicer. As such, the long dsRNA is the substrate of Dice while its product is 21 nt short/small RNA called small interfering RNA (siRNA) (Aravin and Tuschl, 2005). It is now known that Dicer is a specific enzyme of RNAse III family that cut dsRNA. Dicer commits further processing of the originally

double-stranded SiRNA to a protein termed Agonaute (AGO) to form RNA induced silencing complex (RISC) in which only one strand of the siRNA remains while the other strand is cleaved by the AGO and later degraded. RISC will in turn find its target mRNA solely by base pairing and then cleaves the target mRNA into two fragments which are subsequently destroyed in cells, leading to silencing of the target mRNA(Hannon, 2002).

2.1.2 MicroRNAs a new class of gene regulators

Slack and colleague (Slack and Weidhaas, 2006) submitted that miRNAs are 22 nucleotide (nt) noncoding RNAs that belong to a novel class of regulatory molecules found in plants and animals, which control essential developmental processes such as cellular differentiation, proliferation, apoptosis and metabolism (Bartel, 2004). miRNAs control gene expression by binding to complementary sites on target messenger (m)RNA transcripts and repressing their translation, stability or both. miRNAs that bind to exactly complementary mRNA sequences induce the RNA-mediated interference (RNAi) pathway. Targets of mRNA are cleaved by ribonucleases in the RNA-induced silencing complex (RISC). This mechanism of miRNA-mediated gene silencing has been observed mainly in plants (Reinhart *et al.*, 2002), Bartel and his colleagues, equally observed it in animal (Yekta *et al.*, 2004). Reports from a number of groups, showed that concerning the second mechanism, miRNAs that bind to imperfect complementary sites on 3´-untranslated regions (UTRs) of mRNA transcripts direct gene regulation at the post-transcriptional level, but do not cleave their mRNA targets (Lee *et al.*, 1993;Moss *et al.*, 1997;Slack *et al.*, 2000).

2.1.3 MicroRNA Biogenesis

MicroRNA (miRNA) is a novel family of endogenous small RNA (~21 nt) present in both plants and animals. The miRNA genes are located often in intergenic regions (between

coding genes in the genome) and sometimes in the intron of a coding gene. Like mRNA, miRNA is initially transcribed by RNA polymerase II (pol II) into primary miRNA (pri-miRNA) that is also modified to have a cap structure at the 5' end and a poly(A) tail at the 3'end.

The pri-miRNAs are processed in the nucleus by the RNase III enzyme, Drosha, and the double-stranded- RNA-binding protein, Pasha (also known as DiGeorge syndrome critical region 8 (DGCR8), into ~70-nucleotide pre-miRNAs, which fold into imperfect stem-loop structures (Lagos-Quintana *et al.*, 2001). The pre-miRNAs are then exported into the cytoplasm by the RAN GTP-dependent transporter exportin-5 and undergo an additional processing step in which a double-stranded RNA of ~22 nucleotides in length, referred to as the miRNA:miRNA***** duplex, is excised from the pre-miRNA hairpin by another RNAse III enzyme, Dicer (Lagos-Quintana *et al.*, 2001;Lagos-Quintana *et al.*, 2003). Subsequently, the **miRNA:miRNA*** duplex is incorporated into the miRISC complex. Slack and Weidhaas (2006), pointed out the mature miRNA strand is preferentially retained in the functional miRISC complex and negatively regulates its target genes.

In plants, pri-miRNA is processed first into miRNA precursor (pre-miRNA) and then mature miRNA duplex by Dicer in nucleus. The mature miRNA duplex is then transported by Hasty, a miRNA transporter, from nucleus into cytoplasm where the mature miRNA duplex is assembled into RISC termed miRNA associated RISC (miRISC). Similarly, miRISC finds its specific target mRNA by base pairing and then cleaves and destroys the target mRNA in cells. Consequently, specific cellular mRNA is silenced by specific miRNA. In plants, hundreds of miRNAs have been cloned and their target genes were predicted. Many plant miRNA target genes are transcription factors that control plant development and metabolism (Bartel and Bartel, 2003).

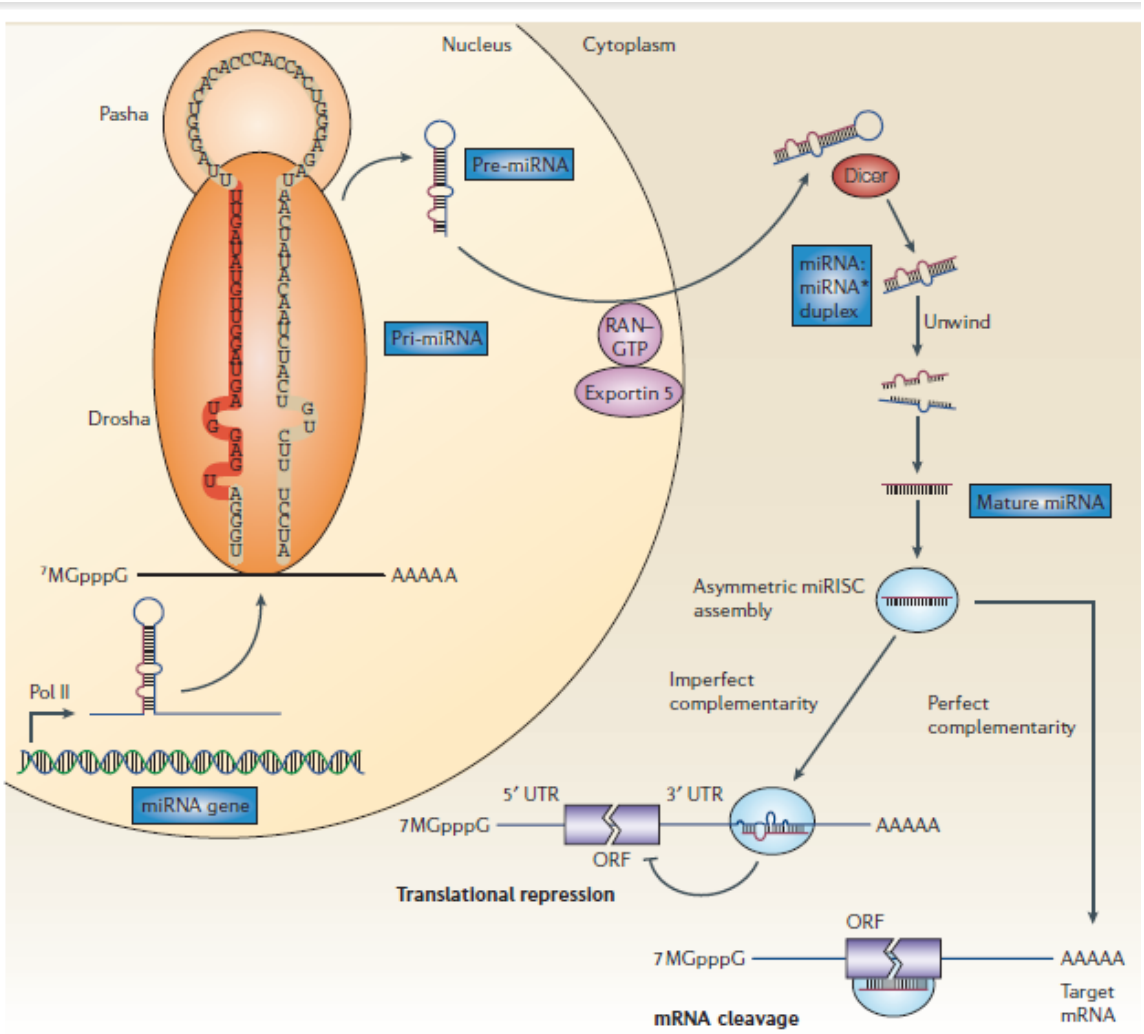


Figure 2.1. 'The biogenesis of microRNAs. MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (7MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human let-7a-1 miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.) RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22- nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target'. (Esquela-Kerscher and Slack, 2006).

2.1.3.1 Genomics and Biogenesis of MIR-21

According to published sequence data in miRbase, The MIR-21 gene is located in an intergenic region. The length of the gene is reported as 3433 nucleotides long. It overlaps with the 3' UTR end of the Transmembrane Protein 49 (TMEM 49) (also known as Human Vacuole Membrane Protein 1, VMP-1). It is suggested that RNA Pol II is the most likely enzyme involved in miRNA transcription. However, studies provide evidences for RNA Pol III dependent transcription of few miRNAs interspersed among repetitive Alu elements (Bartel, 2004).

For MIR-21, the major RNA polymerase is believed to be RNA Pol II due to the presence of 5' cap and 3' poly (A) tail of the pri-MIR-21. That belief is strengthened by Chromatin immunoprecipitation (ChIP) analysis of upstream sequences of MIR-21 which showed enrichment for Pol II order that Pol III (Buscaglia and Li, 2011).

MIR-21 gene was reported to have a 5' promoter element. 1008 bp DNA fragment for MIR-21 gene was cloned (-959 to +49 relative to T1 transcription site). Analysis of the sequence showed a candidate "CCAAT" box transcription control element located approximately about 200 nt upstream of the T1 site. T1 transcription site was found to be located in a sequence similar to "TATA" box ATAAACCAAGGCTCTTACCATAGCTG). To test the activity of the element, about 1kb DNA fragment was inserted into the 5' end of firefly luciferase indicator gene and transfected into 293T cells. The sense orientation insert, unlike antisense, induced luciferase activity (Aigner, 2011).

Pri-MIR-21 gene was reported to have two transcription sites, T1 and T2. T1was shown to be the minor transcription site and T2 reported as the major transcription start site. Based on the data of pmiR-21-luc expression plasmid, the endogenous pri-MIR-21 was suggested

to utilize T1 and T2 sites for initiation of transcription. The maturation of miRNA gene involves sequential process (Bonci, 2010).

2.1.4 Pri-miRNA

The miRNA genes are first transcribed in nucleus as long primary transcripts called pri-miRNA. The primary transcript for MIR-21 is found to be 3433-nt long.

For localization of the pri-MIR-21 transcript, total, nuclear and cytoplasmic RNA fractions from HeLa cells were oligo-dT primed and reverse transcribed into cDNA. Pri-MIR-21 transcript was found mainly in the nucleus as well as modest levels in the cytoplasm (Ahmed *et al.*, 2011).

Sequence: NCBI cDNA clone: BC053563.

Length: 3389bp

2.1.5 Pre-miRNA-21

The primary transcripts of microRNAs are processed by enzymatic microprocessor Drosha (RNase III enzyme) and DGCR8 (dsRNA binding protein) from their 3' and 5' cleavage sites into an intermediate stem-loop precursor or pre-miRNA in the nucleus(Bartel, 2004).

The precursor of MIR-21 is 72 bases long (pre-MIR-21), forms a secondary structure, and contains the mature miRNA sequence, stem and terminal loop structures with 2-nt 3'overhang. The precursor is then transferred from nucleus to cytoplasm by the enzyme Exportin 5. In cytoplasm, a second RNase III enzyme, Dicer, removes terminal loop generating about 20-bp RNA duplex.

Length: 72 bases

Sequence:

UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACAC
CAGUCGAUGGGCUGUCUGACA.

2.1.6 Mature MIR-21

The mature miRNA forms one strand of the RNA duplex. One strand is degraded and other is incorporated in to a protein complex, RNA induced silencing complex (RISC), targeting a partially complementary target mRNA. MIR-21 is 22 nucleotides long (Cheng *et al.*, 2010).

Sequence: UAGCUUAUCAGACUGAUGUUGA .

2.2.7 The Argonaute (AGO) Protein Family

2.2.7.1 Domain Structures and Functions

It is believed that Argonaute (AGO) family proteins are the only small RNA binding proteins identified in all RISCs and RITS complexes. Most AGO proteins are about 100 kDa and highly basic and have two unique signature domains: the PAZ domain shared by Piwi, Argonaute and Zwille/Pinhead proteins and the PIWI domain initially found in Piwi protein from *Drosophila*.

As reviewed by Slack and weidas (Slack *et al.*, 2006), members of the PAZ family of proteins such as Argonaute, have also been shown to co-immunoprecipate and complex with miRNAs and to be involved in miRNA processing (Carmell *et al.*, 2002). There is evidence that genes encoding Argonaute proteins are linked to cancer. hAgo3, hAgo1 and hAgo4, which reside in region 1p34–35, are often lost in Wilms' tumors, and Hiwi, is

located on chromosome 12q24.33, which has been linked to the development of testicular germ cell tumors (Carmell *et al.*, 2002),(Esquela-Kerscher and Slack, 2004)

2.2.1 MicroRNAs and Cancer

'The central dogma' of molecular biology (set of rules which describe how the blueprint of life is encoded on DNA, passaged through RNA and eventually read out as protein) drove the understanding of biology for many years (Slack *et al.*, 2006). Proteins have long been known to be the workhorses of the cell and it is proteins that in turn regulate the use of DNA and RNA. At least one of these central tenets has required a revision in the past decade or so, as biologists begin to appreciate the regulatory roles played by RNAs themselves. Long relegated to the passive role of messenger for DNA, it is now clear that RNA performs many of the functions once thought to be controlled solely by proteins. One class of regulatory RNAs leading the charge is made up of very small molecules called micro (mi)RNAs (Ambros *et al.*, 2003). These miRNAs control gene expression and may protect man from the ravages of viral infections and cancer, and other diseases. Their recent discovery excited the biological community as it tries to understand how small RNAs work in gene regulation, and determine how to harness this technology to provide therapies for human diseases such as cancer.

Cancer has recently surpassed heart disease as the leading cause of death in Americans under the age of 80 years. This staggering statistic is highlighted in the case of lung cancer.

Lung cancer is the leading cause of cancer deaths in the USA with approximately 170,000 newly diagnosed cases each year (Forgacs *et al.*, 2001a). Despite the use of surgery, chemotherapy and radiation in the treatment of lung cancer, the survival rate for patients remains extremely poor. Misregulation of genes that control cell-cycle and cell-fate determination often contributes to cancer.

The expression signatures of miRNA and their targets in cancer therapy were extensively evaluated in breast, prostate lung, gastric, ovarian cancers, and colon,(Barbarotto *et al.*, 2008;Calin and Croce, 2006;Dai *et al.*, 2012;Lim *et al.*, 2005). Miller and colleagues reported a relationship between increased tamoxifen resistance and reduced level of p27, with increased miR-221/222 expression in breast cancer (Miller *et al.*, 2008). Furthermore, Kovalchuk and co-workers, showed that miR-451 was involved in the regulation of multidrug resistance 1 (MDR1) expression in breast cancer cells (Kovalchuk *et al.*, 2008). Likewise Xia et al.,(2008) observed that in gastric cancer cells, miR-15b and miR-16 directly regulated the expression Bcl-2, hence modulating the susceptibility of cancer cells to anticancer drug-induced apoptosis (Xia *et al.*, 2008). In their study, Zhu and others noticed that the expression of miRNA-181b was significantly downregulated in gastric and lung cancer cells with multidrug resistance, which in turn was connected with robust expression of Bcl-2 (Zhu *et al.*, 2010).

The following miRNAs, let-7e, miR-30c, miR-125b, miR-130a, and miR-335, were found by Eitan and colleagues to be diversely expressed in resistant cell lines in Ovarian Cancer (Eitan *et al.*, 2009).

2.2.2 The miRNA-related Genes and Cancer

Some miRNA-machinery has been implicated in carcinoenesis. Karube, *et al.* showed that the expression of Dicer was downregulated in lung cancer (Karube *et al.*, 2005). They further examined RNA expression levels of *DICER* and *DROSHA* in 67 non-small-cell lung cancer samples. They found that reduced expression of *DICER* correlates with shortened post-operative survival, which indicates that Dicer might be able to prevent the

transformation of lung cancer (Karube *et al.*, 2005). It is believed that since Dicer is associated with heterochromatin maintenance and centromeric silencing, reduced protein levels might directly result in genomic instability and lead to tumour formation (Bernstein *et al.*, 2003;Kanellopoulou *et al.*, 2005)

2.2.3 Therapeutic Importance of miRNAs in Cancer Management

The role of miRNAs in various facets of human tumorigenesis, (such as cell proliferation, differentiation, invasion, apoptosis, angiogenesis, migration, and motility) begs the question whether reverting these anomalies of the miRNome can be effectively harnessed for therapeutic gains? Promising preclinical data from many groups encourage this hypothesis and provide the biological enthusiasm for clinical studies in this direction. Re-introduction of miRNAs down-regulated in cancer (e.g. miR-15a and miR-16 in BCL2 positive CLL) and/or silencing of miRNAs up-regulated in the tumor (e.g. miR-34 and *let-7* in lung cancer) may drive apoptosis in cancer cells and lead to a desirable therapeutic outcome (Slack *et al.*, 2006).

2.2.4 Challenges of miRNA Therapeutics

Before miRNA therapeutics becomes a reality, several issues need to be resolved.

Firstly, there is a need to know the full spectrum of targets and effects that a given miRNA has on a given genome. Calin and colleagues estimated that a single miRNA cluster (such as, the miR-15a/16-1 cluster) is able to affect, directly and indirectly, the expression of about 14 per cent of the whole human genome (Calin *et al.*, 2008). It is obvious now that each miRNA is able to target both oncogenes, OGs and Tumor suppressor genes, TSGs, and it is equally apparent that the phenotype induced by the external manipulation of a miRNA is the result of the combined targeting effect on several genes. In view of this,

preclinical research would aim at fully clarifying this aspect before proceeding to clinical application (Fanini *et al.*, 2011).

Secondly, there is need to address tumor-specific delivery of the miRNAs of interest. This has continued to taunt the whole field of gene therapy- delivery, delivery, delivery! The advent of nanoparticles, able to target tumor-specific antigens will hopefully address this concern and allow tumor specific targeting.

It is equally important to understand how the modulation of miRNA expression can leverage the existing anti-cancer therapies (chemo-therapy, radio-therapy, hormonal therapy)?

Finally, studies have shown that miRNAs can restore sensitivity to current therapeutic options to which the tumors became resistant, and this fuels optimism on miRNA-based combination therapy.

2.2.5 MicroRNA in Solid Cancers

2.2.5.1 Lung cancer

As noted by Forgas and co workers, Lung cancer continues to be the leading cause of cancer death globally. Using microarray data, Gao et al. compared miRNAs expression profiles in primary squamous cell lung carcinoma with normal cells and highlighted miRNA potential relevance to clinical events and patient survival periods. They observed that miR-21 was upregulated in nearly 75 per cent of cancer specimens and that this signature was significantly correlated with shortened survival (Gao *et al.*, 2011) Using similar procedure another group correlated miRNA expression profiles with survival of lung cancer, showing that high miR-155 and low let-7a-2 expression were correlated with poor survival. They further found a molecular signature for a subset of lung cancer:

they discovered six differentially expressed miRNA in adenocarcinoma and squamous cell cancer (namely: miR-205, miR-99b, mir-203, mir-202, mir-102, and mir-204-Prec). They noted that the expression of miR-99b and miR-102 were higher in adenocarcinoma (Volinia *et al.*, 2006a).

Another five-microRNA signature (let-7a, miR-21, miR-137, miR-372, miR-182*) associated with survival and cancer relapse in NSCLC (non-small cell lung cancer) patients were reported by another group (Abdurakhmonov *et al.*, 2008). Lebanony and colleagues used microarray data to show that miR-205 is another specific marker for squamous cell lung carcinoma, they found a strong association between the expression levels of miR-205 and squamous cell lung carcinoma (Barshack *et al.*, 2010).

Furthermore, miR-31 according to Anand and co-workers is an oncogenic miRNA targeting mRNAs encoding two anti-tumor proteins, LATS2 (large tumor-suppressor 2) and PPP2R2A (PP2A regulatory subunit B alpha isoform) (Anand *et al.*, 2010). Choudhry et al, (2011) discovered that miR-7 drives EGFR-mediated tumorigenesis in lung cancer by targeting ERF (Ets transcriptional repressor) hence modulating cell growth (Choudhry and Catto, 2011). Interestingly, miR-7 tends to have dual function as oncogene/tumor-suppressor miRNA. Xiong and colleagues showed that over-expression of miR-7 in A549 (NSCLC) cells inhibits cells proliferation and induces apoptosis by targeting anti-tumor Bcl-2 (Xiong *et al.*, 2011).

Wang and others observed that miR-451 displays a tumor-suppressor role in lung cancer. They showed that in addition to being the most downregulated in NSCLC tissues, that it regulates survival of cells partially through the downregulation of the oncogene RAB14 (Ras-related protein 14) (Bian *et al.*, 2011).

2.2.5.2 Breast Cancer.

Breast cancer is the second leading cause of cancer deaths in the developed world and the most commonly diagnosed cancer in women (Bonev *et al.*, 2011). Croce group conducted miRNA expression profile study for breast cancer and found 13 miRNAs differentially expressed between tumor and normal tissues: among the upregulated ones there were oncogenic miR-21 and miR-155, while miR-10b, let-7 miR-125b, miR-145 and miR-205 were found down-regulated (Calin *et al.*, 2005). The latter directly targets HER3 receptor and blocks the activation of downstream Akt, inhibiting cell proliferation. Moreover, miR-205 sensitizes cells to Gefitinib and Lapatinib, two tyrosine-kinase inhibitors, promoting apoptosis (Iorio and Croce, 2009).

Akao et al,(2011), found that miR-301 has an oncogenic role in breast tumor by targeting FOXF2, BBC3, PTEN and COL2A1. Its upregulation promotes proliferation, migration, invasion and tumor formation. Moreover, by cooperating with its host gene SKA2, miR-301 promotes the aggressive breast cancer phenotype with nodal or distant relapses (Akao *et al.*, 2011).

Heyn and co-workers identified miR-335 as a tumor-suppressor gene. It controls different factors of the upstream BRCA1 regulatory pathway (such as ERa, IGF1R, SP1), inducing an upregulation of the tumor suppressor gene BRCA1 (Heyn *et al.*, 2011).

2.2.5.3 Colorectal Cancer

Schetter *et al*., (2008) performed miRNA microarray expression profiling comparing 84 pairs of tumors (colon adenocarcinoma) and adjacent non-tumoral tissues (Schetter et al., 2008). They found 37 differentially expressed miRNAs; among them miR-20a, -21, -106, -181b and -203 levels were higher in tumor specimens. The overexpression of miR-21 and

its role in tumor proliferation in several kinds of cancers has already been described before. Also miR-20a belongs to the miR-17-92 cluster, whose overexpression promotes cell proliferation (Hayashita *et al.*, 2005) and increased tumor size.

One of the most recent tumor suppressor miRNAs found in colorectal cancer is miR-137. Balaguer et al. reported that this miRNA is constitutively expressed in the normal colonic epithelium but during the early events of colorectal carcinogenesis it is silenced through promoter hyper-methylation. Moreover, its re-expression *in vitro* inhibits cell proliferation in a cell specific manner. These findings suggest a prognostic role for miR-137 (Balaguer *et al.*, 2010).

It was demonstrated that miR-183 has an oncogenic role in colon cancer (but also in synovial sarcoma and rhabdomyosarcoma) through its regulation of the expression levels of 2 tumor suppressor genes, EGR1 and PTEN. The authors also provided evidence that knockdown of miR-183 affects cellular migration and they suggest that pharmaceutical intervention on tumor characterized by the upregulation of miR-183 may be useful as anti-cancer therapy (Chen et al., 2010).

2.2.5.4 Hepatocellular carcinoma

Hepatocellular carcinoma is one of the most common malignant tumors. Murakami et al., (2006) analysed the miRNA expression profiles in 25 specimens of hepatocellular carcinoma compared with adjacent non-tumoral tissues and nine chronic hepatitis specimens (Murakami *et al.*, 2006). “miR-222, miR-17-92 and miR-106a exhibited higher expression in tumor tissues than in the normal ones and were found associated with the tumor differentiation status.

They found a 12 miRNA signature that characterizes tumor progression starting from normal liver, to cirrhosis to full blown tumor. Among them, miR-21, miR-221/222, miR-34a and miR-224 were found overexpressed in the progression signature. Other tumor suppressor miRNAs are: miR-122, normally downregulated in hepatocellular carcinoma, whose overexpression induces apoptosis and cell cycle arrest through targeting of BCLW; miR-198, which inhibits migration and invasion in a c-MET dependent manner (Akao *et al.*, 2011); miR-125b, which suppresses tumor cell growth *in vitro* and *in vivo* and induces cell cycle arrest at G1/S acting as a tumor suppressor gene through the suppression of LIN28B (Bates *et al.*, 2010), a promoter of cell proliferation and metastasis through regulation of c-MYC and E-Cadherin" (Ai *et al.*, 2010).

2.2.6 Lung Cancer

Lung cancer is the leading cause of cancer deaths in the USA with approximately 170,000 newly diagnosed cases each year (Forgacs *et al.*, 2001a). Tobacco smoking is accepted as the major cause of lung cancer. Lung cancer is classified into two major types, Small Cell Lung Cancer, SCLC and Non-small cell lung cancer, NSCLC. Squamous cell carcinoma, adenocarcinoma, and large cell carcinoma are the major histological types of NSCLC.

Many studies have shown that multiple genetic changes (about 10 to 20) are found in clinically evident lung cancers involving known tumor suppressor genes and several dominant oncogenes, including *myc* family members, K-*ras*, *TP53*, *RB*, *p16-CDKN2*, and candidate tumor suppressor gene regions on chromosomes 3p, 5q22 (*APC-MCC* region), 8p, 11p, and others (Forgacs *et al*.,2001).

Despite the use of surgery, chemotherapy and radiation in the treatment of lung cancer, the survival rate for patients remains extremely poor. Misregulation of genes that control cell-cycle and cell-fate determination often contributes to cancer.

Earlier in the 20th century lung cancer was a rare malignancy. At the moment, it is occurring in epidemic proportions worldwide. It has become the most common cause of death from malignancy in the United States, the United Kingdom, and a number of other countries. The rate of lung cancer in women in the United States is increasing, while the rate in males is beginning to decline, especially in those less than 50 yr of age.

As reviewed by Forgacs (Forgacs *et al.*, 2001b), expression of gastrin releasing peptide receptor in human respiratory tissues is associated with a proliferative response of bronchial epithelial cells to GRP/BN and with prolonged cigarette smoking and may be a risk factor for development of lung cancer. There is significant evidence that women are more susceptible to tobacco smoking leading to lung cancer than men. Thus, it is of interest that the GRP receptor is located on the X chromosome but does not undergo X inactivation so that women have two but men only one gene for the GRP receptor. Women express GRP receptor more frequently than men in the absence of smoking or after shorter smoking histories.

Women account for 40 per cent of all lung cancer cases, and lung cancer has surpassed breast cancer as the most common cause of death from malignancy. Smoking is the predominant risk factor associated with lung cancer. The risk is related to the number of cigarettes smoked, age at starting smoking, and the duration of smoking (Gao *et al.*, 2011).

It is estimated that smoking accounts for 80-90 per cent of all cases of lung cancer. The vast majority of cases could be prevented by never smoking, and the risk of developing lung cancer could be lessened by smoking cessation. Lung cancer is easier to prevent than cure.

2.2.6.1 Histological Classification of Lung Cancer

According to American Thoracic Society, the major histologic subtypes of lung cancer include squamous cell carcinoma, adenocarcinoma, SCLC, and large-cell carcinoma. Now adenocarcinoma has surpassed squamous cell carcinoma as the most common histologic subtype of lung cancer in the United States.

Squamous cell carcinoma is recognized by the histologic features of intercellular bridging, squamous pearl formation, and individual cell keratinization. Adenocarcinomas may take the form of several histologic subtypes, including acinar (gland forming), papillary, bronchioloalveolar, and solid with mucus formation. Cases with mixed adeno- and squamous differentiation are called adenosquamous carcinoma. Large-cell carcinoma is a poorly differentiated NSCLC. It is a diagnosis of exclusion made after ruling out the presence of adenocarcinomatous or squamous differentiation. Large-cell carcinoma can be difficult to separate from poorly differentiated squamous cell carcinomas, adenocarcinomas, or small-cell carcinomas. Histochemical stains such as mucicarmine or Periodic-acid Schiff after diastase digestion may be necessary to separate solid adenocarcinomas with mucus formation from some large cell carcinomas. Bronchioloalveolar carcinoma (BAC) is an adenocarcinoma of the lung that grows in a lepidic fashion along the alveolar septa.

2.2.7 Oncogenic microRNA.

2.2.7.1 MiR-21

Liu and workers stated that "miR-21 is one of the well characterized miRNAs and overexpressed in various solid tumors including prostate cancers" (Volinia *et al.*, 2006b), (Liu *et al.*, 2011). A number of reports from many groups show that "several tumor

suppressors including phosphatase and tensin homolog known to be deleted on chromosome ten (PTEN) (Meng *et al.*, 2007a), tumor suppressor gene tropomyosin 1 (Zhu *et al.*, 2007), programmed cell death 4 (Asangani *et al.*, 2008;Frankel *et al.*, 2008), maspin (Zhu *et al.*, 2008), and matrix metalloproteinases inhibitors RECK and TIMP3 (Gabriely *et al.*, 2008) were targets of miR-21, the whole data put together suggest that miR-21 is an important oncogenic miRNA which is closely related to tumor growth and metastasis. The expression of miR-21 is also associated according to Blower and others, with prognosis, chemosensitivity and therapeutic outcome in colon adenocarcinoma"(Blower *et al.*, 2008;Schetter *et al.*, 2008).

Schetter and colleagues particularly stated that they "selected three microRNAs previously implicated in cancer, miR-21, let-7i, and miR-16, and tested the effect of their expression on the potencies of a number of compounds with anticancer activity. All microRNAs are thought to target multiple mRNAs that are known to be relevant to cancer biology; let-7i targets RAS, mir-16 targets BCL2, and miR-21 targets PTEN. However, new targets continue to be discovered.

Furthermore, these findings show that those microRNAs may have multiple mechanisms of action. MiR-21, in particular, is known to be highly expressed in various cancer types. MicroRNA functions can be cell context and tissue-dependent. For example, inhibition of miR-21using anti- sense oligonucleotides increased the growth of HeLa cells (derived from cervical cancer) but did not significantly affect the growth of A549 cells (non–small cell lung cancer; (Lu *et al.*, 2005). The inhibition of miR-21 resulted in increased apoptosis in human glioblastoma cells.

As noted by Cheng and colleagues, (Chen *et al.*, 2012), micro-RNA-21 promoter contains highly conserved regions with consensus binding sites for several transcriptional factors,

including activator protein 1 (AP-1) and factors of the forkhead family (FOXO), such as FOXO3a.

Working with Side population (SP) cells, Misawa and coworkers, showed that microRNA-21and its upstream regulator activator protein-I (AP-1), composed of c-Jun and c-Fos family transcription factor were frequently upregulated in SP cells. 'The side population (SP) of cancer cells is a minor population of cells that were identified in a variety of cancers and known to harbor many cancer stem cell (CSC)-like properties, such as self-renewal potential, tumour-forming capacity, and chemoresistant phenotype. CSCs are regarded as the root of cancer origin and recurrence Sp cells' (Misawa *et al.*, 2010a).

Misawa (Misawa *et al.*, 2010b) further asserted that downregulation of tumor suppressor, programmed cell death 4, one of the miR-21 target gene products, confirmed miR-21 overexpression in SP cells. They reported that the treatment of the cells with the 'AP-1 inhibitor SP600125 attenuated miR-21 levels and increased topotecan sensitivity'. Interestingly, they further showed that, 'specific inhibition of miR-21 by an anti-miR-21 locked nucleic acid (LNA), increased drug sensitivity and decreased colony forming ability, thereby underscoring the role of miR-21 in maintenance of the chemoresistant phenotype of SP cells. They believed, giving their findings that 'targeting miR-21 may provide a new strategy for cancer therapy by impairing resistance to chemotherapy in Cancer stem cells (CSCs)'.

In another study, Talotta and fellows (Talotta *et al.*, 2009a), pointed out that, 'homodimerization or heterodimerization of epidermal growth factor receptor may activate the ras/ mitogen-activated protein kinase/mitogen-activated protein kinase pathway and then enhance the AP-1 activity and subsequently stimulate the transcription of pri-miR-21 (Talotta *et al.*, 2009b).

Wang et.al, reported that, FOXO transcription factor represses the promoter activity of miR-21, and phosphorylated activation of Akt may relieve the repression of the promoter and upregulate the function of miR-21(Wang and Li, 2010). The targets of miR-21 are also variable. Phosphatase and tensin homolog, a key tumor suppressor that modulates cell growth, division, and death, had been noted to be an important target of miR-21; however, the influence of miR-21 on PTEN is inconsistent in the literature. Micro-RNA-21 had been found to induce downregulation of PTEN and the subsequent up-regulation of cell proliferation and drug resistance in human hepatoma cells and bladder cancer cells (Meng *et al.*, 2007b;Tao *et al.*, 2011a). As observed by other workers, "the induction of chemoresistance by miR-21 in cancer cells had been demonstrated to be related to the suppression of PTEN and program cell death 4 (PDCD4) expression, enhancement of the phosphoinositide kinase 3/Akt, increased Bcl-2, decreased Bax/Bcl-2 ratio and caspase 3 activity, and reduction of the G2/M arrest and apoptosis (Bai *et al.*, 2011a;Giovannetti *et al.*, 2010;Gong *et al.*, 2011;Valeri *et al.*, 2010;Yang *et al.*, 2013a).

MiR-21 is thought to play a significant role in the drug resistance to cisplatin in the neuroblastoma cells. Many reports suggest, miR-21 is important for the resistance to various kinds of drugs in different types of cancer cells, including resistance to doxorubicin in bladder cancer cells (Tao *et al.*, 2011b); to trastuzumab, topotecan, or taxol in breast cancer cells (Corcoran *et al.*, 2011;Gong *et al.*, 2011); to arabinosylcytosine or daunorubicin in leukemia cells (Bai *et al.*, 2013;Bai *et al.*, 2011b); to 5-fluorouracil in colorectal cancer cells (Valeri *et al.*, 2010); to interferon α/5-fluorouracil in hepatocellular carcinoma cells (Tomimaru *et al.*, 2010); to VM-26 or temozolomide in glioblastoma cells and to gemcitabine in pancreatic ductal carcinoma cells (Giovannetti *et al.*, 2010).

Higher levels of miR‑21 are reported in the serum and tumours of patients with cancer(Medina *et al.*, 2010b). Upon returning miR‑21 to endogenous levels the mouse tumours disappeared. Notably, this was the first report indicating the addiction of tumours to a single oncogenic miRNA (termed ‘oncomir addiction’). Ubiquitous expression of miR‑21, fourfold to sixfold over endogenous levels, resulted in no obvious phenotypes; however, miR‑21 overexpression could potentiate the phenotype of mice with a latent KrasG12D allele (KrasLA2), a constitutively activated version of the KRAS proto-oncoprotein. Doubly transgenic animals had an increased lung tumour burden relative to the KrasLA2 mice, but no increase in the rate of conversion from adenoma to adenocarcinoma. By contrast, the lung tumour burden was decreased in KrasLA2; miR‑21animals, relative to the KrasLA2 controls. Contrary to earlier report (Hatley et al., 2010), Liu and co-workers (Liu et al., 2011) showed that miR‑21 is involved in the later stages of tumorigenesis and not in tumour promotion, as it has no effect on tumorigenesis in the absence of oncogenic KRAS. Another work (Zhang et al., 2010) reported an oncogenic role for miR‑21 in skin carcinogenesis using DMBA–TPA model, that study, wildtype animals developed early skin papillomas that progressed into invasive carcinomas. In identically treated mir‑21 animals, papilloma multiplicity and incidence were reduced. The molecular explanation probably involves increased expression of the pro-apoptotic miR‑21 target genes sprouty homologue 1 (SPRY1), PTEN and programmed cell death protein 4 (PDCD4), which negatively regulate the RAF, PI3K and RAL guanine-nucleotide dissociation stimulator (RALGDS) pro-survival signalling pathways, respectively. In accordance, mir‑21 loss was associated with enhanced cellular apoptosis and a moderate reduction in proliferation. These studies, combined with human

tissue data and cell culture experiments, confirm that miR‑21 is an oncogene and provide a rationale for the therapeutic inhibition of miR‑21.

According to Liu and co-workers (Liu et al., 2012), MiR-21 is a miRNA which has been reported to be overexpressed in many human malignancies including NSCLC. In their previous studies, they showed that upregulation of miR-21 in NSCLC serum is correlated with TNM stage and lymph node metastasis (Wang et al., 2011; Fu et al., 2011). Furthermore, the status of serum miR- 21 expressions is found to be an independent prognostic factor for NSCLC patients. They further found that miR- 21 could regulate Adriamycin (ADR) resistance of breast cancer cells, at least in part, by targeting the tumor suppressor gene PTEN. However, the associations of miR-21 expression with malignant phenotypes of NSCLC are still poorly understood.(Stahlhut Espinosa and Slack, 2006)

2.2.7.2 miR-155

MicroRNA-155 is encoded in B-cell integration cluster (BIC), a conserved region of the non-coding gene (Medina and Slack, 2008). BIC was originally recognized as a common integration site for the avian leucosis virus, inducing B-cell lymphomas together with MYC (Zhang, Nie, & Tam, 2008). Overexpression of miR-155 has been observed in both hematological (Eis et al., 2005), (Kluiver et al., 2005) and solid tumors (Zheng et al., 2012). miR-155 is regarded as “masterregulator” of many biological processes. It is a prominent microRNA (miRNA) that regulates genes involved in immunity and cancer-related pathways. MiR-155 is said to be overexpressed in lung cancer, which correlates with poor patient prognosis. It is unclear how miR-155 becomes increased in lung cancers and how this increase contributes to reduced patient survival.

The crucial role of miR-155 is further underscored by independent generation of transgenic, mir-155-overexpressing mice and mir-155-knockout mice which demonstrated that this gene is important in the immune system. Wild-type levels of miR-155 are essential for preserving normal immune system function, including the maintenance of both major classes of cells (B and T lymphocytes) of the adaptive immune response and dendritic cells, which are involved in the innate immune response. Although miR-155-knockout mice are immunocompromised owing to defects in these cell lineages, overexpression of miR-155 specifically in the B cell lineage results in pre-leukaemic pre-B cell proliferation in the spleen and bone marrow, followed later in life by B cell malignancy.

2.2.7.3 Tumour Suppressor miRNAs

If several miRNAs are known for their pro-oncogenic role, then other miRNAs represent their counterpart by acting as a TSG. Their silencing due to mutations, chromosomal rearrangements or to promoter methylation (Calin *et al.*, 2002; Calin et al., 2005; Ishii and Saito, 2006; Arisawa *et al*., 2007) contributes to the initiation and progression of cancer. MiR-15a and miR-16-1 represent a typical example of TSG miRNA. Encoded as a cluster at the level of chromosome 13q14.3, a region frequently deleted in chronic lymphocytic leukemia (CLL) (Bullrich *et al.,* 2001), miR-15a and -16 display expression levels inversely correlated to the *BCL2* ones. These miRNAs in fact induce apoptosis in leukemic cells by directly targeting the anti-apoptotic gene (Calin *et al.,* 2005). Moreover, it has been demonstrated that miR-15a and -16 exert a tumor-suppressor role also in prostate cancer by targeting BCL2, CCND1(cyclin D1) and WNT3A (encoding a protein which promotes cell survival, proliferation and invasion) (Bonci *et al.,* 2008). Taken together,

these findings harbor therapeutic implications and bring new insights to the comprehension and treatment of cancer.

Chromosome 7q32 hosts the miR-29 family (comprising miR-29a, -29b and -29c), which is downregulated in lung cancer, CLL, AML, breast cancer and cholangiocarcinoma (Calin *et al.,* 2005;Calin *et al.,* 2008).Â It has been demonstrated that in lung cancer the expression of miR-29 family members is inversely correlated with DNMT3A and -3B (DNA methyltransferases 3A and 3B) and that these miRNAs directly target these enzymes, inducing global hypomethylation of tumoral cells (Calin *et al.,* 2007) and reactivation of methylation-silenced TSGs such as WWOX, FHIT, MCL1 and TCL1 (Costinean *et al.,* 2006; Mott *et al.,* 2007).

Among the tumor suppressor miRNAs there is the let-7 family.Johnson et al. demonstrated an inverse correlation between the expression of the let-7 family members and the expression of the oncogene RAS in lung cancer tissue (Adai *et al.,* 2005). Let-7 family targets as well other oncogenes such as C-MYC (Sampson *et al.,* 2007), HMGA2 (high mobility group A2) (Barakat *et al.,* 2007) and MYCN (Buechner *et al.,* 2011). However, not all the members of this family display a tumor suppressor role since in lung adenocarcinoma let-7a-3 has an oncogenic function and promotes tumor cell proliferation (Brueckner *et al.,* 2007).

The miR-34 family (comprising miR-34a, -34b and -34c) is downregulated in lung cancer tumor cells with respect to normal tissue and their re-expression in pancreatic cancer cell lines inhibits cell growth and invasion, and induces apoptosis and cell cycle arrest in G1 and G2/M (Gallardo *et al.,* 2009). Similarly to the tumor suppressor miRNAs described above, the miR-34 family exerts its function by targeting anti apoptotic mRNAs such as BLC2 and MYCN (Camps *et al.,* 2008).

The list of miRNAs having a tumor suppressor function ends with the cluster miR-143 and -145. These miRNAs, downregulated in several tumors (Akao *et al.,* 2007; Banaudha et al., 2011), have been found to target ERK5 (extracellular signal-regulated kinase 5) and c-MYC with consequent inhibition of tumor proliferation and increased apoptosis (Akao *et al.,* 2007; Ibrahim *et al.,* 2011).

2.3. CHEMOTHERAPEUTICS AND LUNG CANCER

Slack and co-worker, expressed optimism in the ability of miRNAs to target signaling pathways that are often perturbed in cancer, as well as the potential of miRNAs or antagomirs to sensitize resistant cells to commonly used cancer therapies currently being evaluated (Kasinski *et al.,* 2011). Although miRNA-mediated Chemo-sensitization is an emerging area, with limited in vivo analysis at the moment; but, based on promising in vitro experiments, more miRNA-based sensitization studies in preclinical animal models will probably be reported soon.

2.3.1 Cisplatin [CIS-DIAMMINEDICHLOROPLATINUM(II)]

According to Moufarij *et al*.,(2003) (Moufarij *et al.,* 2003) "Cisplatin (*cis*-diamminedichloroplatinum II) is one of the most frequently used anticancer drugs. It is used as a single agent and in combination regimens to treat a variety of solid tumor types, including head and neck, ovarian and non– small-cell lung (NSCLC) cancers (Cooley *et al.,* 1994). The therapeutic efficacy of cisplatin derives from its ability to form complexes with DNA (Cohen and Lippard, 2001), where it binds the N7 reactive center on purine residues to form both monofunctional and bifunctional DNA adducts. The 1,2-intrastrand GG cross-link (65 per cent) and the 1,2- intrastrand AG cross-link (25 per cent) are the predominant lesions; minor lesions include the 1,3-intrastrand GNG cross-link (6 per cent), the interstrand GG cross-link (1–3 per cent), monoadducts, and protein-DNA cross-links

(Eastman, 1986). Cisplatin-DNA adducts are suggested to exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis (Meyn *et al.*, 1995). Perez, (1998) observed that the key clinical problem associated with cisplatin therapy is that although many tumors may be initially responsive to platinum therapy, these often relapse and become refractory to further platinum agent treatment (Perez, 1998). Akiyama *et al.,* (1999) pointed out that several mechanisms of cisplatin resistance have been identified, including reduced intracellular drug accumulation, elevated levels of cellular thiols, increased tolerance to cisplatin DNA damage, and enhanced DNA repair (Akiyama *et al.*, 1999). Reed, (1998) proposed that "increased capacity of cells to repair and/or tolerate cisplatin-induced DNA damage was the major mechanism of acquired resistance to Cisplatin (Reed, 1998). Several studies have shown that cisplatin resistance is associated with the increased capacity of cells to repair drug-induced DNA damage both in the overall genome (Johnson *et al.*, 1994). Furthermore, elevated levels of DNA repair proteins have also been shown both in cell lines and in patient tumors resistant to cisplatin (Reed, 1998). Inhibition of DNA repair pathways may therefore increase the sensitivity of a tumor to cisplatin therapy.

2.3.1.1 Cisplatin and inhibition of miR21

MicroRNA-21 (miR-21) has been implicated in many cancers and has contributed to chemo-resistance. Its role in gastric cancer was investigated by Yang and colleagues (Yang *et al.*, 2013c). They investigated whether miR-21 mediated resistance of the gastric cancer cell line SGC7901 to the chemotherapeutic agent cisplatin (DDP). They found that the expression of miR-21 upregulated in the cisplatin resistant cell line SGC7901/DDP compared to its parental line SGC7901. They further showed that, over-expression of miR-21 significantly decreased antiproliferative effects and apoptosis induced by cisplatin,

while knockdown of miR-21 dramatically increased antiproliferative effects and apoptosis induction by cisplatin. Yang et al.,(2013) also noted that miR-21 induced cell survival and cisplatin resistance through downregulating the expression of phosphatase and tension homolog deleted on chromosome 10 (PTEN) and activation of Akt pathway. That inhibition of Akt using PI3K inhibitor LY 294002 could abrogate miR-21 induced cell survival (Yang *et al.*, 2013c).

Using microRNA analysis softwares, Yan et al (2012) and colleagues predicted that MSH2 -3′-untranslated region (3′-UTR) was targeted by miR-21. MSH2 is known to be involved in the DNA mismatch repair (MMR) system and over-expression of MSH2 can induce cell apoptosis,(Yan *et al.*, 2012). To further explore the roles of miR-21 and MSH2 in A549 cells, they constructed pcDNA-GFP-msh-UTR vector (including MSH2-3′-UTR) to transfect A549 cells with miR-21. They estimated GFP positive cells using fluorescence microscopy and flow cytometry. Zhang and co-investigators found that miR-21 downregulated the expression of MSH2, which they validated with western blotting. On treating A549 cells with cisplatin they reported that cisplatin inhibited A549 cell growth in vitro and in vivo. They showed further that cisplatin could downregulate miR-21 expression, while increase MSH2 expression in A549 cells. Taken together, their study demonstrated that cisplatin could upregulate the expression of MSH2 through down-regulating miR-21 to inhibit A549 cell proliferation, which provides new gene targets for drug design or cancer therapy. miR-21 can act as an oncogene.

Using microarray, Vargas et al., (2010) found that miR-21, miR27b and miR-622 were upregulated in the cisplatin resistant ovarian cancer cells (A2780CP20) compared with the A2780PAR cells, which are sensitive to cisplatin treatment. Real-time PCR studies confirmed these findings. Opposite, the c-Jun transcription factor was upregulated in this

microarray analysis. They used TargetScan and miRBase tools to identify miR-21, miR-27b and miR-622- target genes. Interestingly, several of these miRNA-target genes were identified as downregulated in microarray study. These results were corroborated by Western blot analysis. Moreover, cell survival experiments in the presence of a miR-21 mimic, made A2780PAR cells more resistant to cisplatin treatment. These results indicate that dysregulation of various miRNAs and their target genes contribute to the cisplatin resistance of ovarian cancer cells.

2.3.2 Etoposide

Etoposide is said to be an epipodophyllotoxin employed in the therapy of a wide spectrum of cancers (Tomida and Tsuruo, 1999). Robert et al.,(1998) in an vitro studies showed that Etoposide increases Topo II-mediated DNA breakage primarily by inhibiting the ability of the enzyme to religate cleaved nucleic acid molecules (Robert and Larsen, 1998).The formation of a topoisomerase–drug–DNA ternary complex appears to be critical for nucleic acid cleavage. The drug enters the complex through a direct interaction with the ATP-bound enzyme monomer in such a way that each drug molecule stabilizes only a single-stranded break (Baguley and Ferguson, 1998a;Leung *et al.*, 2014). Thus, depending on the molar ratio between etoposide and topoisomerase II either singlestranded or double-stranded DNA breaks are generated with the enzyme covalently attached to the 50-end of DNA through a phosphotyrosine bond.

As noted by Fan and colleagues, many anticancer drugs, including etoposide, *m*-AMSA, doxorubicin and mitoxantrone, act through a common cytotoxic mechanism by induction of topoisomerase II cleavable complex (TOP2cc). TOP2cc represents a unique form of DNA damage characterized by reversible TOP2-DNA covalent-linkage andDNAdouble-strand breaks (DSBs). Due to its reversibility, TOP2cc is generally believed as an

important determinant but not the only determinant for the cytotoxic effect of TOP2 drugs. In this regard, a variable success has also been observed in clinical treatment (Hande, 1998). Furthermore, the TOP2-based chemotherapy is often associated with chromosome

translocations connected to the development of therapy-related secondary acute myeloid leukemia (tAML) (Baguley and Ferguson, 1998b). Therefore, comprehension of cellular responses to TOP2- targeting drugs is expected to increase the anticancer efficacy and/or decrease the carcinogenic side effect of such drugs. TOP2 belongs to an ubiquitous class of enzymes that is responsible for solving the topological problems of DNA during replication, transcription and other DNA transactions.

Woessner and colleagues showed that in mammalian cells, TOP2 exists as two isoforms with TOP2 predominantly functioning in relaxation and decatenation of DNA during replication as well as chromosome condensation(Woessner *et al.*, 1991). 'Different from those of TOP2α, the functions of TOP2β are primarily associated with transcription' (Lyu *et al.*, 2006). Both isozymes perform their functions via transesterification reactions involving transient cleavage and religation of two

DNA phosphodiester bonds . Notably, during this reaction, TOP2 dimer covalently links to two 5 prime ends of cleaved DNA strands, resulting in transient formation of TOP2cc. Many anticancer drugs target such a key step by stabilization of TOP2cc (Hande, 1998). For example, etoposide blocks the relegation step and therefore shifts the equilibrium to the formation of TOP2cc (Hande, 1998).

2.3.3 Paclitaxel

Paclitaxel was discovered in 1962 as a result of a U.S. National Cancer Institute-funded screening program; Monroe Wall and Mansukh Wani isolated the drug from the bark of the Pacific yew, *Taxus brevifolia*, and named it "taxol".

Paclitaxel is a mitotic inhibitor used in cancer chemotherapy; together with docetaxel they represent the taxane family of drugs. Developed commercially by Bristol-Myers Squibb (BMS), the generic name changed to "Paclitaxel" when BMS sold the compound with trademark, *Taxol*. Paclitaxel is also available as an injectable suspension of albumin-bound drug, where it is sold under the trademark *Abraxane* (Celgene).

Paclitaxel's mechanism of action involves its stabilization of cellular microtubules; as a result, it interferes with the normal breakdown of microtubules during cell division. Paclitaxel is used to treat patients with lung, ovarian, breast, and head and neck cancers, and advanced forms of Kaposi's sarcoma. It is also used for the prevention of restenosis.

Paclitaxel is approved in the UK for ovarian, breast and lung, bladder, prostate, melanoma, esophageal, and other types of solid tumor cancers as well as Kaposi's sarcoma.

Rowinsky and Donehower,(1995) noted that**,** Paclitaxel is the first member of a new class of chemotherapeutic agents which interact with polymerized tubulin to both promote the formation of microtubules and to prevent their disassembly (Rowinsky and Donehower, 1995)**.** As highlighted by Liebmann *et.al*. (1994), this mechanism of action is believed to cause cells to be arrested in G2-M, and this cell cycle specificity may, in turn, result in a schedule-dependent effect on cytotoxicity (Liebmann *et al.*, 1994). According Rowinsky *et al.*, (1994) preliminary *in vitro* data suggest that paclitaxel cytotoxicity may be a function of the duration of paclitaxel exposure to cancer cells. In an earlier study, Rowinsky *et al.*,

(1988) used a clonogenic assay system to evaluate the cytotoxicity of paclitaxel against four leukemia cell lines. They found increasing cytotoxicity in three of the four cell lines as the duration of exposure to Paclitaxel (0.1-10 p.M) increased from 2 to 22 h (Rowinsky *et al.*, 1988). Unfortunately, these studies evaluated the schedule effect only to maximum exposure duration of 22 h. Liebrnann *et al.*, (1993) evaluated Paclitaxel cytotoxicity in a clonogenic assay with three Adenocarcinoma cell lines. In A549 (lung adenocarcinoma cell line), they noted a 100-fold increase in cytotoxicity as the duration of exposure to paclitaxel (0.05 p.M) increased from 24 to 72 h. A similar effect was seen with both a breast and a pancreatic adenocarcinoma cell line (Liebmann *et al.*, 1993). A report utilized infusion durations of 24 h to minimize hypersensitivity reactions from one component of the paclitaxel vehicle, Cremophor EL(Rowinsky *et al.*, 1995).

2.4 RECOMBINEERING

2.4.1 What is Recombineering

As highlighted by Sharan *et al*, (2009), (Sharan *et al.*, 2009a) 'Recombineering is an efficient method of *in vivo* genetic engineering applicable to chromosomal as well as episomal replicons in *Escherichia coli*.'(Sharan *et al.*, 2009b). This method is said to circumvent the need for most standard *in vitro* cloning techniques. Recombineering allows construction of DNA molecules with precise junctions without constraints being imposed by restriction enzyme site location. Bacteriophage homologous recombination proteins catalyze these recombineering reactions using double-and single-stranded linear DNA

substrates, so-called targeting constructs (eg Zinc finger nucleases, TALENS, CRISPR), introduced by electroporation or nucleofection. Gene knockouts, deletions and point mutations are readily made, gene tags can be inserted and regions of bacterial artificial chromosomes or the *E. coli* genome can be subcloned by gene retrieval using recombineering. Most of these constructs can be made within about one week.

Using phage recombination to carry out genetic engineering has been called recombinogenic engineering or recombineering. Recombineering offers exciting new opportunities for creating mouse models of human disease and for gene therapy.

2.4.2 Recombineering Systems

As reviewed by Copeland et.al (Copeland *et al.*, 2001a) "limitation of most of gene modification technologies is that they require the production of complicated targeting and selection constructs. The ability to generate these constructs is often limited by the availability of appropriate and unique restriction-enzyme cleavage sites in both cloning vectors and genomic DNA. Other types of functional genomics experiment also require the manipulation of large segments of DNA, which is difficult to do using standard recombinant DNA techniques, because many cloning vectors do not have sufficient capacity or do not tend to tolerate large inserts (Liu *et al.*, 2003). The development of PHAGEBASED HOMOLOGOUS RECOMBINATION systems in the past couple years, has greatly simplified the generation of transgenic and knockout constructs, making it possible to engineer large segments of genomic DNA, such as those carried on Bacterial artificial chromosomes (BACs) or P1 artificial chromosomes (PACs),that replicate at low-copy number in Escherichia coli" (Warming *et al.*, 2005).

2.4.3 Galk Recombineering System

2.4.3.1 SW102 strains as a Recombining Vector.

SW102 (equivalent to DY380) is a special strain for use in recombineering. The strain has been modified such that it expresses the proteins needed for recombination under the control of a promoter that can be induced by heat shock. Therefore, two batches of cells are used: one batch that has been exposed to heat shock (experimental) and a batch that has not been heat shocked (control). SW102 does not have the galK gene normally, so that permits the use of galK as a selection scheme.

2.4.4 GalK Expression Cassette



Figure 2.3 GalK Expression Cassette (Sigma compozr)

2.4.5 Recombineering using ssDNA

As reviewed by Copeland and co-workers, Recombineering can also be done using single-stranded oligonucleotides as the targeting cassette. They noted that, in addition to being simpler to construct, such single-stranded oligos are 10–100-fold more efficient than recombineering with dsDNA; up to 6 per cent of electroporated cells can be recombinant. These and other features discussed below make this technology very useful for modifying BAC DNA. In *E. coli*, the *galK* gene encodes the bacterial galactokinase, which is required for metabolizing galactose. Using ssDNA oligos, a single base change has been introduced into *galK* and a 3.3-kb insertion in *galK* has been removed. As reported by Yu and others (Yu *et al.*, 2003)Single-stranded oligos have also been used to cure five different *Tn10* insertions at different places on the *E. coli* chromosome(Yu *et al.*, 2003).Whereas Exo, Beta and Gam are required for dsDNA recombination, only Beta is required for ssDNA recombination. Maximum recombination requires oligos of 70-nucleotides in length, although 40–60-nucleotide-long oligos are only fivefold less efficient (Copeland *et al.*, 2001a).

2.4.6 Strand bias.

As noted earlier by Yu and colleagues, ‘recombination with either of two complementary DNA oligos has shown that one DNA strand is more competent for recombination than the other (Yu *et al.*, 2003). This strand bias from work on bacterial chromosome, show that the preferred strand for recombination correlates with the lagging strand of DNA replication at each site tested. These results indicate that this strand bias might be associated with the direction of DNA replication at the region being targeted, and that ssDNA recombination occurs near the replication fork. DNA replication results in transient regions of ssDNA that

might be accessible to the Beta-mediated annealing of the ssDNA oligo.DNA polymerase and DNA ligase could then complete the joining of the annealed oligo to the lagging strand. The increased recombination efficiency of the lagging-strand oligos might reflect the increased frequency of single-stranded regions during lagging- versus leading-strand synthesis (Copeland *et al.*, 2001a).

2.4.7 Cloning DNA by Gap Repair

Following the lead of earlier studies in yeast, gap repair can also be used to directly subclone sequences from purified total genomic DNA using the RecET recombination functions. As expected, it is more difficult to subclone fragments from mouse genomic DNA than from less complex DNAs, such as yeast or *E. coli* genomic DNA. This is thought to reflect the physical limitation imposed by co-electroporation of the linear cloning vector with total genomic DNA (Copeland *et al.*, 2001a).

2.4.8 Strategies for DNA engineering in E. coli

To understand the various strategies for DNA engineering certain concept are important such as RAC PROPHAGE Escherichia coli and other bacteria contain, in their chromosomes, remnants of viruses or prophages, such as Rac in E. coli, that often are defective and contain only a few genes of the original virus. Two Rac genes, recE and recT, encode homologous recombination functions, and are normally silent, but the sbcA mutation activates their constitutive expression.

As noted by Copeland (2001), ‘unlike in yeast, linear dsDNA is unstable in E. coli due to

the presence of the ATP-dependent, linear-dsDNA exonuclease, RecBCD. However, E. coli strains that lack RecBCD by virtue of a recBC mutation can be transformed by linear

dsDNA, provided that they also have sbcB and sbcC mutations, which restore recombination'

2.4.9 Homologous recombination

As noted by Shibata, (2001) 'Homologous recombination is the exchange ("crossing over")

or replacement ("gene conversion") of a DNA region by its homologous DNA sequence from the homologous chromosome or the sister chromatid.

Homologous recombination he further observed also occasionally occurs between similar DNA sequences on nonhomologous chromosomes or within a chromosome. A single gene conversion event changes one of the pair of homologous sequences without changing any other parts of the genome.'

RecA is central to recombination in Escherichia coli, and all organisms have RecA homologues. It allows two homologous DNAs to find each other, and to trade DNA strands by binding to a single-stranded region in one of the DNAs and by using that strand to search for its double-stranded DNA (dsDNA) homologue. It then binds to a homologue, causing the single strand to pair with its complement in the dsDNA, displacing the identical strand of the duplex and generating a key intermediate in the recombination process (Asami *et al.*, 2011).

Genetic engineering steps to generate a BAC recombinant include:

• Cleaving a cassette DNA with a restriction enzyme.

• Cleaving a target DNA on a plasmid with a restriction enzyme.

• Ligating the cassette to the plasmid.

• Transforming the plasmid into competent bacterial cells.

• Selecting drug-resistant (*drugR*) clones.

• Isolating the transformant and verifying the cloned cassette.

• Transforming the cloned cassette into a BAC-containing bacterial strain to introduce the cloned cassette into the BAC by homologous recombination.

Recombineering steps to generate a BAC recombinant include:

• Amplifying a cassette by PCR with flanking regions of homology.

• Introducing phage recombination functions into a BACcontaining bacterial strain, or introducing a BAC into a strain that carries recombination functions.

• Transforming the cassette into cells that contain a BAC and recombination functions.

• Generating a recombinant *in vivo*.

• Detecting a recombinant by selection, counterselection or by direct screening (colony hybridization) (Asami *et al.*, 2011).

2.4.10 Bacterial Artificial Chromosome (BAC)

Asami et al, (2011) stated that 'Bacterial Artificial Chromosome (BAC) contains an Escherichia coli derived F-factor replication origin, allowing stable propagation of a large exogenous DNA fragment (average size:200 kb) by a single copy per a bacterial cell in a supercoiled circular form'(Asami *et al.*, 2011).

YACs versus BACs and PACs for recombineering

As discussed by Copeland *et al*, (2001), Yeast artificial chromosomes (YACs) were developed for cloning large fragments of genomic DNA into yeast, whereas bacterial artificial chromosomes (BACs) and P1 artificial chromosomes (PACs) were developed for cloning large genomic fragments into *Escherichia coli*. Each vector type has its own inherent advantages and disadvantages. They include the following:

Advantages

YACs can contain megabase-sized genomic inserts,whereas BACs and PACs carry inserts of ~200–300 kb or less.

Disadvantages

I. YAC DNA is difficult to purify intact and at a sufficiently high concentration for generating YAC transgenic mice. BAC and PAC DNA are easy to purify intact and BAC and PAC transgenic mice can be produced in large numbers. A single yeast cell might contain both wild-type and modified YACs. A bacterial cell usually carries a single BAC.

II. YACs are often chimeric; a single YAC can contain DNA from multiple locations in the genome. BACs and PACs are rarely chimeric.

III. Yeast recombination is very potent and always active, and so can generate deletions and other rearrangements in a YAC. *E. coli* recombination is prevented in RECA mutants and is turned on when needed, so reducing unwanted rearrangements in BACs or PACs.

IV. Manipulating recombinant YACs that are generated in yeast is laborious and usually requires YACs to be transferred to *E. coli* for subsequent manipulation.

BAC and PAC modification occurs directly in *E. coli*, eliminating the need for DNA transfer (Copeland *et al.*, 2001b).

2.5 DNA BREAK REPAIR:

Double Stranded Break is induced by ZFN which is a designed, sequence specific endonuclease that can be customized to cleave a user-chosen DNA target. It is used to disrupt native loci in model organisms such as rats and *Arabidopsis thaliana*, to drive trait stacking in a crop species, to engineer HIV-resistant human T cells and haematopoietic stem cells (HSCs), and to drive targeted integration in human embryonic stem (ES) cells, induced pluripotent stem (iPS) cells and mesenchymal stem cells.

2.5.1 Genome Editing

This is an approach that enables efficient and precise genetic modification via the induction of a double-strand break (DSB) in a specific genomic target sequence, followed by the generation of desired modifications during subsequent DNA break repair.

2.5.2 Gene Disruption

This is the simplest genome editing method. It takes advantage of errors introduced during DNA repair to disrupt or abolish the function of a gene or genomic region. This approach has been applied in various species and cellular contexts to knock out user-specified genes in a single step and without selection for the desired event.

2.5.3 Homology-Directed Genome Editing (HDr)

Another more complex mechanism of a ZFN-induced DSB is called HDr. Whether spontaneous or induced by the I-*Sce*I homing endonuclease or a ZFN a DSB is recombinogenic in cells of higher eukaryotes. Homology-based genome editing requires the simultaneous provision of a suitably designed, homology-containing donor DNA molecule along with the locus-specific ZFNs. This enables two related modes of genome editing that are a function of donor DNA design and distinguished by the type of allele being generated.

2.5.4 Zinc Finger Nucleases

Zinc finger nucleases are composed of an engineered zinc finger DNA binding domain fused to the non-specific nuclease domain of the restriction enzyme *Fok*I. Each zinc finger interacts with three nucleotides and multiple (N) fingers can be assembled together to specifically bind a targeted sequence of 3N bases. Importantly, the *Fok*I nuclease domain must dimerize to achieve a double strand cleavage in the DNA. Thus, ZFN pair is required to bind and cut the targeted site.

ZFNs are used to create a targeted double strand break that stimulates the process of HDR.

ZFN-mediated targeted integration is important to eliminate the effects genomic context has on the expression of delivered transgenes. Most transgenic cell lines are constructed via random integration of a plasmid. This results in a collection of clones with greatly varied expression levels and expression stability.

It is believed that AAVS1 site is present in every human cell line, unlike phage recombination sites such as Cre and FLP. This provides a consistent site for transgene delivery and reduces the likelihood of expression variability across experiments.

As noted by Urnov et al, (2005), an endonuclease is required to exhibit an extraordinary combination of qualities to be used in genome engineering. Such qualities include: specific recognition of long target sequences coupled with sufficient adaptability for retargeting to user-defined sequences. The ZFN architecture is known to address these specifications by linking the DNA-binding domain of a versatile class of eukaryotic transcription factors — zinc finger proteins (ZFPs) — with the nuclease domain of the *Fok*I restriction enzyme. By virtue of their structure, ZFNs combine the favourable qualities of both components — the DNA binding specificity and flexibility of ZFPs and a cleavage activity that is robust but restrained in the absence of a specific binding event (Gupta *et al.*, 2011).

The ZFP region is said to provide a ZFN with the ability to bind a discrete base sequence. This region according to Miller and colleagues contains a tandem array of Cys2-His2 fingers (Miller *et al.*, 2007), each recognizing approximately 3 bp of DNA. In early studies individual ZFNs used three fingers to bind a 9-bp target, which enabled ZFN dimers (the active species) to specify 18 bp of DNA per cleavage site. Further studies have added more fingers (up to six per ZFN) to specify longer and rarer cleavage targets. A variety of strategies have been described for making ZFPs with new, user-chosen binding specificities. The first emerged from observations of the initial ZFP–DNA co-crystal structure, which suggested a substantial degree of functional autonomy in the interaction of

individual fingers with DNA (Gupta *et al.*, 2011). The approach, which has been termed ‘modular assembly’, generates candidate ZFPs for a given target sequence by identifying fingers for each component triplet and linking them into a multifinger peptide targeted to the corresponding composite sequence. Fingers used for modular assembly have been developed for most triplet sequences (Gupta *et al.*, 2011). The method has been used to

develop the zinc finger component of active ZFNs for a number of endogenous targets in higher eukaryotic cells.

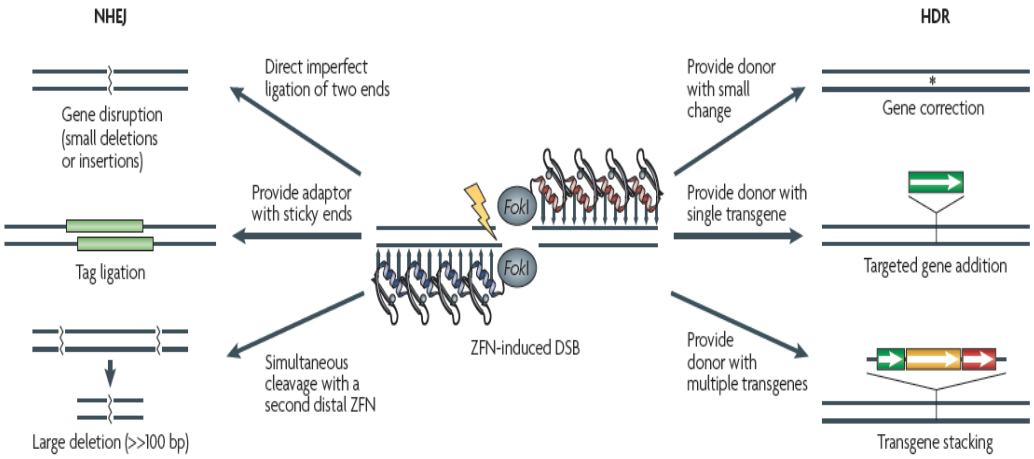


Figure 2.3. Types of genome editing made possible using zinc finger nucleases. A zinc finger nuclease (ZFN)-induced double-strand break (DSB) allows a range of alleles to be generated at endogenous loci, as specified by the investigator. The diagram shows the different outcomes that can result from the introduction of a site-specific DNA break. A ZFN pair is shown bound to a genomic target site (the two different DNA binding domains are shown in red and blue). The DSB generated by ZFN cleavage induces DNA repair processes that may be influenced by the addition of an investigator-designed donor DNA. As shown on the left, if the break is resolved via non-homologous end joining (NHEJ) (which will occur in the absence of donor DNA), this can lead to the following outcomes (from top to bottom): gene disruption — the two ends can be ligated back together, frequently with loss or gain of genetic information at the site of the break, resulting in small insertions or deletions; tag ligation — if a double-stranded oligonucleotide is provided with overhangs complementary to those left by the ZFNs (an adaptor), it will be ligate ligated into the chromosome, thus producing, for example, a tagged allele; large deletion — two simultaneous DSBs made on the same chromosome can lead to a deletion of the entire intervening stretch. As shown in the panel on the right, if the break is resolved via homology-directed repair (HDR) (which will occur in the presence of donor DNA), this can result in (from top to bottom): gene correction — if the donor specifies solely a single-base-pair change (for example, a restriction fragment length polymorphism encoding a novel allele), this will result in 'gene correction' that subtly edits the endogenous allele; targeted gene addition — if a donor is provided that carries an ORF or a transgene at the position corresponding to the site of the break, its sequence will be transferred to the chromosome.(Urnov *et al.*, 2010)

2.5.1 ZFN and Gene Targeting

Therapeutic application of ZFNs Editing of native loci is, in principle, a superior strategy in certain clinical settings. For example, a corrected allele of a disease-causing gene could be curative in several monogenic diseases.

2.6 ADENO ASSOCIATED VIRUS AAV AND AAVS1 LOCUS

2.6.1 Molecular Biology of Adeno Associated Virus AAV

The AAVs are parvoviruses of the *Dependovirus* genus that all share the dependence on unrelated DNA viruses (i.e., adenovirus, herpesvirus, papilloma virus and vaccinia virus) for completion of the productive part of their lifecycle (Walz *et al.*, 1997). They are small non-enveloped single-stranded DNA viruses with icosahedral capsid symmetry. The first serotype isolated was AAV2 and, since then, eight more have been characterized (Srivastava *et al.*, 1983). Recent discoveries of AAV isolates derived by PCR from nonhuman primate and human tissues have extended the list of capsid variants to over 120 (Gao *et al.*, 2003).

The AAVs are genetically very simple, with two major open reading frames, encoding for the nonstructural Rep proteins and structural Cap proteins, respectively.

Linden group (Henckaerts and Linden, 2010) reviewed that AAV is unique among animal viruses in that its latent state involves insertion into the host genome at a preferred site. This site, referred to as AAVS1 (Philpott *et al.*, 2002), is found at chromosome 19q13.4qter and has been mapped to the first exon of myosin binding subunit 85 of protein phosphatase 1 (Calcedo *et al.*, 2009). Although the molecular mechanisms of AAV integration have not been fully elucidated, the sequence requirements necessary in both the

virus and the host are known. McLaughlin *et al*, (1988) observed that in the host, a 34-bp sequence is both necessary and sufficient for efficient integration (McLaughlin *et al.*, 1988).

2.6.2 Molecular characterization of AAV Integration

Since the discovery of site-specific integration of AAV in the latently infected cell line (Surosky *et al.*, 1997), numerous latently infected cell lines have been generated, and attempts have been made to uncover the molecular organization of the integrated provirus (Dutheil *et al.*, 2009). The presence of site-specifically integrated AAV is usually determined by Southern blot analysis using an *MBS85*-specific and virus-specific probe. Disruption of the *MBS85* gene and comigration of virus-positive and disrupted *MBS85*-positive band(s) indicate site-specific integration of AAV. Extensive rearrangements of the *MBS85* gene have been observed; however, they are always confined to one allele (Dutheil *et al.*, 2009). The integration event is often further analyzed by the identification of the viral–cellular junctions, and vast majority of integration events are scattered throughout the first exon and intron of the *MBS85* gene (Dutheil *et al.*, 2009).

2.6.3 Strategies for Site-Specific Gene Modification

As noted by Dutheil *et al*, (2009), Adeno-associated virus is impressive in that it seems to have built a near-perfect relationship with its host as the virus is only detrimental to the host cell when it has already suffered an insult caused by the helper virus. In the absence of helper factors, the virus can integrate its genome into a specific locus on human chromosome 19 without causing any apparent adverse effects (Dutheil *et al.*, 2009). Whether the co-evolution of virus and host also applies to the phenomenon of site-specific integration remains a question. Does this particular genetic environment, combined with specific host factors, provide something unique, potentially beneficial to the virus, and *vice*

versa, does the host cell profit from this viral integration?- are some of the question begging for answers.

These questions remain largely unanswered as most observations concerning the lifecycle of AAVs were made in tissue culture and very few data have been generated that could contribute to our knowledge of the *in vivo* lifecycle of the virus. However, attempts have been made to identify a reservoir of latent AAV. Grossman et.al, observed that the virus is present in a small percentage of hematopoietic cells (Grossman *et al.*, 1992), in the human genital tract (Friedman-Einat *et al.*, 1997) and large percentage of muscle biopsies (17%) (Tezak *et al.*, 2000). However, these studies did not present any data on integration of AAV in chromosome 19. Schnepp *et al*.,(2005), using sensitive, unbiased PCR techniques, were able to show that the viral genome predominantly exists as a circular double-stranded episome and showed that tonsils contain AAV integrated into chromosome 1, arguing that site-specific integration may not be part of the *in vivo* lifecycle of the virus (Schnepp *et al.*, 2005).

Furthermore, the mechanism by which AAV establishes latency is rather precise and elaborate, which calls the hypothesis that AAV site-specific integration is a 'tissue culture artefact' into question.

2.6.4 **Human Target Sequence**

The preintegration site is located on the long arm of chromosome 19, at position 19q13.42 (Surosky *et al.*, 1997) and has several interesting features, including a high number of short repetitive sequences, a unique minisatellite sequence, the presence of CpG islands and putative *cis*-acting DNA elements, as well as a DNaseI hypersensitive site (Surosky *et al.*, 1997). Most intriguingly, the first 500 bp of *AAVS1* contain TRS and RBS sequences very

similar to those found in the AAV genome , which were shown to support Rep-mediated replication of the *AAVS1* sequence.

As predicted from its characteristics, a gene was identified within *AAVS1*, and the translation initiation start codon is located only 17 nt downstream from the RBS (Schnepp *et al.*, 2005). This 27-kb gene encodes the protein phosphatase I regulatory inhibitor subunit 12C (PPP1R12C), also termed Mbs85 or myosin-binding subunit 85. Transcription of the 22 coding exons results in a unique 3-kb mRNA, which is ubiquitously expressed in adult human and mouse tissues (Schnepp *et al.*, 2005).

2.6.5 AAV as a vector for gene therapy

As noted by Dutheil *et al., (*2009), the natural propensity of AAV to persist in human cells and its nonpathogenic character has sparked an enormous interest in the development of AAV as a gene therapy vector. Recombinant AAV (rAAV) vectors typically consist of the gene of interest and the elements that regulate its expression, flanked by the inverted terminal repeats (ITRs) of AAV, resulting in a vector genome of approximately 4.5 kb, which is unable to replicate or integrate site specifically as it is missing the viral proteins necessary to direct these events (Dutheil *et al.*, 2009). The ITRs are the only viral elements that are present in the vector and constitute the signals necessary for AAV DNA replication and packaging, which can take place when the required helper virus genes and AAV *rep* and *cap* genes are provided in *trans*. AAV production schemes are mostly based on dual or triple transfections of 293T cells with vector plasmid and helper and/or *rep-cap* plasmids. Approximately 48 h post-transfection, the cells are lysed and recombinant virions can be purified from the lysate (Dutheil *et al.,* 2009). Other strategies used for the production of

2.7 Towards Insulin Replacement

Treatment of diabetes typically requires regular injections of insulin. Due to the inconvenience of insulin injections, various approaches have been attempted to formulate insulin for administration by non-injectable routes.

Kitao and colleagues (Kitao, Abe, and Ito, 1982) proposed pharmaceutical compositions for rectal administration of insulin. The pharmaceutical compositions include insulin and fatty acids having 8 to 14 carbon atoms and nontoxic salts thereof. Kidron and colleagues, (Kidron et al., 2004) suggested pharmaceutical compositions consisting of, a bile acid or alkali metal salt for the oral administration of insulin. The composition is provided with an enterocoating to assure intra-gastric passage and release in the intestine.

Backstrom et al., (1997) proposed a therapeutic preparation for inhalation that includes insulin and a substance, which enhances the absorption of insulin in the lower respiratory tract. The enhancer is preferably a sodium salt of a saturated fatty acid of carbon chain length 10, (Backstrom, Hinkle, & Flicker, 1997).They noted that if the carbon chain length is shorter than about 10, the surface activity of the surfactant may be too low, and if the chain length is longer than about 14, decreased solubility of the fatty acid in water limits its usefulness.

2.7.1 Therapeutic Targets of Diabetes

Diabetes mellitus, the most commonly encountered endocrinopathy, continues to increase dramatically in prevalence. Diabetes is the sixth most common cause of death in the United States and significantly affects other more common causes of death such as cardiac disease and stroke (Varshosaz *et al.*, 2005).

Diabetes mellitus (commonly referred to simply as diabetes) is a disease characterized by disregulation of metabolism, particularly glucose metabolism. In normal individuals, a rise in blood glucose levels (such as that which occurs immediately following eating) triggers the islet beta cells of the pancreas to secrete insulin, a peptide hormone, into the bloodstream. The insulin binds to insulin receptors located on a number of cell types, notably muscle cells, and thereby signals the cells to increase the rate of glucose uptake into the cells. As the blood glucose returns to normal pre-prandial levels, the amount of insulin in the blood also drops. In the absence of insulin, blood glucose levels would rise to dangerously high levels (a condition termed hyperglycemia), possibly resulting in death. Too much insulin causes abnormally low blood glucose levels (hypoglycemia), which is also dangerous and possibly fatal. In a normal individual, built-in feedback loops regulating the secretion of insulin and its clearance from the systemic circulation prevent both hyperglycemic and hypoglycemic conditions from occurring (Varshosaz *et al.*, 2007).

2.7.2 Novel Drug Delivery Systems for Insulin

As noted by Varshosaz *et al.*,(2007) "biologically active agents such as nutritional supplements, hormones, and a variety of pharmaceutical preparations, which will generally be referred to as 'drugs' are typically provided in oral (liquids or solids) or injectable dosage formulations, however there are many disadvantages associated with this type of administration. Many of the ingredients are degraded within the gastrointestinal (GI) tract or undergo first-pass metabolism in the liver. In addition, there exists a segment of the population who experience difficulty in swallowing pills or are unable to tolerate any solids. During the past three decades, however, formulations that control the rate and period of drug delivery (e.g., time-release medications) and target specific areas of the

body for treatment have become increasingly common and complex (Varshosaz *et al.*, 2006).

Some have provided solutions to the problem of administering different types of drugs but there are still a large number of medications that do not achieve maximum pharmaceutical effect because they do not reach the intended tissue targets either fast enough or in high enough concentrations.

The potency and therapeutic effects of many drugs are limited or reduced because of the partial degradation that occurs before they reach a desired target in the body.

Further, injectable medications could be made less expensively and administered more easily if they could simply be dosed by other routes such as the oral mucosa, the pulmonary mucosa or through the vaginal and intestinal tract. However, this improvement cannot happen until methods are developed to safely shepherd drugs through these specific areas of the body, where different physiological environments (e.g. low pH values in the stomach) can destroy a medication or where absorption is not rapid or complete, or through an area where healthy tissue might be adversely affected. The goal of all drug delivery systems is to deploy medications intact to specifically targeted parts of the body through a medium that can control the therapy's administration by means of either a physiological or chemical trigger (Pardakhty *et al.*, 2007).

2.7.3 Transdermal Delivery

The efforts to develop a non-injectable transdermal insulin delivery system for the treatment of diabetes have not been very successful to date. While insulin can be systemically delivered to a patient by the topical application of an insulin-containing vehicle, the systemic blood levels of insulin that are achievable using this delivery method

have proven to be generally inadequate for meeting the demands of the diabetic patient (Varshosaz *et al.*, 2010). 'Various methods have been developed for enhancing the transdermal delivery of insulin including improved passive diffusion carriers for increasing the permeability of the epidermis, sonophoresis, iontophoresis and ionosonic transport. Passive diffusion through the outer layer of skin has been used successfully for the delivery of low molecular weight lipophilic drugs such as scopolamine, estradiol and nitroglycerine, but has been largely unsuccessful for the transdermal delivery of hydrophilic peptides such as insulin due to the low skin permeability of such peptides. Thus, mechanical vibrational energy and/or iontophoresis are employed to increase skin permeability and facilitate transdermal insulin delivery' (Varshosaz *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS FOR RECOMBINEERING

The following materials were used for the recombineering work flow.

Media (from Current Protocols in Molecular Biology)

3.1.1 M9 Medium (1 liter)

1X 6 g Na_2HPO_4

3 g KH_2PO_4

1 g NH_4Cl

0.5 g NaCl

Autoclave

M63 minimal plates

1L 5X M63 10 g $(NH_4)2SO_4$

68 g KH_2PO_4

2.5 mg $FeSO_4 \cdot 7H_2O$

Adjust to pH 7 with KOH

Autoclave

0.2 mg/ml d-biotin (sterile filtered) (1:5000)

20% galactose (autoclaved) (1:100)

20% 2-deoxy-galactose (autoclaved) (1:100)

20% glycerol (autoclaved) (1:100)

10 mg/ml L-leucine (1%, heated, then cooled down and sterile filtered)

25 mg/ml Chloramphenicol in EtOH (1:2000)

1 M $MgSO_4 \cdot 7H_2O$ (1:1000)

3.1.2 Materials for PCR Amplification of *galk* Plasmid

The following materials were used for the PCR amplification of *galk* plasmid;

iProof high fidelity polymerase kit(Biorad)

Water

5X Buffer (HF or GC)

10µM CAGGS -GalK-F

10µM CAGGS -GalK-R

dNTP mixture

Template (pGalk) (10ng)

3.1.3 Sequences of Primers used

The sequences of the primers used are;

pSA-2A-GFP-PA plasmid,

pAAVS-CAGGS-GFP

Verification primers

SA2A-GalK-F

SA2A-GalK-R

AAVS-GalK-F

AAVS-GalK-R

pAAVS1-CAGGS

3.1.4 Materials for Nucleofection of Dermal fibroblast

The following materials were used for nucleofection of dermal fibroblast;

Nucleofection

5 x 10^5 Human dermal fibroblast cells

Plasmid DNA (in H_2O or TE) or 2 μg pmaxGFP Vector

100 μl Human Dermal Fibroblast Nucleofector Solution

Amaza Nucleofector Device

Supplemented Nucleofector Solution at room temperature

Supplied certified cuvettes

Supplied plastic pipettes

Supplied pmaxGFP Vector

Template

6-well culture dish

For trypsinization

Trypsin/EDTA, HEPES Buffered Saline Solution (HBSS) and Trypsin Neutralizing Solution (TNS) [Lonza, Cat.No. CC-5034].

Culture medium: FGM-2 BulletKit [Lonza; Cat. No. CC-3132].

3.1.5 Cell line for Recombineering.

SW102 (equivalent to DY380) is a special strain for use in recombineering. The strain has been modified such that it expresses the proteins needed for recombination under the control of a promoter that can be induced by heat shock. Therefore, two batches of cells: one batch that has been exposed to heat shock (experimental) and a batch that has not been heat shocked (control) were used. SW102 does not have the *GalK* gene normally, so that permits the use the *GalK* scheme for selections.

3.2. METHODOLOGY

3.2.1 Method of Replacing GFP with *GalK* in Targeting Plasmids.

The Goal of this project was to replace GFP with *GalK* in targeting plasmids.

To do this, a large batch of SW102 competent cells were prepared, Induced and un-induced. They were stored frozen in 15 per cent glycerol for use in all downstream steps. Efficiency was verified using the test plasmids. *GalK* gene was PCR-amplified from p*galk* plasmid, DPnl digested and gel purified. 0.1-100ng plasmid DNA was co-electroporated into SW102. A total of 3 electroporations were carried out.

1. Induced, plasmid, PCR product, II Uninduced, plasmid, PCR product, III Induced, plasmid.

Cells were recovered after electroporation in LB rich medium for 2 hours. Cells were pelleted and washed 2 times in1mL M9 medium. They were later grown in 10mL culture overnight in selective medium (M63 minimal medium with galactose, leucine, biotin and ampicillin). The culture was minipreped, linearized with sall-HF enzyme and ran out on 0.8% Agarose gel (long slow run) to separate by size- Galk band was 500bp larger than FP band. The larger DNA fragment was gel purified and a ligation of several dilute amount of DNA was set up (favoring intramolecular ligation). Re-circularized DNA was transformed back into uninduced SW102 (no-ligation control was equally included in the study). They were recovered 2 hours in rich media, washed 2 times in 1mL M9 medium, plated on M63 plates with ampicillin and allowed to grow at 30°c for 2-3 days. Colonies were re-streaked onto McConkey agar plates, individual red colonies were picked into 100ul of deionized water. 2 ul of diluted colony was used as PCR template. PCR was done with confirmation and FP primers to identify colonies with the desired recombineered product and no detectable FP. For colonies that seem to be pure desired plasmid mini cultures in M63 medium were set up, miniprep,and confirmed by diagnostic digest.

3.2.3Preparation of Competent Cells

500 ml of an overnight culture of SW102 was diluted in 25 ml Luria–Bertani (LB) medium with or without ampicillin selection (12.5 mg/ml) in a 50 ml baffled conical flask and grown at 30°C in a shaking waterbath to an OD600 of 0.6. Then, 10 ml was transferred to another baffled 50 ml conical flask and heat-shocked at 42°C for exactly 15 min in a shaking waterbath. The remaining culture was left at 32°C as the uninduced control. After 15 min the two samples, induced and uninduced, were briefly cooled in an ice/water slurry and then transferred to two 15 ml Falcon tubes (BD Biosciences) and pelleted using 5000 r.p.m. (Eppendorf centrifuge 5804R, rotor A-4-44) at 0°C for 5 min. The supernatant was

poured off and the pellet was resuspended in 1 ml ice-cold ddH_2O by gently swirling the tubes in ice/waterbath slurry. Subsequently, 9 ml ice-cold ddH_2O was added and the samples pelleted again. This step was repeated once more. After the second washing and centrifugation step, the supernatant was removed, and the pellet (~50 ml each) was kept on ice until electroporated with PCR product, gel-purified fragment

3.2.4 Electroporation of Competent Cells

An aliquot of 25 ml of prepared competent cells was used for each electroporation in a 0.1 cm cuvette (BioRad) at 25 mF, 1.75 kV and 200 V. After electroporation the bacteria was recovered in 1 ml LB (15 ml Falcon tube) for 1 h in a 30°C shaking waterbath. For the counter-selection step, the bacteria were recovered in 10 ml LB in a 50 ml baffled conical flask and incubated for 4.5 h in a 32^{o}C shaking waterbath. After the recovery period, the bacteria were washed twice in 1X M9 salts as follows: 1 ml culture was pelleted in an eppendorf tube at 13,200 r.p.m. for 15 s and the supernatant removed with a pipette. The pellet was resuspended in 1 ml of 1x M9 salts, and pelleted again. This washing step in turn repeated again. After the second wash, the supernatant was removed and the pellet resuspended in 1 ml of 1x M9 salts before plating serial dilutions (100 µl, 100 µl each of 1:10, 1:100 and 1:1000 dilutions) on minimal medium plates. Washing in M9 salts was necessary to remove any rich media from the bacteria prior to selection on minimal medium. The uninduced samples routinely had a higher degree of lysis/bacterial death after electroporation, so the uninduced samples was diluted in 0.25–0.75 ml 1x M9 salts in the final step to make up for the difference.

3.2.4 PCR Reaction for the Amplification *Galk* Gene from *Galk* Plasmid

CAGGAS-GALK-F and CAGG-GALK-R were resuspended at 100µM (by adding 40µl of molecular biology grade water to the plasmid, a working solution of each primers were

prepared at 10µM by mixing 10µl of 100µM of stock solution with 90µl of water. PCR reaction was set up as follows:

Table 3.1, PCR set up for the amplification *galk* gene from *galk* plasmid using primers with homology to pAAVS-CAGGS-eGFP

Component	**Amount**
Water	32.5µl
5X Buffer (HF or GC)	10 µl
10µM CAGGS -GalK-F	2.5 µl
10µM CAGGS -GalK-R	2.5 µl
dNTP mixture	1 µl
Template(pGalk)(10ng)	1ul
iProof enzyme (Biorad)	0.5 µl
Total	50µl

A negative control reaction was set up for each primer pair, using 10ng pGalK as a template. A total of 4 reactions, 2 tests and 2 negative controls PCR runs were set up on PCR machine using the following reaction conditions:

a. 98^{o}C, 30 sec
b. 98^{o}C, 10 sec
c. 55^{o}C, 30 sec
d. 72^{o}C, 1 minute
e. (repeat b-d 35 times)
f. 72^{o}C, 5 minutes
g. 4^{o}C, for ever

At the end of PCR Run, PCR products were ran on 0.8% Agarose gel to evaluate the efficiency of the reaction. The gel was photographed using BioRad Gel documentation station and thereafter the gel was discarded.

3.2.5 PCR Set Up to Detect Recombined Plasmid DNA in Miniprep Population

Verification primers were resuspended at 100μM concentration, 10μM working solutions were prepared (one for the pSA-2A-GFP-PA plasmid, and one for pAAVS-CAGGS-eGFP).For each miniprep, one PCR reaction was set up as follows:

Table 3.2 PCR scheme for detection of recombined plasmid DNA in miniprep population

Component	**Amount**
Water	32.5ul
5X Buffer (HF or GC)	10 μl
10μM CAGGS -GalK-F	2.5 μl
10μM CAGGS -GalK-R	2.5 μl
dNTP mixture	1 μl
Template(pGalk)(10ng)	1ul
iProof enzyme (Biorad)	0.5 μl
Total	50μl

The following PCR cycling conditions were used:

a. 98^{o}C, 30 sec
b. 98^{o}C, 10 sec
c. 55^{o}C, 30 sec

d. 72^{o}C, 1 minute

e. (repeat b-d 35 times)

f. 72^{o}C, 5 minutes

g. 4^{o}C, for ever

At the end of PCR Run, PCR products were ran on 0.8% Agarose gel to evaluate the efficiency of the reaction. The gel was photographed using BioRad Gel documentation station and thereafter the gel was discarded.

3.2.6 Preparation of alga plates

Fifteen (15) g agar in 800 ml H_2O was autoclaved in a 2 liter flask and was allowed to cool down a little. 200 ml autoclaved 5X M63 medium was added and then 1 ml 1 M $MgSO_4 \cdot 7H_2O$.

The volume was adjusted to 1 liter with water when necessary. It was Let to cool down to 50°C ("touchable hot"). Ten (10)ml carbon source was added (final conc. 0.2%), and then 5 ml biotin (1 mg), 4.5 ml leucine (45 mg), and 500 ml Chloramphenicol (final conc. 12.5 mg/ml). The plates were poured, 25-40 plates per liter.

2.6 Preparation of MacConkey indicator plates

MacConkey agar plus galactose was prepared according to manufacturer's instructions. After autoclaving and cooling to 50°C, 500ml Ampicillin was added (final conc. 50mg/ml) to one liter of the agar, and the plates were poured, about 25-40 plates per liter.

3.2.7 Sigma compozr Targeted integration kit.

The CompoZr Targeted Integration Kit –AAVS1 is designed to integrate a user specified

Gene of Interest (GOI) into the adeno-associated virus integration site 1 (AAVS1) on human chromosome 19. The use of a well-validated pair of zinc finger nucleases (ZFNs) engineered to target the AAVS1 locus enables the efficient targeted integration. When the provided AAVS1 ZFN pair is co-delivered into a human cell line along with a donor plasmid, coding for a user specified gene, the cell is stimulated to go through the natural process of homology directed repair (HDR). HDR directs the targeted integration of the user specified gene of interest into the AAVS1 locus. ZFN-aided targeted integration can achieve 20% efficiency in some cell types, representing an improvement of three to four orders of magnitude in efficiency over normal homologous recombination events.

3.2.8 Isolation of SW102 DNA Using Qiagen Miniprep Kit

DNA from SW102 cultures were minipreped using Qiagen miniprep kit by Spining down bacterial cells at 3000rpm for 5 minutes in large table-top centrifuge. Cells were re-suspended in 250ul P1 buffer in each tube (stored in refrigerator) and each transferred to an Eppendorf tube (6 tubes total). 250µl P2 (lysis) buffer was added, and the tubes were inverted 10 times to mix well. Thereafter 350µl N3 (neutralization) buffer was added, the tubes were inverted 10 times to mix well. The tubes were spurned in microcentrifuge for 10 minutes at maximum speed. Supernatant was poured onto labeled miniprep column and spurned through 30 seconds at maximum speed, flow through was discarded. 750µl of PE (wash) buffer was added to column and centrifuged through 30 seconds at maximum speed, flow through was discarded. An empty column was spinned for 1 minute at maximum speed to dry column. The column was transferred to a fresh eppendorf tube and 50µl water was added to each column, and incubated at room temperature for 1 minute.

DNA was eluted by spinning for 2 minutes at 9500 rpm. DNA was quantitated on nano-drop.

3.2.9 PCRs to Check for Integration of GFP Gene into AAVS1 Locus

Table 3.3: Set up for PCRs to check for integration of GFP gene into AAVS1 locus

Component	**Volume**
1 **Negative control**	
Water	32.7µl
5X Buffer (HF or GC)	10 µl
10µM CAGGS -GalK-F	2.5 µl
10µM CAGGS -GalK-R	2.5 µl
dNTP mixture	1 µl
Template(Neg. ontrol)(200ng)	0.8ul
iProof enzyme (Biorad)	0.5 µl
Total	50µl
2.(pMax-GFP control 200ng)	
Water	30.7µl
5X Buffer (HF or GC)	10 µl
10µM CAGGS -GalK-F	2.5 µl
10µM CAGGS -GalK-R	2.5 µl
dNTP mixture	1 µl
Template(pMax-GFP)(200ng)	1.8ul
iProof enzyme (Biorad)	0.5 µl
Total	50µl
3.(pSA-2A-GFP(200ng)	
Water	30.6µl
5X Buffer (HF or GC)10µM CAGGS -GalK-F	10 µl 2.5 µl
10µM CAGGS -GalK-R	2.5 µl
dNTP mixture	1 µl
Template(pSA-2A-GFP (200ng)	2.9µl
iProof enzyme (Biorad)	0.5µl
Total	50µl
4.(pAAVS1-CGGS-GFP**)**	
Water	30.9µl
5X Buffer (HF or GC)	10 µl
10µM CAGGS -GalK-F	2.5 µl
10µM CAGGS -GalK-R	2.5 µl
dNTP mixture	1 µl
Template(pAAVS1-CGGS-GFP	2.6µl

(200ng) iProof enzyme (Biorad) Total	 0.5 µl 50µl

The PCR reactions were run using the following reaction conditions:

a. 98^{o}C, 30 sec,
b. 98^{o}C, 10 sec
c. 55^{o}C, 30 sec
d. 72^{o}C, 1 minute
e. (repeat b-d 35 times)
f. 72^{o}C, 5 minutes
g. 4^{o}C, for ever

PCRs products were loaded on 0.8 percent agarose gel and photographed.

3.2.10 Determination of Transformation Efficiency of Colonies.

Competent cells were tested to determine transformation efficiency using one tube each of experimental and control cells that were electroporated with 100ng of pGalK plasmid. Cells were recovered for 1 hour in 1mL LB-Amp at 30°C and plated on LB-Amp plates. In order to plate 1 or 10 µl of cells, 100µl water or LB was mixed first with the cell suspension so that the cells were spread evenly. 1ul of the cell suspension was diluted in 1mL fresh LB. Then 1, 10, and 100µl of the suspension were platted on each of 3 plates, as well as 1, 10, and 100 µl of the undiluted cell suspension on each of 3 plates. The cells were spread on the plates using glass beads. A total of 12 plates were made, (6 each for experimental and control cells).Plates were then incubated at 30°C over night for colonies to grow.

3.2.11. Calculation of Transformation Efficiency of The Competent Cells

Efficiency is expressed as colonies per µg of DNA. Colonies on one plate were counted and expressed as a ratio of colonies per µg to determine the Transformation efficiency of the competent cells. The amounts of DNA represented by the colonies were estimated using the calculation below by converting from ng to µg:

(# Colonies on plate/ng DNA transformed) X (1000ng/1µg) = # colonies per ug DNA.

The efficiency of the cells were of the order of 10^8-10^10 colonies per µg DNA. When it is much lower (10^5-10^6), that could lead to difficulty with recombineering. The table show the transformation efficiency obtained.

3.3. CELL CULTURE OF HUMAN DERMAL FIBROBLAST

Seeding of cells were done with 2 x 10^5 cells/75cm^2 flask. The cells were maintained in media. The media was changed every 2 days; Cells were passaged after reaching 80 – 90 percent confluency. For Nucleofection cells were preferably passaged 6 days before. To avoid lower gene transfer, cells after passage number 9 were used. For Trypsinization media was removed from the cultured cells and cells washed once with HBSS; using at least same volume of HBSS as culture media. Cells were harvested by incubating the cells 1 – 3 minutes at 37°C with trypsinization reagent. Trypsinization was neutralized using trypsinization Neutralization solution (TNS) once the majority of the cells (>90 percent) were detached.

3.3 .1 Transfection using Amaza Nucleofection

The human dermal fibroblast cells were seeded at a density of 2 x 10^5 cells/ml, the day before transfection, cell density was between 2 x 10^5 cells/ml. 2 million cells per transfection were Centrifuged at 200 X g for 5 minutes. Cells were washed twice with 20

ml of HBSS, and then experimental tubes prepared: each 6-well plate was filled with 2 ml of medium in each well and pre-warmed in a CO_2 incubator at 37 °C for at least 20 minutes or until needed in the next step. The cells were then nucleofected with ZFN mRNA as described in the Amaza manual, as follows:

Cell pellet were carefully resuspended in 100 µl room temperature Nucleofector Solution per sample, avoiding storing the cell suspension longer than 15 minutes in Nucleofector Solution, as this reduces cell viability and transfection efficiency. 100 µl of cell suspension was combined with 2 µg pmaxGFP Vector. The cell/DNA suspension was transferred into certified cuvette ensuring that the sample covered the bottom of the cuvette, without air bubbles. The cuvette was closed with the cap and the Nucleofector Program (U-023) was selected for high transfection efficiency or P-022 for high cell viability. (Here U-23 was selected for Nucleofector I Device).

The cuvette with cell/DNA suspension was inserted into the Nucleofector Cuvette Holder and the selected program was applied. The curvette was removed from the holder once the program was finished.500 µl of the pre-equilibrated culture media were added to the cuvette and the sample was gently transferred immediately into the 6-well plate (final volume 1.5 ml media per well/sample). Using the supplied pipettes and avoiding repeated aspiration of the sample.

3.3.2 Isolation of genomic DNA from Nucleofected Human Dermal Fibroblast using Sigma Genelute Mammalian DNA Prep Kit.

As per the manufacturer's manual: cells were harvested by detaching cell with trypsin. Cells were pelleted by centrifuging for 5 minutes at 300 × g; the culture medium were completely removed and discarded. The cell pellets were thoroughly resuspended in 200 mL of Resuspension Solution. The cells were lysed by adding 20 mL of the Proteinase K

solution to the sample, followed by 200 mL of lysis solution C. It was vortexed thoroughly (about 15 seconds), and incubated at 70 °C for 10 minutes to give a homogeneous mixture indicating efficient lysis.

3.4 Targeted integration workflow

As contained in the Sigma Compozr manual: Sigma Compozr workflow consists of:

i. Supplied pZDonor with a multiple cloning site (MCS) used to subclone in the Gene of Interest (GOI) in this case GFP.
ii. GFP-modified pZDonor, which contains a left and right homology arm to the AAVS1 genomic integration site.
iii. Co-transfection of the GFP-modified pZDonor and supplied mRNA encoding a pair of ZFNs that target the genomic integration site of AAVS1.
iv. ZFNs bind and cut the genomic AAVS1 site in the cell type of interest.
v. ZFN stimulated homology directed repair leads to targeted integration of the GOI into the genome of the cell type of interest.

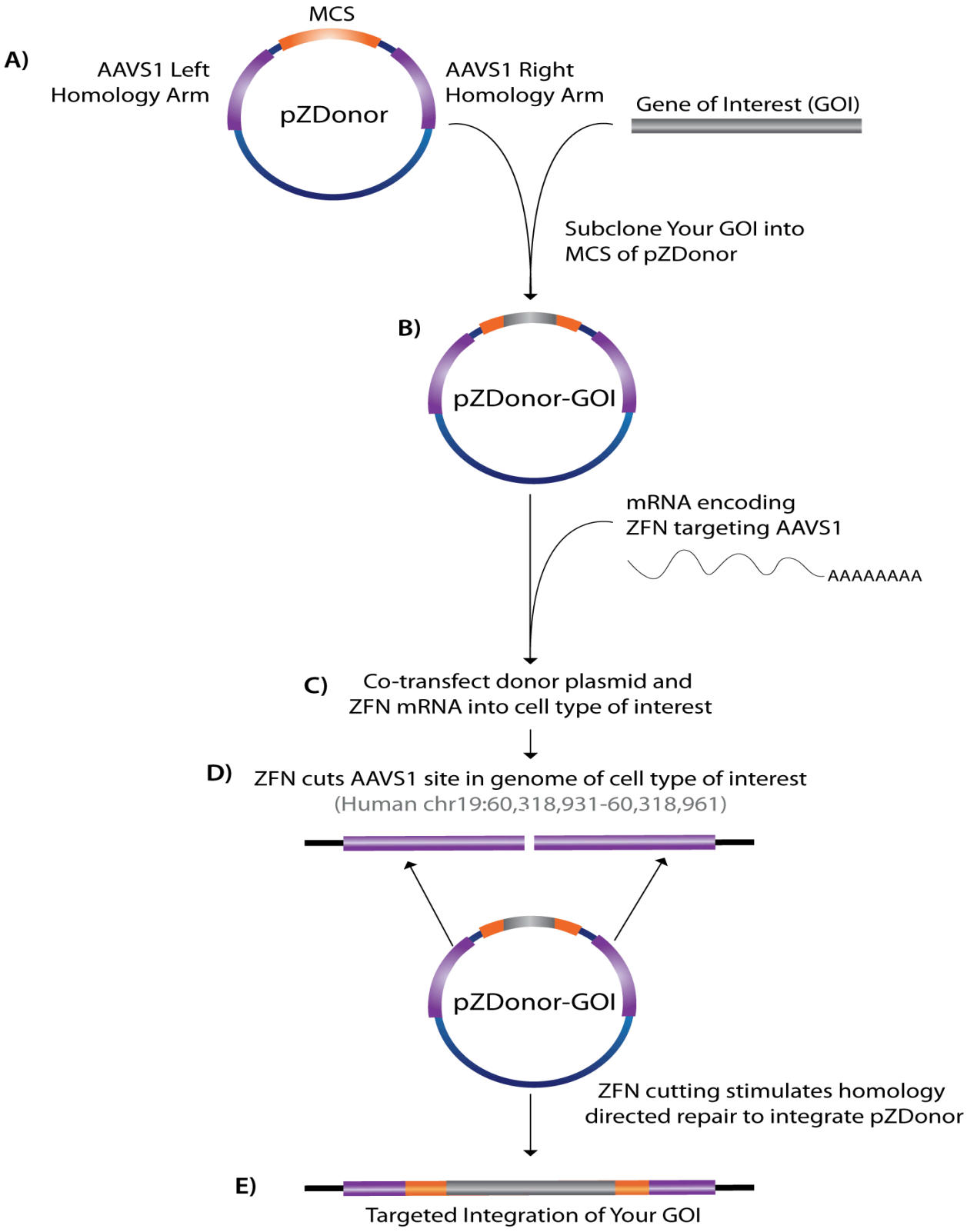


Figure 3.1 Targeted integration workflow using Sigma Compozr Zinc finger nucleases (from Sigma Compozr manual)

3.4. MATERIALS FOR CELL CULTURE

3.4.1 Cell lines:

A549 cells were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/ Streptomycin;

H358 cells were cultured in RPMI-1640 media + 10% FBS

H441 cells were cultured in RPMI-1640 media + 10% FBS

H23 cells were cultured in RPMI -1640media + 10% FBS

Normal lung -FC 3KT, bronchoaveolar cells were cultured with keratinocyte media (Gibco), containing Bovine Pituitary Extract, BPE, and Epidermal Growth Factor, EGF. Cells were maintained in an incubator at 37 degrees with 5 percent CO_2 according to standard procedures. Cells were obtained from ATCC, which authenticates cells using short tandem repeat (STR) profiling, morphological analysis, karyotyping, and isoenzyme analysis. The media were changed every 2 days. Cells were subcultured at about 70 to 80 percent confluency using Trypsin-EDTA.

3.4.2 Cell Transfection

All microRNA transfections and chemotherapeutics dose- response studies were done in triplicate. Ambion mirVana anti-miR microRNA inhibitors, and Ambion mirVana anti-miR microRNA Negative control were used. Anti-miR inhibitors are chemically modified, single-stranded nucleic acids designed to bind specifically to, and inhibit, endogenous microRNA molecules. Pre-miR precursors are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNA molecules. The anti-miR negative control no. 1 was used here, which is a random sequence anti-miR molecule that

was validated to produce no identifiable effects on microRNA function. To transfect cells with microRNA inhibitor, or control, 12.5µL of 2 mmol/L oligomer to 100 µL with Opti-MEM (Invitrogen Corp.), diluted 10 µL of Dharmafect1 (Life Technologies) to 100 µL with Opti-MEM, mixed the transfection components, added 2 µL of diluted cell suspension containing 20,000 cells, and incubated the plate at 37°C. After 24 h, the medium was removed, and the cells were trypsinized. For the growth inhibition assay, the cells were treated with chemotherapeutics and the further processed for SRB assay.

3.4.3. SRB Assay.

Cell growth and drug potency were tested using the sulforhodamine B proliferation assay In each experiment, 3,000 to 5,000 cells per well were seeded in 96-well plates after transfection with microRNA Inhibitors and incubated for 24 and 48h. Anticancer drugs (Cisplatin , Etoposide and Paclitaxel) were added in a seven-point dilution series, three replicate plate columns per treatment. After 48 h, incubation was terminated by replacing the medium with 100 uL of 10% trichloroacetic acid (Sigma-Aldrich) in 1X PBS and incubating at 4°C for 1h. The plates were then washed with water, air-dried overnight, and stained with 50 µL of 0.4% sulforhodamine B (Sigma) in 1% acetic acid for 30 min at room temperature. Unbound sulforhodamine B was washed off with 1% acetic acid. After air- drying and resolubilization of the protein-bound dye in 10 mmol/L of Tris-HCl (pH 8.0), absorbance was read in a Wallace victor2 multilabel plate reader at 570 nm. To determine IC_{50} values, the absorbance of control cells without drug was set at 1, and dose-response curves were plotted using Prism software (GraphPad Software, Inc.). Each experiment was done independently at least thrice.

After incubation of cells in chemotherapeutics Cisplatin, Etoposide and Paclitaxel or transfection with microRNA inhibitors(Ambion) without discarding the media cells were

fixed in situ by adding 50 μl per well of 50% cold TCA (w/v) (to give a final concentration of 10% TCA); plate were incubated for 1 hour at 4^{o}C;supernatant was discarded and plates washed 5 times with water and air dried overnight.Then,100 μl of sulforhodamine B (SRB) solution 0.4% (w/v) in 1% acetic acid were added to each well; and incubated for 30 minutes at room temperature. After staining, unbound dye was removed by washing 5 times with 1% acetic acid and plates were air dried. After air- drying and resolubilization of the protein-bound dye in 10 mmol/L of Tris-HCl (pH 8.0), absorbance was read in a Wallace victor2 microplate reader at 570 nm. To determine IC50 values, the absorbance of control cells without drug was set at 1, and dose-response curves were plotted using Prism software (GraphPad Software, Inc.). Each experiment was done independently at least thrice.

3.4.4. Renilla Luciferase Reporter Assay

The knockdown of 3'UTR-luciferase activity in the presence of mir-21 was measured by co-transfecting a miR-21 sensor reporter with a synthetic miRNA. DharmaFECT DUO was used to transfect H 358 cells in triplicate in 96-well format with 100ng of miR-21 sensor reporter and serial dilutions (50nM- 1.53nM) of antimiR-21 inhibitor or scrambled control miRNA. After 24 hours of incubation, 100μL of LightSwitch reagent was added to each well and luminescence was recorded, plates were incubated at room temperature for 30 minutes and read on a Wallace Victor 2 Multilabel plate reader.

3.4.5. Isolation of Total RNA

Cultured cancer cells were washed once with PBS and then lysed directly in the well by addition of 1ml of TRIzol reagent; lysed cells were removed from the well by scraping with a cell scraper. This mixture was transferred to a 1.5ml Eppendorf tube, RNA was then extracted according to manufacturer's instructions. Briefly, 200 μl chloroform was added

to the sample,and mixed by inverting the tube for 15 sec. Leave for 5 min on ice and spin at 12,000 x g for 15 min at 4^{o}C, which was then mixed and centrifuged. After centrifugation the sample had separated into three phases, the upper, aqueous RNA-containing phase was transferred to a 1.5ml Eppendorf and precipitated by addition of isopropanol. The sample was centrifuged, pelleted RNA washed with 75 percent ethanol and re-suspended in 32µl of sterile RNase free ddH20 (Ambion).

3.4.6 Quantitative Real Time Reverse Transcription PCR

According to the manufacturer, the miScript SYBR Green PCR Kit is based on the

QuantiTect SYBR Green Kits. The miScript SYBR Green PCR Kit includes QuantiTect SYBR Green PCR Master Mix and the miScript Universal Primer which is specific for the universal tag sequence on the 5' end of the cDNA.

The cDNA serves as the template for real-time PCR analysis using the miScript Primer Assay in combination with the miScript SYBR Green PCR Kit. miRNAs are amplified using the miScript Universal Primer together with the miRNA-specific primer (the miScript Primer Assay). Briefly, qPCR was done using the QuantiTect or miScript SYBR green PCR kit (Qiagen, Valencia, CA) using Roche Light cycler 480 real-time PCR system. RNUB6 was used as a control for miR-21-5p and miR-155-5p (miScript Primer Assay; Qiagen). Quantification was done using the ΔΔCt method, and then expressed as fold changes. PCR was performed in 384-well optical plates.

3.4.7 cDNA Synthesis using miScript II RT Kit (Qiagen, Valencia, CA)

Briefly Total RNA was reverse transcribed using the miScript II RT Kit (Qiagen, Valencia, CA), as per the manufacturer’s protocol

Table 3. 4 Qiagen miScript Reverse Transcription PCR scheme for H358 and FC 7333 3KT Cells for Experiment 4

	M1+	M1-	M2+	M2-	M3+	M3-
1. 5X Hiflex Buffer	4	4	4	4	4	4
2. 10X miScript Nucleics Mix	2	2	2	2	2	2
3. Rnase-free water	9.8	11.8	8.9	10.9	8.93	10.93
4. miScript Reverse Transcriptase Mix	2	0	2	0	2	0
5. Template RNA	2.2	2.2	3.1	3.1	3.07	3.07
Total	20	20	20	20	20	20

3.4.8 MiScript Primer Assay

MiScript Primer Assays are miRNA-specific primers used in combination with the miScript SYBR Green PCR Kit for real-time PCR of miRNA using SYBR Green detection. The miScript SYBR Green PCR Kit includes the miScript Universal Primer which is necessary for successful amplification.

3.5 Inhibition of MicroRNA Function

Transfection was done using Dharmafect1 (Life technologies) according to manufacturer manual as follows; H358 cells were trypsinized and counted. Cells were diluted in antibiotic-free complete medium to achieve the appropriate plating density in 100 µL of media solution. 100µL of cells were then plated into each well of a 96-well plate and incubated at 37°C with 5 percent CO_2 overnight.

Table 3.5 Dharmafect transfection protocol (from Dharmafect manual)

		Tube 1: diluted siRNA (µL/well)		Tube 2: diluted DharmaFECT (µL/well)			
Plating Format (wells/ plate)	Surface Area (cm_2/ well)	Volume of 5 µM siRNA (µL)	Serum-free Medium (µL)	Volume of DharmaFECT reagent (µL)*	Serum-free Medium (µL)	Complete Medium (µL/well)	Total Transfection Volume (µL/well)
96	0.3	0.5	9.5	0.05 –0.5	9.95 - 9.5	80	100
24	2	2.5	47.5	0.25-2.5	49.75 – 47.5	400	500
12	4	5	95	0.5-5.0	99.5 - 95.0	800	1000
6	10	10	190	1.0-10.0	199.0 - 190.0	1600	2000

(Source: Dharmafect, Life Technologies)

Table 3.6 Transfection Protocol

Tube A				
	Wells	Dharma	Media	Total
Anti-21	48	0.4	9.6	10
extra 20%		19.2	460.8	480
total	0.2	23.04	552.96	576
		Tube B		
	Vol(µL)	wells	extra	total
Anti-21	5	48	48	288
media	5	48	48	288
Total	10	96	96	576

3.5.1 Chemotherapeutics treatment

The schedule for chemotherapeutics treatments were as follows. Three commonly used drugs for NSCLC were used-Cisplatin, Etoposide (Sigma) and Paclitaxel.

Table 3.7 Chemotherapeutics treatment of H358 Cells (9X wells)

			Cisplatin			
			3µM stock		30µM stock	
	0	0.1µM	1µM	5µM	10µM	15µM
Cisplatin	0	29.7	299.7	150.3	299.7	450
Media	900	870.3	600.3	749.7	600.3	450
Total	900	900	900	900	900	900
			Etoposide			
			1.01 µM stock Conc.		10.1 µM stock conc.	
	Dmso*	0.01µM	0.05µM	0.1µM	1µM	5µM
Dmso	4.5					
Etoposide	0	9	45	90	90	250
Media	895.5	891	855	810	810	650
Total	900	900	900	900	900	900
	(0.5% DMSO)*					
			Paclitaxel			
			23.4 nM stock conc.			
	Dmso*	0.1 nM	0.5 nM	1 nM	5 nM	10 nM
Dmso	3.87					
Paclitaxel	0	3.8	19.3	38.5	192.5	385
Media (µl)	896.13	896.2	880.7	861.5	707.5	515
Total (µl)	900	900	900	900	900	900
	(0.0004% DMSO)*					

3.6 Statistical Analysis

Data were expressed as means ± SD of the experiments outcome, performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student's t-test, while One-Way ANOVA was used for multiple means comparison. $P < 0.05$ was considered significant. Graphpad Instat version 3 and Graphpad-Prism version 6 softwares were used for analysis. For relative quantification of gene expression using qPCR; the data were analyzed by comparative Ct method ($\Delta\Delta$Ct).

CHAPTER FOUR

4.0 RESULTS

4.1 H358 CELL CULTURE

Plate 1, H358, lung adenocarcinoma cells, showed rapid proliferation in culture following plating. The cells were seen spreading and forming intercellular networks within days of seeding the cells. They were adherent cells. A and B lung cancer cells one day after plating, C and D are the cells after 5 days in culture, showing massive proliferation of the cells, using Axioimager Epi-Flourescent microscope (Carl Zeiss).

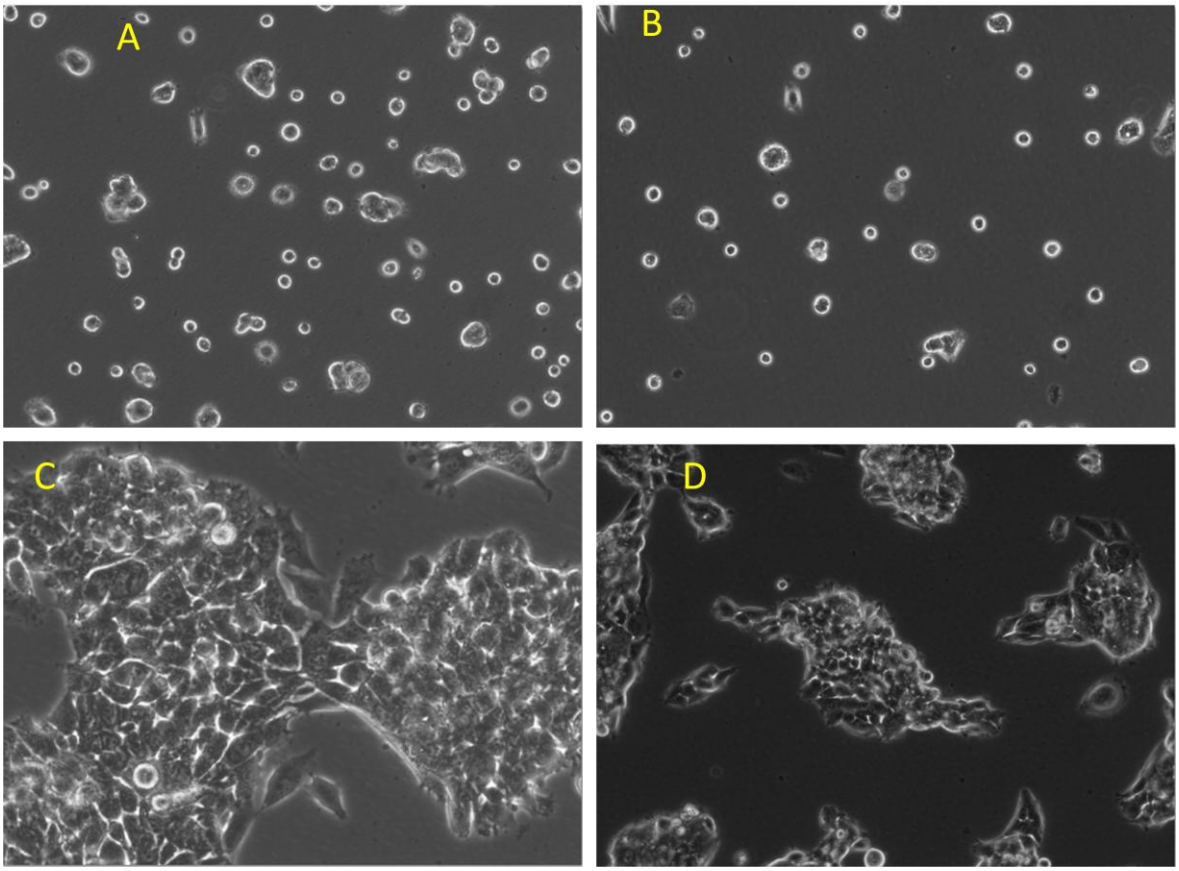


Plate 1: Phase contrast micrographs of H358 lung adenocarcinoma cells. A and B Lung cancer cells one day after plating, C and D are the cells after 5 days in culture, showing massive proliferation of the adherent cells.

4.2 MICRORNA PROFILING OF H358, A549, USING MISCRIPT QUANTITATIVE REAL TIME-PCR ASSAY (QIAGEN)

4.2.1 Mir-21 is Up-Regulated In H358, A549 Lung Cancer Cell Lines

Figure 4.1 demonstrates the relative miRNA expression profile of miR-21 in H358 and A549 (cancer) compared to normal lung cells (Bronchio-aveolar cells), miR-21 was up-regulated in Cancer cells, (H358 and A549), using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. The level of miR-21 in H358 cells were significantly higher than the normal lung cells ($P<0.05$).

Figure 4.2 also shows the expression level of miR-21 in H358 (cancer) and compared to normal lung cells; here equally miR-21 was significantly up-regulated in Cancer cells, (H358) using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. ($P=0.07$)

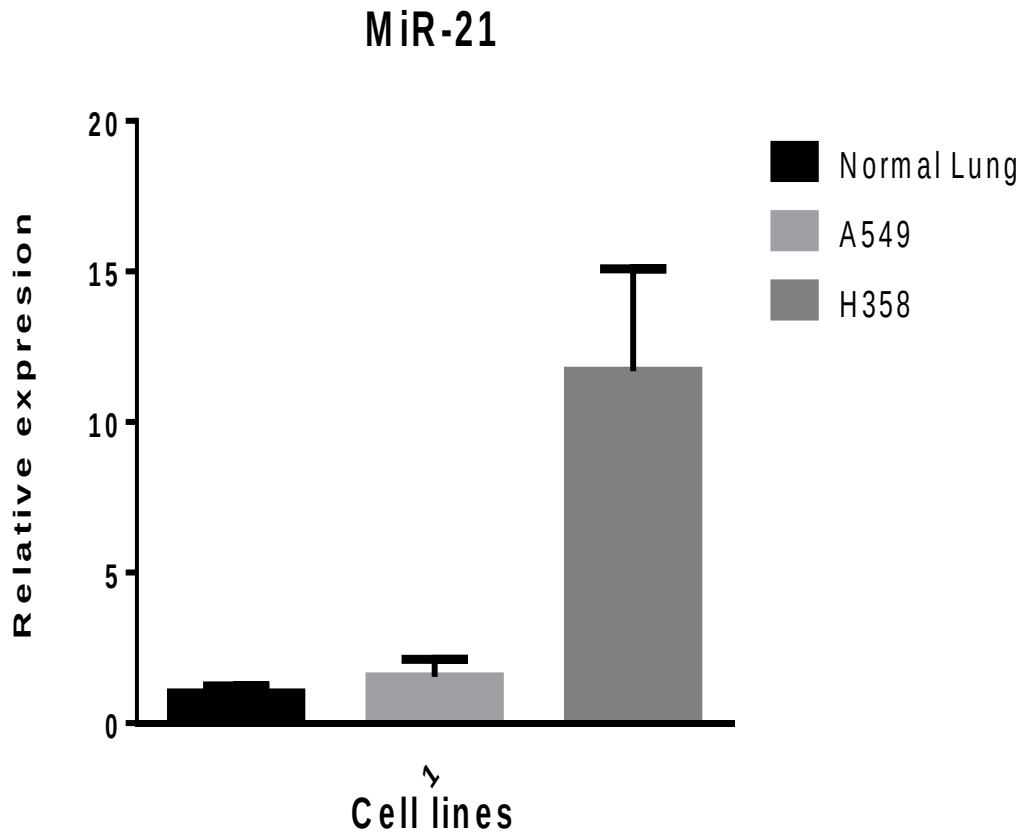


Figure 4.1 Expression level of miR-21 in H358 and A549 (cancer) and normal lung cells, miR-21 is up-regulated in Cancer cells, H358 and A549, using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. ($P<0.05$)

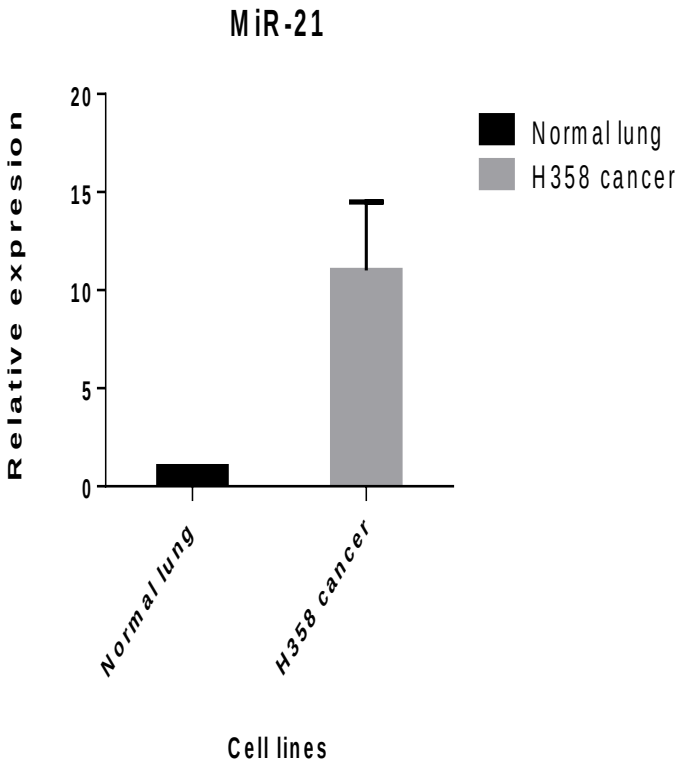


Figure 4.2 Expression level of miR-21 in H358 (cancer) and normal lung cells, miR-21 was up-regulated in Cancer cells, (H358) using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. (P=0.07)

4.2.2 MiR-155 is Down-regulated in H358 lung cancer Line.

Figure 4.3 shows relative miRNA expression level of miR-155 in H358 and A549 (cancer) compared to normal lung cells, miR-155 was down-regulated in Cancer cells (H358 and A549).

While figure 4.4 shows the expression level of miR-155 in H358 (cancer) and normal lung cells, miR-155 was down-regulated in Cancer cells H358, (from cDNA C2 as well as cDNA M2), using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. The result showed down regulation of miR-155 in two biological replicates of H358 lines.

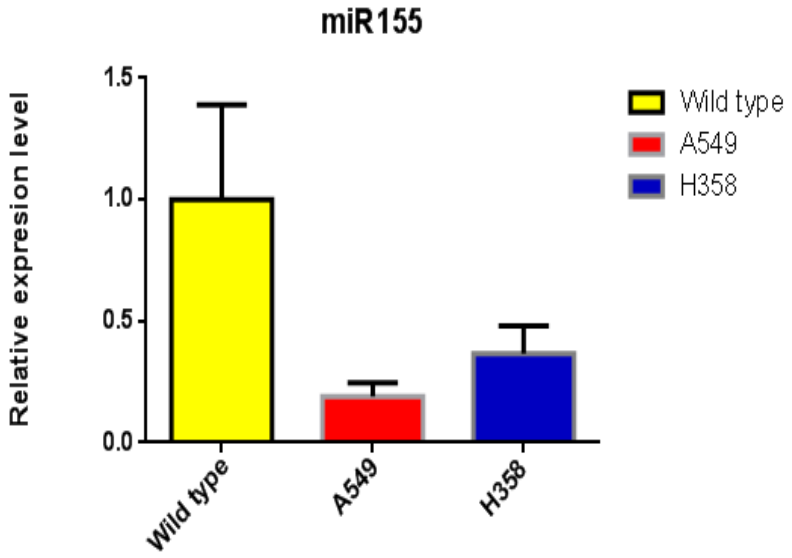


Figure 4.3 Expression level of miR-155 in H358 and A549 (cancer cells) and normal lung cells, miR-155 is downregulated in H358 and A549 cells using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 and a549 compared to FC 7333 3KT (normal lung) Cells.

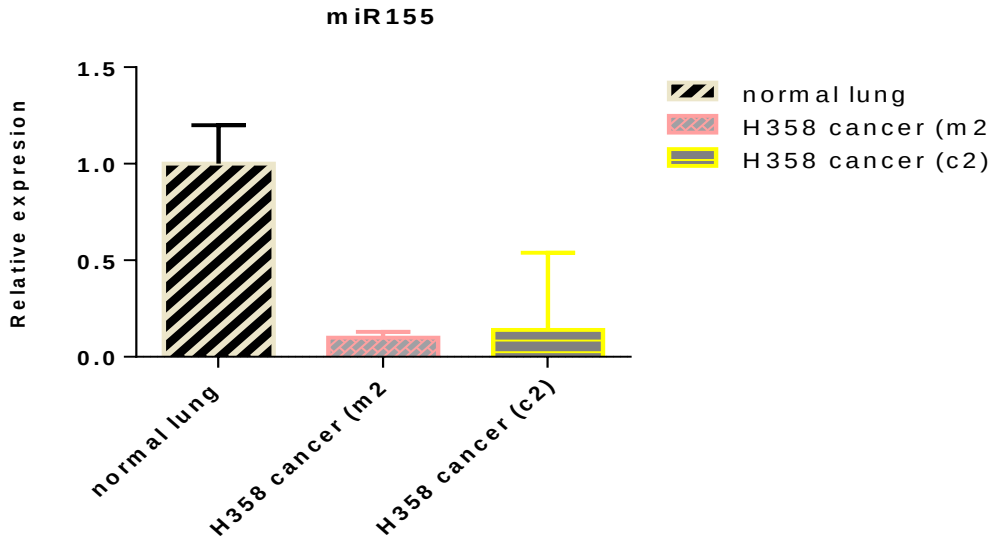


Figure 4.4 Expression level of miR-155 in H358 (cancer) and normal lung cells, miR-155 is down-regulated in H358 (M2 and C2) cells using qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 and FC 7333 3KT (Normal Lung Cells) ($p<0.05$)

4.3 Determination of IC_{50} of the Chemotherapeutics used

The dose-response curve of treating H358 cancer lines with three commonly used Chemotherapeutics in the management of Lung cancer were determined by SRB assay after 72h and 96h incubation with Etoposide, Cisplatin and Paclitaxel. The results are shown below;

4.3.1 The IC_{50} of Cisplatin following incubation of H358 cells in Cisplatin.

Figure 4.5 is the Dose-response curve Inhibitory Concentration, IC_{50} (defined as the drug concentration causing a 50% decrease of absorbance over that of untreated control) of Cisplatin following 96h incubation of H358 in serial concentrations of Cisplatin. The cells were fixed with 1% TCA and analyzed using SRB assay. The IC_{50} of Cisplatin (72h incubation) is 10 µM and 5.47µM for 96h incubation. The data were generated using Graphpad Prism v 6.

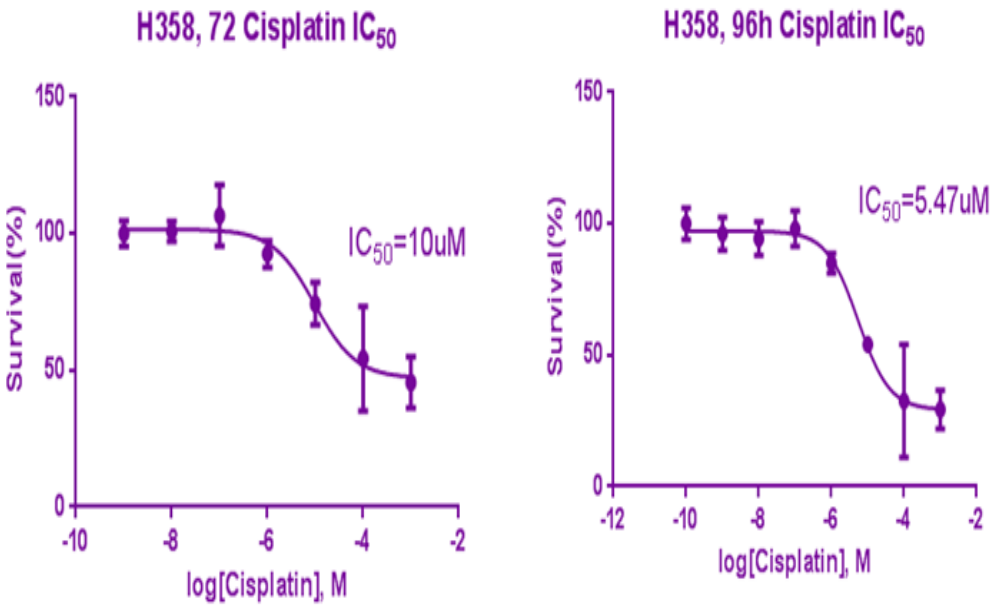


Figure 4.5: The Dose response curve IC_{50} of Cisplatin following 72h and 96h incubation of H358 in various concentrations of Cisplatin and analyzed using SRB assay. The data generated were plotted with Graphpad Prism version 6.

4.3.2 The IC_{50} of Etoposide following incubation of H358 cells in Etoposide.

Figure 4.6 is the Dose-response curve IC_{50} of Etoposide following 72h and 96h incubation of H358 in serial concentrations of Paclitaxel. The cells were fixed with 1% TCA and analyzed using SRB assay. Data were generated using Graphpad Prism 6

The IC_{50} of Etoposide (72h incubation) is 15.8 µM and 0.1µM for 96h incubation. The data were generated using Graphpad Prism v 6.

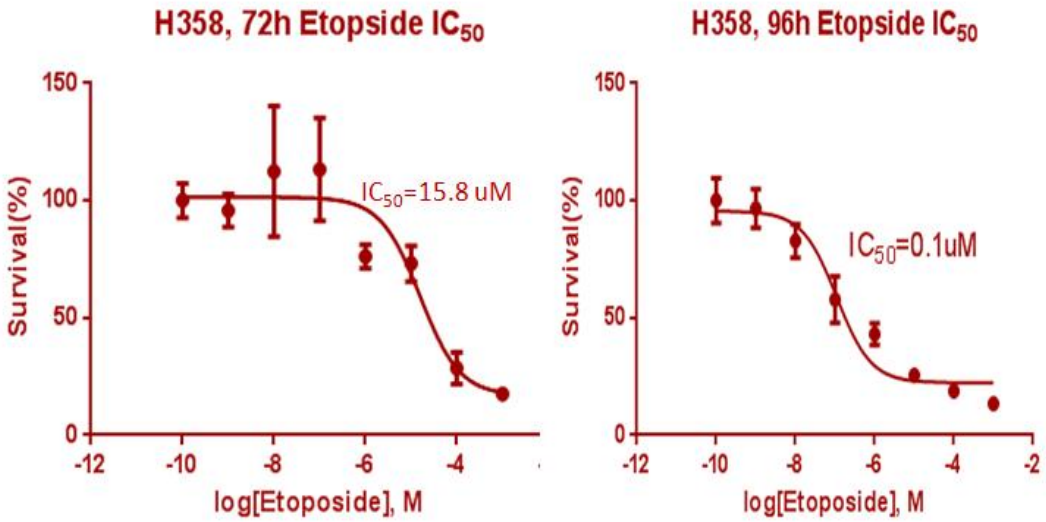


Figure 4.6: The Dose response curve IC_{50} of Etoposide following 72h and 96h incubation of H358 in various concentrations of Etoposide and analyzed using SRB assay. The data generated were plotted with Graphpad Prism version 6.

4.3.3 Determination of IC_{50} of Paclitaxel following incubation of H358 cells in Paclitaxel.

Figure 4.7 is the Dose-response curve IC_{50} of Paclitaxel following 72h and 96h incubation of H358 in serial concentrations of Paclitaxel. The cells were fixed with 1% TCA and analyzed using SRB assay. The IC_{50} of Paclitaxel (72h incubation) is 3.0 µM and 0.7µM for 96h incubation. The data were generated using Graphpad Prism v 6.

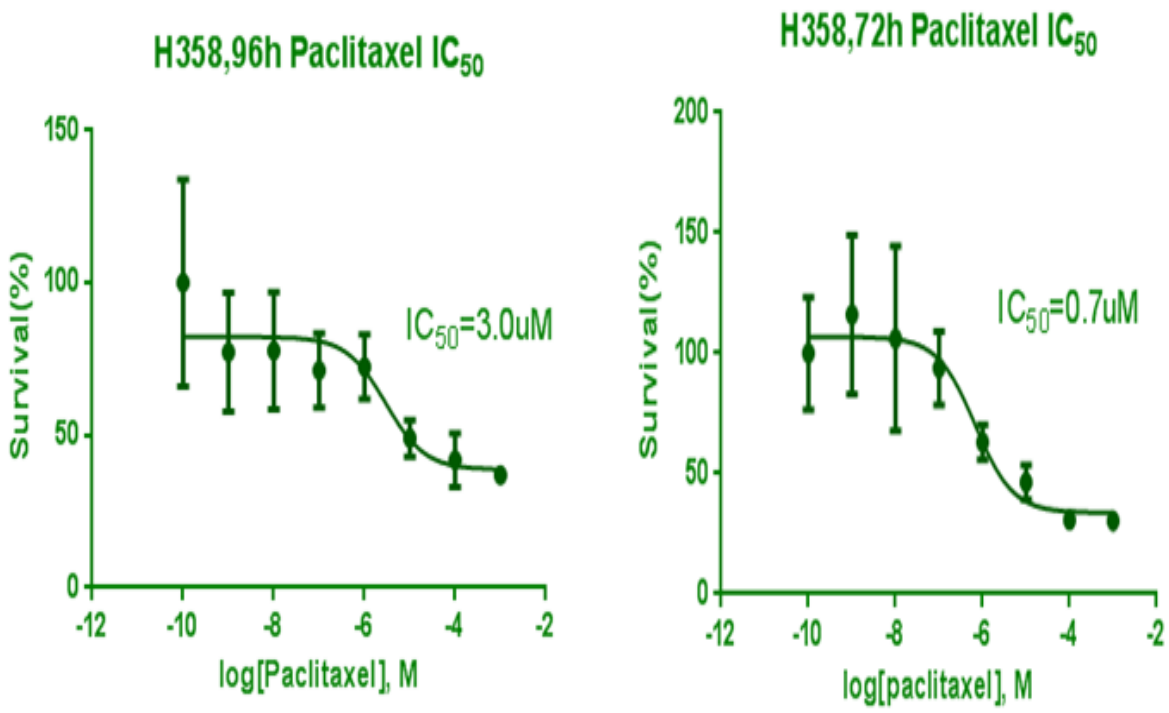


Figure 4.7 Dose-response curve, IC_{50} of Paclitaxel following 72h and 96h incubation of H358 in various concentrations of Paclitaxel and analyzed using SRB assay. The data generated were plotted using Graphpad Prism version 6.

4.4 Down-regulation of miR-21 Inhibited Survival Capacity of H358 Cells after Chemotherapeutics treatment

To assess whether miR-21 down-regulation could sensitize NSCLC H358 cells to chemotherapeutics, H358 cells were transfected with either anti-miR-scrambled Control(Ambion) or anti-miR- 21(Ambion). The following serial concentrations were used for both the control and the miRNA inhibitor- (5nM, 10nM, 20nM, 40nM). After 48h incubation the cells were in turn treated with various concentrations of Etoposide which was the selected chemotherapeutic due its robust activity from the previous IC_{50} experiments. Their response was analyzed using SRB assay. In SRB survival assay, there was decreased survival capacity of H358 cells transfected with 40nM anti-miR-21; 7 days post transfection and 7 day post Etoposide treatment (Figure 4.7). Forty-eight hours after transfection, H358 cells were treated with various doses of Etoposide (1.25, 2.5, 5, 10, 20μM) and the survival percentage upon incubation were detected. As shown in figure 4.9, the survival fraction of H358 cells in 40nM anti-miR-21-transfected group was significantly lower than that in the DF control transfected group. There were clear decrease in survival of all antimiR-21 transfected cells (5, 10, 20, 40nM respectively) at the lower doses of Etoposide indicating that down-regulation of miR-21 could significantly enhance the sensitivity of H358 cells to Chemotherapeutics (figure 4.8).

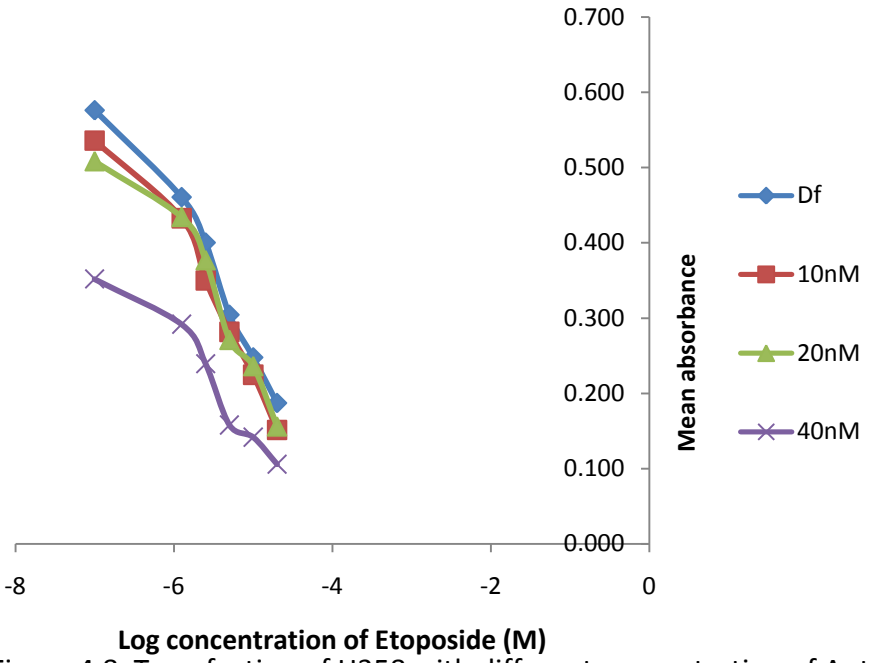


Figure 4.8. Transfection of H358 with different concentration of Anti- miR21 Inhibitor (D5 post transfection and D3 post Chemo-treatment), compared to Dharmafect alone treated control(DF). 40nM of antimir-21 significantly reduced the survival of H358 cell Increasing the sensitivity of the cells to chemotherapeutics Etoposide, in a dose dependent fashion.

4. 5 Does inhibition of miR-21 and 155 functions in H358 cells lead to reduced survival of cancer cells?

In order to answer this question H358 cells were transfected with 25nM and 50nM of antimiR-21 and 155 using Dharmafect1 transfection solution and incubated for 3days, 5 days and 6days post-transfection and analyzed using SRB survival assay. Figure 4.10 shows the result obtained:

Transfection of H358 with 50nM of antimiR-21microRNA inhibitor (Ambion) significantly inhibited the growth of H358 cells in all the three times points (3 days, 5days, 6days) compared to antimiR-155, which seem to support qPCR data showing down-regulation of miR-155 in H358 cells relative to normal lung cells. The effect of 50nM concentration of the anti-miR seem to be very marked compared to the 25nM concentration showing that the effect could be dose dependent. This observation could also be a preliminary validation of the qPCR data suggesting that miR-155 could be acting as a tumour suppressor in lung cancer cells (figures 4.10 and 4.11).

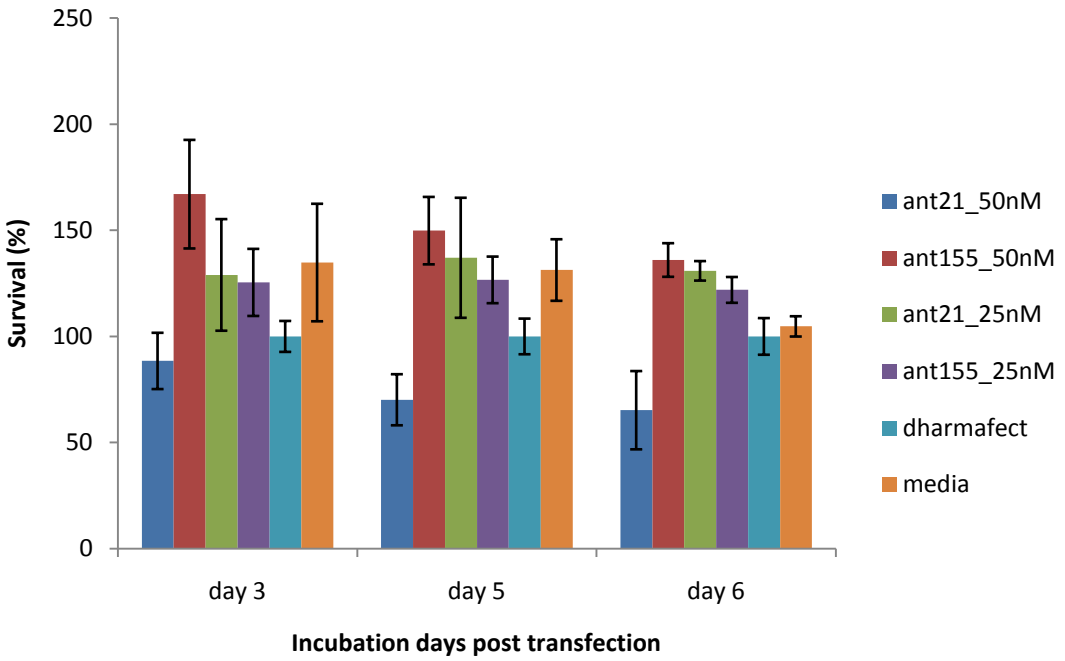


Figure 4.9 Inhibition of H358 with AntimiR-21 and 155. H358 cells were transfected with 50nM and 25nM of Ambion microRNA inhibitors (antimiR -21 and 155). 50nM of antimiR-21 significantly inhibited the growth of H358 cells in all the 3 times points.

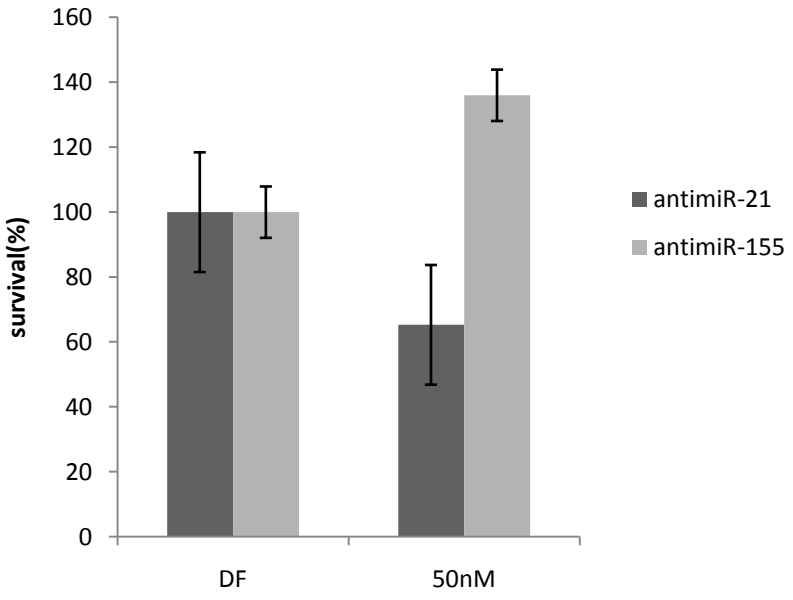


Figure 4.10 H358 was transfected with 50nM of Ambion microRNA inhibitors (antimiR -21 and 155) using Dharmafect 1 transfection reagent (Thermoscientific). 50nM of antimiR-21 significantly inhibited the growth of H358 cells 6 days post-transfection.

4.6 Luciferase assay

Inorder to verify transfection efficiency and to show that the antimiR-21 specifically binds target, Renilla Luciferase using miR-21 sensor (Switchgear Genomics) was co-transfected with antimiR-21 microRNA inhibitor(Ambion) as well as with Scrambled anti-miR control(Ambion) using Dharmafect Duo(Life Technologies). Serial dilution of AntimiR-21 and negative control(Scramble) were made from 50nM to 1.53nM. Figure 4.11 shows the result obtained: which clearly demonstrates de-repression of reporter activity in H358 cells indicating the functioning or transfection efficiency of the antimiR-21 used. The Scramble control are seen not evoking similar responses like anti-miR21. This shows that the killing seen in the scramble control could be normal toxicity of nucleic acid.

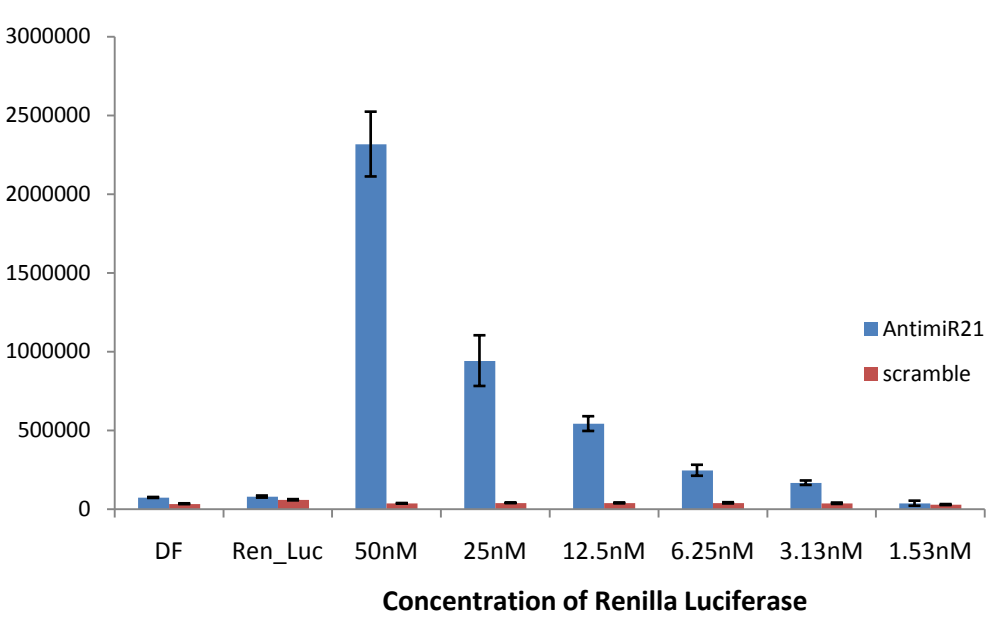


Figure 4.11: Co-transfection of H358 cells with 100nM of Renilla MiR-21 sensor and various concentrations of anti-mir21 and scramble control serially diluted from 50nM to 1.53nM solutions using Dharmafect duo transfection reagent. Result shows increased reporter activity indicating the functioning or transfection efficiency of the antimiR-21 used.The Scramble control are seen not evoking similar responses like antimir21. which shows that the killing seen in the scramble control could be normal toxicity of nucleic acid.

4.7 Recombineering in SW102 and Zinc finger Nuclease mediated dermal fibroblast Targeting result

The goal of this work was to express GFP in human Dermal Fibroblast using Zinc finger nucleases known for precise genome editing using homology directed repair mechnism called recombineering in a strain of Bacteria called SW102 which is like a BAC system with a defective galk gene which makes it unable to metabolize galactose as only carbon source. As such this is a desirable selection scheme in recombineering as reintroduction of Galk in trans shows efficeint recombineering event. Here the results of the recombineering and targeted integration of GFP in the AAVS1 locus of the dermal fibroblast are shown.

4.7.1 Amplification of pGal*K* gene

PGalK were amplified using the following primers and with plate 2, depicts the Amplification of *galK* gene, showing excised 1.2 kb band corresponding to galk,

The two buffers were used to optimize the buffer to be used tat would yield better

amplification result. The galk gene amplified was later purified and used for transformation of Sw102 strain of E. coli used in recombineering. The result showed that the amplification of the plasmid with the primers with 50 bp homology to target plasmid (pSA-2A-GFP-PA) actually ampliflied Galk gene and its promoter from the pGalk plasmid.

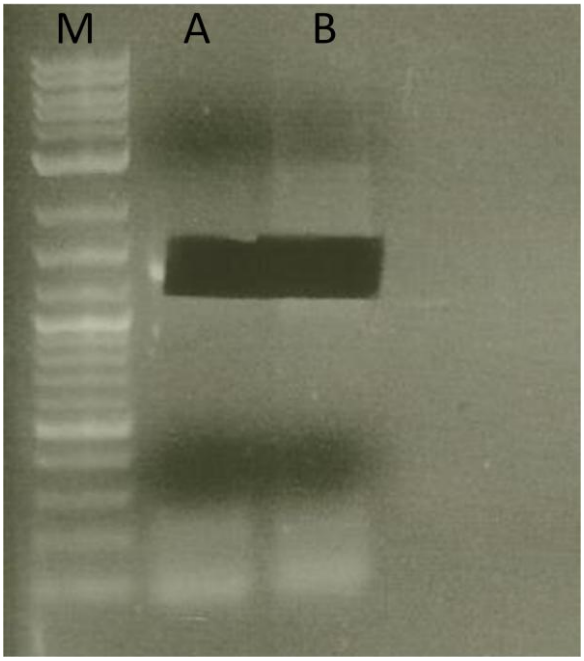


Plate 2: Amplification of *galK* gene, showing excised 1.2 kb band corresponding to galk, M is 1Kb Plus DNA ladder, lane A is HF (buffer) pgalk plasmid PCR product, while B is GC(buffer) p*Galk* plasmid PCR product. The two buffers were used to optimize the buffer to be used for better amplification.

4.7.2 LB Plate Showing Successful Expression of *Galk* Gene SW102 Strain of E.*Coli.*

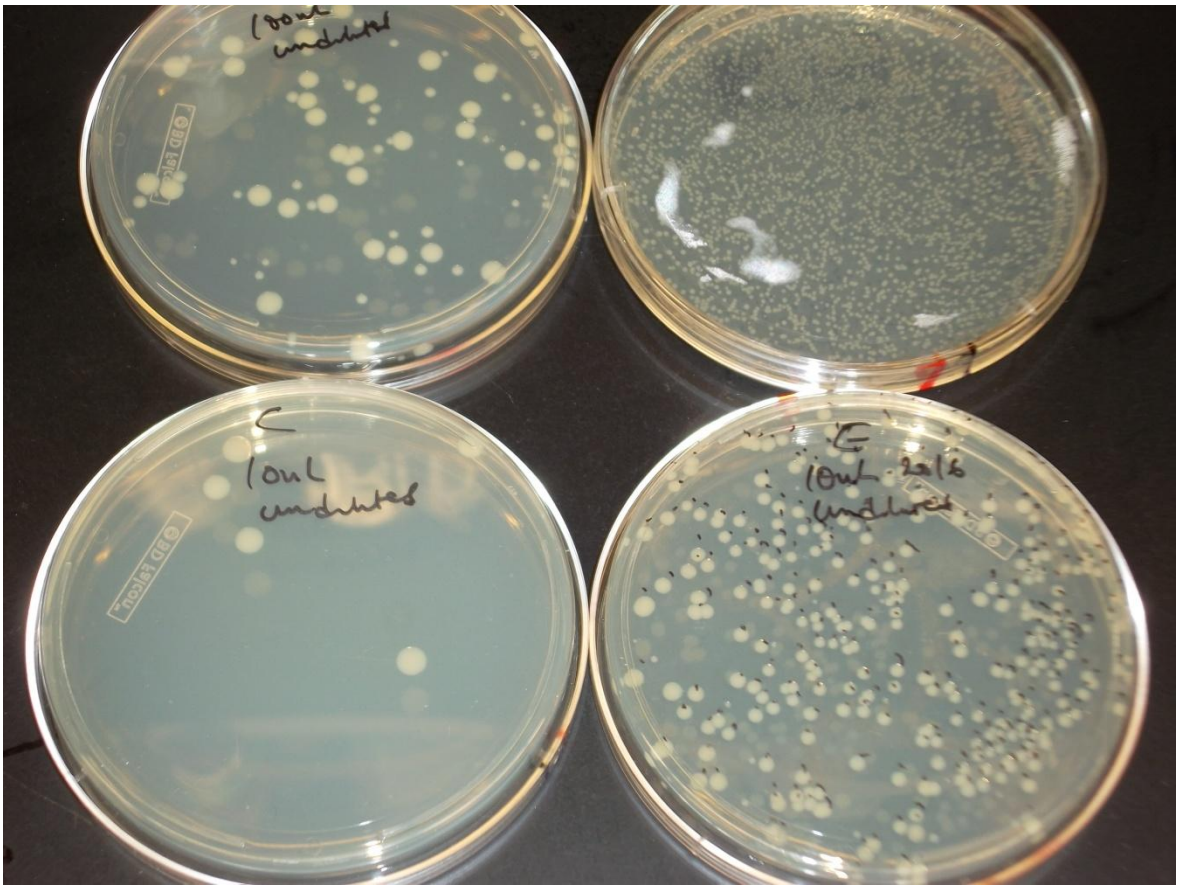


Plate 3: LB plate showing successful expression of *galk* gene in SW102 strain of E.*coli*.

4.7.2 Transformation of SW102 with Psa-21-GFP-2P Plasmid and PCR Products in Both Control and Experimental Cells Show Successful Recombineering.

There was cell growth in E1 plate the cells there grew at 90µl concentration while at 10µl showed less growth compared to the former. Furthermore the E2 cells had more growth than the E1 at 90µl concentration. There was equally growth in the 10µl plate.

Plate E3 (pGalk plasmid) had growth but one or two colonies. This was the positive control. The negative control plate C4 showed no cell growth at both 90µl and 10µl concentrations

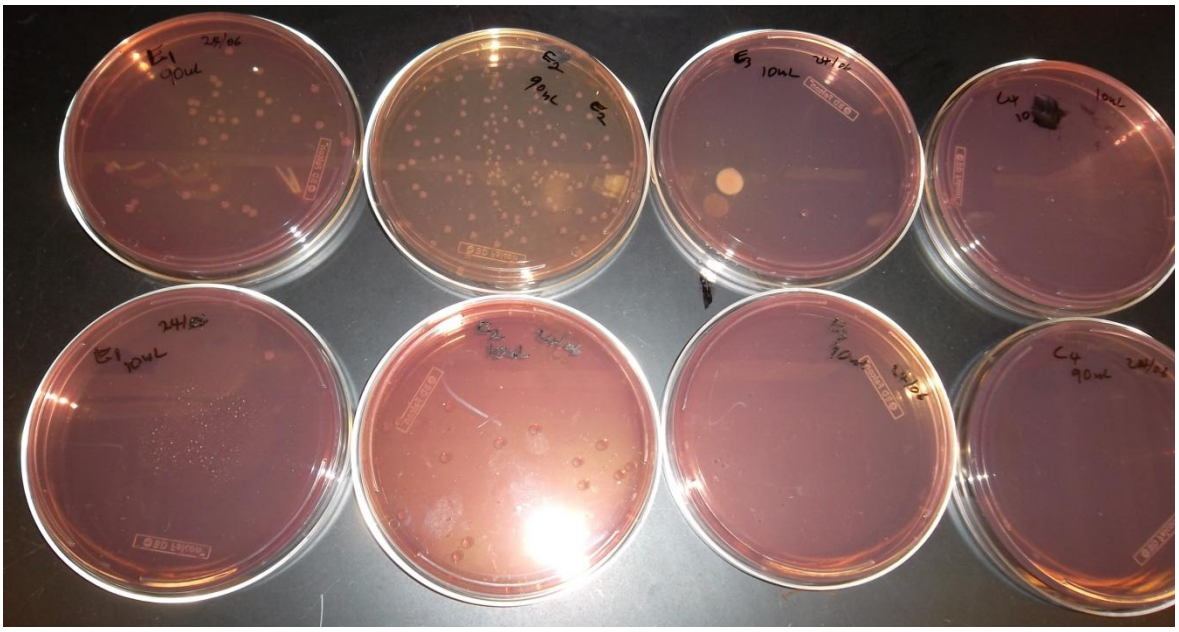


Plate 4: McConkey plates showing expression of *Galk* gene in trans that enables transformant colonies to survive media with galactose as the only carbon source. Only successfully transformed cells expressing the *galk* gene can grow on this minimum indicator plate. There was cell growth in the experiment group (E1) plate/ the cells there grew at 90µl concentration while at 10µl showed less growth compared to the former. Furthermore, the E2 cells had more growth than the E1 at 90ul concentration. There was equally growth in the 10µl plate. There was no colony in C4 the negative control group.

4.7.3 Transformation Efficiency of Competent Cells

Efficiency is expressed as colonies per microgram, µg of DNA. Plate 2 shows the result obtained.

Table 4.1Transformation efficiency on McConkey plate

Experiment group	No of colonies	Amount DNA(ng)	Transformation efficiency (colonies/µg)
E1	21	0.1	210000 =2.1x10^5
E2	351	0.1	3510000=3.5x10^6
E2	985	0.1	985000=9.9x10^5

4.7.4 PCR to Confirm Integration of GFP into the AAVS1 Site

Plate 5 shows PCR to confirm integration of GFP into the AAVS1 site. SW102 Cells were transfected via nucleofection and harvested 1 day after transfection. DNA was prepared, and PCR and digestions were done as previously described. The gel showed the expected 300bp detecting GFP containing FP-detection-F and FP- detection –R primers as well as 1.8kb band corresponding to FP-detection-F and reverse AAVS1 primers successfully detecting junction between GFP and genomic locus AAVS1 (at chromosome -19). This result demonstrated integration and expression of GFP in the locus of interest, following a successful recombineering event.1A and 1B (negative control) 2A and 2B (positive control) 3A and 3B (GFP detection experiment group) 4A and B(Integration detection).

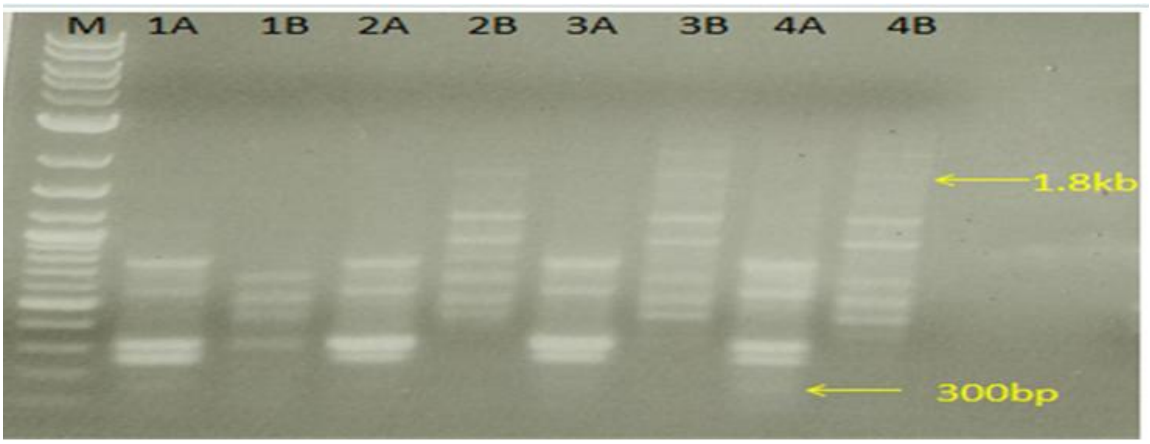


Plate 5: Gel of PCR to confirm integration of GFP into the AAVS1 site. SW102 cells were transfected via nucleofection and harvested 1 day after transfection. DNA was prepared, and PCR and digestions were done as previously described. the gel show the expected 300bp detecting GFP containing FP-detection-F and FP- detection –R primers as well as 1.8kb band corresponding to FP-detection-F and reverse AAVS1 primers successfully detecting junction between GFP and genomic locus AAVS1 (at chromosome -19). 1A and 1B (negative control) 2A and 2B (positive control) 3A and 3B (GFP detection experiment group) 4A and B(Integration detection).

4.7.5 Diagnostic PCR to verify recombined and GFP expressing clones from Mini-prep.

Here, PCR was set up to verify or confirm integration of GFP into the AAVS1 site. SW102 Clones expressing the GFP from the nucleofected dermal fibroblast were mini-prepared. PCR and digestions were done as previously described. The gel showed the expected 200bp verifying GFP containing SA2A-GalK-verif -F and SA2A-GalK-verif–R primers as well as 600bp band corresponding to CAGGS-GalK-verif-F and CAGGS-GalK-verif-R primers successfully verifying junction between GFP and genomic locus AAVS1 (at chromosome-19). This result demonstrates integration and expression of GFP in the locus of interest, following a successful recombineering event.

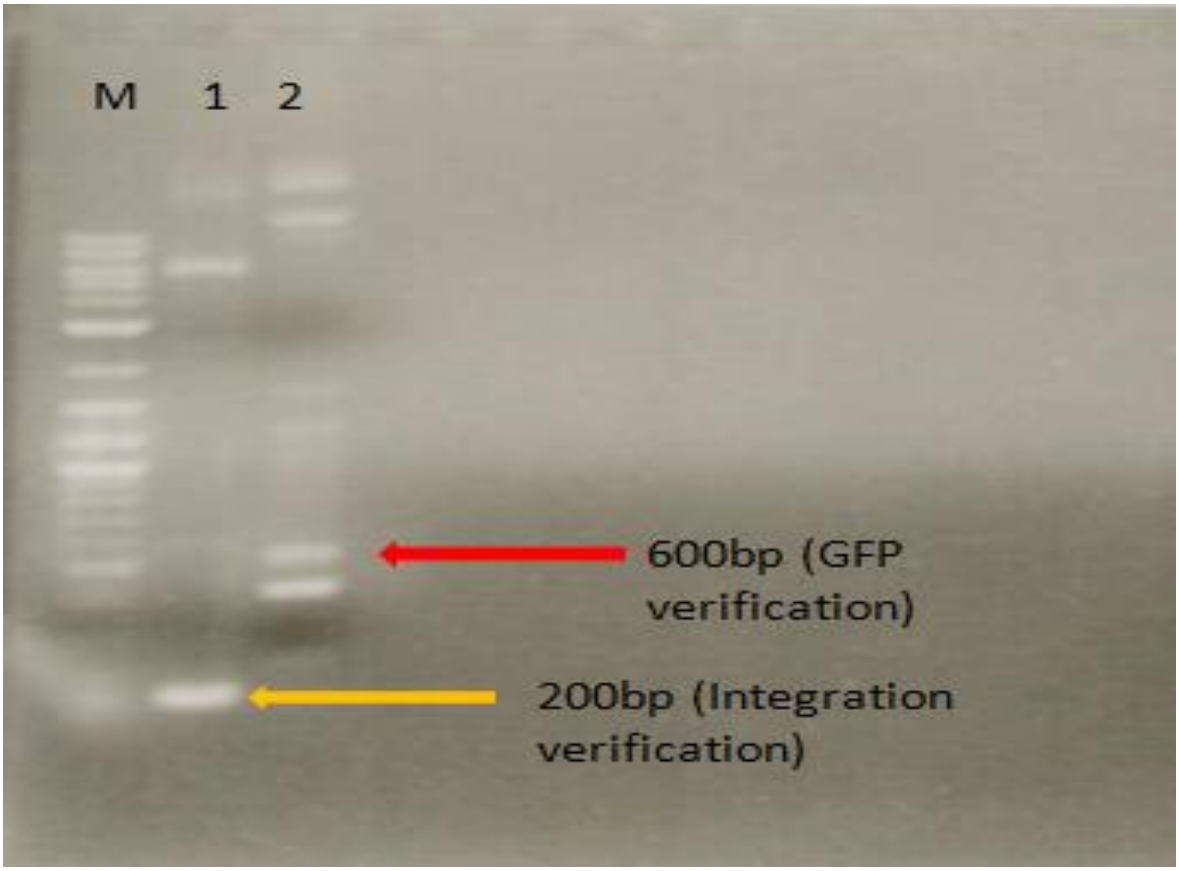


Plate 6: Gel of diagnostic PCR to verify recombined and GFP expressing clones from miniprep.SW102 Cells expressing the GFP from the nucleofected dermal fibroblast were minipreped. DNA was prepared, and PCR and digestions were done as previously described. The gel showed the expected 200bp verifying GFP containing SA2A-GalK-verif -F and SA2A-GalK-verif–R primers as well as 600bp band corresponding to CAGGS-GalK-verif-F and CAGGS-GalK-verif-R primers. M(1Kb DNA Plus ladder), 2 (CAGGS-GalK-verif-F), 3 (CAGGS-GalK-verif-R).

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 MICRORNA MEDIATED SENSITIZATION OF LUNG CANCER CELLS TO CHEMOTHERAPEUTICS

Lung cancer continues to be the leading cause of cancer death globally. As opined by Slack and Weidhaas, (2006), the role of miRNAs in various facets of human tumorigenesis, (such as cell proliferation, differentiation, invasion, apoptosis, angiogenesis, migration, and motility) begs the question whether correcting these anomalies of the miRNome can be effectively harnessed for therapeutic gains? Promising preclinical data from many groups encourage this hypothesis and provide the biological enthusiasm for clinical studies in this direction. Re-introduction of miRNAs down-regulated in cancer and/or silencing of miRNAs up-regulated in the tumor may be hoped to drive apoptosis in cancer cells and lead to a desirable therapeutic outcome (Slack *et al.*, 2006).

Using microarray data, Gao *et al*., (2011) compared miRNAs expression profiles in primary squamous cell lung carcinoma with normal cells and highlighted miRNA potential relevance to clinical events and patient survival periods. They observed that miR-21 was upregulated in nearly 75% of cancer specimens and that this signature was significantly correlated with shortened survival (Gao *et al.*, 2011). Furthermore, aberrant expression of miRNAs and their roles in malignant transformation and tumor development has been a focus of research in recent times. MiRNA expression signatures seem to hold great promise in cancer diagnosis and treatment, and strategies to interfering with miRNA function are considered to offer novel opportunities for cancer treatment

This work was designed to look at the expression profile of two known oncomir, miR-21 and miR-155 in lung cancer, and to see whether re-introduction or inhibition of these would affect progression or aid sensitivities of the lung cancer cells to major chemotherapeutics used in the management of lung cancer.

The finding of this study showed that miR-21 in lung adenocarcinoma cells lines H358 and A549 were highly expressed than that in normal human bronchial epithelial cell line. This finding is in line many reports from other laboratory showing upregulation of miR-21 in many cancers than the normal cells. In fact, miR-21 is thought to play a significant role in the drug resistance to cisplatin in the neuroblastoma cells. Many reports suggest, miR-21 is important for the resistance to various kinds of drugs in different types of cancer cells, including resistance to doxorubicin in bladder cancer cells (Tao *et al.*, 2011b); to trastuzumab, topotecan, or taxol in breast cancer cells(Corcoran *et al.*, 2011;Gong *et al.*, 2011); to arabinosylcytosine or daunorubicin in leukemia cells(Bai *et al.*, 2013;Bai *et al.*, 2011b); to 5-fluorouracil in colorectal cancer cells(Valeri *et al.*, 2010); to interferon α/5-fluorouracil in hepatocellular carcinoma cells (Tomimaru *et al.*, 2010); to VM-26 or temozolomide in glioblastoma cells and to gemcitabine in pancreatic ductal carcinoma cells (Giovannetti *et al.*, 2010).

However, expression of miR-155 was down- regulated in the Lung cancer lines compared to the normal cells. This result seems to suggest that miR-155 could be acting as tumour suppressor in some Lung cancer lines. Earlier report by Slack and colleagues showed that hypoxia leads to the upregulation of miR-155 in lung cancer cells.

They specifically, identified a role for HIF-1α in the induction of miR-155. They believed their data were supported by the observation that miR-155 was induced in renal cancer

cells lacking functional Von Hippel-Lindau (VHL), and that this induction depends in part on HIF-1α (Babar *et al.*, 2011).

Furthermore, the Slack group (Babar *et al.*, 2012) equally showed that 'overexpression of miR-155 in lymphoid tissues resulted in disseminated lymphoma characterized by a clonal, transplantable pre-B-cell population of neoplastic lymphocytes. Again that the 'withdrawal of miR-155 in mice with established disease resulted in rapid regression of lymphadenopathy, in part due to apoptosis of the malignant lymphocytes, demonstrating that these tumors are dependent on miR-155 expression. They equally showed that systemic delivery of antisense peptide nucleic acids encapsulated in unique polymer nanoparticles inhibits miR-155 and slowed the growth of "addicted" pre-B-cell tumors in vivo, suggesting a promising therapeutic option for lymphoma/leukemia'(Babar *et al.*, 2012). From these report, down regulation of miR-155 in H358, an equally non-small cell lung cancer cell line seem to support two reports suggesting that miR-155 the so called "master-regulator" of numerous biological processes, most notably those involved in immune function and cancer development could also be acting as a tumour suppressor.

This finding that miR-155 could be acting as a potential tumour suppressor in H358 lung cancer line is supported by the work of Gasparini in Triple negative breast cancer (Gasparini *et al.*, 2014). They showed that 'overexpression of microRNA 155 (miR-155) in human breast cancer cells reduced the levels of RAD51 and affects the cellular response to irradiation, IR. MiR-155 directly targets the 3′- untranslated region of RAD51. Overexpression of miR-155 decreased the efficiency of homologous recombination repair and enhanced sensitivity to IR in vitro and in vivo' (Gasparini *et al.*, 2014).

This observation therefore supports the 'double-edged role of miR-155: from oncomir in most of the cancer models to protective in TNBCs' and in H358 lung cancer line that this

current work reported. Their proposed rationale for this completely unexplored role of miR-155 is believed to be based on TNBC's high dependency on the Homologus Recombination repair pathway (Gasparini *et al.*, 2014)

In another study, that seem to support miR-155 as a potential tumor suppressor, Levati and others, reported that 'microRNA-155 (*miR-155*) was down-regulated in melanoma cells as compared with normal melanocytes and that its ectopic expression impaired proliferation and induced apoptosis' (Levati *et al.*, 2011).

They further investigated whether *miR-155* could mediate melanoma growth inhibition via *SKI* gene silencing. Using Luciferase reporter assays they demonstrated that *miR-155* interacted with *SKI* 3′UTR and impaired gene expression (Levati *et al.*, 2011).

As noted by Croce and colleagues, 'therapies combined with adjuvant radiotherapy have been demonstrated to improve the survival of triple-negative breast cancer patients; however, such therapy is challenged by the emergence of resistance in tumor cells. It is, therefore, essential to develop novel therapeutic strategies to overcome both radio-resistance and chemo-resistance and improve radio- and chemo-sensitivity' (Gasparini *et al.*, 2014).

Development of resistance to chemotherapy or radiotherapy is a serious problem in the management of cancer, greatly increasing patient morbidity and mortality. Thus, this work set out to investigate whether miR-21 and miR-155 could affect the sensitivity of lung cancer cells to chemotherapy. Data from this work showed that inhibition of miR-21 significantly enhanced the sensitivity of Lung Cancer cells to chemotherapeutic agents (Etoposide) Figure 4.4.

From IC_{50} (defined as the drug concentration causing a 50% decrease of absorbance over that of untreated control) data of various chemotherapeutics used such as Etoposide, cisplatin and Paclitaxel, effective dosage of the drugs and antimiR were used to set up experimental matrix to assess the effect of the chemotherapeutics and miRNA-inhibition on cell death or cell survival. The results showed that in H358 lines, IC_{50} of Etoposide following 72h incubation was 15.8μM, while after 96h incubation it was 0.1μM. Furthermore, the IC_{50} for Cisplatin after 72h incubation was given as 10μM and 5.45μM after 96h of incubation in Cisplatin. For Paclitaxel the IC_{50} after 72h incubation was 0.7μM and 3.0μM after 96h of incubation in Paclitaxel.

Inhibition of microRNAs using Antimirs in this study demonstrated that knock-down of miR-21 could significantly inhibit growth of lung cancer cells by inducing apoptosis enhancement. This observation is supported by various reports in other human cancers, showing that miR- 21 up-regulations promote the growth of tumor cells. Furthermore other laboratories have shown that miR-21 could affect the metastatic behavior of tumor cells. Yang et al. (Yang *et al.*, 2013b) reported that miR-21 regulates the metastatic behavior of B16 melanoma cells by promoting cell proliferation, survival, and migration/invasion as well as by suppressing IFN action.

Moreover, Zhu et al. (Zhu *et al.*, 2012) showed that miR-21 functions in both invasion and tumor metastasis by targeting multiple tumor/ metastasis suppressor genes, suggesting that suppression of miR-21 may provide a novel approach for the treatment of advanced cancers. Therefore inhibition of miR-21 in H358 in this current study would provide this novel tumour targeting approach in discuss.

It is important however, to keep in mind that microRNA functions can be cell context and tissue-dependent. For example, inhibition of mir-21 using anti- sense oligonucleotides increased the growth of HeLa cells (derived from cervical cancer) but did not significantly affect the growth of A549 cells (non–small cell lung cancer) (Lu *et al.*, 2005). The later finding was also observed in this study in relation to A549.

5.2 ZINC FINGER NUCLEASE-MEDIATED EXPRESSION OF GFP INTO HUMAN DERMAL FIBROBLAST AAVS1 LOCUS

The initial idea that Zinc finger nucleases, ZFNs could stimulate gene targeting in mammalian somatic cells came from the work of Porteus and colleagues (Porteus *et al.*, 2005a). In these experiments, recognition sites for known zinc finger DNA-binding domains were inserted into a green fluorescent protein (GFP) reporter gene, which was integrated as a single copy into the genome of the human embryonic kidney cell line. In this system, gene targeting is measured by the correction of an integrated mutant GFP target gene by a transfected donor plasmid and the resultant conversion of GFP-negative cells into GFP-positive cells. In addition, this work showed that targeting with ZFN could be induced simultaneously at both the site of the break and at a distance of 400 bp from the break, demonstrating that a single pair of ZFNs can stimulate targeting in a relatively large region surrounding a DSB.

Urnov and workers reported that designed ZFNs can cleave an endogenous human gene in cultured cells and lead to targeted gene replacement in up to 20% of the cells (Urnov *et al.*, 2005). The target was the gene for interleukin (IL) - 2Rγ, a cytokine receptor that is required for T-cell development and the establishment of a functional immune system. Until now, ZFNs have been primarily applied to transformed mammalian cell lines that are relatively resistant to apoptotic stimuli. An important advance, therefore, will be to develop

ways of using ZFNs in primary cells (like the human dermal fibroblast that this research addressed) known to be more sensitive to DNA damage.

The second theme of this work was to perform targeted integration of a gene into human dermal fibroblasts, using the GFP versions of the plasmids of interest. In order to achieve gene integration in the AAVS1 locus, SW102 cells, a strain of E.*coli* with defective *galk* gene were transfected with two components: a GFP-encoding plasmid with homology to the AAVS1 locus, and RNA encoding the zinc finger nucleases that target the locus, using a technique called Nucleofection to introduce the DNA and RNA molecules into the cells.

The galk recombineering used in this work according to Copeland *et al.,* (2001) involved *galK* positive and negative selection scheme which be used to make point mutations, deletions, and insertions. SW102 as noted above contains the lamda prophage recombineering system; the galactokinase gene (*galK*) is defective. The *galK* selection scheme is a two-step system: First, a cassette containing *galK* flanked by arms that are homologous to the target site AAVS1 locus was inserted into the BAC. Recombinant bacteria were then selected by being able to grow on minimal medium like M63 with glycerol or MacConkey Plates with galactose as the only carbon source. Second, the *galK* cassette was substituted by a GFP, a PCR product, with 50 bp homology flanking the cassette. Recombinants were identified by selecting against *galK* by resistance to 2-deoxy-galactose (DOG) on minimal plates with glycerol as the carbon source. DOG is harmless unless phosphorylated by GalK, which turns it into a non-metabolizable, toxic intermediate (plate 6). The observed background clones (which were DOG resistant clones without the desired mutation) seen in the control group arose by spontaneous deletion of the *galK* gene and flanking DNA. As also noted by Copeland *et al.,* (2001) increasing the length of the homology arms (33 bp gives an efficiency of 50% correct; 50 bp is said to give 80%).

Consistent with their finding this work utilized 50 bp left and right homology arms targeting the AAVS1 and achieved the expected 80% expression of the desired clones (plates 5 and 6).

The whole essence of this project was to build an intelligent protein, GFP in this case that would be able to monitor sugar level to trigger precise release of insulin, in order to control sugar levels in diabetics. The data obtained in this work showed that the expression of GFP in the AAVS1 site, the so-called 'safe-harbour' of transgenes in the human genome located in Chromosome-19 using zinc finger nucleases were very successful. This could be seen from the result of the FP forward/reverse PCR, where the expected band of ~300bp, and for the FP-detection-F/AAVS1-R reactions, where the expected band of approximately 1800bp where achieved from the gel read-out in figure 22. This detection was further confirmed by a diagnostic PCR to verify the integration at AAVS1 site as well as the expression of the GFP at the precise locus by detecting the ~200bp band verifying GFP expression and 600bp band verifying integration, plate 6.

Data presented in this work were in line with the work of Pincelli and her colleagues (Coluccio *et al.*, 2013) who used alternative gene transfer strategy aimed at targeting a "safe harbor" locus, the adeno-associated virus integration site 1 (AAVS1), by zinc-finger nuclease (ZFN)-induced homologous recombination (HR) similar to this very work. However, in their case, they focused on the 'delivery of AAVS1-specific ZFNs and a GFP-expressing HR cassette by integration-defective lentiviral (LV) vectors (IDLVs) or adenoviral (Ad) vectors which according to them resulted in targeted gene addition with an efficiency of >20% in a human keratinocyte cell line, >10% in immortalized keratinocytes, and <1% in primary keratinocytes. The finding of this work showed that the

recombineering event known to be between 3 to 30% was equally successful judging by transformation efficiency of $3.5x\ 10^6$ and above obtained in the experiment groups.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY AND CONCLUSION

This present study supported the demonstrated biological functions of miR-21, with the ability to promote growth, migration, and invasion, chemo-resistance of NSCLC cells. These results indicated that inhibition of miR-21 might be a rational therapeutic strategy for the treatment of NSCLC in the future. It remains to be investigated whether the other differentially expressed miRNAs also participate in NSCLC progression. More importantly this work has shown that miR-155 could be acting as a tumour suppressor. Which will change therapeutic approach of miRNA mediated targeting of Lung cancers.

Green fluorescent protein, GFP was successfully expressed in dermal fibroblast targeting the AAVS1 site using *galk* recombineering scheme in SW102 strain mediated by zinc finger nucleases, a homology repair genome editing tool that holds sufficient promise for insulin- signaling that will enable trans-dermal insulin therapy in diabetics in the near future. This work therefore explored targeted therapies in two very challenging medical conditions: cancer and diabetes with novel molecular approaches; microRNAs and Zinc finger nucleases.

6.2 RECOMMENDATIONS

This work further recommends the following line of action:

- Over-expression of miR-155 using Pre-microRNA mimics.

- Development of efficient *in-vivo* delivery system for miRNA.
- Scale up from cell lines to mice and other higher animal models.
- Replacement of Green fluorescent protein, GFP with Red fluorescent, RFP and Yellow fluorescent protein, YFP in targeting plasmid.
- Also to incorporate targeting with CRISPR CAS-9 system to improve targeting efficiency and to align with the latest trend in genome engineering.

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APPENDICES

APPENDIX I

Recombineering using the modified DH10B strain DY380

by Søren Warming, Ph.D.

Overview

1. Design primers with 50 bp homology suited for either targeting (insertion of a selectable marker into a specific site) or gap repair (retrieving a piece of DNA into a linear piece of vector backbone).

2. Run PCR and digest residual plasmid template away with *Dpn*I (1-2 □ l in a standard 25 □ l PCR reaction, @37°C for 1 h). *Dpn*I works fine in PCR buffers. Gel purify the PCR product and elute in ddH_2O. If concentration is low, EtOH precipitate and dissolve in small volume of ddH_2O. You need 2 x 100-300 ng rather concentrated PCR product per experiment.

3. Alternatively, design primers to amplify "mini-arms" of 250-500 bp length, and clone these arms into an appropriate vector using conventional cloning techniques. The vector is prepared by digestion/linearization and gel purification of the desired band.

Background will be higher than with *Dpn*I digested PCR fragments, but the efficiency is very high when the arms are longer.

4. Transform your target plasmid (or BAC) into electrocompetent DY380 cells, see below. Alternatively, co-transform target plasmid and PCR product (see note below).

5. From a single isolated colony, inoculate a 5 ml o/n culture with appropriate selection

6. Perform the heat shock induction, followed by transformation (the Recombineering experiment).

Transformation (electroporation) of DY380

1. Make a 5 ml o/n culture either from the DY380 freeze stock or from a colony containing a target plasmid. **IMPORTANT**: Keep the cells @ 32°C or lower. The
DY380 strain is resistant to tetracycline (12.5 □ g/ml), but it is not necessary to include selection in this step – unless you are afraid of contamination. If a target plasmid/BAC is already in the strain, use the appropriate antibiotic in order not to lose the plasmid/BAC *(you don't have to double select using both tet and the other marker – the plasmid marker selection is enough).*

2. Dilute the o/n culture 1:50, i.e. transfer 500 □ l into an autoclaved 50 ml Erlenmeyer baffled flask with 25 ml LB. Incubate for 3-5 hours with appropriate antibiotic selection in a 32°C shaking waterbath until the density reaches an OD_{600} of 0.6. Remember to save 1 ml LB before adding the 500 □ l o/n culture. Use this as a reference when measuring the OD. Put a bottle of 10% glycerol or ddH_2O in an ice/waterbath slurry. *(If the competent cells are to be used right away, use ddH_2O. If the competent cells are going to be stored @ -*

70°C for later use, you must use 10% glycerol. In the following ddH_2O is used, but this should be substituted with 10% glycerol if appropriate).

3. When the OD_{600} is 0.6 the flasks containing the bacteria are cooled down in the ice/waterbath slurry for a minute or two and subsequently transferred into pre-cooled 15 ml Falcon tubes.

4. Spin down the bacteria in a cold (0°C) centrifuge for 5 min @ 5000 RPM (standard Beckman or Eppendorf centrifuge)

5. Pour off all supernatant and briefly invert tube on a paper towel, and add 1 ml icecold ddH_2O while keeping the tube in the icewater. Resuspend the pellet in the ddH_2O by gently shaking the tube in the ice/waterbath *(Gently move the tubes around in circles while keeping them in the ice/water slurry. This can take a while the first time, around 5 minutes. When the cells are prepared for electroporation after heat shock, see later, you might notice that the un-induced half is more difficult to get into suspension than the induced one).* When resuspended, fill up to 10 ml with ice cold ddH_2O, invert a couple of times, and spin again for 5 minutes.

6. Pour off supernatant, resuspend as in step 5 *(this time resuspension is much faster),* and spin once more.

7. Gently remove ALL supernatant by inverting the tube on a paper towel or shake out the final bit – be careful so you don't lose the pellet. Store the competent cells on ice.

Gently resuspend the pellet as before, but in the residual small amount of ddH_2O left in the tube *(volume should be around 50 □l).*

8. Transfer 25 □ l of the freshly made electrocompetent cells to a pre-cooled eppendorf tube and mix with the DNA to be transformed (1-5 □ l DNA). Transfer to a pre-cooled 0.1 cm cuvette. To put in a target plasmid for subsequent recombination, use 1-10 ng supercoiled plasmid. To put in a BAC, use 1-5 □ g relatively freshly prepared BAC DNA *(DNA made same day works best, but even DNA stored a couple of months in your –20°C freezer will work).*

9. Transform by electroporation. Transfer the bacteria to a tube with 1 ml LB medium. Incubate @ 32°C in a shaking waterbath for 1 h.
10. Plate the transformed bacteria on selective medium (see note below), *e.g.* 1 □ l, 10 □ l, 100 □ l, and the rest*(transfer to an eppendorf tube, spin down @ full speed for 10 seconds, and remove most of the supernatant. The bacteria will become "hairy" or "fluffy" after the 1 hour incubation. This is quite normal and doesn't influence the outcome of the experiment. When plating "the rest", it might be an advantage to pipet up and down BEFORE the 10 sec spin. The cells don't become "fluffy" when heatshocked, see below).*
11. Incubate @ 32°C for 18-24 hours.

Heat shock induction of the □Red recombination proteins – addition to the transformation protocol

1. When the bacteria containing the target plasmid/BAC have reached an OD_{600} of 0.6, transfer 10 ml to an autoclaved 50 ml Erlenmeyer flask, and put the rest (10-12 ml) back @ 32°C. The 10 ml are incubated in a shaking waterbath @ 42°C for *exactly* 15 min.

2. Cool down the bacteria by gently shaking the flask in the ice/waterbath for a minute or two. Pour the bacteria into 15 ml Falcon pre-cooled tubes (one for the bacteria grown @ 32°C and one for the heat-shocked bacteria incubated @ 42°C) and place in the ice/waterbath slurry. Go to step 4 in the transformation protocol above.

3. Transformation of the bacteria grown @ 32°C only (*i.e.* no heat shock) is the control experiment. No or very few colonies should appear on this plate. When using longer arms and preparation of the vector by digestion/gel preparation, instead of PCR + *Dpn*I digest, the background will be higher – still the ratio between the heat-shocked and the control plate should be significant.

4. Check a number of colonies (miniprep followed by digestion or PCR). For targeting experiments using plasmids, the bacteria might contain both un-recombined and recombined plasmid. You can purify correct clones by re-transforming the purified plasmid into e.g. DH10B and select for the antibiotic resistance present only in the targeted plasmid *(often this would be kanamycin)*. Alternatively *(works with 100% efficiency)*, linearize the miniprep DNA, dilute, selfligate, and transform bacteria – select for the antibiotic resistance present only in the targeted plasmid *(this way, if the targeted and un-targeted plasmids were entangled, the targeted plasmid is released and can be purified after being re-transformed).*

Notes

(1) Antibiotics and selection: Plate on LB plates with appropriate selection: For gap repair *(also known as "retrieval")* select for the resistance present in the backbone of the PCR product/retrieval vector and NOT the resistance of the target plasmid (MUST be different). For targeting, co-select for the resistance genes present on BOTH the PCR product/targeting vector and the target plasmid. We use the following concentrations:

- BACs
  - chloramphenicol (cm): 12.5 μg/ml
  - kanamycin (kan): 25 μg/ml
  - ampicillin (amp): 50 μg/ml
  - tetracyclin (tet): 12.5 μg/ml
- Plasmids
  - cm: 25 μg/ml
  - amp: 100 μg/ml,
  - kan: 50 μg/ml

(2) LB media: For 1 liter: 10 g Tryptone, 5 g Yeast extract, 5 g NaCl, ddH_2O up to 1 liter. Autoclave.

(3) Co-transformation: Instead of first introducing the target plasmid into DY380 followed by heat-shock and transformation with a PCR product/targeting cassette, the target plasmid and the PCR/targeting cassette can be co-transformed into +/- heatshocked and electro-competent DY380. The efficiency will be lower, but with this strategy you don't have to first introduce the target plasmid, and you often don't have to purify by re-transformation. With BAC modifications/retrieval, however, you should always put the

BAC in the strain first, followed by transformation with your PCR product/retrieval vector/targeting cassette *(the efficiency of linear DNA + BAC cotransformation is simply too low)*. Using 50 bp homology arms and PCR products for targeting, I suggest that the target plasmid is introduced into the strain first. However, using the longer homology arms, co-transformation can be very efficient.

(4) Oligos for recombineering experiments: Use your favorite oligo-provider for standard primers for PCR, say up to 30-40 bp. However, when using longer primers, up to 100 bp, choosing a good oligo-provider can be crucial. We are pretty happy about the oligo quality from Invitrogen, so we always use them for our long oligos. No special purification, just desalting.

APPENDIX II

PROTOCOL FOR TOTAL RNA ISOLATION

REAGENTS/EQUIPMENT

RNase/DNase free 2.0/1.5/0.5 ml eppendorf tubes

Rnase/DNase free pipette tips of appropriate size

TRI-reagent (Sigma, T-9424) – protect from light, store 40C

Chloroform

Isopropanol

Ethanol 100 %

0.1 % Diethyl Pyrocarbonate (DEPC) -treated ddH_2O, store 40C

Liquid Nitrogen

Ultra Turrax tissue homogeniser

Refrigerated Centrifuge (up to 12,000 x g)

Ice bucket

Gloves

Eye protection

Fume hood

0.1 % DEPC water

500 □ l DEPC

500 ml millipore H_2O

Cover and leave to stir overnight in fume hood. Autoclave next morning and store at 4 ^{o}C.

METHOD

1. Keep samples frozen during weighing (work in liquid nitrogen). Weigh out ~ 50 - 100 mg tissue into a 2 ml eppendorf tube.

2. Add 0.5 ml TRI-reagent to frozen tissue and keep on ice. Homogenise (using the ultra turrax) for 30 sec at level 5.

3. Add an additional 0.5 ml TRI reagent. Can use this to wash off excess sample from the homogeniser down into the tube.

4. Wash the homogeniser between each sample. Set up four 25 ml plastic tubes with 10-15 ml 0.1% DEPC water. Switch on the probe in first tube and leave for several seconds making sure probe is well washed, switch off and move onto next tube, repeat. Repeat with remaining tubes. Change 0.1% DEPC water regularly. Wipe probe before starting next sample.

5. Incubate the homogenised sample for 5 min on ice to permit the complete dissociation of nucleoprotein complexes.

6. Add 200 µl chloroform. Mix by inverting the tube for 15 sec. Leave for 5 min on ice and spin at 12,000 x g for 15 min at 4 ^{o}C.

7. Transfer the aqueous phase (upper phase) to a new 1.5 ml tube and precipitate the RNA with 500 µl Isopropanol. If extracting DNA also see appropriate protocol – do not throw away interphase of lower phase, can keep at 4^{o}C overnight.

8. Incubate the samples for ~ 2 hours or overnight at -20 ^{o}C.

9. Centrifuge at 12,000 x g for 10 min at 4 ^{o}C.

10. Remove the supernatant by pipetting and wash the pellet with 1 ml 75% ethanol-DEPC water. Gently flick the tube to dislodge to pellet allowing

the ethanol to circulate round the pellet. Incubate for 10 min on ice to remove trace of TRI-reagent, and then spin at 5,000 x g for 5 min at 4 ^{o}C.

11. Remove the supernatant carefully by pipetting, and allow the pellet to air dry for ~15 min on ice. Another quick spin will help to remove residual liquid.

12. Resuspend pellet in 40 – 70 µl DEPC-water.

13. Check the RNA by:

i) NanoDrop to determine RNA concentration:

2µl RNA + 2 µl DEPC-water in a 0.5 ml tube

ii) RNA gel analysis for RNA quality:

5□ g/well

iii) Agilent for RNA quality:

Neat or 1:10 dilution (DEPC-water) – if using RNA for micro array or real time PCR, RNA will need to be DNase treated before Agilent.

12. Store the RNA at –80 ^{o}C. Aliquot the samples in RNase/DNase free tubes in appropriate volumes depending on next stage. This will help reduce freeze/thaw cycles and RNA denaturation.

APPENDIX III



APPENDIX IV

Yale GRADUATE SCHOOL
OF ARTS AND SCIENCES

June 11, 2013

PO Box 208236
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Mr. Nnaemeka Ndodo
Human Anatomy Department,
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Dear Mr. Ndodo:

On behalf of the Faculty, I am pleased to offer you admission to the Yale Graduate School as a full-time Visiting Assistant in Research from September 3, 2013 to May 31, 2014. You will be enrolled in the Division of Special Registration (DSR) as a visiting assistant in research for the purpose of pursuing research under the direction of Professor Frank Slack in the Department of Molecular, Cellular, and Developmental Biology. You will not be eligible to take courses for either credit or audit and no fellowship aid will be available.

Visiting Assistants in Research who are only doing research are charged tuition based on the length of their proposed stay at Yale University. You will be charged tuition of $1,520 for the period of September 3, 2013 to December 31, 2013 and $1,900 for the period of January 1, 2014 to May 31, 2014. It is my understanding that your tuition and health insurance will be funding by a Fulbright Scholarship. Fulbright will also cover your living expenses in the amount of $21,060. As a student in the DSR, you may enroll in the Yale Health Billed Associate Plan by paying a monthly premium fee.

Upon arrival, please check in with the Office of International Students and Scholars at 421 Temple Street. Your second stop should be to inform the Registrar, Ms. Marrisa Delise, and your advisor that you have arrived. After notifying your department, please go to the Graduate Registrar's Office at 246 Church Street, first floor, to obtain instructions about receiving your ID card, Net ID and e-mail access.

The Department and the Graduate School hope that you will accept this offer of admission and support. To start at Yale on the projected start date, you must respond to this offer by faxing a copy of this offer letter and the completed reply form to 203-432-6724 on or before July 15, 2013. If you are unable to respond within this period, you should contact your department or program to arrange for a later start date. Yale Graduate School housing is generally unavailable for Visiting Assistants in Research. However, you may search for off-campus housing through the Yale Housing website at www.yale.edu/och.

Should you have any questions or concerns, please contact the Registrar, Ms. Marrisa Delise at Marrisa.delise@yale.edu.

With best wishes,

Sincerely yours,

Richard G. Sleight
Associate Dean

cc: Professor Christine Jacob-Wagner, DGS
Professor Frank Slack
Ms. Christine Chan, Fulbright Program Officer