Folate Receptor-Targeted Delivery of Small Interfering RNA to Cancer Cells

Yilin Zhang
University of Southern Mississippi

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FOLATE RECEPTOR-TARGETED DELIVERY OF SMALL INTERFERING RNA TO CANCER CELLS

by

Yilin Zhang

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

December 2010
ABSTRACT

FOLATE RECEPTOR-TARGETED DELIVERY OF SMALL INTERFERING RNA TO CANCER CELLS

by Yilin Zhang

December 2010

The vitamin folic acid (folate, FA) has been extensively explored as a targeting ligand to deliver a variety of diagnostic/prognostic/therapeutic agents into various tumors through the assistance of its receptor – the folate receptor (FR). FR is over-expressed in many types of human cancer and can mediate internalization of FA-conjugates through an endocytic pathway. The discovery of small interfering RNA (siRNA), which is capable of inducing potent gene silencing in a sequence-specific manner, provides an excellent molecular tool to suppress aberrant gene expression in malignancies, and therefore siRNA has the potential to revolutionize cancer therapeutics. Towards the goal of developing an efficient and cancer-specific siRNA strategy, three types of FA-conjugated molecules have been synthesized to investigate targeted siRNA delivery to cancer cells in vitro.

In the first section, FA-linked siRNA was synthesized through our one-step in vitro transcription using FA-HAD-AMP as a transcriptional initiator. FR-dependent cellular uptake and moderate specific gene down-regulation (50%) were observed in a stable cell line (Gluc-KB), which was established in this work by integrating Gaussia luciferase (Gluc) gene to the genome of KB cells (human nasopharyngeal carcinoma). Gluc-KB provides a platform to better evaluate gene expression changes upon siRNA treatment.
In the second section, a FA-functionalized, multivalent copolymer (FAPol13) was introduced to: (i) complex with siRNA to form a FAPol13/siRNA complex, (ii) protect siRNAs from enzymatic degradation, (iii) enhance cellular uptake by conjugating several FA molecules to copolymer, and (iv) increase RNAi efficacy eventually. A typical FAPol13 compound has three functional groups: cationic, hydrophilic, and FA moieties for providing siRNA packaging site, water solubility, and cell-selectivity, respectively. This nontoxic polymer successfully delivered siRNAs against *Gluc*, *survivin (Sur)*, *Caspase 8 associated protein 2* (*Casp8ap2*) genes in KB cells and efficiently reduced their expression (i.e., 62% and 68% downregulation from siSv and siGLuc treatments, respectively). Strikingly, treatment of KB cells with FAPol13/siCasp8ap2 significantly repressed cell growth and induced cells into apoptosis (24.5%, \( p = 0.01 \)). Furthermore, a real-time imaging system, employing fluorescence approaches (time-lapse, z-stacking, and colocalization analysis), has been developed to evaluate siRNA binding, cellular uptake, and escape from compartments, respectively. In particular, Pearson’s correlation coefficient (PCC) was employed to quantify siRNA endosomal escape, one of the crucial steps of siRNA intracellular trafficking. This method was further applied to the analysis of siRNA delivery in HeLa (human cervical carcinoma) and SKOV3 (human ovary carcinoma).

In the third section, a gold nanoparticle (AuNP) capable of packaging and protecting siRNAs was utilized to transport siRNA to cancer cells. Cytotoxicity assay, cellular uptake, and gene down-regulation studies of the AuNP-siRNA system indicate that AuNP can be an efficient siRNA platform.
In the fourth section, a correlation between FR expression level and the delivering effectiveness of FA-conjugates was established by comparing cellular absorption, cellular uptake, and RNAi efficiency among KB, HeLa, SKOV3 and A549 (human lung carcinoma) cells.

Collectively, we have demonstrated effective FR-mediated and cancer cell-specific siRNA delivery by several approaches. Further studies may lead to the development of therapeutic siRNA delivery systems.
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by

Yilin Zhang

A Dissertation
Submitted to the Graduate School of The University of Southern Mississippi
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<tbody>
<tr>
<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>HS-RNA</td>
<td>5’-thiolated RNA</td>
</tr>
<tr>
<td>Ab</td>
<td>absorbance</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblast leukemia</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5’-monophosphate</td>
</tr>
<tr>
<td>ADA</td>
<td>adenosine deaminase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>As</td>
<td>antisense</td>
</tr>
<tr>
<td>As-ODN</td>
<td>antisense oligodeoxyribonucleotide</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid peptide</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BHQ</td>
<td>black hole quencher</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BBB</td>
<td>brain blood barrier</td>
</tr>
<tr>
<td>BB</td>
<td>bromophenol blue</td>
</tr>
<tr>
<td>CPT</td>
<td>camptothecin</td>
</tr>
<tr>
<td>CSCs</td>
<td>cancer stem cells</td>
</tr>
<tr>
<td>CASP8AP2</td>
<td>caspase 8 associated protein 2</td>
</tr>
<tr>
<td>CPPs</td>
<td>cell penetrating peptides</td>
</tr>
<tr>
<td>CPC</td>
<td>chromosome passenger complex</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cy3</td>
<td>cyanine 3</td>
</tr>
<tr>
<td>CDK9</td>
<td>cyclin dependent kinase 9</td>
</tr>
<tr>
<td>pCMV</td>
<td>cytomegalovirus promoter</td>
</tr>
<tr>
<td>DEDs</td>
<td>dead effector domains</td>
</tr>
<tr>
<td>DISC</td>
<td>death-inducing signaling complex</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>K_d</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
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</table>
dsRNA  double-stranded RNA
DRBD  dsRNA binding domain
ESCs  embryonic stem cells
EGFP  enhanced green fluorescence protein
EPR  enhanced permeability and retention
EGFR  epidermal growth factor receptor
EtOH  ethanol
EtBr  ethidium bromide
EDTA  ethylenediaminetetraacetic acid
FCS  fetal calf serum
FGFR  fibroblast growth factor receptor
FLUC  firefly luciferase
FAD  flavin adenine dinucleotide
FMN  flavin mononucleotide
FLASH  FLICE-associated huge protein
FAM  fluorescein
FITC  fluorescein isothiocyanate
FRET  fluorescence resonance energy transfer
FR  folate receptor
FAPol13  folate-block copolymer
FA  folic acid, folate
GLUC  Gaussia luciferase
GPI  glycosylphosphatidylinositol
AuNP  gold nanoparticle
GFP  green fluorescence protein
G  guanosine
HSPG  heparin sulfate proteoglycan
HBXIP  hepatitis B X-interacting protein
HSV  herpes simplex virus
HAD  hexamethylenediamine
HAT  hour after transfection
Dh  hydrodynamic Diameter
IAPs  inhibitor of apoptosis proteins
MS  mass spectrosocpy
MFI  mean fluorescence intensity
mRNA  messenger RNA
miRNA  microRNA
MAPK  mitogen-activated protein kinase
MW  molecular weight
HPMA  N-(2-hydroxypropyl)methacrylamide
APMA  N-(3-aminopropyl)methacrylamide
DMAPMA  N-[3-(dimethylamino)propyl]methacrylamide
NHS  N-hydroxysuccinimide
NAD  nicotinamide adenine dinucleotide
N/P  nitrogen to phosphate ratio
NT  nucleotide
ODNs  oligo deoxyribonucleic acids
ORF  open reading frame
OD$_{260}$  optical density unit at 260nm
PABA  p-aminobenzoate
PCC  Pearson's correlation coefficient
PBS  phosphate buffered saline
PLC  phospholipase C
piRNA  piwiRNA
PGF  placental growth factor
PAGE  polyacrylamide gel electrophoresis
PEI  polyethylenimine
PCR  polymerase chain reaction
PTGS  post-transcriptional gene silencing
PI  propidium Iodide
PSMA  prostate-specific membrane antigen
PTD  protein transduction domains
PA  pteroic acid
RFP  red fluorescence protein
RFC  reduced folate carrier
RLUC  renilla luciferase
ra-siRNA  repeat-associated siRNA
RT  reverse transcription
RAFT  reversible addition-fragmentation chain transfer
NTP  ribonucleoside 5'-triphosphate
RNAi  RNA interference
RISC  RNA-induced silencing complex
SCID  severe combined immunodeficiency
siRNA  small interfering RNA
SELEX  systematic evolution of ligands by exponential enrichment
ta-siRNA  trans-acting siRNA
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>TBE</td>
<td>Tris/borate/EDTA</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UTRs</td>
<td>untranslated regains</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. food and drug administration</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIS</td>
<td>visible</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked IAP</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescence protein</td>
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CHAPTER I
LITERATURE REVIEW
Cancer and Cancer Treatment

Cancer is a class of diverse diseases associated with uncontrolled growth of abnormal cells where a series of genetic alterations leads to either activated oncogenes (gain of function) or inactivated cancer suppressor genes (loss of function).\textsuperscript{1} Currently, cancer is the second leading cause for human death with more than 10 million new cases every year.\textsuperscript{2}

After decades of persistent effort, cancer research has achieved rapid progress and generated a large body of knowledge, revealing that malignant cells are transformed from normal cells after a succession of genetic changes that confer cell growth advantages.\textsuperscript{1} Through progressive conversion, cancerous cells gain the capabilities to proliferate aberrantly (anti-apoptosis, growth signal autonomy), to stimulate the formation of blood vessels (angiogenesis), and to travel to adjacent tissues (invasion, metastasis).\textsuperscript{3}

Characteristics of Cancer

Cancer cells are transformed from normal cells that have defects in regulation on cell proliferation and homeostasis.\textsuperscript{4} A large and diverse collection of cancer-associated genes is involved in this conversion, resulting in the observed complexity and heterogeneity of cancer \textsuperscript{1}. However, most tumors share well-defined characteristics, such as unrestrained cell growth, deregulated cell cycle control, anti-apoptosis (immortality), invasion, metastasis, and angiogenesis (formation new vessels from the old ones to supply oxygen and nutrients through blood stream).
Proliferation of normal cells is under a strict homeostatic control of growth signals and anti-growth signals, while in malignant cells, this balance is broken due to genetic alterations. As a result, tumor cells obtain the capability to produce growth signals, thereby reducing their dependence on stimulation from the normal tissues nearby, and simultaneously becoming insensitive to anti-growth signals. This process is designated growth factor autonomy — cells responding to the signals produced by themselves. To achieve growth factor autonomy, cancer cells either overexpress receptor, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor, (FGFR), folate receptor (FR), or alter transcellular/intracellular signal transducers (e.g., mitogen-activated protein kinase, MAPK pathway).

Normal cells are under strict regulation associated with the homeostatic balance between pro- and anti-apoptotic signaling molecules. Cancerous cells, however, acquire abilities from genetic alternations to destruct the pro-apoptotic regulators (e.g., p53) and induce anti-apoptotic regulators (e.g., inhibitor of apoptosis proteins, IAPs). Apoptosis — a process of programmed cell death — causes a series of cellular/molecular changes in organisms, including cell blebbing, cell shrinkage, chromatin condensation, and nuclear fragmentation. Apoptosis is conducted by a class of intercellular proteases termed caspases. Caspases harbor a critical cysteine residue at the active site and hydrolyze target proteins on the carboxyl-terminal side of specific asparagine residues. Under apoptosis, cells were triggered to release cytochrome C (a potent catalyst of apoptosis) from mitochondria, destroy subcellular structures, organelles, and genome (defragmentation). Numerous data have revealed that many apoptotic genes (e.g.,
caspase 8 and caspase 9) are suppressed and anti-apoptotic genes (e.g., survivin, and livin) are activated in various types of tumors.\textsuperscript{11} For example, survivin (an apoptosis inhibitor), which expresses at low to undetectable levels in most normal tissues, is found overexpressed in nearly every human tumor tested.\textsuperscript{12}

Unlike normal cells but similar to stem cells, cancer cells have the ability to migrate through the bloodstream, allowing a small population of cancer cells (presumably cancer stem cells) to invade adjacent tissues and form tumors. This process causes 90\% of human cancer deaths.\textsuperscript{13} After decades of investigation, significant progress has been made to characterize several classes of protein components involved in this invasive and metastatic process, such as cell adhesion molecules (e.g., E-cadherin, integrin)\textsuperscript{14} and chemokine receptors.\textsuperscript{15} For instance, ectopic expression of integrin $\alpha_4\beta_1$ in mouse melanoma is sufficient to retain tumors \textit{in situ}.\textsuperscript{16} In addition, downregulation of E-cadherin and upregulation of vimentin (one type of intermediate filaments) enable the migration of cancer cells through a process of epithelial-mesenchymal transition. Moreover, several blood and solid cancers overexpress chemokine receptors to favor this process.\textsuperscript{17}

In a normal tissue, cellular function and proliferation are restricted by the vasculature which supplies oxygen and nutrients to cells, whereas tumor tissue gains the capability of angiogenesis, which leads to a sustained vasculature through which cancer cells evade the limitation of nutrient supply and grow consistently. Due to the rapid and defective angiogenesis, the vessels become leaky and increase the permeability to some macromolecules, which facilitates the passive delivery of various anticancer drugs.\textsuperscript{18} This phenomenon is termed enhanced permeability and retention (EPR) effects.\textsuperscript{19} Two protein
families play critical roles in the acquisition of angiogenic capability for tumor: vascular endothelial growth factor (VEGF) family,\textsuperscript{20} and acidic and basic fibroblast growth factor (aFGF and bFGF) family,\textsuperscript{21} which are upregulated in various types of tumors.\textsuperscript{20a}

\textit{Cancer Treatments}

Although tremendous efforts have been dedicated to cancer biology research and substantial progress has been made, approximately 1.5 million new cancer cases, and more than 0.5 million deaths from cancer were reported in the United States in 2009.\textsuperscript{2a}

Effective cancer therapy confronts at least four challenges: (1) Tumor can originate from various tissues and as a result there are more than 100 distinct types of cancer; (2) The fact that tumorigenesis is a combinatory outcome of genetic and epigenetic alterations makes it impossible to have a universal treatment suitable to every scenario of tumor; and therefore, it is proposed that cancer therapies should be as diverse as the diseases themselves;\textsuperscript{22} (3) Current prognostic and diagnostic techniques are insufficient; (4) Classic chemotherapy drugs have undesired cytotoxicity, which causes the death of both normal and cancerous cells.

Generally speaking, there are three standard approaches for cancer treatment: surgery, radiation and chemotherapy. The type, size, location, and stage of cancer determine which approach to be used, and in most cases, they are combinatorially employed. Surgery is a physical process to remove a tumor if it can be clearly localized. For several types of cancer, surgical removal of a tumor may be sufficient to cure the cancer patient in the case that cancer cells have not invaded into other tissues and tumor tissue has been incised as much as possible. Cancer metastasis contributes to the tumor recurrence and is the major reason for death of cancer patients.
Radiation therapy takes advantage of high-energy radiation (i.e., X-rays, gamma rays, and charged particles) to cause DNA damage and thereby killing cancer cells. Radiation may be employed in the form of beams outside the body (external-beam radiotherapy) or be implanted into the tumor (internal radiotherapy). More than half of all cancer patients receive radiotherapy during the course of treatment. In most cases, radiotherapy is used in a combination with other types of therapeutics. However, radioactive illumination, no matter from outside or inside the body, will do harm to DNA, evoking mutagenesis or cell death at the nearby tissues. The mutated cells have a high likelihood to give rise to a new tumor, which undermines the effectiveness of this approach. The severity of side effects is dependent on the distance between the illuminated tumor and normal tissue, and the duration/intensity of radiation beams.

Chemotherapy is perhaps the most commonly used means for cancer therapy. It is often combinationally applied to post-surgery patients to treat the remaining or migrated cancer cells that render tumor recurrence. Cancer drugs are hypersensitive to the rapidly proliferating cells, including cancerous cells and a small portion of normal cells (e.g., blood cells). This non-targetability of chemotherapy leads to certain undesired symptoms (side effects) including pain, diarrhea, hair loss, nausea, anemia, etc.

Most traditional chemotherapeutic drugs are derived from natural sources or synthetic structural modifications. For example, paclitaxel (Taxol) was originally derived from the bark of Pacific yew tree and is used for treatment of ovarian and breast cancers. Camptothecin (CPT) is isolated from the bark and stem of happy tree. Taxol can impair the microtubule function which is critical for cell division, and therefore serves as a mitotic inhibitor. CPT is the inhibitor of DNA topoisomerase I and shows remarkable
anticancer activity. Currently, two CPT analogues, topotecan and irinotecan, hold great promise in treatment of several types of tumors for their better water solubility and less cytotoxicity.\textsuperscript{25} Taxol and CPT are two representative chemotherapeutic drugs of this approach.

Besides these three standard approaches, several novel strategies have emerged and shown great potential in cancer therapy. Proton therapy derived from radiation therapy is the latest advancement in the treatment of various types of tumors. It uses a beam of protons to destroy cancer cells without harming healthy cells and thereby reduces the side-effects of conventional cancer treatments.\textsuperscript{26} Gene therapy was initially designed as a means to correct gene defects in hereditary diseases,\textsuperscript{27} and currently is one of potential strategies for cancer treatments. The first gene therapy clinical trial began in 1990 by introducing adenosine deaminase gene to the T cells of two children with severe combined immunodeficiency (SCID).\textsuperscript{28} From then on, hundreds of clinical studies have been conducted with almost 70\% of these studies being in the area of cancer gene therapy.\textsuperscript{29} Because malignant transformation initiates from genetic alterations occurred in oncogenes and tumor suppressor genes, we can in principle manipulate these genes through cancer gene therapeutic techniques to restore the cell’s normal gene function.

Other innovative cancer therapeutic approaches include photodynamic therapy (destroying cancer cells through a photosensitizing drug activated by specific wavelengths of light),\textsuperscript{30} vaccine therapy (treating cancer cells by boosting immune response),\textsuperscript{31} and targeted therapy (tumor-directed drug delivery).\textsuperscript{32}

Targeted gene therapy, one of cancer therapy strategies, combines the strengths of gene therapy and targeted therapy, thereby leading to some advantages over conventional
cancer treatment approaches in experimental and clinical studies. This treatment may have significant impact on future diagnostics, therapeutics of cancer, and others genetic diseases.

*Cancer Stem Cells*

The hypothesis of cancer stem cells (CSCs) originated more than a hundred years ago when it was proposed that the “misplaced” embryonic stem cells (ESCs) during the embryonic development could be the source of cancer. Later evidence in mice and humans has indicated that a single cancer cell can generate a heterogeneous tumor, suggesting the existence of cancer stem cells. CSCs harbor the self-renewal ability and can give rise to the neoplasm at high efficiency. Meanwhile, over the past few years, researchers have isolated and identified CSCs from human and mouse leukemia, as well as from epithelial tumors such as the skin, breast, brain, colon, head and neck tumors.

Three models have been proposed to explain the formation of CSCs. The first states that one critical mutation (e.g., the one that breaks the asymmetric division pattern) triggers the “cascading transformation” from stem cells to cancer cells. The second model regards that the accumulation of genetic alterations in stem cells leads to the transformation of CSCs. The third considers that the dedifferentiation of cancer cells through epithelial-mesenchymal transition results in CSCs. Each theory rationalizes under a certain scenario.

Although researchers have successfully identified, isolated, and cultured the hemacytometric CSCs, and several critical components in acquiring self-renewal of CSCs have been demonstrated, the mechanism underlying CSCs acquired capabilities of metastasis, and apoptosis-resistance is rarely known. To address this issue, efforts have
been dedicated by conducting gene profiling techniques (e.g., microarray to systematically compare cancer cells and CSCs, or stem cells and CSCs.\textsuperscript{39-40}

CSCs might lead to tumor recurrence because of their drug resistance and capability of metastasis. Therefore the identification and characterization of CSCs from various tumors are of significant importance for the improvement of cancer therapy.\textsuperscript{41} Further understanding of CSCs has implications for the development of more innovative and efficient approaches for cancer treatment.\textsuperscript{41a}

\textit{Cancer and microRNA (miRNA)}

miRNA is a class of evolutionarily-conserved, endogenous, noncoding, small RNA with 20-23 nucleotides (nt) in length.\textsuperscript{40c} Since its discovery in the early 2000s,\textsuperscript{42} miRNA has walked its way into virtually every corner of the biology field. It regulates the expression of genes involved in the control of development, proliferation, apoptosis, and stress response at translational and posttranslational levels. miRNAs manipulate more than 60\% of human genes\textsuperscript{43} by inhibiting ribosome function, decapping the 5’-cap structure (cleavage of 5’-cap of mRNA cause translation repression), deadenylating the poly(A) tail, and degrading the target mRNA.\textsuperscript{44}

By 2006, more than 400 miRNAs have been experimentally identified in mammalian genomes;\textsuperscript{45} whereas an estimate shows 1000 miRNAs or more exist in vertebrate and about 100 in invertebrate.\textsuperscript{45-46} In addition, bioinformatics prediction shows more than 30\% human genes may be under the regulation of miRNAs.\textsuperscript{47} Strikingly, an individual miRNA has the ability to control the expression of several to hundreds of mRNAs it binds to through an imperfect sequence complementarity.
Altered miRNA expression profile and several other lines of evidence indicate the critical role of miRNAs in cancer biology. For example, it has been found that the global miRNAs expression in cancer is downregulated and inhibited biogenesis of miRNAs will lead to tumorigenesis, which suggests the suppressive role of miRNAs in cancer. How miRNAs regulate tumorigenesis has remained poorly understood. However, recent research starts to yield clues. Hannon and colleagues identified a new family of miRNAs, miR-34a-c, through comparing miRNA expression profiles of wide-type and p53-dificient (p53 is a tumor suppressor) cells. Their findings suggested miR-34 may act with other effectors to inhibit cancer proliferation through participating p53 network that inhibited tumor formation. Additionally, Piccolo and coworkers found another miRNA family, miR-103/107, which reduces the miRNA biosynthesis by inhibiting Dicer (a key component of siRNA/miRNA process) expression. The data demonstrate a new pathway where cancer cells downregulate miRNAs syntheses through Dicer, which in part explains the reduced expression of global miRNAs.

In addition, other miRNAs have been identified and demonstrated as tumor suppressors. For instance, downregulation of let-7 is associated with tumor progression and poor prognosis of lung cancer patients. Expression of let-7 also prevents tumor sphere formation of breast cancer cell lines and inhibits tumorigenicity in an in vivo xenograft tumor assay. On the contrary, miRNAs, such as miR-21, miR-106a, and miR-155, have oncogenic activities.

Overall, miRNA holds potential to influence cancer therapy in different ways. First, along with the technological advances that facilitate miRNA expression profiling in tumors, miRNA expression signatures could be identified and utilized as helpful
biomarkers for cancer diagnostics, which would render a powerful tool for cancer prevention and therapeutics.\textsuperscript{52} Second, increasing lines of evidence suggest that sequence-specific knockdown of oncogenic miRNAs would result in favorable anticancer effects, demonstrating the promising potential of miRNA-based cancer therapy.\textsuperscript{50}

RNA Interference (RNAi)

Since its discovery,\textsuperscript{53} RNAi research within biology and medicine have enjoyed rapid development.\textsuperscript{54} RNAi process is triggered by short RNA, such as small interfering RNA (siRNA), microRNA (miRNA), piwiRNA (piRNA), that is homologous to the target genes being suppressed.\textsuperscript{55} Due to its sequence-specificity and knockdown potency, RNAi has been applied to gene function and therapeutic applications.

The Discovery of RNAi

Small RNA-mediated gene silencing phenomenon was first observed in petunia when an exogenous transgene designed to increase gene expression actually induced the gene silencing of the homologous endogenous gene.\textsuperscript{56} Unfortunately, this unexpected and intriguing result was ignored and explained by co-suppression or post-transcriptional gene silencing (PTGS).\textsuperscript{57} It was not until 1998 that the secret of RNAi was uncovered by Andrew Z. Fire and Craig A. Mello when they injected long double-stranded RNA (dsRNA) into the nematode \textit{C. elegans} as well as single strand sense or antisense RNA.\textsuperscript{53} They found that only dsRNA initiated a potent and specific degradation of endogenous mRNA containing the same sequence as dsRNA trigger. The groundbreaking discoveries of RNAi in nematodes let researchers rethink of PTGS in plants, and proved that RNAi existed in both nematodes and plants.\textsuperscript{58} However, investigators could not find RNAi in
mammals because long dsRNA induced the activation of interferon response pathway and the inhibition of global protein synthesis. Tuschl and colleagues demonstrated that short dsRNA rather than long one could inhibit gene expression. Soon RNAi was observed in *Drosophila melanogaster*, *trypanosima*, and vertebrates. We now understand that RNAi is an evolutionally conserved mechanism in eukaryotes. The discovery of RNAi has revolutionized gene function research and biomedical research.

**RNAi Mechanism**

The RNAi pathway can be divided into two major steps: initiation and activation as shown in Figure I-1. In the first step, a RNase III type enzyme termed Dicer cleaves the longer dsRNA to smaller RNA that is typically portrayed by ~21 base pair (bp) in length containing 2 nt overhangs with 5’ phosphate and 3’ hydroxyl terminals. Subsequently, one strand of siRNA duplex (guide strand) is incorporated into a complex called RNA-induced gene silencing complex (RISC), which binds to complementary mRNA substrates and manipulates gene function. If the guide strand binds to the corresponding sequences in the open reading frame (ORF) of mRNA with a complete base paring (e.g., siRNA or miRNA), it will induce site-specific mRNA degradation. More precisely, RISC cleaves mRNAs at 10 nt from the 5’ end of the guide strand. If the guide sequence is not fully complementary to the sequence of mRNA (i.e., miRNA), which normally occurs at the 3’ untranslated regains (UTRs), the RNA will trigger translational repression. In another word, RNAi can resulted from either siRNA or miRNA, depending on the degree of the complementarity between the guide RNA and its target mRNA. If the complementarity is low, the guide RNA will go into miRNA pathway, leading to translational inhibition; whereas the perfect base pairing match will
lead to siRNA pathway, causing the cleavage of mRNA. Actually, miRNA process is biochemically or functionally indistinguishable from siRNA pathway because they share some essential factors which are often encoded by multigene families conserved among eukaryotes.\(^\text{67}\)

**Figure I-1.** Mechanisms of RNA inference.

In addition to siRNA and miRNA, a new class of small, non-coding but functional RNA termed piRNA (piwi-interacting RNA) has been discovered recently.\(^\text{68}\) piRNA is typically 26-31 nt in length, longer than 21-23 nt of miRNA and 21 nt of siRNA, with an important role in mammalian spermatogenesis.\(^\text{68}\) Taken together, the vital role of miRNA, siRNA, piRNA, and other RNA species, such as repeat-associated siRNA (ra-siRNA), trans-acting siRNA (ta-siRNA) etc. in the regulation of chromatin structure, gene expression, mRNA processing and splicing, mRNA stability and translational control demonstrate the importance of uncovering and understanding the RNA world.\(^\text{69}\)

**siRNA Design**

Although any target-complementary siRNA could theoretically silence its cognate gene,\(^\text{63b}\) only a small fraction of siRNAs has high effectiveness and specificity.\(^\text{70}\) A positional shift on the target mRNA can alter siRNA function in a dramatic manner.\(^\text{71}\)
Moreover, siRNA may nonspecifically target unrelated genes with only partial sequence-complementarity. Therefore, a rational design of siRNA sequence is a prerequisite for performing reliable gene knockdown.  

In the early 2000s, a set of empirical rules based on the first identified functional siRNAs was proposed and influenced siRNA design profoundly. Today, some of these criteria are still being utilized in several siRNA selection/design tools. However, more powerful, comprehensive tools have emerged based on the following advances: (i) further understanding of RNAi mechanism, (ii) statistical analysis of libraries of siRNAs with experimentally determined efficiency; and (iii) the rapid development of bioinformatics. Some research groups have collected published functional siRNA sequences and established open-access databases; while a few biotechnology companies, such as Ambion, Qiagen, and Invitrogen, provide pre-designed or custom siRNAs synthesis services.

Despite the availability of these sources, it is necessary for investigators to know some criteria in the case of choosing an appropriate tool or selecting a siRNA candidate from a list. There are four typical rules as an important guideline in the considerations of effective and specific siRNA: (1) Sequence asymmetry: siRNAs symmetry in structure but not in sequence shows improved efficacy and specificity. (2) siRNA duplex stability: siRNAs with G+C content between 30% ~ 52% keep the balance between structure stability and release flexibility. Typically, G+C content lower than 30% will destabilize siRNA duplexes and reduce the affinity for target mRNA binding; whereas higher than 52% may impede RISC loading and/or cleavage-product release. (3) Target accessibility: the local secondary structures of target mRNAs might restrict the
accessibility of RISC, reducing or abolishing siRNA efficiency.\textsuperscript{76} (4) Sequence features: single nucleotide at different positions will affect siRNAs performance dramatically. For instance, U/A at position 1, A/U at position 10, C/G at position 19.\textsuperscript{55} Additionally, some other factors, such as thermodynamic consideration and BLAST search, are contributing to the rational design of siRNAs.

\textit{RNAi Application}

The ability of siRNA to silence gene expression as a powerful investigational tool has been intensively explored in dissecting gene function and blocking disease-causing gene expression.\textsuperscript{65, 77}

RNAi has been successfully applied to systematic genome-wide screening in many species, such as \textit{C. elegans}\textsuperscript{78} and \textit{Drosophila}\textsuperscript{65}. High-throughput techniques combining the strengths of RNAi, bioinformatics and genomics, offer rapid annotation of genomes with functional information on each gene readily.\textsuperscript{79} In addition, siRNAs hold great potential as gene therapeutic agents for treating a wide range of diseases, such as cancer, neurodegenerative disorders, and viral infection, where aberrant gene expression occurs.\textsuperscript{80} For instance, downregulated expression of VEGF, an important factor for pathological angiogenesis,\textsuperscript{81} through RNAi technique suppressed tumor angiogenesis and growth in PC-3 (a human prostate cancer cell line) xerograph model.\textsuperscript{82}

Overall, compared to the classic genetic and reverse genetic approaches, siRNA has incomparable advantages in functional genomics and as therapeutic applications.\textsuperscript{83} Classical genetic approaches study the gene function by identifying gene mutations, and conventional reverse genetic methods involve the disruption of a known gene (i.e., knockout) to study the effect on a function or pathway. Both are time-consuming and
expensive. In contrast, the function of an unknown gene through RNAi technique could be revealed in several days.\textsuperscript{80} siRNA possesses the high specificity and potency in inducing gene silencing with a sequence-dependent manner. The accessibility of genomics sequencing of many key organisms accelerates the development of nucleic acid-based RNAi in gene function research.\textsuperscript{84}

\textit{siRNA Delivery}

An appropriate delivery strategy that enables siRNA translocation and balances siRNA stability and activity is the key to a successful siRNA application. siRNA delivery can be divided into two classes: \textit{in vitro} and \textit{in vivo} delivery. The latter include two major routes according to biodistribution: local and systemic administration. Local administration, as the name implies, is a route by which siRNA or drug is directly delivered to a localized area of the body (e.g., tumor) with topical effects. This strategy uses a low dose of substance but achieves relative high concentration at the intended site; and therefore, reduces the risks of systemic side effects.\textsuperscript{85} In contrast, systemic administration refers to deliver drugs or substances through either enteral (digestive tract) or parenteral (intramuscular or intravenous injections) route. The choice between these two administrations depends on tissues location and cell type being targeted.\textsuperscript{86}

According to the endurance of RNAi effects, siRNA delivery can be sorted into transient and stable delivery. In other words, siRNA can be delivered to cells either exogenously as synthetic agents or endogenously as gene-encoding siRNAs.\textsuperscript{87} For the former, RNAi effect is achieved through exogenous delivery of chemical or transcripted siRNA and normally exists for a few days before the degradation of siRNAs. As for the latter, siRNA is often delivered by viral plasmid vectors which have the capability of
being integrated into the host’s genome, and thereby, RNAi effects can last for weeks or even longer. This strategy is preferred for siRNA library construction and systematic genome-wide functional screening. Besides, for some chronic diseases which need lasting therapeutics and low toxicity, the stable transfection is advantageous. Short hairpin siRNAs (shRNAs) are commonly used under this strategy. However, those methods based on the plasmid which will be expressed under the host’s transcription system pose a safety issue.

siRNA delivery systems can be divided into viral- and nonviral-based delivery. Due to the high efficiency of ferrying DNA/RNA into the cells, viral delivery has been intensively investigated for gene therapy. There are at least four classes of virus in this arena: retrovirus, adenovirus, baculovirus, and lentivirus. Despite of high transduction efficiency, viral-based delivery system *in vivo* is limited by safety concerns. For example, retroviruses could be integrated into the genome of the host cells, which potentially increases the risk of carcinogenesis. Another issue is the transduction efficiency of the retroviral vector is correlative with the activity of cell division.

The lack of genomic integration of adenoviral vectors provides a safety advantage over retrovirus vectors. As a result, many adenovirus-based clinical trials occur, and several studies report that the local administration of adenoviral vectors has achieved significant therapy effects. However, therapeutic effects from adenoviral vectors, which are generally lost after several cell divisions, are moderate and short-lived compared to those from the retrovirus vectors. In addition, this approach is impaired by dose-dependent liver toxicity. In contrast to baculovirus, lentiviral vector is a promising strategy owing to its relative bio-safety, less mutagenesis risk, and less pathogenesis.
Nonviral based delivery strategies, such as (co)polymer, Au nanoparticle (AuNP), liposome, polyarginine, polylysine, polyethylenimine (PEI), hydrogel, dendrimer, hold tremendous promise.\textsuperscript{92} Some liposome-based, commercial reagents (e.g., Lipofectamine, DharmaFECT) have been routinely used for siRNA delivery into various types of cancers \textit{in vitro}.\textsuperscript{93} However, their applications \textit{in vivo} are hurdled by cytotoxicity and instability in serum.\textsuperscript{85} PEI is another widely used transfection agent for many years.\textsuperscript{94} Despite its variable performance, it is a useful tool for delivering antisense oligodeoxyribonucleotide (As-ODN), DNA plasmid, and siRNA etc.\textsuperscript{95} Furthermore, modified PEI increases the efficiency and specificity of delivery.\textsuperscript{96}

Most of these nano-scale carriers contain cationic groups which can bind to anionic phosphate backbone of the nucleotides via electrostatistics and facilitate the cellular uptake of complexes without target specificity. The complexes (i.e., lipoplex, polyplex) can be adjusted to be cationic, anionic, or neutral in terms of nitrogen (cationic group) to phosphate (anionic group) ratio ($N/P$ ratio). By the nature of negatively-charged cell membrane, cationic complexes ($N/P > 1$) have the tendency to penetrate the membrane easily; whereas anionic complexes ($N/P < 1$) normally have poor transfection efficiency. As for neutral complexes, they normally need hydrophilic groups, such as hydroxyl group, to maintain water solubility.\textsuperscript{97}

Delivery of siRNA is intended to downregulate abnormal gene expression through RNAi machinery in diseased cells. Although cellular penetration of siRNA has been achieved by efficient delivery strategies, therapeutic effects of siRNA cannot be achieved until it is in the desired cells. For this reason, the development of cell- or tissue-specific delivery must be resolved prior to clinical development of siRNA therapeutics.
Targeted siRNA Delivery

Although significant progress has been made in cancer prognosis, diagnosis, and treatment, cancer is still one of the most devastating diseases. One of the challenges we are facing is the non-targetability of the conventional cancer treatments that cause cell death indistinguishably in both normal and malignant tissues. This Achilles’ heel elicits side effects and compromises the effectiveness of common treatments. To address this issue, a number of novel strategies that aim at homing of drugs to malignant cells through the mediation of targeting moieties have emerged and offer great promise. Targeting ligands applicable to cancer therapy mainly include antibodies, peptides, aptamers, and vitamins.

Antibody-Based Delivery

Antibodies, immunoglobulin proteins, are produced by B lymphocytes to bind foreign objects (antigens), such as viruses, bacteria, exogenous proteins, microorganisms, and toxins. Numerous antibodies have been generated and commercialized by injecting an antigen into a mammal (e.g., rabbit, goats). Successful production of specific antibody depends on several factors of antigen, such as chemical characteristics of antigen, the size of antigen (> 5 KDa), and its difference from an animal’s own proteins.

In the past decades, development of therapeutic and diagnostic antibody is among the fastest growth in biopharmaceutical area by the virtue of its high specificity and affinity to target various antigens. More than 18 monoclonal antibodies have been commercialized. For example, trastuzumab (Herceptin®), rituximab (Rituxan®), and alemtuzumab (Campath®) have been routinely used in clinical treatment of breast cancer and leukemia. Additionally, over 100 therapeutic monoclonal antibodies are currently
under investigation, demonstrating the tremendous biomedical potential of this strategy.\textsuperscript{104}

Due to the exquisite specificity of target recognition and high binding affinity between antibody and antigen, many antibodies have been conjugated to nanoparticle carriers for targeted siRNA/drug delivery. Many therapeutic agents have been transported into diseased cells specifically through this approach. These agents contain chemotherapeutic drugs, siRNAs, and As-ODNs.\textsuperscript{98b, 98d, 105} For example, Song et al. designed a protamine-antibody fusion protein for delivering siRNAs to HIV-infected cells.\textsuperscript{98d} The carrier contains the C-terminus of the heavy chain fragment of an antibody against HIV-1 envelope protein (GP160), and polycationic protamine for complexing siRNA. A cocktail of siRNAs against c-Myc (an transcriptional factor), MDM2 (an negative regulator of the p53), and VEGF had been administered to tumor cells in mice engineered to express GP160, exhibiting potent antitumor effects.\textsuperscript{98d} Despite promising, antibody-based delivery possesses some setbacks, such as immunogenicity, high cost, and difficult conjugation chemistry.

\textit{Peptide-Based Delivery}

Among many strategies for siRNA delivery, peptide-based delivery has received significant attention.\textsuperscript{106} Such cationic and short peptides (motifs) interact with the membrane receptors or proteoglycans which elicit cellular uptake via endocytosis.\textsuperscript{107} Although the underlying mechanisms of internalization remain controversial, those peptides have been applied to deliver a broad spectrum of therapeutic agents.\textsuperscript{99b, 99d, 108}

Peptides with arginine-glycine-aspartic acid (RGD) motif have been used to target drugs, siRNA, imaging agents, and other diagnostic agents to the tumor cells expressing
α,β3 integrin. Integrin is a heterodimeric cell adhesive protein (with two subunits α and β) anchored to the plasma membrane. The ligands of integrin include collagen (a fibrous protein constituent of bone, cartilage, tendon, and other connective tissue) and fibronectin (an extracellular matrix glycoprotein) with characterized RGD motif. Importantly integrin receptors are overexpressed on endothelial cells in a tumor vasculature due to its crucial role in angiogenesis. For this reason, RGD-mediated targeted delivery has been developed to deliver diagnostic or prognostic agents specifically to tumor. For example, PEGylated PEI with RGD group directs the delivery of siRNA against VEGF into tumor with significant inhibitory effects of the tumor growth and reduction of angiogenesis after intravenous administration.

TAT peptide, a fragment of ligands which can be incorporated into the HIV-infected cells and concentrated in nuclei, is characterized by cationic peptides, YGRKKRRQRRR. TAT-siRNA conjugate has been successfully synthesized and effectively delivered with silencing the expression of exogenous enhanced green fluorescence protein (EGFP) and endogenous cyclin dependent kinase 9 (CDK9). TAT peptide has been applied to deliver DNA and protein.

Cell penetrating peptides (CPPs) which are also known as protein transduction domains (PTD) or membrane transduction sequences, are short (~30 amino acids) cationic peptide chains rich in arginine and/or lysine residues. CPPs have the ability to transport linked antisense and siRNA molecules as cargoes into cytosol thereby achieving oligonucleotide-based therapeutics. Several lines of evidence have shown that CPP-antisense oligonucleotide conjugates can be taken up by cells and effectively regulate gene expression in vitro and in vivo. Although not as potent as cationic lipid agents in
complexing siRNAs, CPP-based delivery strategies have substantial advantages in cell penetration potential. This was highlighted in a recent study where the systemically delivered siRNA-peptide conjugates crossed blood-brain barrier (BBB).

*Aptamer-Based Delivery*

Aptamers are normally short nucleic acids that have stable three-dimensional shapes and can bind to targeted molecules tightly and specifically. An aptamer against a target (such as ligands, protein, chemicals, etc.) is selected and identified from combinatorial nucleic acid libraries through a technique called systematic evolution of ligands by exponential enrichment (SELEX). In the past two decades, SELEX has been widely used, and numerous aptamers have been explored in both diagnostic and therapeutic applications.

Aptamer targeting cell surface proteins has been confirmed a promising delivery strategy because of its advantages, such as lack of immunogenicity in vivo, convenient synthesis and modification, relatively small size, rapid in vitro selection process, and large quantities produced with relatively low cost. By virtue of these properties, a wide range of cargoes has been successfully delivered in a cell-specific manner, including siRNAs, enzymes, radionuclide, virus, toxins, anti-cancer drugs.

Aptamer against the prostate-specific membrane antigen (PSMA) is one of the classical aptamer. PSMA is a cell surface antigen overexpressed in prostate cancer cells and in tumor vascular endothelium. Several groups have constructed distinct aptamer-siRNA conjugates that have been delivered into tumors with potent efficacy (Figure I-2). In another example, Neufeld and colleagues have used aptamer against a transferrin receptor (a protein regulates the importation of iron into cells) for delivering α-L-
iduronidase (an enzyme to remove mannose 6-phosphate) into the lysosomes of cells deficient in this enzyme.\textsuperscript{122}

Figure I-2. Representative strategies for targeted delivery of small interfering RNA.

Recent advances in aptamer-targeted siRNA therapeutics make it a promising strategy for clinical application. Some other strategies for siRNA targeted delivery are exemplified in Figure I-2, including cholesterol targeted,\textsuperscript{127} and folate targeted.\textsuperscript{128}

Folate Receptor-Mediated Specific Delivery

Folate receptor (FR)\textsuperscript{101s} is a confirmed tumor-associated antigen that binds folate/folate conjugates with high affinity and transfers them into FR-bearing cancer cells by endocytosis.\textsuperscript{101j} Folate (folic acid, FA) is a crucial vitamin (Vitamin B9) for metabolic maintenance of carbon pathway.\textsuperscript{129} FA harbors three moieties that are pteridine, \textit{p}-
aminobenzoate (PABA), and glutamate moieties (Figure I-3). Pteroic acid (PA) is analogous to FA without glutamate group. The pteridine ring of folate can exist in tetrahydro-, dihydro-, and fully oxidized forms. Plant folates have $\gamma$-linked polyglutamyl tails of up to approximately six residues.\textsuperscript{130}

![Figure I-3. The structure of folate.](image)

Folate is a vital cofactor to transfer 1-carbon groups in de novo synthesis of nucleotides. Therefore, the deficiency of folate will induce DNA strand breakage, increase uracil misincorporation into DNA, impair DNA repair and stimulate apoptosis.\textsuperscript{131} In addition, folate also plays an important role in amino acid metabolism, including serine, glycine, methionine and histidine metabolism. That is probably why some cancer cells overexpress FR to acquire more vitamin folate, so that they can escape the limitation of cell proliferation.\textsuperscript{132}

Receptor-mediated folate uptake was first found about two decades ago.\textsuperscript{133} Cellular uptake of folate in animals is through either FR or reduced folate carrier (RFC).\textsuperscript{7a} FR is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein\textsuperscript{101s} and includes three homologous isoforms, FR-$\alpha$, FR-$\beta$ and FR-$\gamma$, with distinct expression patterns.\textsuperscript{134} FR-$\alpha$ is
the major type expressed by tumors. RFC is a membrane-spanning protein, and most of the antifolate drugs, such as methotrexate, ralitrexed, and lometrexol, are rapidly transferred across the plasma membrane by RFC, leading to inhibition of their target in both tumors and normal proliferating tissues.

Endocytosis mediates the internalization of folate or folate conjugates in FR-expressing cells (Figure I-4). Firstly, folate conjugates bind to FR on cell surface and induce the formation of an intracellular compartment called an endosome, to which they internalize and traffic. Once an endosome transports to cytosol, it triggers acidification under the aid of proton pumps, which decrease pH to 4.3~6.9 (most frequently, pH~5.0). The change of pH results in the release of folate-drug conjugates from FR and the breakdown of compartments (i.e., endosomal escape). FR on the compartment could be recycled back to the plasma membrane and facilitate the next round transportation.

Figure I-4. The process of folate conjugate uptake via receptor-mediated endocytosis.
FR responsible for the internalization of FA conjugates has been supported by experiments where the uptake is abolished by: (i) excess free FA, (ii) FR antibody, (iii) FR negative cells, (iv) the cleavage of GPI anchor with phosphatidylinositol-specific phospholipase C (PLC).\textsuperscript{137} On the other hand, inhibitor studies on several endocytic proteins have suggested that receptor-mediated endocytosis is engaged in the entry of folate conjugates.\textsuperscript{138}

FR-mediated delivery has some great advantages: (i) Normal cells use RFC to transport reduced FA; whereas cancer cells utilize FR to uptake oxidized folates, or folates conjugates,\textsuperscript{139} (ii) The cargo of FA, no matter how big or small, will not affect binding affinity ($K_d \approx 10^{-10}$ M)\textsuperscript{137,140} and endocytosis, (iii) FA conjugate could be released into the cytosol respond to the pH changes, and consequently exert their functions (Figure I-4),\textsuperscript{101j} (iv) Due to the low molecular weight of folate, FA-conjugates facilitate the cellular penetration in solid tumors,\textsuperscript{86} (v) The density of FR in malignant cells appears to increase as the stage/grade of the cancer worsens,\textsuperscript{139} (vi) The carboxyl group of glutamate(s) (Figure I-3) has structural flexibility for chemical conjugation and release,\textsuperscript{141} (vii) FA can be alternatively substituted by Pte (Figure I-3) without compromising binding affinity and delivery efficacy.

High expression levels of FR-α in tumor, such as ovary, cervical, mammary gland, breast, colon, prostate, lung, kidney, nose, throat, and brain, make FR-based delivery attractive for biomedical application. For example, MORAb-003, a monoclonal antibody against FR-α, is under phase II clinical trial in ovarian cancer patients, which has shown cellular cytotoxicity via non-immune mediated cell growth inhibition under folate-deficient conditions.\textsuperscript{142} In addition, several FA-linked drugs are under phase I to
phase II clinical trials. Taking FA-tubulysin derivatives as an example, this group includes tubulysin A hydrazide (EC0510), tubulysin B hydrazide (EC0305), tubulysin B ester (EC0302), and N,O-acetal derivative of natural tubulysin (EC0317). Tubulysin is a type of tetrapeptide that has high cytotoxicity and effective antiproliferative activity against multiple cancer cell lines. EC0305, one of the promising conjugates of the group, shows dose-dependent antitumor activity ($IC_{50} = 1-10$ nM in M109) and favorably low toxicity. Two other FA conjugates, EC131 (FA-maytansionid) and EC145 (FA-desacetylvinblastine monohydrazide), act as tubulin inhibitors by interfering with the formation of microtubulin and exhibit great antitumor potential. These undergoing investigations have demonstrated the FR-targeting delivery holds great potential in biomedical applications.

Additionally, FA has been applied to deliver a wide range of drugs or therapeutic or diagnostic agents into various tumors. The cargoes include siRNAs, ribozymes, anti-cancer drugs, radioactive/magnetic resonance imagining agents, oligonucleotides, radiotherapeutic agents, protein toxins, chemotherapeutic agents, immunotherapeutic agents, and liposomes with entrapped drugs.

Typical FR-positive cancer cells include KB (human nasopharyngeal epidermoid carcinoma cell line), SKOV-3 (human ovarian carcinoma cell line), SW620 (human colorectal carcinoma cell line), etc. However, A375 (Human malignant melanoma cell line), A431 (Human epithelial carcinoma cell line), and A549 (Human lung carcinoma cell line) express little FR and are normally used as FR-negative control cells in studies.
siRNA Targets for Cancer Therapeutics

Sequence-specific gene silencing through siRNAs has become a standard approach for therapeutic application. Many diseases result from abnormal gene expression. siRNA technique has the ability to restore cell’s normal gene function. In cancer cells, genes that are associated with cell proliferation, apoptosis, metastasis, angiogenesis, and drug resistance are potential siRNA targets for therapeutic intervention.\(^{152}\) Three genes, \textit{VEGF}, \textit{survivin (Sur)}, and \textit{caspase 8 associated protein 2 (CASP8AP2)}, are involved in angiogenesis and apoptosis, and are discussed below.

\textit{VEGF and Tumor-Specific Angiogenesis}

VEGF, EGF, and FGF represent the most widely recognized oncogenic growth factors.\(^ {86}\) Among them, VEGF plays as a crucial regulator for blood vessel formation including vasculogenesis (the formation of new blood vessels when there are no pre-existing ones) and angiogenesis (the formation of new blood vessels from pre-existing endothelium),\(^ {153}\) showing pleiotropic responses on migration, proliferation, tube formation, and survival of endothelial cells.\(^ {154}\) Knockout of VEGF in embryonic cells results in despaired vasculogenesis and embryo lethal.\(^ {155}\) Angiogenesis is the second phase of embryological development under physiological conditions.\(^ {20b}\) Recent evidence indicates that VEGF signaling plays a vital role in tumor growth, intraocular neovascular disorders and other conditions due to induced angiogenesis.\(^ {156}\)

VEGF, characterized by the intrachain and interchain disulfide bonds between eight cysteine residues at conserved positions,\(^ {153}\) has two monomers which form a dimer in an anti-parallel fashion with the receptor-binding sites located at each pole of the dimer.\(^ {157}\)
In mammals, there are five members in this family, termed VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PGF). Each member has several different splice variants and processed forms with distinct expression pattern. Human VEGF-A gene contains eight exons separated by seven introns, and has at least five isoforms designated by their expected amino acid length, which are VEGFA121, VEGFA145, VEGFA165, VEGFA189 and VEGFA206. VEGFA121 lacks the residues encoded by exon 6 and 7; while VEGFA165 lacks the residues encoded by exon 6 and is the predominant isoform. The longest VEGF-A transcript in human is 3665 nt long and encodes VEGF206. VEGF-A is a key mediator of blood vessel growth; whereas VEGF-C and VEGF-D regulates lymphatic angiogenesis. In addition, VEGF-A, specifically acting in endothelial cells, has various effects, such as mediating increased vascular permeability, stimulating angiogenesis, vasculogenesis and endothelial cell growth, promoting cell migration, and inhibiting apoptosis.

VEGF receptor family includes VEGFR1, VEGFR2, VEGFR3, and two coreceptors, heparin sulfate proteoglycan and neuropilin, which are defined as VEGF-binding molecules, but lack of VEGF-induced catalytic function. VEGFR1, a soluble or membrane-bound protein, is the receptor of PGF during angiogenesis; whereas VEGFR2 and VEGFR3 are essential for the functions of vascular endothelial and lymphendothelial cells, respectively.

Malignant cells up-regulate the expression of VEGF(R) to induce the formation of blood vessels through which oxygen and nutrients are transported to tumor and eventually facilitate it escaping the limitation of growth supplies. Therefore,
downregulation of VEGF or/and VEGFR expression can diminish angiogenesis by cutting down blood vessel development, and ultimately shrink a tumor. 

This rationale has been applied to treat various cancers and neovascularization age-related macular degeneration (AMD) with pharmaceutical success. Two drugs aimed at VEGF, bevacizumab (Avastin), a humanized monoclonal antibody, and pegaptinib (Macugen), a PEGylated aptamer has been approved by U.S. Food and Drug Administration (FDA) to treat various types of cancer and AMD, respectively. Two other drugs, sorafenib (Nexavar) and sunitinib (Sutent) as molecular antagonists of VEGFRs have also been approved by FDA for treatment of kidney and liver cancer.

Apart from these commercialized drugs, a multitude of VEGF(R)-targeted, siRNA-based therapy are currently being investigated. For example, a siRNA compound, Sirna-027, has disrupted the VEGF pathway and reduced neovascularization by a direct intravitreal (inside an eye) administration. Moreover, Kim and colleagues reported that cocktailed siRNAs targeting three genes (VEGFA, VEGFR1, and VEGFR2) could block angiogenesis from herpes simplex virus (HSV) infection, which caused neovascularization or stromal keratitis. Taken together, VEGF and its receptors are key regulators of the process of angiogenesis, which makes them attractive therapeutic targets with demonstrated success.

Survivin and Anti-Apoptosis

Since the discovery of survivin in 1997, a keen interest and intensive attention have been focused on this unique protein because of its dual-function as a suppressor of apoptosis and a regulator of cell division. Survivin is a member of inhibitor of apoptosis proteins (IAPs) family and a component of a chromosome passenger complex
Survivin, a homodimer, is the smallest member (142 amino acid) of IAP family. IAP regulates apoptosis by blocking the activities of caspases (i.e., caspase 8, caspase 9), contains eight other members: X-linked IAP (XIAP), cIAP1, cIAP2, neuronal apoptosis inhibitor protein, melanoma IAP, IAP-like protein 2, livin, and apollon.

The anti-apoptotic functionality of survivin needs two cofactors, hepatitis B X-interacting protein (HBXIP) and XIAP. Activated by pro-apoptotic signaling, survivin is released from the mitochondria into cytosol and form survivin/HBXIP and survivin/XIAP, which cooperatively inhibit the activity of caspase 9, and eventually downregulate apoptosis (Figure I-5).

**Figure I-5.** The role of survivin in anti-apoptotic pathway. Survivin activates with cofactors, HBXIP (hepatitis B X-interacting protein) and XIBP (X-interacting inhibitor of apoptosis protein) and inhibit the function of Caspase 9, one of effectors of apoptosis.

Survivin expresses during embryonic development and is absent in differentiated tissues. However, it is highly expressed in most human tumors with unveiled mechanisms accompanied by aggressive phenotype, shorter survival time, and decreased response to chemotherapy. Moreover, its expression exhibits prognostic relevance with
some tumors and it appears to be involved in cancer resistance. Based on these findings, survivin has been proposed as an attractive target for cancer therapy. Some preclinical studies have demonstrated that downregulation of survivin expression reduced tumor growth potential, increased the apoptotic rate, and improved the sensitivity of malignant cells to chemotherapeutics. In addition, several survivin inhibitors have been developed as anticancer therapeutic tools and entered clinical trials. YM155, one of them, was discovered through screening assay with a survivin-promoter luciferase experiment. It specifically suppresses survivin transcription and translation in several cancer cell lines with robust antiproliferative activity in vitro and in vivo.

However, in some preclinical experiments, downregulation of survivin expression and function through RNAi technique results in modest antitumor activity, indicating the need of synergic treatment. Following this notion, several components, such as topoisomerase inhibitors, alkylating agents, and UCN-01 (a small molecule inhibitor for protein kinase C, and cyclin-dependent kinase), have been identified and combinatorially utilized with survivin-specific siRNA to increase the inhibitory effects on cancer cell proliferation.

Inhibition of survivin expression does not induce significant apoptosis. Instead, it leads to aberrant chromosome and aneuploidy, suggesting its role as a mitotic regulator associated with CPC. CPC contains at least four proteins: survivin, aurora, borealin, and INCENP. The interaction between aurora and survivin is supported by the correlation of decreased survivin and mislocalized aurora B in dying cells. Data also show that repression of survivin expression will render cell mitotic catastrophe, a form of cell death.
that results from aberrant mitosis.\textsuperscript{179} In these cells, the location of survivin on chromosome is altered and severe spindle defects emerge.\textsuperscript{4,179}

\textit{Caspase 8-Associated Protein 2}

Caspase 8-associated protein 2 (CASP8AP2) is a human apoptotic protein, homologous to FLASH (FLICE-associated huge protein) in mouse and CED-4 in \textit{C. elegans}. Currently, we know little about CASP8AP2, and major knowledge about this protein comes from studies of FLASH.

FLASH was first identified in 1999 by screening a mouse T-cell complementary DNA (cDNA) library with two-hybrid system and tandem dead effector domains (DEDs) of caspase 8 as a probe.\textsuperscript{180} FLASH is constitutively expressed in adult mouse tissue as two transcripts of approximate 4.7 kb and 7 kb, and translated into a \textasciitilde{}220 kDa protein.\textsuperscript{180-181} FLASH contains a DED-recruiting domain, which is able to bind to DED(s) of caspase 8 and Fas-associated protein with dead domain (FADD).\textsuperscript{182} Studies suggested that FLASH may be a component of the death-inducing signaling complex (DISC)\textsuperscript{183} that includes Fas receptor, Fas-binding adapter, and caspase 8, and plays a regulatory role in Fas-mediated apoptosis.

Presently there is some research on CASP8AP2. For example, Kittler et al. generated a genome-scaled, endoribonuclease-prepared short interfering (esi)RNAs library to screen the genes essential for the cell division.\textsuperscript{184} CASP8AP2 was identified through cell viability screening followed by video-microscopy assay with 36 other genes that are critical for cell mitosis.\textsuperscript{184} Downregulation of CASP8AP2 caused cell death, indicating the role of CASP8AP2 as an apoptotic co-repressor. Interestingly, the function of CASP8AP2 in acute lymphoblast leukemia patients appears different. Flotho et al.
found that low expression level of CASP8AP2 was correlative with a high rate of leukemia relapse. By contrast, high expression level was associated with a reduced capacity of leukemic lymphoblasts to grow in vitro. Their interesting results implied that CASP8AP2 was a prognostic factor for leukemia relapse.

These “contradictory” results indicate the dual function of CASP8AP2, similar to FLASH which is capable of regulating apoptosis induction and activation of the anti-apoptotic NF-κB (an important transcriptional factor) pathway. The data from FLASH studies indicate that the subcellular compartmentalization of this protein could play a critical role in its function determination. When FLASH locates in cytoplasm, it mediates apoptotic function in association with caspase at mitochondria; while in the nucleus, it mediates the NF-κB pathway or is assembled into Cajal bodies (a sub-organelle found in the nucleus of proliferative cells), fulfilling the non-apoptotic function.

Transcription-Coupled RNA Conjugation System

Our lab has been interested in RNA catalysis, conjugation, and function. A versatile transcription system has been developed recently using a functional group linked adenosine analog (R-A, R: functional group, A: adenosine) as a transcriptional initiator (Figure I-6). Through this approach, more than 80% transcript RNA may be linked with R through one-step in vitro transcription under the T7 Φ2.5 promoter. Moreover, a number of R-A conjugates have been synthesized for 5’-RNA labeling. The R groups include folate, amines, coenzyme, FAD, NAD, biotin, and fluorophore.
Figure I-6. Adenosine derivative-initiated, one-step in vitro transcription.

In addition, our lab has discovered an RNA bioconjugation method under aqueous solutions where thio-RNA (HS-RNA) can be linked to functional groups by imidazole catalysis with up to 100% yields in 10 minutes. The preparation of thio-RNA gives us another way to modify RNA simply and efficiently by transcription under T7 \( \Phi 2.5 \) promoter.

Although chemical synthesis of siRNA is an easy way with high purity, in vitro prepared RNA is cheaper and more flexible. Typically, the procedure is described as following: (i) DNA templates possessing the T7 \( \Phi 2.5 \) promoter and the sequence for siRNA are rational designed and chemically synthesized; (ii) DNA then is utilized as transcriptional templates to generate two strands of RNA by T7 polymerase; (iii) two strands of RNA are annealed to form a duplex.

This strategy has been applied to synthesize a diverse functional group conjugated RNAs for function or imaging studies. For example, a chimeric FA conjugated pRNA-
siRNA has been generated by this method and shown significant RNAi efficacy.\textsuperscript{128, 190}

Overall, this facile and efficient approach combines RNA synthesis with modification/labeling through one-step transcription, thereby making the method particularly useful in RNA bioconjugation and analysis.
CHAPTER II
OBJECTIVES OF RESEARCH

Taking the advantage of the great advantages (i.e., specificity, and efficiency) of RNAi technique, siRNA-based cancer therapeutics has progressed rapidly and moved into clinical trials. Nevertheless, RNAi clinical application is still impeded by lacking of cell- and tissue-specific delivery. Capitalizing on overexpressed FR in cancer cells but not in normal cells, RNA labeling/conjugation chemistry by one-step transcription, and synthesis of FA-block copolymer, we have a great opportunity to combine these advances to develop cancer cell-targeted siRNA delivery technique and achieve specific gene knockdown.

We hypothesize that FA-linked siRNA or siRNA complexes with FA-copolymer would enter cancer cells with FR-dependency and targetability, activate RNAi pathway, and knockdown targeted gene (e.g., survivin and CASP8AP2) expression, through which we can suppress cancer cell proliferation or induce cancer cell into apoptosis. The overall goal of this research is to develop a strategy that can achieve FR-mediated siRNA targeted delivery to cancer cells with demonstrated inhibitory effects on cell growth. The specific objectives of this research are:

1. Preparation of FA conjugated siRNA (FA-siRNA) with potent RNAi efficacy.

   To achieve this goal, each siRNA targeting a specific gene has to be rationally designed through a selective tool that has integrated a set of proper criteria for increasing the efficiency, specificity, and reducing off-target effects. Both FA conjugated and non-conjugated siRNA will be synthesized by our efficient one-step \textit{in vitro} transcription under T7 Φ2.5 RNA promoter.
2. Delivery of FA-siRNA and FA-polymer/siRNA to FR-expressing cancer cells. To better assess siRNA delivery, a luciferase reporter gene was introduced and integrated into the genome of FR positive KB cells to establish a stable transfected cell line. Taking advantages of bioluminescence, we examine and optimize the transfection condition for both FA-siRNA and FA-polymer/siRNA with enhanced sensitivity and accuracy of detection. Furthermore, several analyses will be conducted. For example, fluorophore-labeled siRNA will be utilized to dissect the internalization of siRNAs through confocal laser scanning microscopy (CLSM) and flow cytometer. On the other hand, polymer encapsulation provides protection of siRNA against enzymatic degradation and therefore increases the half-life of siRNA which can be analyzed by gel electrophoresis and UV/vis spectrometry. In addition, FR-dependency of the delivery is one of the predominant advantages of our delivery system over the other non-specific delivery strategies. FR-assisted siRNA delivery will be examined by experiments where the internalization of FA-siRNA or conjugates is blocked by: (i) competition from excess free FA; (ii) substitution of FR negative cells (e.g., A549) for FR positive cells (e.g., KB); (iii) substitution of non-FA conjugated siRNA and polymer for their FA conjugated counterparts.

3. Achieving significant gene knockdown by delivery of FA-polymer/siRNA complexes and cell growth inhibition/apoptosis. Once the complexes enter cells through endocytosis, they will escape from the endosome and release the siRNA cargo, which will be processed by Dicer, incorporated into RISC and trigger homologous mRNA degradation. Therefore
potent gene downregulation is the goal of our targeted delivery, which will be tested by real-time PCR and luciferase assay. Moreover, due to the pivotal role of our selected siRNA targets in tumor angiogenesis (i.e., VEGF), and anti-apoptosis (i.e., survivin, CASP8AP2), downregulation of their expression will inhibit tumor proliferation, which will be evaluated by morphological observation, cell viability assay, and apoptosis assay. *in vivo* (i.e., xenograft model) assessment is one of the future goals.

4. Application of the FA-copolymer/siRNA delivery system.

   Theoretically, rationally designed and efficiently internalized siRNA could knockdown the expression of any gene in the genome; furthermore, our targeted siRNA delivery system could facilitate siRNA regardless of sequence and pattern to enter any FR-expressing cancer cells. To examine its rationality, three types of FR positive cells (KB, HeLa, and SKOV3), and six siRNA targeted genes (*Survivin, CASP8AP2, VEGF, VEGFR, Luciferase, and GFP*) have been investigated.

5. Monitor siRNA uptake and intracellular trafficking processes.

   The efficiency of siRNA cellular delivery is one of the key factors for the efficacy of RNAi. An evaluative system should answer the following questions: (i) whether siRNA complexes have bound to and entered cells; (ii) how to distinguish specific and non-specific binding; (iii) how to confirm siRNA is inside the cell; (iv) whether siRNA has been escaped from the endolysosome; (v) more importantly, how to quantitate cellular events whereby we can optimize siRNA
delivery. A CLSM-based real-time imaging platform will be used to assess these processes.

6. Establishing the correlation between FR expression level and delivery efficiency of FA-conjugates.

FR-dependence is the key for siRNA specific delivery in our investigation. Therefore clarification of the correlative relationship between FR expression and uptake efficiency is of practical importance. To achieve this goal, experiments will be performed that compare the efficiencies of binding, internalization, and gene knockdown upon transfection of FAPol13/siRNA in three cell lines (KB, HeLa, and SKOV3) that exhibit different FR expression levels.

Successful completion of these objectives is outlined in the following chapters. For example, synthesis of FA-linked siRNA and specific delivery of FA-siRNA in Gluc-KB (objective 1 and part of objective 2) are described in Chapter IV. Cancer specific siRNA delivery via FA-conjugated polymer (objectives 2 and 3) is detailed in Chapter V.
CHAPTER III
MATERIAL AND METHODS
Instrumentation and Equipment

_Name – Model (Company)_

Accumet Basic pH meter – AB15 (Fisher Scientific)
Analytical Balance – HR 60 (A & D Co)
Autoclaves – 2021 and 3031 S (Amsco Eagle series)
Balance – XP 1500 (Denver Instrument)
Electrophoresis Power Supply – EC 154 (E-C Apparatus Corporation)
Freezer –20 °C (Frigidaire)
Freezer –86 °C (Forma Scientific)
Geiger Counter – GSM 110 (Wm. B. Johnson and Associates, Inc.)
Gel Documentation System with Transilluminator – EDAS 290 (Kodak)
Gel Documentation System – Universal Hood II (BioRad)
Biohazard Safety Cabinet – LA2-4A2 (ESCO)
CO₂ Incubation – MCO-17AC (SANYO)
Confocal Laser Scanning Microscope – LSM 510 (Carl Zeiss)
Flow Cytometer – C6 (Accuri)
Fluorescent Microscope – Eclipse 50i (Nikon)
Heating Block – 12359030 (VWR)
Incubator (Labline Instruments Inc.)
Liquid Scintillation Counter – 425 034 (Hidex)
Microcentrifuge – 5415 C and 5451 D (Eppendorf)
Microcon Centrifugal Filter Devices – M10 & M30 (Millipore)

Phosphorimager/Molecular Imager FX – 729 (BioRad)

Phosphorimager/Fluorimager – Typhoon 9400 (Amersham Biosciences)

Pipettes – P-1000, P-200, P-20, and P-2 (Gilson)

Pump – D 25 (Precision Scientific)

Pump – RV3 (BOC Edwards)

Scintillation Counter – 425 034 (Hidex)

Spectrophotometer – V 530 (Jasco)

Stirrers and Heaters (Nuova Thermolyne and Corning)

Thermocycler – Mastercycler (Eppendorf)

Vertical Gel Apparatus – V16 (GibcoBRL)

Heating Circulator – WA01A11B (PolyScience)

Horizontal Electrophoresis System – FB-SB-710 (Fisher Scientific)

Horizontal Electrophoresis System – Mini-sub Cell GT (BioRad)

Vortexer – G560 (VWR)

Microplate Reader – Synergy 2 (BioTek)

Real-time PCR Machine – MX3000p (Stratagene)

Spectrophotometer – ND-1000 (Nanodrop)

Steam Sterilizer – 3031-S (Amsco)

Steam Sterilizer – Lab 250 (Amsco)
Chemicals, Reagents, and Kits

Name (Company)

3'-Mercaptopropionic acid (Sigma-Aldrich)

Acrylamide (40%) (BioRad)

Agar (DIFCO)

Agarose (Fisher Scientific)

Beta-Mercaptoethanol (Sigma-Aldrich)

Bromophenol blue (Sigma-Aldrich)

Calcium Chloride (Sigma-Aldrich)

DAPI (Sigma-Aldrich)

DharmaFECT (Dharmacon)

DMSO (Sigma-Aldrich)

DTT (Promega)

EDTA (Sigma-Aldrich)

Ethanol (Sigma-Aldrich)

Folic Acid (Sigma-Aldrich)

Glycerin (Fisher Scientific)

HEPES (Sigma-Aldrich)

Hoechst 33258 (Sigma-Aldrich)

Hydrochloric Acid (Fisher Scientific)

Lipofectamine 2000 (Invitrogen)

Lysotracker Green (Invitrogen)

Magnesium Chloride (Fisher Scientific)
Manganous Chloride (Sigma-Aldrich)
MMLV reverse Transcriptase (Promega)
Opti-Mem cell Medium (Gibco)
pAcGFP-Mem-Hyg (Clontech)
Parafilm (Pechiney)
Paraformaldehyde (Sigma-Aldrich)
pCMV-Gluc (New England Biolab)
Potassium Chloride (Fisher Scientific)
Potassium Phosphate dibasic (Fisher Scientific)
Propidium Iodide (Abcam)
Riboshredder (Epicentre)
RNasin (Promega)
SDS (Sigma-Aldrich)
Sodium Acetate (Sigma-Aldrich)
Sodium Chloride (Fisher Scientific)
Sodium Hydroxide (Sigma-Aldrich)
Sodium Phosphate Dibasic (Fisher Scientific)
Sodium Phosphate Monobasic (Fisher Scientific)
T4 RNA Ligase (Promega)
T7 RNA Polymerase (Epicentre)
Taq DNA Polymerase (Promega)
Tetramethylethylenediamine (TEMED) (USB)
Top10 One Shot Competent E. coli Cell (Invitrogen)
Tris (Fisher Scientific)
Tris/Borate/EDTA (TBE) Buffer (10x) (BioRad)
Triton X-100 (Sigma-Aldrich)
Trizol (Invitrogen)
Trypan Blue (Sigma-Aldrich)
Tryptone (DIFCO)
Urea (Fisher Scientific)
Yeast Extracts (DIFCO)

Cell Culture Materials
100 mm Cell Culture Dishes (FALCON)
35 mm Cell Culture Dishes (FALCON)
6-Well Cell Culture Plates (FALCON)
12-Well Cell Culture Plates (FALCON)
24-Well Cell Culture Plates (FALCON)
48-Well Cell Culture Plates (FALCON)
96-Well Black Plates (Costar)
96-Well Plates (FALCON)
96-Well White Opaque Plates (Costar)
Antibody against Human Folate Receptor (Abcam)
Chambered Slide (Nunc)
Glass-Bottomed Cell Culture Dishes (MatTek)
Fetal Calf Serum (Hyclone)
Folate Deficiency RPMI 1640 Medium (Gibco)
G418 (Sigma-Aldrich)

Heparin (Sigma-Aldrich)

Hygromycin (CalBiochem)

M-PER Mammalian Protein Extraction Reagent (Thermo)

Phosphate Buffered Saline (Hyclone)

Penicillin (Millipore)

RPMI 1640 medium (Gibco)

Streptomycin (Millipore)

Trypsin (Invitrogen)

**Kits**

AmpliScribe T7 High Yield Transcription Kit (Epicentre)

Celltier 96 Aqueous One Solution Cell Proliferation Assay (Promega)

*Gaussia* Luciferase Assay (New England Biolabs)

Micro BCA Protein Assay (Thermo)

T7 Transcription Kit (Epicentre)

Wizard® Plus Minipreps DNA Purification System (Promega)

Wizard® SV Gel and PCR Clean-Up System (Promega)

Dual-Luciferase Reporter Assay System (Promega)
Buffers and Commonly Used Reagents

**Table III-1. 5X RT Buffer (1mL pH 8.3)**

<table>
<thead>
<tr>
<th>Components</th>
<th>5X Concentration</th>
<th>1X Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 µL 1 M Tris, pH 8.3</td>
<td>250 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>250 µL 1 M KCl</td>
<td>250 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>50 µL 1 M MgCl₂</td>
<td>50 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>20 µL 1 M DTT</td>
<td>2 mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>430 µL Water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table III-2. Denaturing Gel Running Dye (10 mL)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2 g Urea</td>
<td>7 M</td>
</tr>
<tr>
<td>1 mL 10X TBE buffer</td>
<td>1X</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.05%</td>
</tr>
<tr>
<td>Xylene Cyanole</td>
<td>0.05%</td>
</tr>
<tr>
<td>4 mL Water</td>
<td></td>
</tr>
</tbody>
</table>
### Table III-3. Native Gel Running Dye (10 mL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mL glycerol 50%</td>
<td></td>
</tr>
<tr>
<td>1 mL 10X TBE buffer 1X</td>
<td></td>
</tr>
<tr>
<td>Bromophenol blue 0.05%</td>
<td></td>
</tr>
<tr>
<td>Xylene Cyanole 0.05%</td>
<td></td>
</tr>
<tr>
<td>4 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

### Table III-4. Denaturing Gel Mix (1 L)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>420 g Urea 7 M</td>
<td></td>
</tr>
<tr>
<td>100 mL 10X TBE buffer 1X</td>
<td></td>
</tr>
<tr>
<td>200 mL 40% Bis-acrylamide (19:1)</td>
<td>8%</td>
</tr>
<tr>
<td>Add water to 1 L</td>
<td></td>
</tr>
</tbody>
</table>

### Table III-5. Native Gel Mix (1 L)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mL 10X TBE buffer 1X</td>
<td></td>
</tr>
<tr>
<td>200 mL 40% Bis-acrylamide (19:1)</td>
<td>8%</td>
</tr>
<tr>
<td>700 mL water</td>
<td></td>
</tr>
</tbody>
</table>
### Table III-6. SOC Medium (250 mL, pH 7.0)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 g tryptone</td>
<td>2%</td>
</tr>
<tr>
<td>1.25 g yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>625 µL 4 M NaCl</td>
<td>10 mM</td>
</tr>
<tr>
<td>156 µL 4 M KCl</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>240 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Autoclave at 121°C for 20 min, cool down to ~40 °C and then add 2.5 mL of filter sterilized 2 M MgCl2 and 5 mL of filter sterilized 1 M glucose. Prior to autoclaving, adjust the pH to ~7.0 with sodium hydroxide.

### Table III-7. LB Liquid Buffer (1 L, pH 7.5~8)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g Tryptone</td>
<td>1%</td>
</tr>
<tr>
<td>5 g Yeast extract</td>
<td>0.5%</td>
</tr>
<tr>
<td>10 g NaCl</td>
<td>1%</td>
</tr>
<tr>
<td>800 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Autoclave at 121 °C for 20 min, cool down and store in 4 °C refrigerator. Prior to autoclaving, adjust the pH of LB with sodium hydroxide. For solid LB medium preparation, just add 15 g of agar (1.5%) to the above liquid (1 L) before autoclaving. When the temperature of medium is approximate 40 °C, add proper antibiotics (i.e. Ampicillin or Kanamycin), and pour it into plates (25 mL/plate) in the hood. Allow it cool down to room temperature. Invert and store in 4 °C refrigerator.
**Table III-8.** RNA Precipitation Buffer (10 mL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µL 0.2 M EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>1 mL 5 M NaAC</td>
<td>0.5 M</td>
</tr>
<tr>
<td>8.95 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

**Table III-9.** 10X DNA Anneal Buffer (10 mL, pH 8.0)

<table>
<thead>
<tr>
<th>Components</th>
<th>10X Concentration</th>
<th>1X Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 µL 0.2 M EDTA</td>
<td>10 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>1 mL 5 M NaCl</td>
<td>0.5 M</td>
<td>50 mM</td>
</tr>
<tr>
<td>1 mL 1 M Tris, pH 8.0</td>
<td>0.1 M</td>
<td>10 mM</td>
</tr>
<tr>
<td>7.5 mL Water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table III-10.** T7 RNA Transcription (100 µL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µL 10X T7 buffer</td>
<td>1X</td>
</tr>
<tr>
<td>5 µL 0.1 M DTT</td>
<td>5 mM</td>
</tr>
<tr>
<td>4 µL 25 mM NTPs</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>10 µL 4 µM DNA template</td>
<td>0.4 µM</td>
</tr>
</tbody>
</table>
Table III-10 (continued).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µL 50 u/µL T7 RNA polymerase</td>
<td>5 unit/µL</td>
</tr>
<tr>
<td>61 µL water</td>
<td></td>
</tr>
</tbody>
</table>

Note. Incubate the above reaction mix at 37 ºC for 4 h. To get RNA with 5’ adenosine-folate, add 1 µL of 40 mM FA-HDAAMP and replace 25 mM NTPs with 1:4 NTPs (25 mM GTP, 25 mM CTP, 25 mM UTP, and 6.25 mM ATP).

Table III-11. High Yield T7 RNA Transcription (100 µL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µL 10X T7 buffer</td>
<td>1X</td>
</tr>
<tr>
<td>10 µL 0.1 M DTT</td>
<td>10 mM</td>
</tr>
<tr>
<td>30 µL 25 mM NTPs</td>
<td>0.75 mM</td>
</tr>
<tr>
<td>5 µL 4 µM DNA template</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>10 µL AmpliScribe Enzyme Solution</td>
<td>10% v/v</td>
</tr>
<tr>
<td>35 µL water</td>
<td></td>
</tr>
</tbody>
</table>

Note. Incubate the above reaction mix at 37 ºC for 2 h.

Table III-12. Reverse Transcription (20 µL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µL 5X RT Buffer</td>
<td>1 X</td>
</tr>
</tbody>
</table>
Table III-12 (continued).

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 µL 25 mM dNTPs</td>
<td>1 mM</td>
</tr>
<tr>
<td>0.4 µL 50 µM Primers</td>
<td>1 µM</td>
</tr>
<tr>
<td>1 µL 1 µM Total RNA</td>
<td>50 nM</td>
</tr>
<tr>
<td>1 µL of 10u/µL MMLV reverse transcriptase</td>
<td>0.5 u/ µL</td>
</tr>
<tr>
<td>12.8 µL water</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Incubate at 85 °C for 2 min. Cool down. Add 4 µL 5X RT buffer and 1 µL of 10u/µL MMLV reverse transcriptase. Incubate at 42 °C for 1 h.

Table III-13. Real-Time PCR (20µL)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 µL 2X SYBR Solution</td>
<td>1 X</td>
</tr>
<tr>
<td>2 µL 2 µM Primers</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>0.2 µL cDNA</td>
<td>N/A</td>
</tr>
<tr>
<td>7.8 µL Water</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Amplification was started at 95°C for 10 min to activate the DNA Polymerase, followed by 40 cycles of PCRs: at 95°C for 10 s, at 60°C for 20 s, and at 72°C for 30 s, and one more cycle of PCR: at 95°C for 60 s, at 55°C for 30 s, and at 95°C for 30 s.
Experimental Protocols

**Cell Culture**

All cell lines were maintained in folate free RPMI 1640 (Gibco) cell medium supplemented with 10% fetal calf serum (FCS) (HyClone), 100 units/mL penicillin, and 100 µg/mL streptomycin (Millipore) at 37 °C in 95% air humidified atmosphere and 5% CO₂.

**Preparation of Polymer/siRNA Complexes**

FAPol13(Pol13)/siRNA. Neutral polyplexes ($N/P = 1$) were prepared as followed. Briefly, 3 µL of 10 µM Cy3-labeled anti-human survivin siRNA or anti-luciferase siRNA (59 nt) was mixed with 1.5 µL of 50 µM FAPol13 or 1 µL of 50 µM Pol13 at an $N/P$ ratio equal to 1.0. Polyplexes were immediately vortexed to ensure homogenous mixing and equilibrated at room temperature for 10 min before addition to the cell medium (300 µL).

AuNP/siRNA. Complexes (Au nanoplexes) were prepared by adding 1.36 µL of a 25 µM survivin siRNA stock to the 6.64 µL solution of AuNPs. Complexation solutions were immediately vortexed following RNA addition and allowed to incubate at room temperature for 30 min before cell testing or electrophoresis analysis.

**Transfection**

The siRNAs were transfected into mammalian cells by commercial agents (i.e., DharmaFECT; Lipofectamine 2000) according to manufacturer’s recommendations as a positive control. Typically, cells were plated into the dish or plate 24 h prior to treatment with approximate 40% confluency. Two microliter of Lipofectamine and 6 µL of 10 µM siRNA were mixed with 50 µL of Opti-Mem medium at room temperature for 5 min; then two solutions were mixture together and sit for 20-30 min before adding into 500 µL
of medium (total 600 µL, therefore, [siRNA] = 100 nM, Lipofectamine = 0.33% (v/v)). The medium was replaced with fresh one and cells were kept culture for predetermined time.

Confocal Microscopy

For imaging, cells were plated in chambered coverglass wells, transfected with fluorophore-labeled siRNAs, rinsed, and visualized by a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss Inc., Thornwood, NY) with 40×/1.3 or 63×/1.4NA oil lens. A 405-nm diode laser was used to excite Hoechst and fluorescence emission was detected with a band pass filter of 420-480 nm. For Cy3, an excitation wavelength of 543 nm (He-Ne laser) was used and fluorescence emission was obtained using a long pass filter of 560 nm. For FAM, an excitation wavelength of 488 nm (Ar laser) was used, and fluorescence was obtained utilizing a long pass filter of 505 nm. Images were processed via LSM image Examiner software (Carl Zeiss Inc., Thornwood, NY). Further analysis of associated fluorescence was carried out using ImageJ software as following: total associated fluorescence was determined from 30-50 cells (for 63× lens), 75-100 cells (for 40× lens) in at least 5 distinct fields per treatment and corrected by subtracting local background fluorescence from nearby cell-free area.

Time-Lapse and Z-Stack Imaging

KB cells were seeded on chambered coverglass to ~45% confluency for 24 h. Polyplexes were then directly added to the cell medium to give a final siRNA concentration of 20 nM. The dish was immediately put into a heated block holder set at 37 °C. The focal plane was fixed on a cleared bright field plane and confocal images were taken every 10 min for a total of 80 min. Prior to z-stack scanning the cells were
allowed to incubate for 40 min, after which the cells were washed 3 times with pre-warmed phosphate buffer saline (PBS) and the cell medium was replaced. Hoechst 33258 was added to stain the nuclei (1 µg/mL) 20 min before observation. Z-stack scanning was then performed by adjusting the focal plane from the bottom to the top of the cells in 2 µm or 1 µm increments.

Colocalization

KB cells were first cultured for 24 h on chambered coverglass before the addition of polyplexes. Polyplexes were added to the cell medium to give a final Cy3-labeled siRNA concentration of 100 nM and allowed to incubate for 1 h. After 1 h treatment the cell medium was replaced, thereby removing any unbound or free polyplexes from solution. Confocal images were taken at different incubation times, 1 h, 6 h, 24 h, and 48 h. In order to visualize the endosomal compartments, lysotracker was added 30 min prior to each imaging event. The chambered coverglass was placed onto a heated sample holder at 37 °C and imaging process was about 40 min each time. After each imaging event the cell medium was replaced and the cells were returned to the cell culture incubator for another time period observation.

Synthesis of siRNA against Gaussia Luciferase

siRNA against Gluc was synthesized through in vitro transcription according to our published procedure. \(^{188e-h}\) DNA template oligonucleotides containing the T7 \(\phi\) 2.5 promoter sequence were purchased from Integrated DNA Technologies. In vitro transcription was conducted with AmpliScribe T7 high yield transcription kits using the following DNA templates (Sequences refer to 2.5). RNA transcripts were gel-purified, quantified by UV/Vis spectrometry, annealed and then stored at -80 °C.
Total RNA Extraction

Adhesive cells were washed with PBS once before being lysed in Trizol by repetitive pipetting. Use 0.5 mL of reagent per 3.5cm dish (∼ 10^6 cells). Incubate the homogenized samples for 5 min at 15-30° C to permit complete lysis. Add 0.1 mL of chloroform per 0.5 mL of Trizol reagent. Transfer the samples into tubes, cap them securely. Shake tubes vigorously by hand for 15 seconds and incubate them for 5 min. Centrifuge the samples at 12,000 × g for 15 min. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol. Transfer the supernatant (the upper phase) carefully to a new tube (the organic phase would be saved for DNA/protein isolation according to the manual). Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.25 mL of isopropyl alcohol per 0.5 mL of Trizol. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at 12,000 × g for 10 min. Remove the supernatant. Wash the RNA pellet once with 1mL of 75% ethanol. Vortex and centrifuge at 7,500 × g for 5 min. Dry the RNA pellet briefly (do not too dry) and dissolved it in DNase/RNase-free H2O and quantitate by Ab_260. The quality of the extracted RNA would be evaluated by A260/280 ratio.

Real-Time PCR

Total RNA was extracted from 4 types of cells with Trizol following the manufacture’s protocol and mRNAs were converted to cDNA through reverse transcription with MMLV reverse transcriptase (Promega). Finally, real-time PCR was performed with on MX3000P (Stratagene). A typical reaction mixture (20 µL) includes:
10 μL of 2X SYBR Green Mix (Sigma-Aldrich), 2 μL of 2 μM primer pair (final concentration = 200 nM), 0.2 μL of cDNA, and 7.6 μL of DNase/RNase-free H2O. Amplification was started at 95°C for 10 min to activate the DNA Taq, followed by 40 cycles of PCRs: at 95°C for 10 s, at 60°C for 20 s, and at 72°C for 30 s, and one more cycle of PCR: at 95°C for 60 s, at 55°C for 30 s, and at 95°C for 30 s.

**DNA/RNA Purification**

Precipitation. If the DNA/RNA is relative pure, we can precipitate them by adding 3 parts of 0.5 M of NaAc (pH 5.2) and 7 parts of ethanol. After mixing and incubation for at least 30 min at -20 ºC, the sample is centrifuged for 6 min at 14,000 RPM. Remove ethanol and dissolve the sample in DNase/RNase-free H2O.

Column filtration. Load sample volume up to 0.5 mL on to microcon M10 or M30 and centrifuge at 14,000 RPM till the filter is almost dry. Wash the filter with two times of 200 μL water. Invert filter into a clean 1.5 mL Eppendorf tube and spin briefly (~10 seconds) to collect the sample.

PAGE purification. DNA or RNA sample is first separated by 8% denaturing PAGE. The objective sample band is visualized, cut from the gel and then transferred to a fresh 1.5 mL Eppendorf tube. The gel is then crushed and DNA or RNA is extracted from the gel by adding 400 μL of 0.5 M NaAc in 1 mM EDTA and heating at 70°C for 7-10 min to melt the gel. Centrifuge and transfer the liquid to a clean tube. Add 900 μL of ethanol to the tube and incubate the solution at -20 °C for at least 30 min. Remove ethanol and dissolve the sample in DNase/RNase-free H2O.
Flow Cytometry

The expression levels of human folate receptor in cells, the absorption of polyplexes in cells, and the inhibition of polyplexes binding to cells were assessed by flow cytometer. For human folate receptor quantification, cells plated in 12-well plate were harvested, washed, and resuspended to approximately $1 \sim 5 \times 10^6$ cells/mL in ice-cold PBS. Add 100 $\mu$L of cell suspension to the tubes and mix with 1 $\mu$g/mL folate binding protein antibody (AbCam). The tubes were put on the ice in dark for 30 min, and the cells were spin down at 2000 g for 5 min and washed with ice cold PBS twice. The cells were then suspended in PBS with 1 $\mu$g/mL (400× dilution) goat anti-mouse FITC-IgG antibody (Santa Cruz Biotechnology Inc) for 30 min at room temperature in dark. The cells were collected by centrifugation at 1,200 × g for 5min, washed with ice-cold PBS three times before flow cytometer detection. The cells were gently vortexed and introduced into a C6 flow cytometer (Accuri, Ann Arbor, MI) equipped with a 488-nm solid state laser. Data for 10,000 fluorescent events were obtained before or after gating by recording forward scatter (FSC), side scatter (SSC) through channel 1 (530/15 nm) for FAM and FITC, or channel 2 (585/20 nm) for Cy3 using CFlow Plus software (Accuri).

For absorption and inhibition experiments, cells were prepared by plating cells in 12-well plates 24 h prior with ~40% starting confluency. Cells were transfected with neutralized ($N/P = 1$) polyplexes of FAM-labeled siRNA or Cy3-labeled siRNA (final siRNA concentration = 100 nM). Two hours of incubation or twenty four hours of incubation were performed for binding and inhibition experiments. After that, the cells were detached, centrifuged, washed with PBS twice, and ready flow cytometer detection.
Preparation of Plasmid DNA

*E. coli* (TOP10, Invitrogen) were transformed with plasmid DNA (i.e., pCMV-Gluc) and selected in an ampicillin rich LB solid medium (or SOC medium) at 37°C overnight. A single clone of transfected *E. coli* was proliferated in LB liquid media in 37°C overnight with vigorous agitation. The cells were harvested and plasmid isolation and purification were performed consequently with Wizard® Plus Minipreps DNA Purification System kit (Promega) according to the manufacturer’s protocol as followed.

Transfer the *E. coli* cells cultured in LB or SOC medium into 1.5 mL Eppendorf tubes, and centrifuge them at 10,000 × g for 1 min. Remove the medium completely, and add 200 µL of cell resuspension solution for 1 min. Add 200 µL of lysis solution and invert the tube 4 times. Then 200 µL of cell neutralization solution was added and mixed gently to avoid break the DNA. You may see the precipitation appearing. Centrifuge the lysate at 10,000 × g for 7 min. During this time, connect the minicolumn with the barrel of a syringe, add in 1 mL Resin solution (*Note*: make sure there is no crystal inside, otherwise heat the solution by 37°C water bath and cool down to 30°C before using). Transfer the supernatant clear solution into the barrel, and use the plunger to push the resin and lysate mixture go through the minicolumn. Wash the plasmid with 2 mL of column wash solution (add 320 mL of 95% ethanol before first usage). Dry the column by centrifuge at 10,000 × g for 1 min. Dissolve the plasmid DNA by adding 40 µL of DNase/RNase free H₂O followed by spinning down at 10,000 × g for 20 seconds.

The purity of plasmid DNA was determined by both A/A₂₈₀ and A₂₆₀ ratio and agarose gel electrophoresis. The concentration of plasmid DNA was determined by spectrometer and the purified plasmid was stored at -20°C until use.
Preparation of Stable Cell Line (Take SKOV3-KB as an Example)

SKOV3 cell line that stably expresses Gaussia luciferase (Gluc-SKO3) was established. Firstly SKOV3 cells were transfected with pCMV-Gluc by DharmaFECT followed by manufacturer's manual. One day later, the medium was replaced with the one containing 0.5 mg/mL antibiotics G418. Cells were first cultured in a 10 cm dish for 2 weeks. At this time point, the majority (>90%) SKOV3 cells were killed off by G418 because of lacking the expression of neo. However, a few percent of SKOV3 cells that had integrated the pCMV-Gluc plasmid detoxified G418 and survived. Because the resistance to the G418 only indicated the insert of neo gene into the genome of SKOV3, but necessarily meant the integrated of Gluc gene, the selection of Gluc activity was carried out by seeding the surviving cells at very low density (~1 cell/0.1 mL) and plating onto 96-microplates (0.1 mL/well). The selection pressure from the G418 remained for another 7 days. Positive colonies expressing Gaussia luciferase were screened by luciferase assay, propagated, and stored for further experiment.

Cell Treatment with FAPol13/siRNA Polyplexes for Gene Downregulation Studies

Gluc-KB cells were seeded in 96-well plates in folate free RPMI 1640 supplemented with 10% FCS 24 h prior to experiments. Cell treatments included free siRNA and FAPol13/siRNA, Pol13/siRNA, and DharmaFECT/siRNA complexes. The concentration of siRNA against Gaussia luciferase is 100 nM. Cells were treated for 6 h, washed by PBS, and cultured for 2 days. For DharmaFECT group, transfection was carried out according to manufacturer's protocol. Briefly, 10 µL of Optimum buffer mixed with 0.25 µL of DharmaFECT solution and 1 µL of a 10 µM siRNA were mixed with 10 µL of Opti-Mem buffer separately. After 5 min, the two solutions were mixed
and equilibrated for 20 min. The resulting solution was then added to 80 µL of cell medium (total 100 µL) and the cells were treated for 6 h before changing medium.

*Gaussia Luciferase Activity Assay*

*Gaussia* luciferase protein level was measured according to the manufacture’s protocol with minor modification. Aliquots (15 µL) of the cell culturing supernatants were transported into a white opaque 96-well plate and mixed with 40 µL of *Gaussia* luciferase assay solution. The resulting bioluminescence was immediately measure by a Synergy 2 microplate reader (BioTek) and analyzed by Gen5 software (BioTek).

*Cell Viability*

Cell viability tests were performed using CellTiter 96 Aqueous One Solution cell proliferation assay (Promega). Five thousands of KB cells were seeded into the 96-well microplate 24 h before incubation with FAPol13. FAPol13 was subsequently mixed with the cellular medium at final concentrations ranging from 10 nM to 6 µM. Cells were cultured for 2 days before adding 20 µL of CellTiter reagent to each well. After reagent addition the cells were further incubated for 2 hours followed by measuring the absorbance at 490 nm by a microplate reader (Synergy2, BioTek) and Gen5 (BioTek).

*Agarose Gel Electrophoresis*

Neutral complexes were prepared at room temperature. Agarose gel (1%) was prepared by: 0.4 g of Agarose mix with 40 mL of 1× TBE buffer, heat them, and pour them into the casting tray. The gel was pre-run for 30 min. The complexes were mixed with gel loading buffer (0.2% bromophenol blue, 1× TBE buffer and glycerol) and loaded into the wells. Gel was run for 30 min at 90 V. After staining by ethidium bromide, the agarose gel was imaged by a Universal Hood II CCD camera (BioRad).
**Imaging Processing**

All confocal images were processed via LSM image Examiner software. Further analysis of Cy3 and lysotracker fluorophores to examine the fluorescence intense or the distribution was carried out using ImageJ software (http://rsbweb.nih.gov/ij/) and JACoP.

**Statistical Analysis**

Analysis of the statistical significance of FR expression was performed by Graphpad Prism software using $t$ test. Individual flow cytometric, confocal microscopic, and folate-binding assays were repeated at least 3 times to ensure accuracy of results, and representative data are shown.

**Primers and siRNAs**

**Table III-14. Primers for Real-Time PCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank ID</th>
<th>Sequences (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Folate Receptor Alpha (hFRα)</td>
<td>NM_016725</td>
<td>GGAGGCTCAGACAAGGATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGTAGAACAGCAGGCATT</td>
</tr>
<tr>
<td>Human Actin Beta (ACTB, ACTβ)</td>
<td>NM_001101</td>
<td>CATGTACGTGCTATCCAGGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCCTTAATGTACGCACGAT</td>
</tr>
<tr>
<td>Human Survivin (Sur, Sv)</td>
<td>NM_00101227</td>
<td>AGCCCTTTTCTCAAGGACCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCCTCTATGGGTCGCATG</td>
</tr>
<tr>
<td>Human Vascular Endothelial Growth Factor A (VEGF-A)</td>
<td>NM_001025366</td>
<td>TTGCTGCTCTACCTCCAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GATGTCACCAGGTCTC</td>
</tr>
<tr>
<td>Human VEGF Receptor 1 (VEGFR-1)</td>
<td>NP_002010</td>
<td>CTTCGAAGCATCAGCATAAGAAACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGGTACGCCCCACTG</td>
</tr>
</tbody>
</table>
Table III-14 (continued).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank ID</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Integrin Alpha V (ITGAV)</td>
<td>NM_002210</td>
<td>GTGGACAGTCCTGCCGAGTA</td>
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<td></td>
<td></td>
<td>GGCTGGGTGGTGGTTTGCG</td>
</tr>
<tr>
<td>Human Integrin Beta 3 (ITGB3)</td>
<td>NM_000203.2</td>
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<td></td>
<td></td>
<td>TGGCAGGTAGCAGTAAAAGAA</td>
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<tr>
<td>Human ATP-Binding Cassette Subfamily B (ABCB1,MDR1)</td>
<td>NM_000927.3</td>
<td>GTCTACAGTTCTGTAATGCTGACGT</td>
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<tr>
<td></td>
<td></td>
<td>TGTATCCACGGACACTCTCTAC</td>
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<tr>
<td>Human Ribonucleotide Reductase M2 (RRM2)</td>
<td>NM_001034.3</td>
<td>GCCGCTGCTGAGAGAAAACC</td>
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<td>TTACTATGGCCTGCTTGGTGC</td>
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<tr>
<td>Human Caspase 8 Associated Protein 2 (CASP8AP2)</td>
<td>NM_012115.3</td>
<td>CTCTGCTAAAAGCAACCC</td>
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<td></td>
<td></td>
<td>TCACTGCTAGCCGAGCAACCC</td>
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<tr>
<td>pCMV-Gluc Vector (Gaussia Luciferase, Gluc)</td>
<td>AY015993</td>
<td>ACGGATCTCGATGCTGAC</td>
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<td>TCCTATTCTCCCTGTCGC</td>
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<td>pGL3 Vector (Firefly Luciferase, Fluc)</td>
<td>U47296</td>
<td>AACACCCAACATCTTTCG</td>
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<tr>
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<td></td>
<td>CGTCCACAAACACAACCTCC</td>
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<td>pRL-tk Vector (Renilla Luciferase, Rluc)</td>
<td>AF362545</td>
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<td></td>
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<td>CGACAGGACTATAAAGATACC</td>
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Table III-15. DNA Templates for siRNA Transcription

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Stands</th>
<th>Sequences (5'-3')</th>
</tr>
</thead>
<tbody>
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<td>VEGF-A</td>
<td>Sense</td>
<td>CGTAATACGACTCACTATTAGGGCAGAATCATCAGGGACGCATCT</td>
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<tr>
<td></td>
<td></td>
<td>AGATGCCGTCGGGCTGATGATTCTGCTCCCTAATAGTGAGTCGATTACG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGTAATACGACTCACTATTAGGGCAGATCCCGGTTCTGATGATTCTGCTGCCCCCTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAGGGCAGAATCATACACGAAACCCGATCTCCTAATAGTGAGTCGATTACG</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Stands</td>
<td>Sequences (5’-3’)</td>
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<td>-----------</td>
<td>----------</td>
<td>----------------------------------------------------------------------------------</td>
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<td>Gluc</td>
<td>Sense</td>
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<td></td>
<td>AGATGCGGTTAGCAAAAGTTGCACATCTAAATAGTGAGTCGTATTACG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGTAATACGACTCACTATTAGAGATGCGGATGCAAAGTTGCACATCTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAAGATGTCACACTTTTGCTACCCGATCTCCTAATAGTGAGTCGTATTACG</td>
</tr>
<tr>
<td>GFP1</td>
<td>Sense</td>
<td>CGTAATACGACTCACTATTAGCGCCACCATCTTTCTCGACCCGATCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGATGCGGTTAGCAAAAGTTGCACATCTAAATAGTGAGTCGTATTACG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGTAATACGACTCACTATTAGAGATGCGGATGCAAAGTTGCACATCTTT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAAAGGCACCATCTTTCTCGACCCGATCTCCTAATAGTGAGTCGTATTACG</td>
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<td>GFP2</td>
<td>Sense</td>
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<td>AGATGCGGATCCGAGGTTGCTGATGCAATAGTGAGTCGTATTACG</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>CGTAATACGACTCACTATTAGAGATGCGGATGCAAAGTTGCACATCTTT</td>
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<tr>
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<td></td>
<td>AAAAGGCACCATCTTTCTCGACCCGATCTCCTAATAGTGAGTCGTATTACG</td>
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<td>EGFP</td>
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<td>AGATGCAATTAGAAACATGTTGCTGTAATAGTGAGTCGTATTACG</td>
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<td>Antisense</td>
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</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGTAATACGACTCACTATTAGAGATGCGGATGCAAAGTTGCACATCTTT</td>
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### Cell Lines

**Table III-17. Cell Lines**

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<td>testing platform</td>
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<td>CCL-185</td>
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CHAPTER IV
DELIVERY OF FA-SIRNA TO CANCER CELLS
AND DOWNREGULATION OF GENE EXPRESSION

Introduction

Conventional anticancer drugs, such as cisplatin, paclitaxel, doxorubicin, and methotrexate, have been the major chemical agents for cancer treatment. Although they all display significant anticancer activities, patients normally suffer side effects due to drugs’ cytotoxicity and non-specificity (no gene-targeting specificity and cell-targeting specificity). In other words, these small molecular drugs treat normal cells and malignant cells indiscriminately, which limits the effectiveness of these treatments. The recently discovered siRNA can trigger RNAi pathway and induce mRNA degradation with high sequence specificity, low cytotoxicity, and potent gene downregulation efficiency. By virtue of these properties, siRNA has been utilized as a molecular drug for diseases treatment.

Although siRNAs hold incomparable advantages and show great promise, the inefficient cellular uptake and poor bioavailability of siRNA remain the major obstacles that compromise siRNA’s therapeutic application. Moreover, without efficient cell-specific delivery, biomedical application of siRNA is limited. Employing targeting moieties thus provides an opportunity for directed siRNA delivery with enhanced efficiency in vitro and in vivo. Valid targeting groups include aptamers, antibodies, peptides, cholesterol, α-tocopherol, and folate. Among them, FA shows unsurpassable advantages as mentioned in Chapter I.
The use of folate as a therapeutic director has been popularized due to overexpression of the folate receptor in several types of tumors (i.e., ovary, brain, lung, breast, kidney). FA has been successfully employed to deliver siRNAs, ribozymes, anti-cancer drugs, imaging agents and antisense oligonucleotides. Several lines of evidence demonstrate FA-directed siRNAs delivery into cancer cells or tumors. For example, a polyvalent, FA-linked, chimeric pRNA (a 117-nt bacteriophage phi29-encoded RNA, playing a novel and essential role in DNA packaging) had been constructed with siRNAs against human apoptosis inhibitor gene — survivin. The effectiveness and specificity of this chimeric siRNA vehicle has been confirmed by both \textit{in vitro} and \textit{ex vivo} experiment. Zhang et al and his colleagues reported a similar strategy involved in the noncovalent attachment of siRNAs to FA-conjugated oligodeoxynucleotides via nucleic acid base-paired interactions, and resulted in cell-type-specific delivery of functional siRNAs into cells with moderate gene knockdown.

In addition to the efforts for improving the targeted siRNA delivery, an accurate, fast, and convenient method for assessing the RNAi effects is prerequisite. Conventional techniques, such as real-time PCR (or quantitative PCR), and Western blot can hardly meet these expectations. Thus, a reporter protein-assisted evaluative system based on fluorescence (e.g., green luciferase protein, GFP) and bioluminescence system (e.g., luciferase) is constructed and described in this chapter.

GFP and its analogs, such as red fluorescence protein (RFP), yellow fluorescence protein (YFP), have revolutionized our understanding of many biological processes at the molecular and cellular levels. With suitable GFP fluorescence probes and instrumentation (e.g., confocal laser scanning microscope) for functional imaging, we can
identify and measure biological events in whole organism in real time. Another rapid and accessible reporter system is bioluminescence where luciferases convert chemical energy into light.\textsuperscript{201} Combining with charge coupled device cameras that detect light transmitted through tissues/cells has opened the door to sensitive measurements of gene expression changes upon RNAi.

Although fluorescence system has several advantages, such as direct detection of fluorescence, easier visualization, it also has some setbacks compared to luciferase system for quantitation of RNAi effects, e.g., non-quantitative, no read-out.\textsuperscript{202} On the other hand, despite indirect read-out, enzymatic assay-based detection, and repeated measurement, luminescence system is comparably convenient in quantifying the signal changes. For these reasons, two systems have been utilized in my research with preference to the luminescence system.

About four decades ago, a series of luciferase that catalyzed luciferin reaction and emitted light in the visible range under physiological conditions was discovered and characterized.\textsuperscript{203} Various luciferases share sequence homology in different organisms, such as bacteria, fungi, dinoflagellates, radiolarians.\textsuperscript{204} Firefly (\textit{Photinus pyralis}) luciferase (FLuc), sea pansy (\textit{Renilla reniformis}) luciferase (RLuc), and Gaussia (\textit{Gaussia princeps}) luciferase (Gluc) are most commonly used luciferases for monitoring gene expression.\textsuperscript{204b, 205} Among them, Gluc catalyzes the oxidation of substrate coelenterazine to produce light (470 nm),\textsuperscript{206} and has been engineered to highly express in mammalian cells.\textsuperscript{206b} In addition, compared to FLuc and RLuc, Gluc is a more attractive reporter for studying gene expression because of some unparalleled features. Firstly, Gluc generates over 1000-fold higher bioluminescent signal intensity than RLuc does. Secondly, Gluc
possesses a secretory signal process, and therefore the expression of Gluc can be detected from cell medium, omitting the usual cell lysis step. Thirdly, secreted Gluc is very stable and can be stored for several days at 4 °C without the loss of activity, which enables the convenient multiple samples measurement at different time points.

Capitalizing on RNA conjugation chemistry through one-step transcription, and FR-mediated siRNA targeted delivery to cancer cells, I propose to prepare and study the cellular delivery of FA-linked siRNA and assess RNAi effects through reporter system. Specifically, I intend to achieve the following goals (Scheme IV-1): (i) rational design of siRNA with potent potential to induce gene silencing through the proper online tools; (ii) synthesis of the FA-conjugated siRNAs taking advantage of in vitro transcription and FA-HADAMP as the transcription initiator; (iii) the specific uptake of FA-siRNA by FR-bearing human cancer cells; (iv) FR-dependent internalization; (v) gene downregulation as the result of the internalized FA-siRNA via RNAi process; (vi) the convenient and fast assessment of RNAi efficiency by introduction of a reporter system. To test these hypotheses, the experiments were rationally designed and performed.
**Scheme VI-1.** Schematic illustration of the internalization of transcripted FA-siRNA conjugate into GLuc-KB cells through FR-dependent endocytosis.

Folate-HDAAMP is chemically synthesized and serves as the transcriptional initiator under the T7 Φ2.5 promoter. Two single-stranded RNA are synthesized separately. FA-HDAAMP could be added in either sense strand or antisense strand; most of time it will be add into the synthesis of antisense strand. Two strands RNA will be annealed to form double-stranded, FA-linked siRNA. The siRNAs specific for Gaussia luciferase, and GFP and a non-silencing control siRNAs used in the studies are generated by this method. Following internalization into the FR-bearing cells (KB cells in this case) via FR-mediated endocytosis, the FA-siRNA is released from the endosome, processed by Dicer, incorporated into RISC, and eventually induces the silencing of targeted genes.
Result and Discussion

*Design and Synthesis of FA Conjugated siRNA*

This first step for specific siRNA delivery is to design siRNA sequences. We can then synthesize them by one-step *in vitro* transcription. Based on the experience of rational siRNA design accumulated in our laboratory, we chose siRNA selection programs to design siRNA against the genes responsible for pathological disorders. This online tool has an option of a searching pattern which can be customized according to users’ purposes with different sequence length and different nucleotide at particular position. As a result, we set down the pattern N2AGN7UN8AN2 in consideration of transcription promoter requirements and base preferences. In this 23-nt search sequence, N, A, G, and U strand for any nucleotide, adenosine, guanosine, and uridine respectively, and the number after the N means the length of the nucleotides. AG at 3 to 4 position is for our transcription system, and U at 12 and A at 21 is used to meet the sequence preferences rules.

After confirmation of the search pattern, we either paste mRNA sequence or input the accession number (e.g., NM_001025366 for human VEGF-A) into a frame and start search candidate siRNAs with some established filters. Subsequently, a list of siRNA candidates with position in the mRNA sequence, thermodynamics, GC% content and other information will pop out for our reference. To minimize the off-target effects of the siRNA, BLAST (basic local alignment search tool) of selected siRNA sequences against Genbank is highly recommended afterwards to ensure that only the interested gene is targeted. Through this procedure, siRNAs against several genes have been designed, and
importantly the significant gene downregulation efficiency demonstrates the feasibility of the siRNA selecting method.

**Figure VI-1.** The formation of folate conjugated siRNAs by *in vitro* transcription. (A) The schematic illustration of FA conjugated dsRNA. Two dsDNA templates bearing T7 Φ 2.5 promoters and well-designed siRNA sequence were chemically synthesized. Two ssRNAs were synthesized, and the conjugation of FA or other functional groups to RNA was controlled simply by adding FA-HDAAMP. The ssRNAs were annealed by complementary sequences and formed FA-siRNAs. (B) PAGE analysis of sense, antisense RNA (lane 1 and 2), FA-antisense RNA (lane 3), and FA-siRNA (lane 4). Likewise, the FA-sense RNA and FA-sense labeled siRNA could also be generated.
After selection of siRNA sequences, their synthesis via our in vitro transcription is followed (Figure IV-1). Firstly, four DNA strands bearing siRNA sequence and T7 Φ 2.5 promoter were synthesized commercially. Next complimentary DNA strands were annealed to form two pairs of double-stranded DNA, which were used as templates to transcript two single-stranded RNAs. The synthesis of FA conjugated and non-conjugated RNA was adjusted by addition of transcriptional initiator: FA-HDAAMP (Scheme IV-1) which was chemically synthesized and purified by HPLC. After gel purification and UV/Vis quantification, equimolar single stranded RNAs were hybridized to form a FA-conjugated or unconjugated RNA as illustrated in Figure IV-1A. The formation of RNA duplex and FA conjugation were confirmed by PAGE analysis (Figure IV-1B).

In Figure IV-1B, we can see 27-nt sense strand RNA in lane 1, 32-nt antisense strand RNA in lane 2. The antisense RNA with FA (MW = ~441) conjugation (in lane 3) is bigger in size compared to the RNA without conjugation (lane 2), and displays about one nucleotide shift. Besides, a small portion of unconjugated antisense strand is seen in lane 3 as expected. The FA-linked RNA duplex is in lane 4. The clear bands suggest the successful purification and hybridization of two strands.

Comparison of FR Expression in KB and A549 Cells

Once FA-siRNA is synthesized, its internalization into FR-expression cells is examined. Two human cancer cells lines, KB cells (human nasopharyngeal carcinoma) and A549 cells (human lung carcinoma), are used because KB cells highly express FR; whereas A549 has non-detectable expression. To test that, real-time PCR and flow
cytometry were utilized to analyze human FR-α (hFRα) expression in both mRNA and protein levels in those two cell lines (Figure IV-2).

To compare hFRα expression in gene level, total RNA was extracted from two types of cells; reverse transcription was conducted; and real-time PCR was carried out. The representative amplification curves of hFRα in two cell lines were shown in Figure IV-2A. Because the amplification curves of human β-Actin gene (internal control) in KB and A549 are nearly overlapped; and therefore they are not displayed in Figure IV-2A. Statistical analysis from three repeats was presented in Figure IV-2B. The data exhibit ~15 Ct value differences in two cell lines, indicating ~ 30,000 times variation in mRNA level. To confirm this result, flow cytometry was applied to quantitate the hFRα protein level in these two cell lines. The primary antibody against the hFRα was added into the medium and specific bound to the FR expressed at the plasma membrane surface. A FITC labeled second antibody which binds to the primary antibody was then added. Cells were washed and collected, followed by detection by flow cytometry. A typical result of FR quantification in KB was presented in Figure IV-2C. The black, red, blue, and yellow lines represented the KB cells without and with antibody staining, A549 cells without and with antibody staining respectively. It is obvious the black and red line lines are not overlapped. The shifted red line indicates the expression of hFRα in KB. By contrast, the blue and yellow lines in A549 are almost overlapped, suggesting the non-detectable protein expression of hFRα in A549. The distinct expression of this protein in two cell lines was further confirmed in Figure IV-2D, where more than 300-fold expression difference was displayed. Through these analyses, the data clearly exhibit the significant expression difference of hFRα in KB and A549 cells. Regarding the hFRα protein is the
direct executor for FA or FA conjugates uptakes, approximate 300 fold difference of this protein might better reflect the real situation. The fold differences in real-time PCR and flow cytometer would partially result from post-transcriptional modification or other reasons. Collectively, the result has shown that KB cells highly express FR while A549 cells have only background FR level. Therefore, KB and A549 cells can be used as FR-positive and negative cells in our experiments.

We propose to use GFP and luciferase system to study gene expression upon FA-siRNA treatment because of its accuracy and sensitivity. Among various luciferases, Gaussia luciferase (GLuc) is utilized because: (i) it is a secreted protein, therefore cell lysis step is escaped; (ii) GLuc has the 1000 fold sensitivity over Fluc. The utility of Gluc was first in a transient transfection system (Figure IV-3) and a stable transfection system where a cell line based on KB was constructed that express Gluc stably (Figure IV-4).
Figure IV-2. Comparison of human FR-α (hFRα) expression in KB and A549 cell lines. (A) Representative amplification plots of hFRα in KB and A549 cells through real-time PCR. (B) Statistic analysis of real-time PCR results shows the relative hFRα gene expression in two cell lines. (C) Representative fluorescence binding plot of anti-hFRα antibody in KB and A549 cells via flow cytometer; (D) Statistic analysis of flow cytometer results shows the relative hFRα protein expression in two cell lines. Each bar represents mean ± S.D. (n=3-4).

Downregulation of Gluc Activity through Transient Transfection

As for transient system, cancer cells were transfected with plasmids (pCMV-Gluc) encoding Gluc and siRNAs against Gluc (siGluc) by Lipofectamine consecutively. Six hours after transfection of plasmid, the second transfection was performed to introduce siRNA against Gluc (siGluc) into the cells. A siRNA control (siCon) that has no targeted gene in human genome is used. As expected, siCon will not cause any gene silencing. In contrast, the cells transfected with siGluc will downregulate Gluc
expression. Luciferase will be expressed after the first transfection within a few days (at least 3 days), and thus provides targeted mRNA for RNAi. The medium samples were collected approximately every 8 hours and measured by luciferase activity assay (Figure IV-3). The cells treated with siCon (the solid circle) serve as the mock group and show increasing Gluc activity along with time (Figure IV-3A). In contrast, the cells upon siGLuc treatment (the solid square) display lower Gluc activity, indicating gene downregulation from RNAi pathway.

**Figure IV-3.** Downregulation of luciferase activity in KB cells by transient transfection. (A) KB cells were transfected with pCMV-Gluc plasmid first, then with either siRNA targeting Gluc (Plasmid + siGLuc, solid square) or siRNA control (Plasmid + siCon, solid circle). Cellular medium containing the secreted was collected at the different time points, and quantified through luciferase activity assay. Due to RNAi effects, the cells transfected with siGLuc showed less luciferase activity; while the control group showed increased luciferase activity. (B) Relative siGLuc efficiency was presented. The relative siGLuc efficiency was plotted in Figure IV-3B. The data show the maximum siGLuc efficiency, 68% of downregulation, occurs at 42 hour after transfection (HAT), and average RNAi efficiency is approximate 60%. Interestingly, the RNAi efficiency of 12 HAT appears to be 70%. That may not reflect the truth regarding of the unequal expression rates of the plasmid and the processing of RNAi. In other words, the plasmid is expressed in control group (plasmid + siCon); whereas RNAi effect is limited
in treatment group (plasmid + siGLuc). In addition, the plasmids are transfected into the cells prior to siRNAs. Likewise, transient transfection of siRNA first prior to plasmids has been conducted. Similar maximum siRNA efficiency (~70%) at around 48 HAT indicates transient transfection is sufficient for determination of the best siRNA efficiency.

Overall, the data have demonstrated that: (i) transcriptionally synthesized siGLuc can silent the Gaussia luciferase expression potently; (ii) under the transient transfection conditions we studied, the max siRNA efficiency is 68% at 42 HAT; (iii) the transient transfection is not an ideal system for dynamic analysis of RNAi effects.

Downregulation of Gluc Activity through Stable Transfection

**Figure IV-4.** The construction of Gluc-KB cell line. (A) Plasmid map of pCMV-Gluc which had been transfected with KB cells. (B) Gel electrophoresis analysis of prepared pCMV-Gluc. (C) The schematic illustration of stable cell line establishment. KB cells were transfected with pCMV-Gluc and then incubated with G418 for 4 weeks. Those cells capable of tolerating G418 and expressing Gluc constitutively are termed Gluc-KB cells.
To address this issue, a stable transfection is applied, through which the Gluc gene is integrated into the genome of KB cells and the Gluc will express stably. The plasmid pCMV-Gluc vector map (Figure IV-4A), electrophoresis analysis of plasmids (Figure IV-4B), and the procedure for establishment of a stable cell line (Figure IV-4C) are presented in Figure IV-4.

Basically there are two important genes encoded within the humanized sequence of pCMV-Gluc (5764 base pairs) (Figure IV-4A) Gaussia luciferase and aminoglycoside phosphotransferase (neo) under the strong promoters of cytomegalovirus (pCMV) and simian virus (pSV40), respectively. Neo is a selectable marker, conferring the transfected cells resistance to antibiotics Neomycin in prokaryotic cells, or genticin (G418) in eukaryotic cells, through which stable cell lines (usually mammalian cell lines) expressing reporter proteins or cloned proteins are established. To get sufficient amount of pCMV-Gluc for transfection purpose, the plasmid preparation was performed via transformation of *E. coli*. The extracted pCMV-Gluc was confirmed by 0.8% agarose/EtBr gel electrophoresis (Figure IV-4B), the first lane is the original plasmid (100 ng, 2 µL of 50 ng/µL), and the other 9 lanes are amplified plasmid. The procedure of the stable expression cell lines is illustrated in Figure IV-4C. Firstly, KB cells were transfected with pCMV-Gluc by DharmaFECT followed by manufacturer’s manual. One day later, the medium was replaced with one containing 0.5 mg/mL antibiotics G418. Cells were first cultured in a 10 cm dish for 2 weeks. At this time point, the majority (> 90%) KB cells were killed off by G418 because of lacking the expression of *neo*. However, a few percent of KB cells that had integrated the pCMV-Gluc plasmid detoxified G418 and survived. Because the resistance to the G418 only indicated the insert of *neo* gene into
the genome of KB, but necessarily meant the integrated of Gluc gene, the selection of Gluc activity was carried out by seeding the surviving cells at very low density (~1 cell/0.1 mL) and plating onto 96-microplates (0.1 mL/well). The selection pressure from Gl418 remained for another 7 days. Positive colonies expressing Gaussia luciferase (Gluc) were screened by luciferase assay, transferred to a 12-well plate for propagation. The cells are designated as Gluc-expressing KB cells (Gluc-KB).

Gene down-regulation studies were carried out in Gluc-KB cells with siGLuc (Figure IV-5) and FA-siGLuc (Figure IV-6). In Figure IV-5A we can see the continuous expression of Gluc in Gluc-KB cells. Without the interference of siRNA, Gluc expression correlates with time (mock group). The cells transfected with different concentration of siGLuc display downregulated expression of GLuc. The data from Figure IV-5A is converted to Figure IV-5B to show the siGLuc efficiency within the 72 hour after transfection (HAT). From the figure, the dose-dependence pattern is obvious: siRNA efficiency from 30 nM > 20 nM > 10 nM. At 72 HAT, 76%, 67% and 66% of Gluc activity has been reduced from treatments with 30 nM, 20 nM and 10 nM of siGLuc respectively. Due to the limitation of cell confluency (cell density in a cell culture container), the longer incubation time will cause error since 40% confluency is start point before transfection. Therefore, we have not observed the disappearance of siRNA efficiency here, which can map out the best RNAi efficiency of siGLuc.

Nevertheless, the results have demonstrated: (i) after being integrated into the genome of KB cells, Gluc functions as an endogenous gene and is expressed by Gluc-KB cells constitutively; (ii) 60%-70% of Gluc activity is reduced by Lipofectamine-mediated transfection of siGLuc within the 36-72 HAT; (iii) dose-dependent downregulation.
Figure IV-5. Downregulation of luciferase activity by siGluc in Gluc-KB cells. (A) Gluc-KB cells were transfected by Lipofectamine with 10 nM, 20 nM, and 30 nM of siGLuc. (B) The relative siGLuc efficiency was plotted by normalization with non-treated group (mock).

Based on the above results, we tested whether: (1) FA-siGLuc would be internalized into Gluc-KB cells and silence the Gluc activity; (2) the delivery of siRNA is FR-mediated and has the cell-specific feature. The Gluc-KB cells are maintained in Folate deficient medium all the time and seeded onto the dished with 40% confluency one day before the addition of FA-siGLuc (final concentration = 20 nM, the same concentration was used afterwards) into the medium. No more additional treatment was performed. Every day the medium samples were collected and measured and the time course data was plotted into Figure IV-6 which suggested the best siRNA efficiency occurred after 5 days of incubation with ~50% downregulation. It is notable that the best siRNA efficiency shows up after a relatively long period of time which is similar to the results of Dassie et al. who deliver PSMA aptamer-modified siRNA chimeras into the human prostate tumors and observe the best siRNA efficiency at 4 days post incubation. This phenomenon would result from the delayed naked siRNA escape from the endosome, which would compromise the efficiency of siRNA.
Several groups of control (Figure IV-6B) were tested along with FA-siGLuc in Gluc-KB cells, including Lipofectamine/siGLuc, FA-siGLuc with 1 mM of free folate, FA-conjugated siRNA control, siGLuc without FA conjugation. The results showed 55% and 23% Gluc activity remained from the FA-siGLuc and Lipofectamine/siGLuc treatment. The presence of high concentration of free FA competed the binding sites (FR) with FA-siGluc and blocked the internalization of FA-siGLuc, thus the Gluc expression from this groups was unaffected. In addition, transfection of siRNA control (siCon) which has no gene target in the mammalian cells showed no RNAi effects. siGLuc without FA conjugated cannot enter Gluc-KB cells and gene downregulation was observed under this condition. Thus combination of controls demonstrated FR-mediated siRNA delivery.

**Figure IV-6.** Downregulation of luciferase activity by FA-siGLuc in Gluc-KB cells (A) The time course of relative Gluc expression was assessed at 3 to 6 days after transfection (siRNA concentration = 20 nM). (B) Several controls including cell without treatment (mock), FA-siGLuc plus 1 mM free folate, FA-linked control siRNA (siCon), Lipofectamine-mediated transfection of siGLuc, and siGLuc, were explored along with the FA-siGLuc. The concentration of siRNA in the study was 20 nM, and the measurement was carried out at the 5th day after incubation. The data represents mean ± S.D (n=3-4).
Reduction of GFP Expression through Transient Transfection

Besides the bioluminescence system, the possibility of utilizing GFP as a reporter was also exploited in our research as shown in Figure IV-7. Similarly, KB cells consecutively expressing humanized GFP (GFP-KB) was established by transfecting KB cells with plasmid pAcGFP1-MemHyg (5846 bp) and selecting with antibiotics Hygromycin B. The screening of GFP expression positive colonies was performed through typhoon scanner. Due to lack of a spectrofluorophotometer, the fluorescent signals cannot be quantified. Instead, confocal imaging (Figure IV-7) and real-time PCR (Figure IV-7B) were applied to measure the GFP expression reduction after transfection of siRNA against GFP. As shown in Figure IV-7, we can see the high expression level of GFP in GFP-KB cells, and the expression is not influenced by transfection siCon. In contrast, siGFPa and siGFPb (two siRNAs both target the GFP gene) can significantly reduce the GFP expression by 97% and 95% (Figure IV-7B). This second example confirms: (1) our designed and prepared siRNAs can efficiently reduce the expression of targeted gene; (2) the reporter-stably transfected cell is an optimal system to assess gene expression, especially upon RNAi.
Day incubation, indicating the internalization of siRNA through FR mediation and RNAi.

**Figure IV-7.** Downregulation of GFP expression in GFP-KB cells by Lipofectamine. (A) Confocal analysis of reduced fluorescence by transfection of two siRNAs against GFP, siGFPa and siGFPb. In contrast, the non-treated cells and the cells transfected with siRNA control (has no targets in human genome) show unaffected GFP expression. (B) Real-time PCR analysis of gene expression.

**Conclusions**

Here we synthesized FA functionalized siRNA through in vitro transcription system. The FA-HAD-AMP as the transcriptional initiator is the key to this conjugation. Subsequently, to better monitor the gene expression changes upon the siRNA treatment, luciferase reporter, especially *Gaussia* luciferase, system was introduced. A stable transfection cell line based on FR-expressing human cancer cell KB was established and denoted Gluc-KB. Firstly, Gluc-KB cells were transfected with siGLuc through transfection agents to test the efficiency of siGLuc; secondly, Gluc-KB cells were incubated with FA-siGLuc. FA-siGLuc at 20 nM reduced Gluc activity to 50% after 5-day incubation, indicating the internalization of siRNA through FR-mediation and RNAi.
pathway-dependent gene silencing (siRNA control group). More importantly, several control groups had demonstrated the FR-dependent delivery because extra free FA could block the gene downregulation, and the siGLuc without FA modification can not enter cells. Moreover, the utilization of reporter systems and the establishment of GFP-KB and Gluc-KB cell lines render a fast and convenient platform for monitoring gene expression visually and quantitatively, and assessing siRNA efficiency.

In spite of FR-mediated siRNA delivery and gene downregulation (50%), the RNAi efficacy is relatively low and may not meet the needs of biomedical application. The key here is to increase the gene downregulation efficiency by improving delivery strategy. Firstly, naked siRNA would be degraded in a short period of time by extracellular and intracellular RNase. It has been reported the half-life of unmodified siRNA is ~10 min in 100% human serum. Secondly, evidence shows that monovalent folate conjugate (containing one attached FA) compared to its multivalent counterpart (with several FAs) has less efficiency in triggering pH changes in early endosome, which is a crucial for siRNA release into cytoplasm. Another important reason for improvement the strategy comes from the consideration of systemic administration. Because of small molecular weight (59 nt, ~20 kDa) and size (~ 3 nm) of siRNA, it will be rapidly excreted through urine when circulated in blood stream.86

To address these issues, we need a system cable of protecting siRNA and increasing the size of siRNA (i.e., form a complex) to achieve enhanced delivery efficiency and RNAi efficacy. Fortunately, through collaboration, we have developed a series of polyvalent, FA-conjugated copolymers with advantageous solubility, protection and complexation functionalities, which will be discussed in the next chapter.
CHAPTER V
CANCER-SPECIFIC SIRNA DELIVERY VIA FAPOL13

Introduction

siRNA therapeutics have to overcome several major challenges including delivery efficiency, target cell specificity, \textit{in vivo} stability, and appropriate intracellular siRNA release mechanism from delivery vehicles before it enters biomedical applications.\textsuperscript{92a, 210}

To address above issues, significant efforts have been dedicated to synthesize various nanoparticles as siRNA carriers which have become particularly attractive because of their flexibility of available conjugation chemistries, and capabilities of increasing siRNA stability, biocompatibility.\textsuperscript{211}

Cationic (co)polymers, such as PEI, polylsine and polyarginine, have been broadly used because they can package siRNA, or other oligo-/polynucleotides, through electrostatic interactions, thus providing the bound polynucleotide protection from detrimental nucleases.\textsuperscript{212} Although cationic complexes can protect siRNA and remain aqueous soluble, the cationic surface charge leads to non-specific cellular adsorption and thus transfection of both healthy and unhealthy cells.\textsuperscript{213} Furthermore, cationic complexes can associate with anionically charged serum proteins resulting in aggregation and eventual phagocytic clearance.\textsuperscript{214} Utilizing hydrophilic-\textit{block}-cationic copolymers for siRNA complexation, neutral complexes can be formed circumventing problems associated with cationic carriers. The cationic block still provides packaging and protection for siRNA, while the hydrophilic block provides biocompatibility and solubility for the complex. This neutral hydrophilic block also prevents unwanted aggregation and non-specific cellular adsorption through steric stabilization. Since neutral
carriers do not electrostatically adsorb to cellular surfaces, the carrier must be directed to or accumulated at a specific tissue or cellular site. A promising route to direct and trigger cellular uptake is to incorporate cellular targeting moieties to either the copolymer backbone or end-groups.

Due to progress in numerous polymerization methods, (co)polymers with advanced architectures and predetermined chain-end and side-chain functionalities can be specifically designed for targeted delivery applications. Incorporation of such functionalities allows facile post-polymerization conjugation of cellular targeting moieties. In principle, targeted delivery systems mitigate side effects, improve bioavailability, and enhance therapeutic efficacy. Targeting ligands applicable to cancer therapy include antibodies, peptides, aptamers, and folate. The overexpression of FR in various types of tumors, such as, uterus, brain, kidney, breast, ovary, makes it as a cancer-cell-surface receptor, and confer the use of folate as a therapeutic director for homing FA-conjugates to tumors. Folate has been successfully employed to deliver diverse drugs, siRNAs, therapeutic, diagnostic and prognostic agents, and at least four FA-linked drugs are under phase I or II clinical trials.

Several research groups, including our own, have successfully developed (co)polymer systems with utility in siRNA delivery. Although these reports demonstrate gene down-regulation, cellular trafficking events (i.e., binding, uptake, and endosomal release) prior to gene suppression are typically overlooked or ignored. Furthermore, evidence shows that efficient internalization of siRNA does not necessarily result in favorable gene down-regulation. For example, trafficking or degradation events after uptake, such as endosomal entrapment, exocytosis or recycling, or enzymatic
inactivation of the siRNA, can lead to suboptimal gene suppression. For these reasons, investigation of cellular trafficking processes, specifically endosomal escape, are crucial for gene down-regulation efficacy.\(^\text{217}\) Real-time monitoring of such events would allow early detection of carrier drawbacks, optimization of delivery and formulation conditions, dynamic visualization, and easy assessment of endosomal entrapment/release. Therefore, there is a need to develop imaging techniques to follow and visualize the siRNA carrier during various cellular trafficking events. Herein, I describe real-time monitoring for siRNA delivery processes utilizing a multivalent folate-functionalized copolymer (FAPol13) as the carrier. Cellular trafficking events monitored via confocal fluorescence microscopy include (1) receptor binding, (2) polyplexes internalization, and (3) endosomal release. siRNA delivery to multiple cancer cell types, copolymer biocompatibility, and gene down-regulation are also demonstrated. In addition to the development of real-time imaging methods, the result also demonstrates the utility of this polymeric carrier in site specific gene delivery applications.

Result and Discussion

*Cellular Binding and Internalization of FAPol13/siRNA Polyplexes in KB Cells*

Previously our laboratories synthesized a series of well-defined block copolymers capable of delivering siRNA specifically to FR-expressing cancer cell lines.\(^\text{101b}\) These copolymers were prepared via a controlled radical polymerization technique known as reversible addition-fragmentation chain transfer (RAFT) polymerization.\(^\text{101a,b,101e,172,188a,218}\) The RAFT-synthesized block copolymer utilized in this report consists of three monomer components. The first block (HPMA\(_{315}\)-stat-APMA\(_{13}\); \(M_n = 47400\) g/mol;
PDI=1.10) was prepared through the aqueous RAFT copolymerization of \( N \)-(2-hydroxypropyl)methacrylamide (HPMA) and \( N \)-(3-aminopropyl)methacrylamide (APMA).\(^{101b}\) HPMA was chosen because its corresponding polymer is biocompatible and water soluble, while APMA was selected to introduce primary amine functionalities for conjugation with folate derivatives. HPMA\(_{315}\)-\( \text{stat} \)-APMA\(_{13}\) macro chain transfer agent was then successfully chain extended with the cationic tertiary amine containing monomer \( N \)-[3-(dimethylamino)propyl]methacrylamide (DMAPMA) to yield (HPMA\(_{315}\)-\( \text{stat} \)-APMA\(_{13}\))-\( b \)-DMAPMA\(_{23}\) (\( M_n = 51300 \) g/mol; PDI = 1.14),\(^{101b}\) thus facilitating the electrostatic complexation between the copolymer and siRNA. The APMA units were then directly conjugated to \( N \)-hydroxysuccinimide activated folate, and 11 to 13 out of 13 possible APMA units were modified.\(^{101b}\) Due to the presence of 13 APMA units, unmodified (HPMA\(_{315}\)-\( \text{stat} \)-APMA\(_{13}\))-\( b \)-DMAPMA\(_{23}\) was given the name Pol13 and multivalent folate conjugated (HPMA\(_{315}\)-\( \text{stat} \)-APMA\(_{13}\))-\( b \)-DMAPMA\(_{23}\) was named FAPol13. FAPol13 allows the formation of neutral (nitrogen to phosphate ratios, \( N/P = 1 \)) complexes, thereby circumventing problems associated with charged complexes, while the presence of multiple folates in the aqueous stabilizing block directs this siRNA carrier to cancer FR-expressing cell lines.

FAPol13 has a small molecular size (hydrodynamic diameter, \( D_h = 10.8 \pm 0.3 \) nm), and cationic change (Zeta potential = 25.4 ± 0.7 mV).\(^{101b}\) After complexation with siRNA (59 nt, \( D_h = 2.95 \pm 0.34 \) nm),\(^{188a}\) the size of FAPol13/siRNA complex is still nanoscale (\( D_h = 15.2 \pm 2.4 \) nm). Compared to macromolecular siRNA carriers (i.e., antibody, aptamer), this property is advantageous in terms of tumor penetration potential.\(^{219}\) Additionally, the neutral complexes (Zeta potential = -3.88 ± 0.21 mV)
remain sterically stable due to the presence of the hydrophilic block, which increases the delivery efficiency compared with anionic complex, and enhanced targetability compared with cationic complex.\textsuperscript{101b} Therefore, the neutral FAPol13/siRNA complex is an ideal model for study the delivery process (Scheme V-1), including cellular binding, entry (endocytosis), endosome escapes.

After successful preparation of FAPol13 and subsequent formation of neutral FAPol13/siRNA polyplexes, cell binding and intracellular uptakes of these carriers were confirmed by confocal laser scanning microscopy. Fluorescence and thus visualization of the uptake process was made possible by employing either a FAM-labeled or a Cy3-labeled siRNA. To elucidate the specificity of delivery, several control studies were first performed (Figure V-1). Time-lapse analyses were carried out to monitor siRNA binding to KB cells (Figure V-2), followed by z-axis scanning to confirm internalization of the siRNA/FAPol13 polyplexes (Figure V-3). Experiments were performed on polymer/siRNA polyplexes prior to (Pol13) and after FA (FAPol13) conjugation.
**Scheme V-1.** Schematic depiction of siRNA delivery processes via FAPol13.

FAPol13 functionalized with FA carries siRNA into the FR expressing cells and release it once inside. FAPol13 and siRNA form a complex electrostatically which will a) bind to the FR+ cells, b) enter the cells through FR-dependent endocytosis, c) escape from the endosome after pH decreasing, and d) dissociate siRNA into the cytoplasm, which it will be processed by Dicer, incorporated into the RISC, and induce the homologous mRNA degradation (RNAi pathway is not shown here).

Control experiments were first carried out to establish the ability of FAPol13 to deliver FAM-labeled siRNA (FAM-siRNA) to KB cells. KB cells were treated with naked FAM-siRNA, Pol113/FAM-siRNA polyplexes, FAPol13/FAM-siRNA polyplexes, or Lipofectamine/FAM-siRNA complexes. Minimal fluorescence upon treating KB cells with naked FAM-siRNA and Pol113/FAM-siRNA polyplexes was observed and attributed
to non-specific binding. In contrast, the fluorescence intensity from FAPol13/FAM-siRNA polyplexes was stronger, suggesting that the conjugated FA promoted specific cellular associations through the FR. As a positive control FAM-siRNA was delivered via Lipofectamine, a commercial transfection reagent that can effectively perform intracellular siRNA delivery independent of the cellular receptors present. In addition to standard fluorescence imaging, siRNA binding and uptake were verified by time-lapse and z-stack scanning analyses. For the remaining imaging experiments Cy3 labeled siRNA was chosen over FAM because of its resistance to photobleaching.

Real-time monitoring of siRNA-cell binding process is important as it allows for dynamic visualization of the cellular receptor binding/adsorption process, determination of the time required for cellular binding and cellular surface saturation by the therapeutic carrier, as well as a rapid a straightforward analysis to prescreen the designed carrier.
Figure V-1. Confocal images of specific binding of FAPol13/FAM-siRNA to KB cells. KB cells were treated with A) no treatment, B) FAM-siRNA, C) Pol13/siRNA, D) FAPol13/siRNA polyplexes, and E) Lipofectamine/siRNA complexes (total [siRNA] = 20 nM) and incubated for 40 min (A-D) or 1.5 h for E. Images were taken via confocal microscopy after washing the cells with pre-warmed PBS two times. The N/P ratio for each micrograph is 1.0. The panel indicated by FAM was FAM fluorescence images, while the DIC panel represented differential interference contrast or bright field images. Scale bar = 100 µm.
During the following real-time monitoring the temperature was maintained at 37 °C by a heated sample holder and micrographs were taken in 10 min intervals for 80 min. Time-lapse images for the incubation of FAPol13/siRNA polyplexes in the KB (+FR) and A549 (-FR) cell lines are shown in Figure V-2. It is apparent that the cell fluorescence from Figure V-2A (KB cells with FAPol13/siRNA polyplexes) was much stronger than Figure. V-2B (KB cells with Pol13/siRNA polyplexes), indicating siRNA-cell binding events due to ligand-receptor interactions between the FAPol13/siRNA polyplexes and the FRs expressed at the cell surface. Further evidence for specific ligand-receptor interactions is provided by comparison of Figure V-2A to Figure V-2C (A549 cells with FAPol13/siRNA polyplexes). The absence of fluorescence in Figure 2C is related to negligible FR-expression in the A549 cell line. The absence of FR expression precludes specific association of the FAPol13/siRNA polyplexes with from A549 cells. For the above three conditions, fluorescence intensities as a function of time are presented in Figure V-2D. Upon treatment of KB cells with FAPol13/siRNA polyplexes, an increase in cell-associated fluorescence was apparent after 20 min. Maximum fluorescence was reached near 40 min and kept for the remainder of the time-lapse. On the other hand, cell-associated fluorescence over the same time course was negligible for both treatments of KB cells with Pol13/siRNA polyplexes (Figure V-2B) and A549 cells with FAPol13/siRNA polyplexes (Figure V-2C).
Figure V-2. Time-lapse imaging of polyplexes in KB and A549 cells. Cells were treated with A) FAPol13/siRNA polyplexes in KB cells (FR-positive), B) Pol13/siRNA polyplexes in KB cells, and C) FAPol13/siRNA polyplexes in A549 cells (FR-negative). siRNA was labeled with Cy3 (red) ([siRNA] = 20 nM). Images were taken every 10 min for 80 min. The overlaps of fluorescence image and bright field image are shown here. Scale bar = 10 µm. D) Associated fluorescence versus time for FAPol13/siRNA polyplexes with KB cells (■), Pol13/siRNA polyplexes with KB cells (●), and FAPol13/siRNA polyplexes with A549 cells (▲). The associated fluorescence is calculated from 10 individual cells and background corrected. Error bars are represented by ± S.D (n=3).

Although Figure V-2 demonstrates binding of FAPol13/siRNA polyplexes to the cell surface of FR-expressing KB cells, subsequent intracellular uptake cannot be verified through time-lapse analysis. Therefore, siRNA internalization was studied separately by scanning cell sections vertically through the z-axis via confocal microscopy, which can verify if the fluorescence signal comes from the interior or exterior of the cell, or both.
Z-axis sectional images were taken by adjusting the focal plane from the top to the bottom of the cells in 3 µm increments (Figure V-3). As a negative control (Figure V-3A), incubation of KB cells with Cy3-labeled Pol13/siRNA complexes (-FA) resulted in weak but observable background fluorescence. In addition, the weak fluorescence was present only on the cell surface. No internalization was visible. On the contrary, internalization of the FAPol13/siRNA polyplexes in KB cells (Figure V-3B) was evidenced by the relative strong fluorescence that was not only associated with the cell surface but also present inside cells. The largest fluorescence difference between Figure 3A and Figure 3B existed not on the cell surface but within cells. While Figure 3A displays no visible fluorescence inside cells, relative strong fluorescence can be seen in the intracellular volume of Figure 3B. As a positive control (Figure V-3C), Lipofectamine was also used to show cellular internalization (no cell specificity) of Cy3-labeled siRNA. KB cells treated with Lipofectamine/siRNA complexes were incubated for 2 h following the manufacturer’s suggestion. As expected, incubation of cells with Lipofectamine-complexed Cy3-siRNA led to strong fluorescence over the whole cell.

Collectively, these results demonstrate both cell binding and intracellular uptake of FAPol13/siRNA polyplexes in KB cells, due to the presence of the conjugated FAs that promote siRNA cell binding and internalization. In addition to cellular uptake, endosomal release of internalized siRNA from carrier complex would be necessary for efficient RNAi/gene downregulation.217a,220 Therefore, further analysis by fluorescence microscopy was utilized to characterize and visualize endosomal release events during FAPol13-assisted siRNA delivery.
Figure V-3. Z-stack scanning analysis of internalized polyplexes in KB cells. Treatment of KB cells with A) FAPol13/siRNA polyplexes (1 h), B) Pol13/siRNA polyplexes (1 h), and DharmaFECT/siRNA (2 h). siRNA was labeled with Cy3 (red) with a concentration of 100 nM for all images. Hoechst 33258 (blue) was added to reach 1 µg/mL 20 min before observation. The focal plane was moved from the bottom to top of the cells in 2 µm increments. Scale bar = 20 µm.
**Endosomal Escape of FAPol13/siRNA Polyplexes**

Inefficient siRNA release from endosomal compartments is the major reason for subpar gene downregulation. Poor release leads to entrapment of the therapeutic carrier and cargo inside the endosome, potentially causing either their recycling back to the cell surface or their degradation by lysosomal enzymes. Therefore methods to directly monitor endosomal escape are critical for gene downregulation assessment and the rational design of gene delivery vehicles. In the current system, we performed co-localization analysis of FAPol13/Cy3-labeled siRNA polyplexes and lysotracker (a membrane permeable dye that only fluoresces in the endosomal or lysosomal compartment) during endocytotic trafficking (Figure V-4). Co-localization is determined by merging the Cy3 (red) and lysotracker (green) fluorescent images. If siRNA is contained within an endosomal compartment this image overlap will produce a yellow signal. Co-localization between two fluorescent probes, Cy3-siRNA and lysotracker, was quantified by the Pearson’s correlation coefficient (PCC) through a toolbox under ImageJ and JACoP (Figure 4E). PCC typically has a scale between -1 to 1, with -1 having no correlation whatsoever and 1 indicating a perfect overlap of fluorescent signals.

KB cells were first treated with FAPol13/Cy3-labeled siRNA polyplexes ([siRNA] = 100 nM) for 1 h after which the cell medium was replaced. Prior to fluorescent imaging, lysotracker (100 nM) was added to the cell medium. Cells were imaged at 1 h, 6 h, 24 h, and 48 h after FAPol13/siRNA polyplex treatment. Co-localization of lysotracker and siRNA in the endosome, after 1 h treatment, is evidenced by the yellow and localized signals seen in Figure V-4A, indicating that most of the
siRNA is confined to endosomal compartments. After 6 h and 24 h treatment (Figure V-4B and 4C), separation between the Cy3 and lysotracker signals become apparent and are marked by arrows in Figure 4. The loss of co-localization, or return to individual red (Cy3 siRNA) and green (lysotracker) signals, suggests endosomal escape of the FAPol13/siRNA polyplexes. After 48 h treatment (Figure V-4D), the fluorescent signal from siRNA has become more evenly distributed throughout the cytosol and more red signal (siRNA) has separated from the lysotracker signal. At the same time, less yellow signal is present, further indicating endosomal escape of FAPol13/siRNA polyplexes. For comparison, similar treatment of FR-negative A549 cells with lysotracker and FAPol13/siRNA polyplexes led to the observation of green signals only, without the presence of red signals within the cells, as expected from the results in Figure V-2.

In addition to visual observation of spatial distribution of Cy3-siRNA and lysotracker (Figure V-4), PCC is utilized to statistically and quantitatively measure the endosomal escape process. A PCC equal to 1 implies that the FAPol13/siRNA polyplexes and the endosome are completely co-localized. In our case, the PCC from 1 h treatment is 0.99 indicating that the FAPol13/siRNA polyplexes were endocytosed and remained in the endosomal compartments. As treatment time increased, the PCC for the 6 h time point decreased to 0.77. This decrease suggests that a portion of the FAPol13/siRNA polyplexes have escaped from the endosome as visualized in Figure V-4B. The PCCs of the 24 h and 48 h images are 0.64 and 0.49, respectively, suggesting that the FAPol13/siRNA polyplexes continually escape the endosome over time. Notably, the decrease from 0.99 (1 h) to 0.77 (6 h) is the largest among two consecutive time points, suggesting that the highest escaping efficiency occurs between 1 h and 6 h.
Figure V-4. Colocalization analysis of polyplexes and endolysosome in KB cells. The cells were incubated with FAPol13/Cy3-labeled siRNA (red) polyplexes for A) 1 h, B) 6 h, C) 24 h, and D) 48 h. The endolysosome was stained with lysotracker green for 30 min prior to imaging. Yellow fluorescence seen in the overlay images indicates colocalization of siRNA and lysotracker in the endosome. Concentrations of siRNA and lysotracker were held at 100 nM. Cells were washed with pre-warmed phosphate buffer solution three times prior to observation. Scale bar = 10 µm. Arrows mark separating or separated red and green signals. E) The Pearson’s correlation coefficient versus time. The calculation is based on 3 independent images, within each containing 7-12 cells in each time point. Error bars are represented by ± S.D.
These co-localization studies establish that FAPol13/siRNA polyplexes are internalized and can subsequently escape the endosome over time. Endosomal escape has been confirmed both visually and quantitatively by fluorescence microscopy and determination of PCC, respectively. Release of siRNA from the endosome was not unexpected since gene down-regulation was observed in our previous report\textsuperscript{101b}. Although the imaging methods were developed utilizing FAPol13/siRNA polyplexes in KB cells, the methods may be applied to other fluorescently labeled siRNA carrier systems and cell lines for direct observation of endosomal escape. This direct monitoring can provide insight into the efficacy of endosomal escape, thereby offering some guidance on development of efficient siRNA carrier systems.

Another factor facing efficient siRNA delivery is the unpackaging of siRNA from the polymeric carrier. In theory, siRNA release from the carrier can be visualized through the development of a FRET reporter system. For example, the conjugation of quencher dye to the copolymer backbone would absorb the fluorescence emission from the Cy3-labeled siRNA. Upon release, the fluorescence becomes visible allowing the researcher to image and determine the cellular location of release. Such a system is currently being developed within our laboratories.

*Internalization of FAPol13/siRNA Polyplexes into HeLa and SKOV3 Cells*

The above studies established both cellular uptake, via FR-mediated endocytosis, and endosomal release of FAPol13/siRNA polyplexes with KB cells (FR+), along with A549 cells (FR-) as the negative control. Additional FR-positive cell lines, HeLa and SKOV3 cells, were utilized to demonstrate the capacity of this polymeric vehicle to
deliver siRNA to other FR-positive cancer cell lines. siRNA delivery to HeLa and SKOV3 cells was studied via confocal microscopy.

Cellular internalization of FAPol13/siRNA polyplexes in both HeLa and SKOV3 cells can be compared by the confocal microscopy images as shown in Figure V-5. For contrast, the nuclei were stained with Hoechst (blue fluorescence). In addition, z-stack analysis clearly indicates internalization of FAPol13/siRNA polyplexes into HeLa and SKOV3 cells. Furthermore, the uniformity of red fluorescence throughout the cytoplasm further suggests effective cellular internalization FAPol13/siRNA polyplexes. Finally, it is noted that the fluorescence signal decreases from KB cells to HeLa cells to SKOV3 cells, which is consistent with the cellular uptake capacity of other folate conjugates in these cell lines.\textsuperscript{224} Quantitation of the relative FR expression at both mRNA and protein levels in KB, HeLa, and SKOV3 cells, indicates FR expression level of decreasing order: KB > HeLa > SKOV3 (refer to Chapter VII).

**Figure V-5.** Confocal analysis of cellular absorption of polyplexes with cancer cells. A) KB, B) HeLa, C) SKOV3, and D) A549 cells were incubated with FAPol13/Cy3-siRNA (N/P = 1, siRNA concentration = 100 nM) for 40 min before imaging. The nuclei were stained by Hoechst 33258 (blue fluorescence) 20 min prior to imaging. Scale bar = 20 µm.

Next, time-lapse, z-axis scanning and colocalization analysis were conducted in HeLa and SKOV3 cells as shown in Figure V-6. Firstly, in Figure V-6A and 6D, the
polypexes bind to the HeLa and SKOV3 cells along with the time, indicating the interaction between the FR and FA moiety. Certain cells exhibit rapid absorbance of polypexes; while some show slower uptake, probably due to different FR expression level and heterogeneity of the cell lines. Moreover, under the condition studied, the fluorescence intensity has not reached the maximum after 60 min incubation (the data not shown here). Secondly, the z-axis scanning (Figure V-6B and 6E) clearly indicates the cytoplasmic distribution of polypexes after 6 h incubation, which is in agreement with the results in KB cells. More importantly, colocalization of polypexes (red) and lysotracker green (Figure V-6C and V-6F) suggests the polypexes escape from endolysosome after 24 h incubation. The endosomal release efficiencies indicated by PCC in HeLa and SKOV3 are 42% and 34% respectively, close to the 36% in KB cells.

Taken together, these results suggest that FAPol13/siRNA polypexes could bind to the FR-positive cells depending on the presence of cell surface FR; and polypexes are internalized by endocytic pathway and released from endolysosome into cytoplasm after 24 h incubation with approximate 40% efficiencies among these three FR+ cell lines. Furthermore, the data reveal the potential application of this useful approach to evaluate the trafficking process of fluorophore-labeled complexes in different cell lines or different drug delivery systems.
Figure V-6. Confocal studies of polyplexes trafficking in HeLa and SKOV3 cells. Time-lapse imaging (A and D), z-axis scanning (B and E), and colocalization analysis (C and F) were applied here. The images of time-lapse were taken every 20 min and presented in an overlap of fluorescence (Cy3 and Hoechst) and bright field. For z-stack analysis, cells were incubated with polyplexes for 6 h before imaging and the overlaps of Cy3 and Hoechst fluorescence are presented here. The 24 h incubation for colocalization imaging was chosen and presented here. Scale bar = 10 µm.
Copolymer Cytotoxicity and Gene Downregulation of Luciferase

Prior to gene down-regulation experiments the cytotoxicity of FAPol13 was determined utilizing a cell viability assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Conversion of MTS to formazan by dehydrogenase enzymes can only be accomplished if cells are metabolically active. Therefore, the absorbance of formazan at 490 nm is a direct measurement of cell viability. As shown in Figure V-7, FAPol13 has low to negligible toxicity over the complete range of concentrations tested, suggesting that the copolymer is biocompatible and suitable for gene delivery applications. These results are consistent with microscope observation (no cell death). This low toxicity and biocompatibility are advantageous when compared to conventional liposome-based vehicles, which have been shown to be cytotoxic. This, in principle, allows for multiple treatments of cells with FAPol13/siRNA polyplexes due to the non-toxic nature of the copolymer.

**Figure V-7.** Cell viability of KB cells at increasing concentrations of FAPol13. KB cells were first seeded into a 96 well plate and incubated for 24 h prior to introduction of FAPol13. Five different copolymer concentrations ranging from 10 nM to 6 µM were tested and allowed to incubate for 48 h. Untreated KB cells were utilized as a control. After incubation, a solution of MTS was added and the absorbance at 490 nm was measured after two hours. All measurements were directly compared to untreated KB cells. All experiments were performed in triplicate, and error bars represent ± S.D (n=3).
To assess gene down-regulation of these polyplexes, Gluc-KB cells, which stably express *Gaussia* luciferase protein, were treated with neutral FAPol13/siRNA polyplexes ([siRNA] = 100 nM; N/P = 1) for 6 h, followed by 48 h incubation. siRNA targeted against *Gaussia* luciferase was utilized for these studies due to its convenience and sensitivity of the measurement. The facile manner in which luciferase protein level can be determined (i.e., by luminescence) makes this protein an ideal target for testing/optimizing gene downregulation. Following 48 h of incubation, the luciferase protein expression level was quantified and presented in Figure V-8.

![Figure V-8](image)

**Figure V-8.** Relative *Gaussia* luciferase protein expression level in Gluc-KB cells. The control (marked Cell) contained free siRNA and Gluc-expressing KB cells. The remaining three experiments (FAPol13, Pol13, and DharmaFECT) were Gluc-expressing KB cells treated with FAPol13/siRNA polyplexes, Pol13/siRNA polyplexes and DharmaFECT/siRNA complexes. siRNA concentration was kept at 100 nM. Polyplexes were prepared at an N/P = 1.0. Protein expression levels were measured 48 h after treatment. All experiments were repeated in triplicate and the error bars represent ± S.D. (n=3).

The luciferase protein level was normalized to untreated Gluc-KB cells. Upon the treatment of FAPol13/siRNA polyplexes, the luciferase expression reduced to 32%, while minimal gene down-regulation for cells treated with polyplexes prior to FA conjugation...
was observed. This result matches the above studies in that the conjugation of FA to the polymeric carrier is required for cellular uptake (Figure V-1 to V-3). Treatment of Gluc-KB cells with DharmaFECT (commercial transfection reagent, positive control) lead to 83% down-regulation of luciferase. The similar gene down-regulation efficiency between DharmaFECT and FAPol13 and the enhanced biocompatibility of the latter demonstrates the potential utility of FAPol13 for specifically delivering siRNA to FR-bearing cancer cells. These results are consistent with gene down-regulation studies of human survivin gene by real-time PCR (Figure V-9).  

![Figure V-9](image)

**Figure V-9.** Real-time PCR analysis of downregulation of human Survivin gene upon treatment with FAPol13 complexed anti-survivin siRNA (siSv). The control contained either KB cells incubated with siSv or A549 cells incubated with siSv. The other three experiments (marked KB, KB/Free FA, and A549) were treated with FAPol13/siSv complexes in the presence (KB/Free FA) or absence (KB, A549) of 1 mM free folic acid (FA). All experiments were done in triplicate and error bars represent ± SD.

**Kinetic Analysis of the Absorption of FAPol13/siRNA Polyplexes in KB Cells**

This low toxicity and biocompatibility of FAPol13 make multiple treatments of cells with polyplexes with minimal side-effects possible. To follow this notion, we conduct the kinetic analysis to determine the effective incubation time (Figure V-10).
KB cells were treated by DharmaFECT (positive control) and FAPol13 complexed, Cy3-labeled siRNA in vitro, and the mean fluorescence intensity (MFI) was assessed by flow cytometry as a function of time. In Figure V-10, the kinetics of KB accumulation is quantified. KB cells appear to saturate by DharmaFECT/Cy3-siRNA in ~10 h (half time, $t_{1/2} = \sim 2.8$ h), by FAPol13/Cy3-siRNA in ~13h ($t_{1/2} = \sim 5.3$ h). The result is consistent with Figure V-1 and V-3, which indicate the higher absorption efficiency of commercial transfection agents.

**Figure V-10.** Kinetic studies of Cy3-siRNA uptake in KB cells by flow cytometry. Cy3-siRNA (final concentration = 100 nM) were delivered by DharmaFECT (solid circles) and FAPol13 (solid squares). The uptake of siRNA by KB cells was monitored as a function of time (1h, 2h, 4h, 8h, and 24h) by flow cytometry. The data represents mean ± SEM (n=3).

*Induction of Apoptosis by Transfecting KB Cells with siRNA against CASP8AP2 (siCAS)*

As discussed in Chapter I, Caspase 8 associated protein 2 (CASP8AP2) is involved in apoptosis. Downregulation of this gene expression through RNAi technique would cause cell death. Therefore we choose this gene as siRNA target to enhance the cellular effects upon siRNA treatment. Firstly, DharmaFECT was used to deliver
transcription-synthesized siRNA against CASP8AP2 (siCAS) into KB cells to test the feasibility of this target (Figure V-11), and subsequently, FAPol13 was used to carry siCAS into the cells specifically (Figure V-12).

Morphological changes, such as, cell shrinkage and DNA fragmentation (pointed by arrows), occur in KB upon siCAS treatment for 24 h (Figure V-11A). This indicates apoptotic cells and demonstrates the important role of CASP8AP2 for cell survival. Furthermore, cell viability (Figure V-11B) and dead cells (floating in the cell medium) (Figure V-11C) were measured via MTS assay and flow cytometry. From Figure V-11B, less than 40% KB cells after 24h-treatments are alive. By contrast, KB cells incubated with and without DharmaFECT are unaffected. Cell death induced by siCAS treatment was further validated by counting the floated cells (death cells) collected from the cell medium. Several thousands of cells died after treatment, compared to a few hundreds of dead cells in normal situation (without any treatment) and DharmaFECT treatment.

Overall, the data demonstrate that CASP8AP2 is a favorable target for inducing apoptosis through siRNA-triggered downregulation.
After confirming the crucial role of CASP8AP2 in maintaining cell survival, FAPol13 was applied to delivery siCAS into KB cells (Figure V-12) and apoptosis was assessed by Annexin V assay. Annexin V has a strong and specific affinity for phosphatidylserine which is normally located at the inner (cytoplasmic) leaflet of the plasma membrane. During the apoptotic process, phosphatidylserine will be translocated from inner to outer leaflet and detected by FITC-labeled annexin V conjugate. Therefore
Annexin V assay is an easy and convenient method to evaluate and validate apoptosis by fluorescence microscopy or flow cytometry. Besides, co-staining apoptotic cells with propidium iodide (PI), a nucleic acid dye, can distinguish the cells that undergo apoptosis at the early stage and last stage.

In Figure V-13A, x-axis is the fluorescence intensity from Annexin V-FITC staining, whereas y-axis is the fluorescence intensity from PI staining. Q1-LL stands for the live cells, Q1-LR and Q1-UR means early apoptotic cells and late apoptotic or necrotic cells respectively. The data shows 93.4%, 44.3%, and 78.5% of cells are alive upon non treatments, DharmaFECT/siCAS, and FAPol13/siCAS 2.5-day after treatment. Statistical analysis of this assay (Figure V-12B) exhibits 92.4%, 45.5%, and 76.5% accordingly. Intriguingly, approximate 24.5% of KB cells have entered apoptotic pathways upon FAPol13/siCAS treatment. By contrast, in normal situations, only 8% of cells are under apoptosis.
siRNA-based therapy holds great potential to treat many types of diseases including cancer. Efficient and target cell-specific siRNA internalization to the cytoplasm is the key for future therapeutic applications of siRNA.
In this chapter we have demonstrated: (1) the efficient siRNA delivery via FAPol13 into FR+ cells (i.e., KB, HeLa, and SKOV3); (2) the dose-dependent and FR-dependent siRNA Delivery; (3) the significant gene down-regulation upon FAPol13 delivered siRNA against human survivin (~60%) and Gaussia luciferase (~70%); (4) the non-toxicity of the FAPol13; (5) induction of KB cells into apoptosis upon FAPol13 delivered siCAS; (6) a CLSM-based effective system to assess siRNA intracellular trafficking processes (cellular binding, endocytosis, and endosomal escape).

Taken together, the non-immunogenic and biocompatible folate-conjugated copolymer FAPol13 may be used to construct efficient siRNA delivery systems for cancer therapeutic applications.
CHAPTER VI
CORRELATION OF FOLATE RECEPTOR EXPRESSION LEVEL
AND FAPOL13/SIRNA DELIVERY EFFICIENCY

Introduction

Folate receptor (FR), a glycosylphosphotidylinositol (GPI)-anchored glycoprotein, specifically binds to its ligand, folate, with high affinity. FR in human has at least three homologous isoforms, hFR-α, hFR-β and hFR-γ with 70-80% amino acid similarity in sequences and distinct developmental expression patterns.

The reduced-folate carrier (RFC) is another folate transporter. RFC is a membrane-spanning protein and transports reduced folate into cells via carrier-mediated mechanism, distinct from receptor-mediated endocytosis of FR. Most of classical antifolate drugs are rapidly transferred across the plasma membrane by this mechanism, leading to inhibition of their target in both tumors and normal proliferating tissues.

FR overexpresses in some epithelial tumors, such as ovary, cervical, mammary gland, breast colon, prostate, lung, kidney, nose, throat, and brain etc; whereas it has a restricted distribution in normal tissues. In addition, FR can bind with FA conjugates with similar binding affinity and assist their transport across the plasma membrane via endocytosis. These unique properties have been utilized to facilitate the delivery of a broad range of therapeutic agents into various cancers.

Thus, FR-mediated system can specifically, efficiently deliver folate-drugs into FR-positive cancer cells to correct or eliminate aberrant gene expression. The expression of FR on cell surface is the prerequisite to the success of therapeutics. Recently, evidence
shows that a subtype of macrophage cell has elevated FR expression when encountered stimuli, and importantly, its expression could vary upon the environment or situation changes accordingly.\textsuperscript{101c} Other finding displays that the expression of FR is also affected by the surrounding FA concentration.\textsuperscript{134} For example, the mRNA level of FR in KB and JEG-3 cells doubles when folate concentration reduces.\textsuperscript{134} In addition, FR expression increases along with tumor progressing,\textsuperscript{230} and FR expression patterns in tumor cell lines could not reflect the virtual FR expression \textit{in vivo}.\textsuperscript{134} These findings suggest that the FR expression level or patterns is critical for evaluating the utility potential of FR-mediated delivery and predicting the outcomes of FR-targeted therapeutics.

A large body of evidence has shown high expression level of FR in KB cells and negligible expression level in A549 cells,\textsuperscript{231} which explains researchers use this pair of cell lines as a model to illustrate the delivery of FA-conjugates. HeLa and SKOV3 are both FR positive cell lines. In Chapter V, we have presented data to support the FR-mediated delivery of FRPol13/siRNA into KB, HeLa, and SKOV3 cells. The findings show the relatively low expression level of FR in HeLa and SKOV3 cells compared to that of KB cells. However, the relationship of FR expression with binding efficiency, uptake efficiency, and the overall RNAi efficacy remains elusive.

To address those issues, comparative studies have been employed among KB, HeLa, SKOV3, and A549 cells. Firstly, FR mRNA and protein levels were quantified through real-time PCR and flow cytometry. Secondly, the binding (2 h incubation) and internalization (24 h incubation) of FAPol13/fluorophore labeled siRNAs were measured via microscopy and flow cytometry. Thirdly, free FA competition assay were carried out to “mimic” less FR availability would abolish the internalization of polyplexes. Lastly,
luciferase assay was utilized to demonstrate that the RNAi effects from the internalized siRNAs were also correlated with the FR expression in both transient and stable transfection systems.

**Result and Discussion**

*Quantification of FR Expression in Human Cancer Cell Lines*

Firstly, real-time PCR was utilized to measure the human FR alpha gene (hFRα) expression in KB, HeLa, SKOV3, and A549 cells (Figure VI-1A). As reported in Figure IV-1, the A549 cells rarely express hFRα. Thus, the Ct value (the number of cycles required for the fluorescence signals across the threshold) of hFRα in A549 about 15 cycles bigger than that of KB cells, indicating the nearly undetectable hFRα in this cell line. Compared to the expression level of a human housekeeping gene, hFRα mRNA level in A549 cells is $\sim 1.5 \times 10^{-4}$ % of β-Actin mRNA level; whereas in KB cells hFRα is 41% (Figure VI-1A).

Because FR protein is the direct executor for FA transportation, after determining the mRNA expression level of hFRα, its protein level in 4 cell lines was further assessed by flow cytometer (Figure VI-1B). To perform this experiment, a monoclonal antibody against the human FR was utilized to recognize proteins on cell membrane, then the secondary antibody, goat anti-mouse FITC conjugate antibody, was sequentially bind to the primary antibody (mouse origin). The fluorescence from the FITC was detected by flow cytomter. Through this procedure, the total FR protein level was obtained. To facilitate the comparison, the ratio of FITC-fluorescence (Signal, S) over the auto-fluorescence (Noise, N, background) was plotted. The ratio should be correlative with the
FR protein level. The S/N ratios in KB, HeLa, SKOV3 and A549 are 329.1, 166.0, 6.9, and 1.7 respectively.

![Graph](image)

**Figure VI-1.** Comparison of human folate receptor expression in cancer cells. (A) Real-time PCR analysis. (B) Flow cytometry quantification. The data represents mean ± S.D. (n=4).

Comparison of the expression of hFRα in mRNA and protein levels shows similarity: KB>HeLa>SKOV3>>A549. Interestingly, in protein level, there is 4-fold difference between SKOV3 and A549 cells. Whereas, in mRNA level, this is 9 Ct value difference. Assuming PCR amplification efficiency is 100%, nine cycles equal to 200-fold. This discrepancy would be explained by post-transcriptional modification, or post-translational modification mechanisms. Additionally, a group reported that hFRα mRNA from KB cells is 4 fold of that in SKOV3 cells quantitated by PCR. In our experiment, there is 4.8 Ct difference (~32 fold). Regarding the heterogeneity of cells, the maintained cell lines, the sensitivity of the regular PCR and real-time PCR, different expression level from two groups is understandable.
Quantification of the Absorption of FAPlo13/Cy3-(FAM-) siRNA in Cell Lines

Once we determined the different FR expression level among cell lines, considering the FR-dependent delivery, we propose that different binding, uptake efficiency of FAPol13/siRNA in cell lines would be variable accordingly. In addition, my previous imaging studies had shown unequal associated fluorescence intensities from these 4 cell lines. To confirm this hypothesis, the absorption (Figure VI-2) and internalization (Figure VI-3) of FAPol13/siRNA polyplexes were evaluated in 4 cell lines via fluorescence microscopy and flow cytometry.

In Figure VI-2, the data show that FAPol13/Cy3-siRNA (Figure VI-2 A-D) and FAPol13/FAM-siRNA (Figure VI-2 E-H) bind with the FR+ positive cells after 2h incubation. The associated fluorescence exhibited different intensities. KB cells have the highest fluorescence intensity (FI), then the HeLa, and SKOV3. There is barely fluorescence seen in A549 cell some from the nonspecific binding, which is common and has been discussed in previous chapters. The associated fluorescence is then quantitated by ImageJ software and plotted into the Figure VI-2 I (Cy3) and Figure VI-2 J (FAM). All the FI has been subtracted to the background FI and normalized to the FI of A549 (FI of A549 = 1). In Figure VI-2I, the relative FIs on KB, HeLa, and SKOV3 cells are 4.2, 3.4, and 1.8 respectively. Likewise, the relative FIs from FAM dyes on KB, HeLa, and SKOV3 cells are 3.2, 2.8, and 2.6, which displays the same trends of absorbed complexes: KB > HeLa > SKOV3 > A549.
Figure VI-2. Fluorescence intensity analysis of cellular bound FAPol13/siRNA. The siRNA is either Cy3 labeled (A-D) or FAM labeled (E-H). KB (A, E), HeLa (B, F), SKOV3 (C, G) and A549 (D, H) cells were seeded onto glass-bottomed wells 24h before transfection. FAPol13/siRNA complexes (final siRNA = 100 nM) incubated the cells for 2 hours. Cells were washed and imaged. (I, J) The associated fluorescence intensities (FI) were quantified by ImageJ software, and plotted. The data represents mean ± SEM (n=4). Scale bar = 10 µm.

Quantification of the Uptake of Polyplexes in Cell Lines

The absorption of the polyplexes by the cells will be followed by internalization if the binding is specific. In Figure VI-2 we have demonstrated the different degrees of binding of polyplexes in 4 cells lines. The distinct uptake/internalization of polyplexes
follows the rationality and is examined here (Figure VI-3). Flow cytometer was applied to quantitate the intercellular FAPol13/FAM-siRNA after 24 h incubation.

In Figure VI-3A, the black lines stand for the cell background (no treatment), whereas the red lines are the samples. If the red line is on the right of black line, that indicates the stronger fluorescence intensity. The internalization of polyplexes accounts for this augment. In addition, the degree of right-shift in KB cells is slightly bigger than that in HeLa cells and in SKOV3 cells. In A 549 cell, two lines are nearly overlapped, suggesting non-internalization of polyplexes occurs in this FR negative cells.

The medium fluorescence intensity (MFI) from three repeats is measured and plotted into Figure VI-3B, displaying 2666.9, 2408.0, 1782.2, 488.4 units in KB, HeLa, SKOV3, and A549 cells respectively. If those numbers are normalized to A549, then the ratios will be 5.5, 4.9, 3.7, and 1. This number can be depicted as, for example, 5.5 fold polyplexes have been internalized into KB cells compared to A549 cells.

The trend of the internalized fluorescence and the trend of the bound fluorescence are the same: KB> HeLa> SKOV3>A549. The difference, however, is not exactly the same. That would result from FR expression variation from different cell passage number, incubation time-associated the FR changes. Nevertheless, the trend from the associated siRNA and internalized siRNA are consistent with FR expression level among those four cell lines, indicating the correlation between the FR levels with the siRNA delivery.
Figure VI-3. Quantification of the internalized polyplexes in human cancer cells. KB, HeLa, SKOV3, and A549 were plated into plates one day before transfection with ~40% confluency. FAPol13/FAM-siRNA polyplexes (final siRNA concentration = 100 nM) treated the cells for 24h before detection. A) The representative histogram plots of 4 cell lines were exhibited. The black lines are the background signal (cellular auto-fluorescence); whereas the red lines are the signals from the samples. B) The mean fluorescence intensity (MFI) from repeated experiments was plotted. The data represents mean ± SEM (n=3).

Inhibition of the Cellular Internalization of Polyplexes by Free Folic Acid

In the previous results, we have demonstrated that the entry of FAPol13/siRNA to KB is FR-dependent because: (1) the polyplexes cannot enter the FR deficient A549 cells; (2) the Pol13 (the copolymer carrier without FA conjugation) cannot deliver the siRNA; (3) the presence of FA (1 mM) blocks gene downregulation. However, the relationship of FA concentration and inhibitive effects are not clear, especially in different cells with various FR expression level.

To examine the correlation, free FA was added into the medium 1h before transfection of FAPol13/FAM-siRNA polyplexes (siRNA concentration = 100 nM) to
make the final concentration of 1 mM, 0.2 mM, and 0.04 mM along with the control (no treatment). After 24 h incubation, the cells were trypsinized and the internalized complexes were evaluated by flow cytometer (Figure IV-4). As expected, the presence of free FA greatly reduced the uptake of the polyplexes in the FR+ cells. In contrast, it showed non-detectable effects in A549 cells. It is noted that a higher concentration of free FA led to stronger inhibitive effects on the internalization.

![Graph](image-url)

**Figure VI-4.** Free folate inhibition assay. KB, HeLa, SKOV3, and A549 were seeded in plates 24 h before transfection with ~40% confluency. One hour prior to the transfection, free FA were added into the medium to make the final concentration 1 mM, 0.2 mM, and 0.04 mM. FAPol13/FAM-siRNA polyplexes treated and incubated the cells for 24h before flow cytometry detection.

A calculation was performed based on the assumption that 1 mM of FA could invariably block >99% of binding/uptake of FA conjugate. The results show that 95.7% and 90.1% internalization of polyplexes in KB cells, 91.5% and 81.9% internalization of polyplexes in HeLa cells; 85.6% and 54.7% internalization of polyplexes in SKOV3 are blocked by incubation of 0.2 mM and 0.04 mM FA respectively. As for the expression levels (Figure VI-1), a correlation is indicated that a higher expression of FR (e.g., in KB)
is correlated with more activated FR-mediated uptake, and therefore stronger inhibition effects from the competitor—FA. In other words, cancer cells with higher FR expression are susceptible to the competition from free FA.

*Studies of Gene Downregulation Efficiency in Cell Lines*

Previous data indicate that the binding/update of polyplexes (Figure VI-2 and VI-3) is correlative with the FR expression level (Figure VI-1), and the inhibition effect from free FA is also relative to the FR expression level (Figure VI-4). RNAi effects from the internalized siRNA should also follow this rule. To test it, both transient (Figure VI-5) and stable (Figure VI-6) transfection have been implemented. The Gaussia luciferase reporter system is utilized here because of its accurate, rapid, and sensitive measurement.

The principle and utility of transient transfection system have been discussed and detailed in Chapter VI. In this case, four cell lines were transfected with the plasmid pCMV-Gluc that encodes the *Gaussia* luciferase by conventional transfection agent — DharmaFECT. The cells were rinsed 6 h later and waiting for second transfection. The polyplexes (FAPol13/siGLuc) were added into the medium and incubated with cells for a period of time (1 to 4 days) before the luciferase detection.
Figure VI-5. Transient transfection of pCMV-Gluc and siGLuc in human cancer cells. KB, HeLa, SKOV3, and A549 were seeded on the plates one day before the transfection with ~40% confluency. The cells were first transfected with DharmaFECT/pCMV-Gluc (1 µg) for 6 h. The medium was replaced and the cells were rinsed and incubated for 2 h. FAPol13/siGLuc polyplexes (siRNA concentration = 100 nM) were prepared and added into the medium. The Gluc activity was assessed at 1, 2, 3, and 4 day. The Relative Gluc activity was normalized to non-siGLuc treated (plasmid transfected) control. The data represents mean ± SEM (n=3).

As shown in Figure VI-5, the relative Gluc activity in A549 is barely affected upon the siGLuc treatment in 4 days because FA-linked polyplexes cannot enter into FR negative cells. In contrast, in all three FR positive cell lines, the relative Glu activities have been reduced. In addition, along with time processes, RNAi effects increase. For instance, in KB cells, 76%, 64%, 54%, 49% of Gluc activity remains after 1, 2, 3, and 4 day treatment. Importantly, at each time point, RNAi effect is correlated with the FR expression level. For example, after 3-day incubation, Gluc activity has been reduced to 59%, 65%, 74%, and 91% in KB, HeLa, SKOV3 and A549 cells respectively. This correlation between the RNAi effects and FR expression level suggests: (1) the FR-
mediated siRNA uptake; (2) the FR expression level determines the therapeutic efficacy of the delivered drugs.

To confirm the correlation between RNAi effects and FR expression, a new stable cell line, SKOV3 cells that constitutively expresses Gluc, is established and denoted as Gluc-SKOV3. Therefore, experiments were conducted in Gluc-KB and Gluc-SKOV3, which have different expression level of FR. If the FR expression level is the key factor for determining the downstream RNAi effects, the degrees of gene downregulation from transfection of polyplexes should be distinct, and KB-Gluc should have better RNAi efficacy.

To test this hypothesis, FAPol13/siGLuc (siRNA concentration = 100 nM) polyplexes were transfected into Gluc-SKOV3 and Gluc-KB and the relative Gluc activity was determined at 1 day and 2 days. As shown in Figure VI-6, after 2d incubation of polyplexes, 35% and 65% Gluc activities remained in Gluc-KB and Gluc-SKOV3 respectively. Approximately, 60% and 80% Gluc activity remains in Gluc-KB and Gluc-SKOV3 cells at 1d incubation. The findings shows better RNAi efficacy in Gluc-KB cells compared to Gluc-SKOV3, suggesting a good correlation of FR expression level with RNAi effects from FR-mediated siRNA delivery.
Figure VI-6. Comparative studies of gene downregulation upon FAPol13/siGLuc treatments in Gluc-KB and Gluc-SKOV3 cells. Cells were plated in plates one day before transfection with ~40% confluency. Cells were transfected with polyplexes (siRNA concentration = 100 nM) and incubated for two days. The Gluc activity was detected after one and two day incubation by Gluc assay. The relative Gluc activity was calculated by normalization of non-transfected controls. The data represents mean ± SEM (n=3).

Conclusions

Increasing lines of evidence show that FR expression on cell surfaces would vary upon condition changes. High expression level of FR might be a key factor for successful delivery of therapeutic drugs in tumors through FR-mediated strategies. To demonstrate this concept, we take advantage of four human cancer cell lines: KB, HeLa, SKOV3 and A549, which have different FR expression level, to study binding, internalization of FAPol13-assisted siRNA delivery. Utilizing fluorescence microscopy and flow cytometry, we have demonstrated a correlation between FR expression and efficiency of binding and internalization of polyplexes in different cells. Subsequently, transient and stable transfection systems combined with luciferase reporter were employed to study the
gene downregulation of Gluc activity upon transfection with siRNA. The findings confirmed the previous conclusion and strongly indicate that high FR expression level is crucial for significant RNAi effects in FR-dependent siRNA delivery systems. The competition studies suggest that low FR expression level or less available FR on the cell surface will compromise the delivery efficiency.

Taking FAPol13 as a model, our results reveal a correlation between FR expression and absorption/internalization of FA-functionalized therapeutic agents; highlighting the importance of clarification of FR expression level when performing FR-dependence drug delivery strategies. The finding suggests that we can increase delivery efficiency and thereby enhance therapeutic effects through inducing FR expression (e.g., stimulation, folic acid starvation).
CHAPTER VII
DELIVERY OF AU NANOPARTICLE-SIRNA IN CANCER CELLS

Introduction

The nonviral siRNA delivery system,\textsuperscript{92b, 233} such as liposome, dendrimers, polycations (i.e., PEI), copolymers, and Au nanoparticles (AuNPs), harbors the several advantages over the viral system, such as bio-safety, non-immunogenicity.\textsuperscript{97a, 234}

Typically, the nonviral siRNA carriers are cationic for the purpose of complexing with anionic nucleic acid and increasing the transfection efficiency. Although the cationic carrier/siRNA complexes ($N/P > 1$) have the higher delivery efficiency by the nature of plasma membrane, their interactions with membrane and serum proteins that are required for cell maintenance would result in non-specific binding or internalization, which compromise the targetability of delivery. While the neutral complex ($N/P = 1$) hold the equilibrium between the transfection efficiency and specificity: having higher delivery efficiency than anionic complex, and higher specificity than cationic complex.

Over the past decades, AuNPs have been widely employed in chemistry, biology, engineering, and medicine\textsuperscript{235} by virtue of controllable size, shape, facile surface modification, and unique optical properties.\textsuperscript{235b} Therefore, they have been applied to imaging, optical and electrochemical sensing, diagnostics, and diseases therapy (e.g., cancer, Alzheimer, AIDS, hepatitis, arthritis, diabetes).\textsuperscript{236} Recently, a few lines of evidence display its great potential in delivering siRNA for cancer treatment. For instance, Mirkin group synthesized and characterized a polyvalent AuNPs that are modified with thiolated RNA duplexes and capable of inducing the RNAi pathway.\textsuperscript{237} These highly anionic particles can enter into cells through absorbing of serum proteins
(opsonization), rather the transfection reagent, which confers the cellular uptake and sequential gene downregulation in HeLa cells. Nagasaki group generated a type of AuNP with poly(ethylene glycol)-block-poly(2-(N,N-dimethylamino)ethyl methacrylate) copolymer (PEG-PAMA), which was further functionalized by immobilization of siRNA bearing a thiol group. The RNAi activity from the uptake of this AuNP-siRNA was demonstrated in HuH-7 cells.

One main advantage of AuNPs as a scaffold for siRNA delivery is its high loading capacity. For example, in case of FAPol13 (refer to Chapter V), there are 2-3 copolymer molecules per one 59-nt siRNA. However, an AuNP with ~13 nm gold core can easily load up to dozens of siRNA cargoes. Through this, we can achieve much higher intracellular siRNA concentration if the equal amounts of siRNA complexes have been internalized.

Although promising, AuNPs have some drawbacks in synthesizing their stabilizing ligands, which have the poorly-defined molecular weight (MW) control of polymerization. Nevertheless, reversible addition-fragmentation chain transfer polymerization, a controlled polymerization technique, has the abilities to control MW, synthesize complex architectures (i.e., blocks, stars, grafts), and maintain a narrow polydispersity. A polymer stabilized AuNP had been rationally designed and synthesized by this technique in our collaborative laboratory.

In this AuNP, a copolymer poly(N-2-hydroxypropyl methacrylamide_70-block-N-[3-(dimethylamino)propyl] methacrylamide_24) (P(HPMA_70-b-DMAPMA_24)) (M_n = 15000, PDI = 1.08) is utilized as a AuNP stabilizing ligand. P(HPMA) block is chosen because: (1) its hydrophilicity prevents AuNP aggregation; (2) its stability confers
the steric protection of siRNAs against the enzymatic degradation; (3) its neutral property
diminishes opsonization. On the other hand, the cationic DMAPMA block renders the
sites for the electrostatic complexation of anionic siRNA. These advantages of copolymer
have been elaborated in Chapter V when we discuss the properties of FAPol13.

As a result, this AuNP can encapsulate the therapeutic potential siRNAs
electrostatically and form neutral a soluble polyplexes, AuNP/siRNA. The AuNP/siRNAs
display significant stability in biological medium because the enzymatic tests
demonstrate approximately 100 times increased protection effects compared with free
siRNAs. Additionally, AuNP/siRNAs readily enter a series of human cancer cells
without assistance from transfection agents, and knockdown Gluc expression in Gluc-KB
cells.

Result and Discussion

The Characterization of AuNP

The surface charge and relevant hydrodynamic diameter (Dh) of the P(HPMA70-b-
DMAPMA24) and AuNP were measured by zeta potential and dynamic light scattering
(DLS) respectively. The zeta potential of P(HPMA70-b-DMAPMA24) and AuNP are 19
mV and -1.1 mV. As expected, P(HPMA70-b-DMAPMA24) has a positive surface change
due to the presence of cationic DMAPMA block. In contrast, P(HPMA70-b-DMAPMA24)
stabilized gold nanoparticle exhibits near-neutral zeta potential, indicating the DMAPMA
repeats are segregated close to the Au and hidden inside AuNPs as depicted in Scheme
VII-1. On the other side, the Dh of AuNP is 29 nm, significantly larger than the Dh of
P(HPMA70-b-DMAPMA24) which is 3.8 nm, further confirming the formation of
complexed architecture as illustrated in Scheme VII-1. The augmented $D_h$ of AuNPs is presumably because after DMAPMA block interacts with the Au core, the polymer chains become extended due to steric repulsion. Additionally, the diameter of Au core ($D_{Au}$) determined by transmission electron microscopy (TEM) and small-angle X-ray scattering is 6.5 nm and 7.8 nm respectively.$^{188d}$

**Scheme VII-1.** Schematic depiction of AuNP/siRNA complexes formation.$^{188d}$

*Complexation of AuNP and siRNA*

To test whether this AuNP has the ability of a gene delivery carrier, the complexation with siRNAs was first explored. Due to the cationic property of the DMAPMA, the siRNA will bind to this block and be close to the gold core as illustrated in the Scheme VII-1. Because a fraction of the DMAPMA groups has been used to reduce and activate Au, the preparation of $N/P_1$ AuNP/siRNA polyplexes can’t be only based on the calculated charge of AuNP. In another words, the actual $N/P$ ratio of complexes should be greater than 1.

To determine the neutral point, a series of AuNP/siRNA polyplexes were complexed and analyzed by gel electrophoresis with ethidium bromide (EtBr) stain. The
data show that the N/P ratio for neutral complexes is between 1.8 and 1.9 where siRNA is restricted in the wells, suggesting approximate 46% of DMAPMA repeats have been utilized for Au reduction, and 54% is available for siRNA complexation.

Once the neutral point is determined, the available cationic change of DMAPAM would be figured out and applied to future neutralization. Besides its ability of complexing siRNAs, AuNP could also form a complex with plasmid DNAs as shown in Figure VII-1. Two plasmids, pCMV-Gluc (5764 bp) and pAcGFP1-MemHyg (5846 bp) which are used for stable cell line establishment discussed in Chapter IV are utilized to exemplify it.

![Figure VII-1](image)

**Figure VII-1.** Agarose/EtBr electrophoresis analysis of AuNP/plasmid complexes. Two plasmids, pCMV-Gluc (left) and pAcGFP1-MemHyg (right), were complexed with AuNP to form neutral complexes according to the cationic change of AuNP. The complexes were analyzed along with the uncomplexed plasmid. Each lane contains 33 pmol siRNA. Each lane contains approximate 166 ng plasmid DNA.

After complexation with the AuNP, siRNA acquires the capability to resist RNase, which is confirmed by degradation studies. Briefly, the free and AuNP complexed siRNA were exposed to a mixture of nucleases, RiboShredder RNase, and
then assessed by UV/Vis spectroscopy monitored at 260 nm wavelength. If the nucleotides are degraded, the absorbance increases. The results demonstrate that AuNP confer ~100 fold long half-life time than that of the free siRNAs.\textsuperscript{188d}

_Uptake of AuNP/siRNA Complexes by Human Cancer Cells_

The internalization of AuNP/siRNA complexes in several human cancer cells was evaluated by fluorescence microscopy (Figure VII-2). The cell lines include KB, HeLa, SKOV3 and A549 (refer to Chapters V and VI). The neutral complexes were prepared by mixing AuNP and Cy3-labeled siRNA against human _Survivin_ gene and added into medium for 40 min. Following incubation, cells were rinsed thoroughly with pre-warmed PBS for three times to remove free complexes and fixed by polyformaldehyde and stained with DAPI. As seen in Figure VII-2 A-D, all cell lines treated with siRNA/AuNP complexes exhibited red fluorescence, indicating internalization of siRNA. Since lacking of FA conjugation, the complexes cannot distinguish the folate receptor positive cells (KB, HeLa, SKOV3) and FR negative cell (A549), which is a principal difference between this AuNP/siRNA and aforementioned FAPol13/siRNA. By contrast, the KB cells treated with uncomplexed Cy3-siRNA display negligible fluorescence (Figure VII-2E), suggesting the prerequisite of AuNP in siRNA delivery. Generally, some nonspecific binding signals from the free fluorophore-labeled siRNA incubation will be observed from such control panel. Here, such background has been normalized by adjusting the contrast settings to all the images.
Figure VII-2. Imaging analysis of the cellular uptake of AuNP/Cy3-siRNA. (A) KB cells, (B) HeLa cells, (C) SKOV3 cells, and (D) A549 cells were incubated with complexes. (E) KB cells incubated with uncomplexed Cy3-siRNA serves as a control. The cells were plated onto the glass-bottom wells 24 h before transfection. Then the neutral complexes ($N/P = 1.85$) were added to the cell media to give 100 nM concentration of cy3-labeled siRNA. After 40 min incubation, the cells were washed, fixed with 4% paraformaldehyde, mounted, and stained with DAPI. The imaging was conduct through Zeiss LSM 510 confocal with 40X/1.3 oil lens. Scale bars = 20 µm.
Our data also show, surprisingly, serum proteins are dispensable for the uptake of AuNP/siRNA complexes from the studies of fluorescence microscope and gene downregulation (Figure VII-3). In contrast to the serum-dependent AuNP reported by Mirkin group, the difference presumably stems from the distinct surface properties of complexes. In their work, thiolated siRNA is covalently conjugated to AuNPs which is further coated with oligoethylene glycol-thiol (OEG-thiol) for stabilization; while in our system, siRNA electrostatically bind to cationic DMAPAM at Au core, and therefore is physically excluded from serum proteins by extended, neutral, hydrophilic HPMA blocks. In terms of that, our AuNP/siRNA is more stable and has less possibility to cause non-specific uptake. Nevertheless, the mechanism for AuNP/siRNA internalization is not well understood here and need to be further exploited.

*Gene Downregulation by AuNP/siRNA Treatments in Gluc-KB Cells*

Gene downregulation of a Gluc gene was performed to determine whether siRNA could be released from the AuNP carrier once internalized. The reasons we choose this reporter system has been elaborated in previous chapter. As well, a KB cell line stably expressing Gaussia luciferase (Gluc-KB) was utilized here.

Complexes of the AuNP and siGLuc were prepared according to the results from electrophoresis studies and incubated the Gluc-KB cells ([siGLuc] = 100 nM) for 6 h before changing the cell medium. To test the potential opsonization effect from serum proteins, cells were treated with complexes in the presence and absence of serum. The luciferase assay was conducted with the medium samples by the virtue of the secretion of the Gaussia luciferase after one-day incubation.
In addition to the AuNP/siGLuc treatment, the cells have been tested with positive and negative controls which contain Gluc-KB cells, siGLuc only, DharmaFECT complexed control siRNA (has no siRNA target in mammalian cells), and DharmaFECT complexed siGLuc. All the results have been normalized to the untreated Gluc-KB cells. As shown in Figure VII-3, the Gluc activity has been reduced to 53% and 52% after AuNP/siGLuc treatment with and without serum protein presence, confirming the update of the complexes and the release of siRNAs from the complexes after internalization. As expected, incubation of Gluc-KB with DharmaFECT/siCon and siGLuc only does not induce the gene downregulation as a result of lacking of siRNA target and internalization respectively. Additionally, siGLuc delivered via transfection agent DharmaFECT reduce the Gluc activity to 48% of non-treatment control after one-day incubation, and to ~25% after two-day incubation \(10^{1b}\), demonstrating the well-designed siRNA.

The moderate RNAi effects could presumably result from: (i) relative low efficiency of uptake of complexes by cancer cells, which is indicated by low associated fluorescence intensity (would be evaluated by flow cytometry and microscopy); (ii) inadequate release of complexes from the compartments, which would be assessed by colocalization analysis and quantified by PCC; (iii) insufficient dissociation of siRNA from the complexes because of the steric hindrance from the polymer chains (i.e., HPMA). On the other hand, the similar gene downregulation efficiency from serum-absence treatment suggests that the delivery of AuNP/siRNA in our case is serum-independent as discussed before.
Figure VII-3. Relative *Gaussia* luciferase (Gluc) protein expression level in Gluc-KB. The Gluc-KB cells were seeded in 96-well plate with ~40% confluency 24 h before the transfection. The preformed complexes or siRNA were then added into the cell medium to make the siRNA concentration of 100 nM. The cell medium was changes 6 h later and the cells were kept culturing for 18 h before luciferase activity assay conducted. The experimental controls included non-treated, DharmaFECT/siCon, free siGLuc, and DharmaFECT/siGLuc treated cells (from left to right). The neutral AuNPs/siGLuc complexes ($N/P = 1.85$) treated the cells at the absence and present of 10% FCS. Protein expression levels were measured 24 h after treatment. All experiments were repeated in triplicate and the error bars represent ± S.D. (n=5).

Time course study shows the best gene downregulation efficiency from AuNP/siGLuc occurs after one day, which is different from the two-day optimum of FAPol13. This discrepancy suggests the distinct internalization mechanism and trafficking processes between these two siRNA delivery strategies.

*Nontoxic AuNP as a siRNA Carrier*

After gene downregulation experiments, the cytotoxicity of AuNP were assessed using a CellTitr 96 Aqueous one solution cell proliferation (MTS) assay, which is a colorimetric method for determining the number of viable cells in cytotoxicity or
proliferation. The principle of method has been discussed in Chapter IV. Basically, the dehydrogenase in the viable cells can convert the MTS substrate to blue/brown formazan which is detectable with a plate reader via monitoring absorbance at 490 nm (Ab$_{490}$); while the dying or dead cells cannot reduce the MTS and change the color. Importantly, the quantity of product as measure by the amount of Ab$_{490}$ is proportional to the number of living cells.

**Figure VII-4.** Cytotoxicity studies of Au nanoparticles (AuNPs). KB cells were first seeded into a 96-well plate 24 h prior to treatment. Six different AuNP concentrations ranging from 0.5 µM to 100 µM were tested and allowed to incubate for 48 h. A small amount (20 µL) of the CellTiter 96 AQueous One Solution Reagent was added directly to wells (100 µL) and incubated for 2 hours before recording Ab$_{490}$ with Synergy 2 multimode microplate reader (BioTeck, Winooski, VT). The relative cell viability has been normalized to the non-treated cells. All experiments were performed in triplicate, and error bars represent ± S.D. The concentration of DMAPMA utilized in gene delivery studies is 1 µM.

As shown in Figure VII-4, AuNP has negligible toxicity over the concentration from 0.5 µM to 100 µM regarding the concentration of DMAPMA in the experiments is 1 µM (to make complexes with N/P=1.85, [siRNA] = 100 nM), suggesting that the AuNP
is cyto-friendly and suitable for gene delivery applications. These results are also in agreement with morphological observations through microscopy. The biocompatibility of AuNP is of fundamental importance for its potential therapeutic, and diagnostic applications.

Conclusions

A rational design Au nanoparticle (P(HPMA\textsubscript{70}-b-DMAPMA\textsubscript{24})) is reported here. This copolymer is synthesized through aqueous RAFT polymerization and has a narrow polydispersity (PDI=1.08), controlled MW (Mn = 15,000), dual-functionalized blocks (HPMA for solubility, DMAPMA for polycation), and small size. This copolymer is employed as stabilizing ligands of neutral Au nanoparticles (zeta potential = -1.1mV), which have a high loading capability for oligonucleotides (siRNA duplexes and plasmid DNA) through electrostatic effects. Additionally, the ability of the AuNPs as a siRNA delivery vehicle has been intensively investigated and characterized by demonstrating cellular uptake of complexes in a variety of human cancer cells without the aid of transfection agents, and the downregulation of luciferase activity in Gluc-KB cells. Taken together, these findings shred lights on the potential biomedical applications of this AuNP.
CHAPTER VIII
FUTURE RESEARCH

Although major aims of my research have been achieved, there are still some aspects of investigations that deserve further exploitation, which are likely to lead to other projects. Some remaining questions include: which mechanism of endocytosis underlies the delivery of FAPol13/siRNA and AuNP/siRNA, how to improve the efficacy of endosomal release of FAPol13/siRNA complexes, when and where does the dissociation of FAPol13/siRNA occur, how to improve of inhibition effects of FAPol13/siCAS in cell level.

Exploration of Cell Entry Pathways

Essentially, there are at least five different routes for the entry of macromolecules via endocytosis pathway: macropinocytosis, phagocytosis, clathrin-dependent endocytosis, caveolae-dependent endocytosis, and clathrin/caveolae-independent endocytosis. Among them, the clathrin-mediated and caveolae-mediated deliveries are the major routes for the uptake of polyplexes and lipoplexes.

In recent years, numerous innovative drug/gene delivery strategies have emerged and exhibited great potential in biomedical applications. In many systems, the mechanism for the cellular internalization of complexes is mainly elusive. For example, in the cases of peptide transduction domain (PTD)/dsRNA binding domain (DRBD)/siRNA delivery system from Dowdy group, the serum protein-dependent AuNP from Mirkin group, and serum protein-independent AuNP from our group, the membrane penetration machinery is not well understood.
To this issue, the specific inhibitors to certain enzymes that play roles in special entry pathways are valuable and commonly utilized.\textsuperscript{240} Besides, the energy- or temperature-dependence are usually characterized. Combining our expertise in fluorescence imaging and colocalization analysis, we might gain plenty of valuable data from conducting it.

Spatiotemporal Analysis of the Dissociation of FAPol13/siRNA

Our data have demonstrated that FAPol13/siRNA polyplexes can release from endosome compartment through PCC-equipped colocalization analysis. However, when and where dissociation of FAPol13/siRNA occurs in a cell is ambiguous. It is one of the important steps for siRNA intracellular trafficking.

We have an opportunity to clarify it by utilizing a NHS-activated black hole quencher (BHQ). BHQ is a dye with absorption range of 550-650 nm and an absorption max at 579 nm. BHQ can be facilely conjugated to the backbone of a copolymer (i.e., FAPol13). Via complexation with Cy3-siRNA electrostatically, BHQ will quench the fluorescence from Cy3 because of their overlapped emission spectrums. Furthermore, this BHQ-conjugated FAPol13 can facilitate fluorescence resonance energy transfer (FRET) analysis (Cy3: donor, BHQ: acceptor). When the Cy3-siRNA is encapsulated with BHQ-FAPol13, the fluorescence from Cy3 is invisible; upon release, fluorescence is restored. According to this change, the dynamic process of dissociation of polyplex could be calculated by FRET. In addition to the colocalization of endolysosome, we can determine the cellular distribution of siRNAs or polyplexes. Taken together, the special and temporal dissociation of polyplexes within a cell will be evaluated.
Improvement of the Endosomal Escape of FAPol13/siRNA

Complex release in the endosome is the result of interplays between the complex, siRNAs, carriers, and compartmental environment, and it is an efficiency-limiting step in many nonviral delivery systems. Additionally, endosomal release plays a pivotal role in connecting siRNA uptake and RNAi pathway, and insufficient release might be the major reason for the fact that efficient binding and uptake of siRNA yield suboptimal siRNA effects. To address this issue, efforts have been dedicated to conjugate pH-stimulable, temperature-sensible, light-tiggerable, fusogenic compounds with nanocarriers for spatiotemporally controllable, enhanced releasing of therapeutic cargoes.

The PCC quantification (PCC_{48h} = 0.51) of endosomal escape of FAPol13/siRNA in KB indicates the possible insufficiency. Thus, the conjugation of some fusogenic peptides with FAPol13 is recommended.
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