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Delivery of small molecule and RNA for the treatment of type 1 diabetes and prostate cancer

Di Wen
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Delivery of small molecule and RNA for the treatment of type 1 diabetes and prostate cancer

By

Di Wen

A DISSERTATION

Presented to the Faculty of the University of Nebraska Graduate College in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Pharmaceutical Sciences Graduate Program (Pharmaceutics)

Under the supervision of Professor Ram I. Mahato
University of Nebraska Medical Center

Omaha, Nebraska

January 2017

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ACKNOWLEDGEMENTS

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ABSTRACT

The aim of this thesis is to develop combination therapy using a small molecule and RNA including siRNA, shRNA, or miRNA inhibitor for the treatment of type 1 diabetes and prostate cancer. New amphiphilic biodegradable polymers capable of co-delivering small hydrophobic molecules and RNAs or human bone marrow-derived mesenchymal stem cell (hBMSC) for co-delivery of an shRNA and a miRNA inhibitor were used as drug delivery platform. The drug delivery properties were evaluated in vitro and in vivo islet transplantation, subcutaneous and orthotopic prostate cancer models.

In Chapter 1, an overview of prostate cancer, the role of miRNA and the way for regulating miRNA as well as the design of delivery systems is given. I also introduce a brief background of type 1 diabetes and the treatment methods. Chapter 2 discussed the treatment of early stage or advanced prostate cancer using a luteinizing hormone release hormone (LHRH) conjugated micelles for target delivery of CBDIV17 (a bicalutamide analog) or combination therapy using CBDIV17 and embelin (a XIAP inhibitor). In Chapter 2, subcutaneous model was used to prove our concept. Our results indicated that LHRH conjugated micelles carrying CBDIV17 or both CBDIV17 and embelin inhibited tumor cell growth in vitro and in vivo.

Chapter 3 reports the newly screened small molecule named rubone as a miR-34a modulator for combination therapy with paclitaxel to treat chemoresistant prostate cancer. This compound was first characterized for miR-34a modulation efficacy in
paclitaxel resistant prostate cancer cell lines including DU145-TXR and PC3-TXR. The miR-34a downstream protein level and combination therapy efficacy were also evaluated. The biodegradable copolymer poly (ethylene glycol)-block-poly (2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD) were used to co-deliver both drugs in an orthotopic prostate tumor model after characterizing the drug delivery properties. This combination therapy using rubone as a miR-34a modulator reversed the chemoresistance of prostate cancer and significantly inhibited paclitaxel-resistant tumor growth in vivo. Finally, we summarized the results for prostate cancer treatment and gives suggestions for further research.

**Chapter 4** provided the background information about islet transplantation for treating type 1 diabetes. In Chapter 4, we constructed plasmid encoding shRNA against Fas receptor and miRNA inhibitor for downregulating miR-375. This plasmid was transfected to hBMSCs as an RNA delivery vehicle and hBMSC transferred these two small RNAs to human islet by direct touch and exosome. This stem cell-based gene therapy and cell therapy suppressed islet apoptosis and promoted islet function in vitro and in a humanized NOD scid gamma (NSG) mouse model. The immune reaction after islet transplantation was suppressed by intravenous injection of hBMSC and peripheral blood mononuclear cells (PBMC) co-cultured exosomes. We obtained a 100% insulin independence after humanization by intraperitoneal injection of PBMC.

**Chapter 5** summarizes the results of this thesis and gives suggestions to further research.
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List of Abbreviations

ACN  acetonitrile
APC  antigen-presenting cells
AR  androgen receptor
ASO  antisense oligonucleotide
CFSE  carboxyfluorescein succinimidyl ester
CMC  critical micelle concentration
CRPC  Castrate-resistant prostate cancer
CSC  cancer stem cell
CTLA-4  cytotoxic T-lymocyte antigen 4
DBU  1, 8-diazabicyclo[5.4.0]undec-7-ene
dc  dendritic cell
DFHBI  3,5-difluoro-4-hydroxybenzylidene imidazolinone
DiI  1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DiO  3,3'-dioctadecyloxacarbocyanine perchlorate
DMF  dimethylformamide
DMSO  dimethyl sulfoxide
DSPE-PEG  1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)
EGFR  epidermal growth factor receptor
EDC  1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPR  enhanced permeability and retention
FBS  fetal bovine serum
FDA  food and drug administration
FRET  Fluorescence Resonance Energy Transfer
H&E  hematoxylin and eosin
hBMSC  human bone marrow-derived mesenchymal stem cell
HCC  hepatocellular carcinoma
Her-2  human epidermal growth factor receptor 2
IAP  inhibitor of apoptosis
IEQ islet equivalent
IHC immunohistochemistry
IRDye infrared dyes
KRB Krebs-Ringer bicarbonate
LHRH luteinizing hormone release hormone
LNA locked nucleic acid
MBC 5-Methyl-5-benzyloxy carbonyl-1, 3-dioxane-2-one
MDR multiple drug resistant
miRNA microRNA
Mtpn myotrophin
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mUA 8-methoxy-urolithin A
NSG NOD scid gamma
oncomiR oncogenic miRNA
P-gp P-glycoprotein
PBS phosphate buffered saline
PBMC peripheral blood mononuclear cell
PEG-b-p(CB-co-LA) polyethylene glycol-b-poly(carbonate-co-lactide)
PEG-PCD poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol)
PEG-PLA poly (ethylene glycol)-polylactide
PHA phytohaemagglutinin
PNF primary non-function
PSMA prostate specific membrane antigen
PTX paclitaxel
PVDF polyvinylidene fluoride
RES reticuloendothelial system
RP-HPLC reverse phase high performance liquid chromatography
scFV single-chain variable fragment
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMIR</td>
<td>small molecule miRNA inhibitor</td>
</tr>
<tr>
<td>SMMR</td>
<td>small molecule miRNA modulator</td>
</tr>
<tr>
<td>STEAP</td>
<td>six-transmembrane epithelial antigen of the prostate</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TAA</td>
<td>tumor associate antigen</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>TK</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis</td>
</tr>
</tbody>
</table>
CHAPTER 1. THERAPEUTIC OF PROSTATE CANCER USING SMALL MOLECULES

1.1. INTRODUCTION

Prostate Cancer is the most frequently diagnosed neoplasm of prostate and the second leading cause of cancer mortality affecting men in the United States. (1) Nearly all prostate carcinomas are initially androgen dependent and further developed into androgen independent. (2) Hence, the disease is classified as hormone-dependent or hormone-refractory depending on the sensitivity of androgen ablation. Androgen ablation or blockade of androgen receptor (AR) is the cornerstone of treating early stage prostate cancer. Among various antiandrogens are used for chemotherapy, bicalutamide has long half-life and tolerable side effects, leading to its wide clinical application for treating early stage prostate cancer (3).

Although most patients respond well to androgen ablation therapy, chemotherapy, and radiotherapy at the beginning, many patients relapse over time and become resistant to chemotherapy (4). For example, prolonged treatment with bicalutamide leads to AR proliferation and mutation, which converts bicalutamide from an AR antagonist into an AR agonist. This is mainly caused by the over-expression of multiple drug-resistant (MDR) transporters in prostate cancer cells. These transporters, including P-glycoprotein (P-gp), breast cancer resistance protein, and multiple drug resistance protein, increase drug efflux and reduce drug accumulation in tumor cells (5, 6). The prognosis of patients with MDR cancer is poor, due to the lack of effective...
clinical interventions. Also, many commonly used chemotherapy drugs such as paclitaxel have inherent toxicity associated with their use(7).

MicroRNAs (miRNAs) are single stranded small non-coding RNAs (21–23 nucleotides) that have been reported as regulators of gene expression by hindering translation and triggering degradation of target mRNA post-transcriptionally. miRNAs play a crucial role in the initiation and development of a variety of human cancers with numerous studies reporting aberrant miRNA expression. miRNAs are not only deregulated in cancers, but are also acting as oncogenes or tumor suppressors. Oncogenic miRNAs (oncomiRs) function by either inhibiting tumor suppressor genes or genes responsible for promoting apoptosis or stimulating cell proliferation and are normally upregulated in cancer (Table 1-1). In contrast, tumor suppressor miRNAs are downregulated in cancers. These miRNAs function by inhibiting genes that hinder apoptosis or cell proliferation. For example, miR-221 has an oncogenic function by suppressing Bmf, a proapoptotic BH3-only protein, to inhibit cell apoptosis(9). In addition, miR-221 enhances cell migration by targeting PTEN and TIMP3(10). In contrast, Let-7g suppresses tumor cell proliferation by targeting both c-Myc(11) and COL1A2(12). Meanwhile, Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is identified as a target of let-7g to induce cell apoptosis(13). Normally, oncogenic miRNAs are overexpressed while tumor suppressor miRNAs are downregulated in cancer. Therefore, two miRNA-based therapeutic strategies used are: (1) miRNA inhibition for addressing oncogenic miRNAs and (2) miRNA replenishment for overexpressing tumor suppressor miRNAs. Similar therapeutic molecules including
oligonucleotides and small molecules may be employed in both approaches to either directly inhibit miRNAs or indirectly by targeting specific genes or transcription factors which modulate specific miRNA expression.

Table 1-1. OncomiRs as targets for cancer therapy

<table>
<thead>
<tr>
<th>OncomiRs</th>
<th>Target genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>PDCD4, PTEN, BCL2, TPM1, RECK,</td>
<td>(14-18)</td>
</tr>
<tr>
<td>miR-17-92</td>
<td>PTEN, Bim</td>
<td>(19, 20)</td>
</tr>
<tr>
<td>miR-221/222</td>
<td>p27, TIMP2, DKK2</td>
<td>(21-23)</td>
</tr>
<tr>
<td>miR-155</td>
<td>DMTF1, annexin 7, LKB1, E2F2, GABA receptor</td>
<td>(24-28)</td>
</tr>
<tr>
<td>miR-223</td>
<td>PAX6, Statmin1, FBXW7/hCdc4</td>
<td>(29-31)</td>
</tr>
<tr>
<td>miR-214</td>
<td>PTEN, p53</td>
<td>(32, 33)</td>
</tr>
<tr>
<td>miR-191</td>
<td>C/EBPβ, checkpoint kinase 2</td>
<td>(34, 35)</td>
</tr>
<tr>
<td>miR-25</td>
<td>CDKN1C, LATS2, RECK</td>
<td>(36-38)</td>
</tr>
</tbody>
</table>

1.2. CURRENT TREATMENT OF PROSTATE CANCER

1.2.1. Small molecule drug

Treatment of prostate cancer might involve surgery, external beam therapy, and small molecule drug. With the advances in drug design and chemical synthesis, small molecule drugs become crucial part in clinical trial. They can be further modified for better therapeutic outcome and oral absorption.

Androgen receptor is a ligand-inducible transcription factor and member of the steroid hormone, which enhances prostate cancer growth and progression at the early stage. For hormone related prostate cancer therapy, abiraterone is a Food and Drug Administratoin (FDA) approved drug by inhibiting CYP17 activity and further suppress androgen synthesis(39). Bicalutamide is another small molecule drug in early stage prostate cancer therapy by inhibiting the binding of androgen receptor (AR)(40). With
potent mutation of AR after prolonged bicalutamide treatment, some bicalutamide analogs including CBDIV17 was developed to overcome the resistance from AR mutation(41).

Almost all androgen dependent prostate cancer will be developed into androgen independent, which is defined as lower levels of testosterone found in tissue and blockage of androgen does not affect the growth of tumor tissue(42). In this advanced stage of prostate cancer, paclitaxel or docetaxel has been extensively used. Their anti-cancer mechanisms for prostate cancer are associated with tubulin and to promote microtubule assembly, which causes mitotic-dependent cell cycle arrest(43). Furthermore, they also induce cell death through activation of caspase and lysosomal pathways(44).

The highly metastatic potential of prostate cancer in advanced stage is another crucial issue to overcome, especially bone metastasis. For clinical efficacy, therapies must target tumor-microenvironment interactions, where several tyrosine kinases, including PDGFR, EGFR, VEGFR, IGF-1R and c-Met, have been implicated to promote metastasis and tumor growth, especially in patients with advanced castrate-resistant prostate cancer (CRPC)(45). There were many small molecules screened as tyrosine kinase (TK) inhibitor to inhibit different TK activities, including imatinib for PDGFR(46) and sunitinib for VEGFR(47). However, only few of them showed promising results in phase III clinical trials. Cabozatinib is an ATP competitive inhibitor with selectivity to c-Met and VEGFR-2 showing high clinical potential(48). Phase II clinical trial exhibited that 86% of patients with documented bone metastasis
experience complete or partial response on bone scans 6 weeks after initiation (45).
Although the clinical tests are not completed yet, researches about c-Met inhibitors are now being rapidly accelerated and clinically useful agent might be identified for prostate cancer treatment.

1.2.2. Immunotherapy

Immunotherapy for the treatment of cancer has made significant progress over the past 20 years. It implies the host response beginning with an antigen presenting cell able to recognize foreign biological threat and processing this into presentable antigens which are delivered to T cells (49). Thus, the basic mechanism of any immunotherapy in cancer is to activate a specific immune response creating tumor cell destruction. Cancer immunotherapy can be broadly classified as vaccines (50), checkpoint inhibitors (51), or adoptive cellular therapy (52). Vaccine-based immunotherapy relies on the innate ability of antigen-presenting cells (APCs), which capture and present prostate tumor associated antigens (TAAs) leading to the generation of humoral and cytotoxic T-cell response (53). Checkpoint inhibition aims to reverse the inhibition caused by signals intended to prevent autoimmunity or tumor microenvironment. Adoptive cellular therapy uses engineered synthetic single-chain variable fragment (scFv) that recognize the TAA in a human leukocyte antigen-independent fashion to facilitate T cell mediated cytotoxicity.

Sipuleucel-T (Provenge) is the only FDA approved therapeutic cancer vaccine. It is an autologous dendritic cell (DC)-based vaccine with a prostatic acid phosphatase-
granulocyte-macrophage colony-stimulating factor fusion protein. A phase III trial published by Kantoff et al. showed significantly improved overall survival in a multicenter, double-blind, placebo controlled clinical study(54). Patient treated with Provenge had an increased mean overall survival by 4.1 months compared with placebo-treated group (25.8 vs 21.7 months).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) normally acts to suppress T-cell activity and therefore the inhibition of CTLA-4 could induce a net activation to strengthen the immune activity for tumor killing. Ipilimumab was a small molecule that was identified that could improve the survival of metastatic melanoma(55). Recently, a phase III trial of double-blind study was performed to determine the effect of ipilimumab on CRPC with at least one bone metastasis and improved overall survival was obtained (11.2m vs. 10m) compared to the placebo group(56). Like CTLA-4, PD-1 might be another therapeutic target for prostate cancer as PD-L1 is overexpressed by tumor cells(57). This PD-L1 could bind PD-1 and inhibit the activation of T cells and further enhance tumor survival.

1.2.3. miRNA

Drug target selection remains a bottleneck in the quest for anticancer therapeutics. The current paradigm where drugs are designed to target cancer-related proteins is flawed for several reasons. Since cancer is a complex process involving multiple factors and multistep processes, the efficacy of anticancer agents designed to target single therapeutic protein is often sub-optimal and less effective in cancer therapy.
Although combination therapy, in which more than one targets are addressed, yields better therapeutic outcomes compared to single drug treatment, it is typically costly and complexed, associated with detrimental drug–drug interactions and involves complicated treatment regimens. Considering their abnormal expression in cancer compared to normal tissues, miRNAs are regarded as high value drug targets (Table 1-1) for cancer therapy and targeting their expression can change cancer phenotype.

One rationale for miRNA-based therapeutics described by Garzon et al., is the notion of cancer networks being miRNA wired(58). The “miRNA wired cancer network” hypothesis indicates miRNAs to be the code that maintains a required connection between all genes and protein networks in normal cells. Thus, normal tissues can be thoroughly characterized and miRNA expression patterns can be established as a coding blueprint. It might then be possible to compare this blueprint to miRNA expression patterns in tumor tissue. Therapeutic approaches can be developed to “reboot” the cancerous tissue by restoring the abnormal expressed miRNA patterns to the default settings observed in the normal tissue. Clearly, such a therapeutic strategy involves targeting more than single miRNA, gene or protein. It may involve simultaneous inhibition or replacement of more than one miRNAs. From an implementation standpoint, it might be tempting to dispose the “miRNA wired cancer network” hypothesis because of its potential complexity. Another argument might be that there is no universal miRNA blueprint for normal tissues. Nonetheless, since abnormal expression of single miRNA may affect hundreds of proteins(59), reprogramming cancer network may be more feasible using miRNAs compared to
proteins. This is also the advantage of miRNA based therapy compared to siRNA therapy since normally siRNA could only target one cancer related gene expression.

Traditional methods to restore the miRNA expression include (Figure 1-1): (1) miRNA mimics or antisense oligonucleotide (ASOs, also known as antagomirs or antimiRs), which includes cholesterol-conjugated antimiRs, locked nucleic acid (LNA) antimiRs and tiny LNA antimiRs; (2) miRNA sponges which contain multiple tandem binding sites to target miRNA; (3) CRISPR/Cas9 based genome editing which modify the genome of cancer cells. Indeed, these three non-small molecule miRNA therapies have the potential to be an efficient method for miRNA inhibition. However, there are still several crucial obstacles need to be overcome. Most of antisense oligonucleotides are perfect complementary to their targets with chemical modifications to improve binding affinity and stability. However, these miRNA inhibitors may not distinguish between miRNAs within the same family, which causes off-target effects (60). Although the off-target effect of miRNA sponges is not reported yet, miRNA sponges always exhibit different degrees of inhibition in different conditions and it is still challenging to evaluate the degree of miRNA silencing under a sponge treatment (61). Similarly, the off-target effect of CRISPR/Cas9 has not been well-recognized and accurately profiled when applied in gene therapy, which significantly limits its clinical application.

Another concern which hinders the clinical development of non-small molecular miRNA inhibitors is the delivery-related issue due to the high instability of oligonucleotide in blood serum. Depending on the diseases and targets, people need to carefully design the delivery systems to achieve optimized clinical effects. Organs,
which are more accessible and responsible for metabolism and excretion including liver, kidney, and spleen, have shown exciting results for antisense oligonucleotide delivery with high accumulation of oligonucleotide. Nevertheless, it is still difficult to ensure an effective dose reaching and entering the tumor cells. Theoretically, liposome or nanoparticle based non-viral delivery system can be used to deliver miRNA sponges or CRISPR/Cas9 based genome editing systems. However, these systems generally suffer from low gene delivery efficiency, especially for in vivo studies.

1.3. Small molecule miRNA therapeutic agents

Due to the above challenges of non-small molecule miRNA inhibitors, it would be promising to develop small molecule drugs to target specific miRNAs and restore their expression (Figure 1-1). Actually, miRNAs have long been neglected as potent drug targets due to their structural flexibility and highly electronegative surface(62). Furthermore, poor understanding of miRNA X-Ray crystallography or NMR structure as well as the limited availability of miRNA-Dicer or RISC complex structure makes the design of small molecule inhibitor or inducer of miRNA much more difficult(63). These might be the reasons why the first reported small molecule miRNA inhibitor (SMIR) by Gumireddy et al.(64) and most following designs were based on non-specific selection assay. For the first SMIR, they selected miR-21 as the target oncogenic miRNA, which is overexpressed in various cancers including breast, ovarian, and lung cancers(65, 66). Lentiviral vector encoding complementary sequences of miR-21 and downstream luciferase reporter gene was constructed for HeLa cell transduction and promising miR-21 inhibitor selection. As a result, diazobenzene was identified for further
modification since 251% increase of luciferase signal was detected relative to untreated cells. Except for this non-specific selection assay, there are also some other screening methods to identify potent small molecule miRNA regulators.

Figure 1-1. Schematic representation of miRNA biogenesis and the inhibition effect of antisense oligonucleotide, miRNA sponges, CRISPR/Cas9 genome editing, and small molecule inhibitor of miRNA (SMIR).

1.3.1. Luciferase (or GFP)-based screening

Luciferase-based vectors, which include a complementary sequence or control sequence of target miRNA linked with downstream luciferase reporter gene, are widely used for SMIR screening. After cloning into lentiviral vectors, they are transduced into culture cells where target miRNA is highly expressed. These genome modified cells are thereby able to determine the efficacy of potent SMIRs. With the presence of effective SMIRs, less target miRNA is available for binding the complementary sequence and luciferase gene is overexpressed as a result. Thus, the more effective SMIR, the more luciferase signals will be detected. Bose et al.(67) used pEZX-MT01
plasmid, which co-express luciferase and PDCD4, a known target of miR-21, to screen SMIRs for miR-21 in the MCF-7 cell line. Streptomycin was identified as the most potent molecule and was characterized as a direct miR-21 inhibitor docking with pre-miR-21 at a region close to the terminal loop. Similarly, this luciferase reporter-based screening method can be used to select specific compounds promoting tumor suppressor miRNA secretion or activity. Xiao et al. (68) constructed miR-34a reporter vector using the Huh7 cell line and found that rubone, which inhibited luciferase activity, was a potent miR-34a promoter. Compared to SMIR selected by the luciferase reporter system, a small molecule modulator (SMMR) of miRNA needs further evaluation to exclude the false-positive phenomenon caused by toxicity since this compound might decrease luciferase activity.

1.3.2. Molecular beacon-based screening

Fluorescent beacons are usually hairpin shaped oligonucleotides which contain a 5′-fluorophore and a 3′-quencher, along with a miRNA targeting sequence (anti-miRNA sequence) in the loop for SMIR selection. Davies et al. (69) first described the design of a fluorescent beacon and forecasted the potent application for screening SMIRs. In a hairpin shape, the base pair of the beacon would bring the fluorophore and quencher closely, leading to quenching of the fluorescence. Thus, a Dicer-dependent increase in the signal would be detected since mature miRNA is generated from Dicer-mediated hydrolysis, resulting in a dissociation of the fluorophore and quencher, and an increase of fluorescence. In the presence of a Dicer inhibitor of pre-miRNA, Dicer activity would be inhibited and the beam showed a lack of fluorescence increase. Vo et al. (70) used
this fluorescent beacon system to screen Dicer inhibitor to inhibit biogenesis of oncogenic miR-372 and miR-373. In their study, neomycin appeared to be the best compound for Dicer inhibition and can thereby be used for further modification.

1.3.3. Structure-based design

One difficulty encountered in the drug development process is the high expense in the process of drug screening. With a more accurate understanding of miRNA (or miRNA protein complex) structure and the simulation of binding affinity of small molecule to miRNA, in silico high-throughput screening is a promising technique to speed up the discovery of SMIRs and decrease the cost during the process. Shi et al.(71) reported AC1MMYR2 as an inhibitor of Dicer-mediated biogenesis of miR-21 using MC-Fold/MC-Sym pipeline for RNA secondary and tertiary structure prediction. In their studies, AC1MMYR2 was proved to be a specific miR-21 inhibitor, which repressed pri-miR-21 expression by approximately 50% after 6 h and inhibited tumor growth in an orthotopic tumor model. However, this computational approach is still challenging and needs further evaluation and recalibration to ensure the efficacy of screened compounds due to the flexible and complicated RNA tertiary structure.

1.3.4. Peptide or peptoid screening

Another category of SMIRs is peptides or peptoids, which are well evaluated for selective RNA binding. Here, we introduce two peptide selection methods, peptoid microarrays and phage display selection. Chirayil et al.(72) performed peptoid microarrays to screen specific ligands for RNA hairpin precursor of miR-21. In their
studies, they used peptoid microarrays as the foundation for RNA ligand discovery to screen a library of 7680 N-substituted oligoglycines. Among them, two compounds were proved to have specific binding affinity to the secondary structure of miR-21 precursor hairpin. A fusion phage is a filamentous virion displaying on its surface a foreign peptide fused to a coat protein(73). In one study, the library of this fusion phage may represent up to billions of peptides(74). If a phage displays a peptide which is a strong ligand of target miRNA, it can be eluted and the peptide sequences responsible for the binding are easily obtained by infecting the specific phage into bacteria and sequencing the relevant part of their viral DNAs(75). Using this method, Bose et al.(76) reported that ‘ALWPPNLHAWVP’ was a potent peptide sequence for binding miR-21.

Figure 1-2. Mechanisms of luciferase/GFP based screening
(A) luciferase/GFP based screening. (B) Molecular beacon-based screening. (C) structure-based design. (D) Peptides or peptoids screening.

1.4. Small molecule restored miRNAs for prostate cancer therapy

miRNAs are considered crucial factors in spectrums of cancers including prostate cancer. In the past decade, various miRNAs have been reported to be associated with
the cancer development process. Drug discovery and development are usually a time-consuming and expensive process, which significantly influences the therapeutic progress of cancer and other diseases, leading to the urgent need for new therapeutic alternatives. SMIRs and SMMRs show another promising approach for the treatment of cancer due to its less time-consuming characteristic for drug development with reduced cost in the whole process. In addition, their exciting results further proved them to be an efficient tool for therapeutic use. Here, we listed several miRNAs that might be therapeutic targets for prostate cancer and introduce some small molecules that might restore the miRNA expression.

1.4.1 miR-21

The oncogenic miR-21, has been identified to be significantly elevated in numerous tumor cells, including prostate, pancreas and liver cancer(77). Abundant expression of miR-21 promotes cell invasion and metastasis in cancer cells in vitro and in vivo, which represses the expression of PDCD4 protein and reactivate the PTEN/PI3K/Akt signaling pathways(78). Zhou et al. demonstrated that 8-methoxy-uroolithin A (mUA) can induce cell apoptosis by down-regulating miR-21 expression and inhibiting PI3K/Akt/β-catenin pathway in DU145 cell line in a PTEN dependent manner(78). For miR-21 inhibition in other cancers, diazobenzene was identified as miR-21 inhibitor by killing Hela cells(64). Streptomycin was screened by Bose et al. as the most potent compound as a direct miR-21 inhibitor(67). Recently, GFP-based screening, which was similar to luciferase based screening, was also developed for SMIR screening. To screen a general miRNA inhibitor, cell line stably expressing lenti-GFP and lenti-shGFP
was developed and a compound was considered potent SMIR if green fluorescence was increased. To screen a SMIR for specific miRNA, EGFP reporter gene expression was under the control of target miRNA through its complementary sequence present at the 3’ UTR. Using this GFP based screening assay, Shum et al. (79) obtained 6 potent miR-21 inhibitors and 6-hydroxy-dl-DOPA was characterized as the most potent SMIR. AC1MMYR2 was identified by Shi et al. as an inhibitor of Dicer-mediated biogenesis of miR-21 using structure design from MC-Fold/MC-Sym pipeline for RNA secondary and tertiary structure prediction(71).

1.4.2. miR-96 & miR-182

miR-96 has been well established to contribute to prostate cancer survival, proliferation, and clonogenicity by reducing FOXO1 expression(80). Furthermore, MTSS1 is another tumor suppressor target of miR-96 for tumor growth, development, and metastasis(81). miR-182 is another oncogenic miRNA which is associated with growth, migration and invasion in prostate cancer via targeting FOXO1(82). To screen small molecule targeting miR-96 and miR-182, Velagapudi et al. reported a new method called informa for sequence based design of SMIR to target pre-miRNAs. Informa integrated a selection-based strategy (Two-Dimensional Combinatorial Screening; 2DCS)(83), a statistical approach (Structure–Activity Relationship through Sequencing; StARTS)(84), and the structural information about the RNA of interest that identified RNA motifs that positively and negatively contributed to binding. After screening and optimization, they selected two compounds for the miR-96 precursor and miR-182 precursor, respectively. The secondary structure was demonstrated by
enzymatic mapping assays and the downstream effect of miR-96 inhibitor was evaluated. Compared to traditional medicinal chemical approaches, Informa provided a reliable prediction of SMIRs that could target specific miRNA.

1.4.3. miR-372

miR-372 is overexpressed in prostate cancer cell lines and its overexpression promotes cell proliferation and migration. Mechanism study elucidated that large tumor suppressor kinase 2 (LATS2) was a direct target of miR-372 using luciferase reporter assays. Decreased expression of LATS2 promoted prostate cancer cell activity just as over-expression of miR-372(85). Cao et al. identified arsenic sulfide as a miR-372 inhibitor to repress the overexpression of miR-372 and they confirmed that arsenic sulfide could suppress tumor growth in a mouse xenograft model(85). Vo et al. screened neomycin as the best aminoglycoside using molecular beacon-based screening for Dicer inhibition to inhibit biogenesis of oncogenic miR-372(70).

1.4.4. miR-34a

Most prostate cancers relapse within two years into hormone refractory due to the presence of tumor initiating cells, known as cancer stem cells (CSCs), which are involved in tumor progression and metastasis, but are resistant to chemotherapy. Recently, miR-34a was reported to be significantly underexpressed in chemoresistant prostate cancer cell line(86) or CD44+ cancer stem cells(87). As a tumor suppressor miRNA, miR-34a is responsible for promoting cancer cell apoptosis, inhibiting cell metastasis(88) and chemoresistance(89). Thus, miR-34a replenishment might be a
novel therapeutic method to reverse PTX-resistance for treating chemoresistant prostate cancer. Among the small molecule drugs, natural and synthetic analogs of chalcones exhibits promising anticancer activity. However, only a few studies have focused on the role of chalcone derivatives on the expression of miRNAs. Xiao et al. first reported rubone, a chalcone analog, as a miR-34a modulator to inhibit hepatocellular carcinoma (HCC) growth(68). In their study, rubone upregulated miR-34a expression, downregulated the downstream target genes expression, and suppressed HCC growth in vivo in p53 dependent manner. Xia et al. identified that genistein could lead to upregulation of miR-34a in pancreatic cancer and further inhibit cell growth and apoptosis. Agostini et al. reported that retinoic acid could enhance miR-34a expression in neuroblastoma cells through Tap73 pathway(90)

1.5. Delivery of small molecule anti-cancer drugs.

Despite their therapeutic potential, clinical applications of small molecule anti-cancer drugs are limited due to their high organ toxicity, poor water solubility, and low bioavailability. To overcome these obstacles, some drug delivery systems have been developed to solubilize these compounds and further deliver them to prostate tumor tissue. These delivery systems could be divided into non-target delivery system and target delivery system.

1.5.1 Non-target delivery system

Polymeric nanomedicines could offer several advantages with the capable of increasing the aqueous solubility of lipophilic drugs. First, they are normally less toxic
compared to traditional solubilizing agents such as DMSO and Cremophor EL. Thus, these nanomedicines could reduce the adverse effects on healthy tissues. Second, these nanomedicines could actively target the tumor site and deliver adequate quantities of drug to enhance the therapeutic efficacy by enhanced permeability and retention (EPR) effect. This negative targeting is caused by the small size of the nanomedicine which ensures preferential accumulation in tumor cells.

Nanomedicines might be lipid-based (liposomes) or polymer-based (micelles, nanoparticles) and the method of drug encapsulation could be chemical conjugation or physical encapsulation. Chemical conjugated nanomedicines are drug deliver platforms composed of a drug chemically linked to a polymeric carrier by a biodegradable covalent bond. This covalent bond is always physiological sensitive to release the drug in cells or tumor tissue. With the chemical linkage, drug release profile always exhibits prolonged release curve(91) and consequently longer circulation and reduced toxicity(92) of the drug are achieved. Furthermore, chemical conjugation might help the drug to circumvent some of the drug resistance pathway of cancer cells(93). To achieve these benefits, this polymer needs to be carefully designed. For example, the polymer needs to be non-toxic and easy to conjugate for decent drug loading capacity. Furthermore, the drug-conjugate platform should be stable in normal organ or blood serum but able to release the drug in tumor site or tumor cells.

Compared to chemical conjugation, physical encapsulation is more widely applied for drug loading since some therapeutic agents do not have functional groups for chemical conjugation and cannot be modified without affecting the therapeutic effect.
Polymeric micelles are self-assembled polymers with spherical structure. They have a hydrophobic core for solubilizing a considerable amount of water insoluble drugs. The hydrophilic component of micelles forms the corona which provides steric stabilization. This shell could also provide stealth properties which prevent the recognition and degradation by reticuloendothelial system and therefore prolong the half-life of micelles in vivo(94). This property of micelles facilitates their wide application in physical encapsulation for drug loading.

1.5.2 Target delivery system

Passive targeting delivery mainly utilize the EPR effect to obtain higher accumulation of drug in tumor site. However, this EPR effect is only efficient for targeting solid tumor and less efficient for spreading or metastatic tumors(40). In some cases where drug loading capacity of the nanomedicine is low, passive targeting is also less effective and may lead to adverse effect on visceral organs before therapeutic levels of drug could reach the tumors. Thus, addition of targeting ligands on the surface of the nanomedicine is crucial to reduce the toxicity to healthy organs and enhance the drug accumulation in tumors.

For prostate cancer, one of the approved clinical trials for target delivery is SGN-15(95) , which is monoclonal antibody-drug conjugates. This SGN-15 delivers doxorubicin to tumor tissues with overexpressed Lewis-y antigen (CD174). For research, several antigens, receptors, and proteins that are overexpressed on the surface of prostate cancer cells could be explored for promising targeting delivery of
Prostate specific membrane antigen (PSMA) is a type II integral membrane glycoprotein(96) which is highly overexpressed in prostate tumors, especially in metastatic and castration resistant prostate cancer(97). Wolf et al. described the design of using a single chain fragment of antibody binding to PSMA for the delivery of an immunotoxin for killing prostate cancer cells(98). The transferrin receptor (TfR) is another membrane associated glycoprotein which is overexpressed 2 to 10-fold in prostate cancer cells compared to normal prostate cells. This might be another targeting method for drug delivery to prostate cancer. Six-transmembrane epithelial antigen of the prostate (STEAP) is a transmembrane protein overexpressed in advanced prostate cancer. Recently, a STEAP binding monoclonal antibody was developed for target delivery and significant tumor growth suppression was observed(99). Human epidermal growth factor receptor 2 (Her-2) is a transmembrane tyrosine kinase receptor and a member of EGFR family which help to induce cell growth, survival, adhesion, and migration(100). Recent report shows that Her-2 is closely linked to the activation of AR pathway and the progression of CRPC(101). Goldstein et al. reported an anti-Her2 immunoemulsion as a targeted drug delivery system for the treatment of prostate cancer and observed significant tumor growth inhibition in immunodeficient mice(102). LHRH is a 10 amino acid peptide hormone secreted by hypothalamus and regulates gametogenesis(103). Overexpressed LHRH receptors are detected in prostate (86%), ovarian (80%), and breast (50%) cancers and have low expression in healthy organs(104). Karampelas et al. reported the design
of LHRH-gemcitabine conjugates for treating androgen-independent prostate cancer(105). Their formulation showed a significant advantage in tumor growth inhibition compared to free gemcitabine.

1.6. Introduction and treatment of type 1 diabetes

Islet graft rejection after islet transplantation is caused by acute immune response, inflammatory reactions, inadequate oxygenation and several other factors collectively contributing to primary non-function.(106) Among the cell apoptotic pathways, Fas/FasL pathway plays an essential role in T1D and is also involved in insulin release.(107) Meanwhile, the inhibition of islet specific miR375 improved insulin release of islet cell line.(108) As a potent approach for inhibiting aberrant protein expression, RNAi is becoming a promising tool to silence genes which are involved in immune reaction and PNF process of transplanted islets. Nevertheless, therapeutic efficacy of gene delivery is hindered by low transfection efficiency in case of non-viral vectors(109) and activation of innate immune response by viral vectors(110). Mesenchymal stem cells support islet viability and function by preventing immune cell infiltration and promoting islet growth.(111) Their potential could be further enhanced by genetically modifying them to express therapeutic proteins while maintaining their stemness and pluripotency. These immunomodulatory properties of MSCs could be mainly attributed to secretion of some anti-inflammatory cytokines which play a central role in induction of regulatory T-cells when co-cultured with lymphocytes.(112)

Exosomes are nanovesicles which serve as shuttle for certain proteins, mRNA
and miRNA into the target cells. MSCs have been used to improve islet transplantation due to their self-renewal potential, promoting islets revascularization, and immunomodulatory effects. For MSC-derived exosomes, Xin et al. reported that systemic administration of MSC-derived exosomes promoted functional recovery after stroke in rats. Lai et al. reported that MSC-derived exosomes reduces myocardial ischemia/reperfusion injury. These studies proved the potential of exosome as immunomodulatory regimens which also induce the regeneration of injured organs. Previous research also indicated that exosomes derived from genetically modified dendritic cells or mesenchymal stem cells (MSCs) can deliver siRNA or miRNA to the mouse brain or cancer cells to inhibit glioma growth.

1.7. CONCLUSIONS

miRNA-based cancer therapy is gaining more and more attention since it could simultaneously control more than one targets in combination therapy. Recently, oncogenic and tumor suppressor miRNAs are being identified in rapid progress. Notably, oncogenic miRNA for one cancer might be tumor suppressor miRNA for another cancer. For example, suppress the expression of miR-27a could inhibit MCF-7 cell growth, while the miR-27a is tumor suppressor miRNA in prostate cancer by targeting MAP2K4. For the treatment of prostate cancer, androgen ablation is the first choice for early stage prostate cancer. However, advanced prostate cancer might develop chemoresistance due to abnormal miRNA expression. Thus, to restore the miRNA expression and miRNA network might be a novel choice for cancer treatment.
Due to the short half-life of oligonucleotide, miRNA delivery platforms are always required for oligonucleotide based therapy. However, obstacles including high efficiency, low toxicity, and high bioavailability still need to be overcome. Recently, small molecules that specifically regulates miRNA expression might generate hope for better future of miRNA-based cancer therapy since small molecules are always easy to formulate with high bioavailability. Furthermore, several lipid or and polymer based drug delivery platforms could efficiently deliver these compounds to the tumor site by physical encapsulation or chemical conjugation, especially when conjugated with tumor specific target delivery ligand. For prostate cancer therapy, several potent compounds have been screened and evaluated to regulate prostate cancer related miRNAs, including miR-21, miR-96, and miR-34a. Although further evaluation is still needed for these compounds, we can expect bright perspective of miRNA based therapy for prostate cancer.

As a novel gene delivery vehicle and immunomodulatory regimen, exosome derived from mesenchymal stem cell could potentially promote islet transplantation by delivering anti-apoptotic siRNA and enhance the regeneration of transplanted islets. However, more persuasive researches are needed to prove the efficacy of this biocompatible vesicle.
CHAPTER 2. LHRH-CONJUGATED MICELLES FOR TARGETED DELIVERY OF ANTIANDROGEN AND XIAP INHIBITOR TO TREAT ADVANCED PROSTATE CANCER.

2.1. INTRODUCTION

Androgen ablation or blockade of androgen receptor (AR) is the cornerstone of treating early stage prostate cancer. Among various antiandrogens for chemotherapy have been developed, bicalutamide has longer half-life and tolerable side effects, leading to its wide clinical application for treating early stage prostate cancer(3). However, prolonged treatment with bicalutamide leads to AR proliferation and mutation, which converts bicalutamide from an AR antagonist into an AR agonist. To overcome this issue, we previously synthesized bicalutamide analog CBDIV17, which was more potent than bicalutamide in inhibiting the proliferation of prostate cancer cells and suppressing tumor growth in vivo(41).

The downregulation of apoptotic and overexpression of antiapoptotic protein is common feature associated with the progression of prostate cancer. The inhibitor of apoptosis (IAP) family suppressing caspase activity is probably key factor of the imbalance between proliferation and apoptosis. X-chromosome-linked inhibitor of apoptosis (XIAP), which binds caspase 9 and further inhibits the activity of caspase-3 and caspase-7, is the most potent IAP protein.(120) Embelin is a well-accepted small molecule inhibitor of XIAP. It binds to BIR3 domain and prevents XIAP binding and inhibiting the activity of Caspase family.(121, 122) Thus, we hypothesize that
Combination therapy using CBDIV17 and embelin has significant potential for treating advanced prostate cancer. However, poor aqueous solubility of CBDIV17 and embelin results in low absorption and less efficacy. Traditional approaches to increase solubility using solubilizing agents including dimethyl sulfoxide (DMSO) and Cremophor EL have associated toxicity limiting their clinical application. (123)

Polymeric micelles are nanosized particles and have spherical structures with a hydrophobic core, which can improve the solubility and stability of hydrophobic anticancer drugs. To enhance the solubility of CBDIV17, we synthesized polyethylene glycol-b-poly (carbonate-co-lactide) (PEG-b-p(CB-co-LA)) copolymer (124, 125) to prepare micelles, which successfully encapsulated bicalutamide, embelin, and some other drugs. In our previous results (41), CBDIV17 and embelin loaded micelles showed high antitumor efficacy and successfully suppressed tumor growth in vivo. However, intratumoral injection of these micelles limited its clinical application to prostate cancer.

Traditional chemotherapy usually employs high dose of anti-cancer drugs, which usually cause severe toxicity to healthy organs. Passive targeting tumors in vivo mainly utilized enhanced permeability and retention (EPR) effect (126, 127) causing preferential accumulation of macromolecules at the tumor site. However, EPR effect is only efficient for targeting solid tumors and is not used for spreading tumors and metastases. Active targeting can be used to make micelles site-specific by coupling a target moiety for receptors overexpressed on cancer cells. Furthermore, targeting cancer cells can diminish the cytotoxicity towards other tissues and the drugs loaded
micelles selectively accumulate to tumor site\(^{(128, 129)}\). For targeted delivery of anti-cancer drugs, several receptors, which are overexpressed by cancer cells, are selected as targets for polymer binding, such as prostate specific membrane antigen (PSMA)\(^{(129)}\), epidermal growth factor receptor (EGFR)\(^{(130)}\), and luteinizing-hormone-releasing hormone (LHRH) receptor\(^{(131)}\). In recent years, LHRH and its analogs have been employed in the clinical trial in the management of prostate cancer. We expect active targeting by LHRH to be safe and efficient after systemic administration. Due to the short half-life of natural LHRH, synthetic LHRH analogue with improved bioactivity has been widely used for targeting LHRH-R\(^{(132, 133)}\).

Therefore, we conjugated LHRH analog to our previously synthesized HOOC-PEG-b-p(CB-co-LA) copolymer and hypothesize that LHRH conjugated micelles would improve the efficacy of antitumor drug in vitro and in vivo and provide targeted drug delivery to suppress tumor growth. In this study, HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) were synthesized, characterized, and used for preparing micelles for targeted delivery of CBDIV17 and embelin. We evaluated the drug therapeutic efficacy of LHRH-conjugated micelles carrying CBDIV17 in an ectopic athymic mouse model of prostate cancer and will further use orthotopic model to evaluate the anti-tumor effect of LHRH-PEG-b-p(CB-co-LA) loaded CBDIV17 and embelin.
2.2. MATERIALS AND METHODS

2.2.1. Materials

2, 2-Bis(hydroxymethyl) propionic acid and benzyl bromide were purchased from Sigma Aldrich (St. Louis, MO). Hydroxyl poly(ethylene glycol) carboxyl (HOOC-PEG-OH, Mn = 5000) was purchased from Jenkem Technology (Allen, TX). SYBR Green, real-time RT-PCR master mix, and reverse transcription reagents were purchased from Roche (Indianapolis, IN). LHRH analog peptide, PYR-His-Trp-Ser-Tyr-DLys-Leu-Arg-Pro-Gly-CONH2, was purchased from Hanhong Group (Shanghai, China). CBDIV17 was synthesized as reported earlier(41). All other chemicals were of analytical grade and used as received.

2.2.2. Synthesis of LHRH-PEG-b-p(CB-co-LA)

5-Methyl-5-benzylloxycarbonyl-1, 3-dioxane-2-one (MBC) and HOOC-PEG-b-p(CB-co-LA) were synthesized as described earlier(134). Briefly, 2, 2-bis(hydroxymethyl)propionic acid (26.8 g, 0.2 mol) and potassium hydroxide (12.72 g, 0.2 mol) were dissolved in 150 mL of dimethylformamide (DMF) and allowed to heat at 100°C for 1 h. Benzyl bromide (41.5 g, 0.243 mol) was then added dropwise and continuously stirred at 100°C for 15 h. DMF was evaporated under vacuum and the crude product was dissolved in ethyl acetate (150 mL), hexanes (150 mL), and water (100 mL). The organic layer was separated, washed with water, and dried with Na₂SO₄. The final solution was evaporated to obtain benzyl 2, 2-bis(methylol)propionate (23.8 g, 56.64%), which was subsequently recrystallized using toluene.
To synthesize 5-methyl-5-benzyloxy carbonyl-1,3-dioxane-2-one (MBC) (base monomer), benzyl 2, 2-bis (methylol)propionate (22.4 g, 0.1 mol) dissolved in pyridine (50 mL) and CH$_2$Cl$_2$ (200 mL), and chilled to -78°C over dry ice. A solution of triphosgene (50 mmol, 14.8 g) dissolved in CH$_2$Cl$_2$ was added dropwise to the above solution and allowed to stir for 1 h at -78°C and for additional 2 h at room temperature. The solution was quenched with saturated aqueous NH$_4$Cl. Organic layer was washed with 1 M HCl, saturated aqueous NaHCO$_3$ and then dried with Na$_2$SO$_4$. The pure MBC (19.7 g, 88.3%) was obtained by evaporating the organic solvent under vacuum and recrystallized using ice-cold ethyl acetate.

To synthesize HOOC-PEG-b-p(CB-co-LA) with the molecular weight of 10000, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (40 μL) as a catalyst was added to the mixture of HOOC-PEG-OH (1 g), lactide (0.6 g) and base monomer (0.4 g) dissolved in 10 mL of anhydrous CH$_2$Cl$_2$ and allowed to react for 3 h under stirring at room temperature. At the end of the reaction, benzoic acid (60 mg) was added and the solvent was removed under vacuum. Crude polymer was purified by dissolving in chloroform, and precipitate in large amount of isopropanol and diethyl ether, followed by drying under vacuum for 48 h. Purified copolymer (100 mg, 0.01 mmol) and LHRH-NH$_2$ (27 mg, 0.02 mmol) were dissolved in a mixture of anhydrous DMSO (3 mL) and anhydrous CH$_2$Cl$_2$ (12 mL). The mixture was allowed to stir for 30 min following which 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide · HCl (EDC · HCl) (3.0 mg, 0.0134 mM) and 4-dimethylaminopyridine (1.0 mg, 0.008 mM) were added. After 48 h, CH$_2$Cl$_2$ was removed under vacuum and the mixture was purified by dialysis molecular mass cutoff,
3,500 Da) using water as a solvent. Purified LHRH conjugated polymer was dried by lyophilization.

Polymers were characterized using $^1$HNMR recorded on a Varian (500 MHz, T=25°C) using DMSO-$d_6$ as a solvent. The chemical shifts were calibrated using tetramethylsilane as an internal reference and reported as parts per million.

2.2.3. Critical Micelle Concentration

Fluorescence spectroscopy was used to estimate the critical micelle concentration (CMC) of HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) copolymer using pyrene as a hydrophobic fluorescent probe. Sixteen samples of HOOC-PEG-b-p(CB-co-LA) or LHRH-PEG-b-p(CB-co-LA) dissolved in water with concentrations ranging from $1.0 \times 10^{-8}$ to 1 g/L were prepared and allowed to equilibrate with a constant pyrene concentration of $6.0 \times 10^{-7}$ M overnight at room temperature with shaking at 200 rpm. The fluorescent spectra of pyrene were recorded at an excitation wavelength of 335 nm and emission wavelength of 373 nm ($I_1$) and 384 nm ($I_3$) using spectrofluorometer (Sunnyvale, CA). Peak height intensity ratio ($I_3/I_1$) was plotted against the logarithm of polymer concentration. Value of the CMC was obtained as the point of intersection of two tangents drawn to the curve at high and low concentrations, respectively.

2.2.4. Formulation and Characterization of Drug-loaded Micelles

CBDIV17 loaded micelles were prepared using the film hydration method as previously described with slight modifications(134). Briefly, 5 mg of CBDIV17 or
embelin and 95 mg of HOOC-PEG-b-p(CB-co-LA) or LHRH-PEG-b-p(CB-co-LA) were dissolved in 5 mL chloroform. Solvent was evaporated under vacuum and resulting film was hydrated in 10 mL of phosphate buffered saline (PBS), and sonicated for 10 min using a Misonix ultrasonic liquid processor (Farmingdale, NY) with an amplitude of 60. Free drug was removed by centrifugation at 5,000 rpm for 5 min and filtration using a 0.22 μm nylon filter. Drug loaded micelles were concentrated by Amicon Ultra-15 Centrifugal Filter Unit (3,000 Da) and stored at 4° C. For further characterization, mean particle size and size distribution of drug-loaded micelles were measured by dynamic light scattering using a Malvern Zetasizer (Worcestershire, UK).

2.2.5. Drug Loading and Encapsulation Efficiency

To determine the drug loading and encapsulation efficiency, drug loaded micelles were dissolved in acetonitrile (ACN). Concentration of CBDIV17 and embelin was measured by reverse phase high performance liquid chromatography (RP-HPLC, Waters, Milford, MA) with a UV detector at 290 nm for CBDVI17 and 288 nm for embelin using a reverse phase C18 Column (250 mm×4.6 mm, Inertsil ODS). The mobile phase was composed of 60:40 V/V of acetonitrile and water for CBDIV17 and methanol: water: acetic acid: tetrahydrofuran=85:15:3:0.1 v/v/v/v for embelin.

2.2.6. In Vitro Drug Release Study

Drug release from HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) micelles was determined after dialysis (3,500 Da cut off) against 25 mL PBS containing 0.1% Tween 80 (pH=7.2) in a thermo-controlled shaker with a speed of 100 rpm (5).
mL samples were taken at specific time points (1, 3, 6, 12, 24, 48, 96, 144, 192 h) and replaced with fresh PBS containing 0.1% tween 80. Drug concentration was measured using RP-HPLC as described for drug loading. Cumulative amount of drug released was evaluated as the percentage of total drug release to the initial amount. All experiments were performed in triplicate and the data reported as the mean of three individual experiments.

2.2.7. Cell Culture and Maintenance

Human prostate cancer cells C4-2 and lymph node prostate adenocarcinoma (LNCaP) were purchased from American Type Culture Collection (ATCC, Manassas, VA). LNCaP and C4-2 cells were cultured in RPMI 1640 media containing 10% fetal bovine serum, 1% antibiotic-antimitotic, and 1% sodium pyruvate at 37°C in humidified environment with 5% CO2. RWPE-1 cell line was kindly provided by Dr. Ming-Fong Lin (University of Nebraska Medical Center, Omaha, NE).

2.2.8. Cellular Uptake of Targeted Micelles

Cellular uptake study was performed as described by Zou(135) and Kutty(136) with minor modification. LNCaP and C4-2 cells were seeded at a density of 1×10^5 cells/well in 96 well plates. After reaching confluence, medium was replaced by coumarin-6 loaded micelles suspensions with concentration of 0.3 mg/ml. After 2 h incubation, the micelles solution was removed and cells were washed twice with 1 × PBS. The cells were examined using inverted fluorescent microscope after DAPI staining. For quantitative study, the cells were immersed in 0.5% Triton X-100 in 0.2 N NaOH
solutions and concentration of coumarin-6 was measured at excitation wavelength of 430 nm, and emission wavelength of 485 nm. The reading of wells with the cells alone represented the background intensity and was set up as a negative control. The reading of the wells with coumarin-6 loaded micelles (0.3 mg/mL) was used as a positive control.

2.2.9. Cell Viability Assay

Cells were seeded in 96-well plate at a density of 5,000 cells/well to determine cytotoxicity of free, HOOC-PEG-b-p(CB-co-LA) or LHRH-PEG-b-p(CB-co-LA) micelles loaded with CBDIV17 at the concentration of 10, 25, 50 μmol/L and embelin at the concentration of 5 and 10 μmol/L for 48 h. The cellular toxicity of the two polymers was also determined in these two cell lines. At the end of the treatment, the original medium was replaced by fresh medium with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for another two hours. The supernatant was removed carefully and MTT crystals were dissolved in 200 μl DMSO and analyzed at a wavelength of 560 nm. To better mimic the in vivo tumor environment, we used 3D on top assays to form sphere-like structure, Briefly, 200 μL of 100% Matrigel was used as the basement and 2x10^5 single cells were suspended in 300 μL 10% Matrigel in RPMI1640 as the growth medium in 24-well plate. After culturing for 48 h, growth medium was exchanged to growth medium containing CBDIV17 and embelin at different concentrations, which was replaced with fresh media every two days for 2 weeks before analyzing the therapeutic effect. Tumor growth was evaluated by H&E staining and immunohistochemistry.
2.2.10. Cell invasion and migration

The effect of CBDIV17 and embelin combination therapy on cell invasion and migration was determined using Transwell membrane filter inserts (pore size 8 μM) in 6-well culture plates. For invasion assay, 200 μL Matrigel (BD Biosciences, CA) was added to each transwell insert where RPMI1640 without FBS was used as the cell culture medium, while RPMI1640 with 10% FBS was added in each well. LNCaP and C4-2 cells were cultured for another 72 h after drug treatment. The number of cells invaded Matrigel was quantified after staining with crystal violet. For migration assay, 1X10^6 cells were seeded into each transwell insert and cultured for another 72 h after adding the drugs. The cell number was counted under a microscope in randomly selected three fields after crystal violet staining for 10 minutes.

2.2.11. Real-Time RT-PCR

Expression of LHRH receptor was determined by quantitative real-time RT-PCR. Briefly, LNCaP and C4-2 cells were seeded in 24-well plate at the density of 5x10^5 cells/well overnight. Total mRNA was isolated from cultured cells using RNeasy mini isolation kit (Qiagen, Valencia, CA) and the concentration was determined by Nanodrop 2000 (Thermo Scientific, Wilmington, DE). 170 ng of total mRNA was converted to cDNA using TaqMan Reverse Transcription Reagents (Life Technologies, Grand Island, NY). cDNA was used as a template and analyzed by SYBR Green universal PCR master mix (Life Technologies, Grand Island, NY) on Roche Real-time PCR instrument. S19 was used as an internal control. All samples were run in triplicate.
The primer sequences were as follows: human LHRH receptor (Type 1) (forward): 5′-GACCTTGTCTGAAAGATCC-3′, (reverse) 5′-CAGGCTGATCACCACCATCA-3′; human S19 (forward): 5′-GGAGCTCTATCCTCTCTATT-3′, (reverse): 5′-CCCAGCATGTTTGTCTAATG-3′.

2.2.1 Western Blot Analysis and ELISA

C4-2 (2×10^6 /well) cells seeded in 6-well plate were treated with CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) with the drug concentration of 25 μmol/L for 48 h. Subsequently, cells were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO) and protein concentration was measured with micro BCA protein assay kit (Thermo Scientific, Wilmington, DE). The lysate was then mixed with 6 × Laemmli Buffer (Bioworld, Dublin, OH) and boiled for 5 min. The samples were loaded to 4–15% SDS-PAGE for electrophoresis and subsequently transferred to immobilon polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in tris buffered saline at room temperature for 1 h and further incubated with primary antibody at 4°C overnight, followed by incubation with secondary antibody conjugated with infrared dyes (IRDye) at room temperature for 1 h. After washing with tris buffered saline and tween 20 for 3 times, the signal of target protein was detected using LiCOR Odyssey infrared imaging system (Li-COR, Lincoln, NE).

LNCaP and C4-2 cells were seeded in 12 well plate with the concentration of 1×10^6 cells/well and treated with free embelin, embelin loaded HOOC-PEG-b-p(CB-co-LA), and embelin loaded LHRH-PEG-b-p(CB-co-LA) for 48 h. Cells were lysed and XIAP
concentrations were detected using human total XIAP ELISA kit according to the manufacturer’s protocol.

2.2.13. Caspase 3 Activity

Caspase 3 activity was analyzed with Caspase-Glo 3 assay kit as per manufacturer’s protocol. To a single cell suspension generated by 0.25% Trypsin-EDTA digestion, 100 μL of Caspase-Glo was added with the concentration of $10^4$ cells/well in 96 well plate and incubated at room temperature for 1 h. The solution was then transferred to culture tubes to determine luminescence by a luminometer (Berthold, Bad Wildbad, Germany).

2.2.14. In Vivo Efficacy of Drug Loaded LHRH Conjugated Micelles in Ectopic Tumor Bearing Mice

All experiments were performed following the NIH animal use guidelines and the protocol was approved by the Animal Care and Use Committee (ACUC) at the University of Nebraska Medical Center. Ectopic flank tumors were induced in 6 weeks old male athymic nude mice (Jackson Laboratory, Bar Harbor, ME) by subcutaneous injection of 2 million C4-2 cells suspended in 1:1 serum free media and Matrigel. The mice were randomized into the following three groups when the tumor size reached to $150 \, \text{mm}^3$: i) untreated control, ii) CBDIV17 loaded non-conjugated micelles, iii) CBDIV17 loaded LHRH conjugated micelles. Formulations were injected intravenously via tail vein at the concentration of 10 mg/kg at 3 day intervals for 25 days. Animal body weight and tumor volume were monitored three times a week. Tumors were
measured with a caliper prior to each injection. To determine apoptosis and proliferation of tumor cells, tumors from mice were excised and fixed in 10% buffered formalin, followed by routinely proceeding to paraffin. For embedding histology, 5 μm thick sections were stained with Hematoxylin & Eosin for detection of tumor architecture. Cell proliferation and apoptosis were determined using an antibody against Ki-67 and caspase-3, respectively. The prostate cancer treatment was evaluated by PSA level.

2.2.15. Statistical Analysis

Statistical significance of difference between two groups was determined by student unpaired t-test.

2.3. RESULTS

2.3.1. Synthesis and Characterization of HOOC-PEG-b-p (CB-co-LA) and LHRH-PEG-b-p(CB-co-LA)

HOOC-PEG-b-p(CB-co-LA) copolymer was synthesized by ring opening polymerization of L-lactide and 5-methyl-5- benzyloxycarbonyl-1,3-dioxane-2-one using HOOC-PEG as the macroinitiator and DBU as a catalyst (Figure 2-1A). The peak (Figure 2-1B) at δ: 10.64 ppm demonstrated the carboxyl group and the multiplet peak at δ: 4–4.5 ppm confirms the successful ring opening polymerization. The following NMR peaks of copolymers were observed of the copolymers at δ: 1.25 (CH₃ in CB unit, s, 3H); δ: 1.60 (CH₃ in LA unit, s, 3H); δ: 3.51 (CH₂ in PEG, m, 4H); δ: 4.15–4.35 (CH₂ in CB main chain, m, 4H); δ: 5.10–5.16 (CH in LA unit q, 1H and CH₂ in CB
side group, s, 2H); δ 7.36 (phenyl, m, 5H).

LHRH-PEG-b-p(CB-co-LA) was synthesized by conjugating NH₂ group of DLys in LHRH peptide to COOH group of HOOC-PEG-b-P(CB-co-LA). Conjugation was confirmed by ¹H-NMR. The peak (Fig. 2-1C) at δ: 10.64 disappeared and new peaks were observed at δ: 6.80, δ: 6.94, δ: 7.18, δ: 7.82, δ: 8.18, δ: 8.31, δ: 8.64, δ: 8.85, and δ: 9.63, which demonstrated the successful conjugation of LHRH peptide.

![Figure 2-1. Synthesis and characterization of LHRH-PEG-b-p(CB-co-LA) copolymer.](image)

(A) Illustration of polymer synthesis (B) NMR of HOOC-PEG-b-p(CB-co-LA) (C) NMR of LHRH-PEG-b-p(CB-co-LA)

2.3.2. Preparation and Characterization of HOOC-PEG-b-p (CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) Copolymer Micelles

<table>
<thead>
<tr>
<th>CBDIV17 formulation</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Drug loading (%)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOOC-PEG-b-p(CB-co-LA)</td>
<td>82.33±3.93</td>
<td>0.237±0.022</td>
<td>4.59±0.01%</td>
<td>91.80%±0.20%</td>
</tr>
</tbody>
</table>
We used film sonication method to formulate micelles of HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) copolymers. Mean particle size of blank and drug loaded micelles was in the range of 72.64-98.91 nm as determined by dynamic light scattering (Table 2-1). Our result showed that the particle size was not influenced by LHRH conjugation. Micelles were further characterized by critical micelle concentration (CMC). Similar CMC values of HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) suggest that the conjugation of LHRH does not influence the self-assembly of the polymer (Fig. 2-2A).

2.3.3. In vitro Drug Loading and Drug Release from Micelles

The amount of CBDIV17 loaded into micelles was calculated using Eq. (1) based on 5% theoretical drug loading. According to our result (Table 2-1), CBDIV17 and embelin loading were not influenced by LHRH conjugation. CBDIV17 loading was 4.59±0.01%. There was 50% of CBDIV17 released from micelles after 24 h. LHRH conjugation did not affect the release of CBDIV17 and embelin (Fig. 2-2B and C).
2.3.4. In Vitro Cellular Uptake

To demonstrate the effect of LHRH conjugation on cellular uptake, we first determined LHRH receptor expression on LNCaP and C4-2 cancer cells and found to be overexpressed by at least two folds as determined by real time RT-PCR, while LHRH receptor was poorly expressed on RWPE-1 cells. We then determined the cellular uptake of coumarin-6 loaded micelles. Figure 2-3A show fluorescent microscope image of LNCaP and C4-2 cells after 2 h incubation with coumarin-6 loaded non-conjugated or LHRH conjugated micelles. The nucleus stained by DAPI was circumvented by green fluorescence of coumarin-6, suggesting that the micelles were internalized in the cytoplasm. Both cell lines incubated with coumarin-6 loaded LHRH-PEG-b-p (CB-co-LA) micelles exhibited brighter fluorescence. Figure 2-3B and C shows fluorescent intensity reading of the two micelles in LNCaP and C4-2 cell lines.
The uptake efficiency of non-conjugated micelles was 22.7% for LNCaP and 28.3% for C4-2. The uptake efficiency of LHRH-PEG-b-p(CB-co-LA) micelles was 33.1% for LNCaP and 35.3% for C4-2. There was no significant difference in the fluorescent intensity between HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) (data not shown). The uptake of micelles in both cell lines, which has overexpressed LHRH receptor, significantly increased with LHRH conjugation. However, LHRH conjugation did not influence cellular uptake of micelles for RWPE-1 cells, which does not have detectable LHRH receptor, according to both fluorescent image and reading (Data not shown).

Figure 2-3. Effect of LHRH conjugated micelles on the cellular uptake of antiandrogen CBDIV17.
(A) Imaging of coumarin-6 uptake in LNCaP and C4-2 cells (B) Fluorescent reading of coumarin-6 uptake in LNCaP cells (C) Fluorescent reading of coumarin-6 uptake in C4-2 cells
2.3.5. Effect of CBDIV17 Loaded LHRH-PEG-b-p(CB-co-LA) and HOOC-PEG-b-p(CB-co-LA) on LNCaP and C4-2 Growth

We determined the anticancer activity of CBDIV17 loaded micelles in LNCaP and C4-2 cells. As shown in Figure 2-4A and B, CBDIV17 exhibited dose dependent anticancer activity in both the cell lines. Drug loaded micelles were more cytotoxic than free drug. IC50 of free CBDIV17, CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA), and CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) was 25.2, 21.9, and 12.8 μM respectively in LNCaP cells. In C4-2 cells, it was 37.1, 30.9, and 19.8 μM respectively for free CBDIV17, CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA), and CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles. Additionally, there was a dramatic increase in the inhibition of prostate cancer cell growth for drug loaded LHRH-PEG-b-p(CB-co-LA) micelles compared to drug loaded HOOC-PEG-b-p(CB-co-LA) micelles, suggesting...
LHRH conjugated micelles increased cellular uptake of CBDIV17 compared to non-conjugated micelles. Similarly, we observed significant cytotoxicity of embelin formulation on LNCaP and C4-2 cells (Figure 2-5A) as well as XIAP inhibition (Figure 2-5B). To mimic the therapeutic procedure of early stage prostate cancer, we introduced androgen ablation in CBDIV17 and embelin combination therapy. Androgen ablation inhibited LNCaP cell growth (Figure 2-6A) while promoted C4-2 cell growth (Figure 2-6B) in vitro, and the effect could be reversed by DHT. In 3D cell culture model, our results indicated that combination therapy using CBDIV17 and embelin significantly inhibited LNCaP and C4-2 tumor growth determined by Ki67 staining and tumor morphology (Figure 2-7). Furthermore, CBDIV17 and embelin combination therapy suppressed tumor metastasis according to tumor invasion and migration assay (Figure 2-8).

2.3.6. Caspase 3 and AR Activity

We next examined the influence of CBDIV loaded micelles on Caspase 3. Drug loaded micelles, especially LHRH conjugated micelles, induced more cell apoptosis.
than free drug according to Caspase 3 activity (Figure 2-4C).

![Figure 2-6. Effect of combination therapy on LNCaP (A) and C4-2 (B) cell viability under androgen ablation.](image)

Furthermore, we determined the effect of drug loaded micelles on the transcriptional activity of AR by measuring protein expression of AR and prostate specific antigen (PSA) after treating LNCaP and C4-2 cells with 25 μmol/L LHRH-PEG-b-p(CB-co-LA) for 48 h. As shown in Fig. 2-4D, PSA protein expression was significantly downregulated with CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles treated, while there was little effect on AR expression.
2.3.7. In Vivo Efficacy of CBDIV17 Loaded Micelles

Since LHRH-PEG-b-p(CB-co-LA) significantly enhanced the effect of CBDIV17 in vitro, we further determined the effect of CBDIV17 loaded micelles in C4-2 ectopic tumor bearing athymic mice. CBDIV17 were formulated to HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) with the concentration of 2 mg/mL. The formulation was injected intravenously 4 times at the dose of 10 mg/kg at 3 day intervals. Changes in the relative tumor volume and body weight are shown in Figure 2-9. Tumor growth was significantly inhibited by both CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) micelles. CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles were more effective in inhibiting tumor growth compared to CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) micelles. No significant loss in body weight was observed in the whole study, suggesting acceptable toxicity of the treatment (Figure 2-9B).
Figure 2-8. Effect of combination therapy on LNCaP tumor growth and morphology by 3D model

On day 17, the tumors from these three groups were excised and incubated with Ki-67 and Caspase-3 antibodies to elucidate the mechanism of tumor suppression (Figure 2-10). The number of cell proliferation marker Ki-67 positive cells in the control group was significantly higher compared to the mice that received CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-B-P(CB-co-LA) micelles, indicating that micellar delivery of CBDIV17 efficiently suppressed tumor growth. There were only minor Ki-67 positive cells in tumor tissues from the mice injected with CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles. CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles were the most efficient in inducing cell apoptosis to the tumor tissues, leading to high Caspase-3 expression. Furthermore, nucleus intensity of CBDIV17/LHRH-PEG-b-p(CB-co-LA) group was obviously less other two groups, suggesting tumor growth has been successfully suppressed. Enhanced Caspase-3 expression was also observed in CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) treated tumor compared to control group, which proved antitumor efficacy of non-target delivery of CBDIV17.

Figure 2-9. Effects of CBDIV17/HOOC-PEG-b-p(CB-co-LA) and CBDIV17/LHRH-PEG-b-p(CB-co-LA) micelles on tumor growth in vivo. (A) Tumor volume (B) Body weight
2.4. DISCUSSION

Androgen inhibition is the first choice for treating early stage prostate cancer. However, AR mutation and drug resistance after prolonged treatment makes this approach less effective. Thus, more potent drugs to block androgen activity or inhibiting multiple targets including IAP are necessary to treat advanced prostate cancer. We have previously shown that CBDIV17 was more potent than bicalutamide in inhibiting the proliferation of LNCaP and C4-2 cells (41). However, the poor solubility of CBDIV17 and embelin (less than 50 mg/L) limit their potential applications. To solve this issue, we thereby have formulated polymeric micelles, which solubilize the drugs with their hydrophobic core. Furthermore, polymeric micelles exhibit passive targeting due to the EPR effect causing preferential accumulation in tumors and inflammation sites. For targeted delivery of drug loaded micelles to the tumors, LHRH peptide was conjugated to HOOC-PEG-b-p(CB-co-LA) copolymer for targeting delivery of CBDIV17 to tumor site after systemic administration. LHRH peptide provides an effective targeting ligand to LHRH receptors overexpressed in prostate cancer cells and limited drug accumulation in normal organs, where LHRH receptors are not expressed.
detectably. Due to the wide application of LHRH analog, such as leuprolide, buserelin and histrelin, in the clinical trials, active targeting by LHRH analog are expected to be safe and efficient.

**Figure 2-10. Effects of CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) and LHRH-PEG-b-p(CB-co-LA) micelles on Ki-67, Caspase-3, and PSA expression in tumor.**

A modified synthetic analog of LHRH, which has free amine group to be linked with polymer without affecting its function, was used as a targeting moiety to LHRH receptors. Therefore, the choice of conjugation with –COOH or –NHS was offered(137, 138). According to our NMR spectra and cytotoxicity results, the conjugation of LHRH with HOOC-PEG-b-p(CB-co-LA) was successful and there was significant difference in cytotoxicity when LHRH was conjugated to the micelles. Our results suggest that—COOH is efficient to conjugate with LHRH for targeting LNCaP and C4-2 cells. LHRH conjugation did not affect the release profile of CBDIV17 (Fig. 2-2B). After LHRH conjugation, cellular uptake was significantly enhanced in LHRH receptor overexpressed cell lines (Fig. 2-3).

LHRH has been an effective systemic treatment for prostate cancer for the past 7 decades(139). Thus, we first demonstrated the non-cytotoxicity of free LHRH analog,
HOOC-PEG-b-p(CB-co-LA), and LHRH-PEG-b-p(CB-co-LA) (data not shown). We observed significant cytotoxicity caused by LHRH conjugated micelles with 10 μM of CBDIV17 in LNCaP cells and 25 μM in C4-2 cells after incubating for 48 h (Figure 2-4A and B). Significantly lower IC 50 demonstrated higher efficacy and cellular uptake of CBDIV17 after coating with LHRH-PEG-b-p(CB-co-LA). Blockage of androgen activity did not show high efficiency in C4-2 cells, which is androgen independent. Significantly higher Caspase 3 activity was also observed upon treatment with CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles as compared to free drug and drug loaded by non-conjugated micelles, suggesting higher cell apoptosis.

PSA, which is a pivotal downstream target gene of androgen receptor(140), is always elevated in the presence of prostate cancer and other prostate disorders. Blocking AR would reduce the transcription activity of AR, which reduces the expression of PSA. Our data (Figure 2-4D) showed that AR protein expression was not influenced by CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles while PSA expression was significantly inhibited. These results were similar to previously published results of bicalutamide(141, 142). It means CBDIV17 does not downregulate AR expression but binds to AR and prevent AR activation, which is same as the mechanism of bicalutamide.

Following in vitro characterization, CBDIV17 were formulated and concentrated to HOOC-PEG-b-p(CB-co-LA) or LHRH-PEG-b-p(CB-co-LA) to evaluate tumor suppression efficacy in mice bearing C4-2 xenografts. Consistent with in vitro data, CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) micelles was more potent in repressing
prostate tumor growth compared to CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) and control groups. To further determine the mechanism of antitumor effect, we evaluated the expression of cell proliferation marker Ki-67 and apoptosis marker Caspase-3 to elucidate cell proliferation and apoptosis level in tumor tissue. Few Ki-67 positive cells and high Caspase-3 expression level demonstrated significant in vivo antitumor efficacy of CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA). We also observed similar but less effect of CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) and tumor growth suppression after CBDIV17 loaded HOOC-PEG-b-p(CB-co-LA) treatment, which is probably caused by EPR effect.

2.5. CONCLUSION

In conclusion, we have successfully conjugated LHRH peptide to HOOC-PEG-b-p(CB-co-LA) and demonstrated that LHRH conjugation did not affect CMC, CBDIV17 loading, and drug release profile. We also revealed the mechanism of CBDIV17 as an androgen receptor antagonist. Finally, CBDIV17 loaded LHRH-PEG-b-p(CB-co-LA) showed significant potential to suppress tumor cell growth in vitro and in vivo.
CHAPTER 3. MICELLAR DELIVERED RUBONE AS MIR-34A MODULATOR FOR TREATING PACLITAXEL RESISTANT PROSTATE CANCER

3.1. INTRODUCTION

Most prostate cancers relapse within two years into hormone refractory due to the presence of tumor initiating cells, known as cancer stem cells (CSCs), which are involved in tumor progression and metastasis, but are resistant to chemotherapy. Recently, aberrant expression of miRNAs is critically implicated in cancer initiation, progression, migration, and chemoresistance (143, 144). Among these miRNAs, miR-34a is significantly underexpressed in chemoresistant prostate cancer cell line (86) or CD44+ cancer stem cells (87). As a tumor suppressor miRNA, miR-34a is responsible for promoting tumor cell apoptosis, inhibiting tumor metastasis (88) and chemoresistance (89). Thus, miR-34a replenishment might be a novel therapeutic method to reverse PTX-resistance for the treatment of chemoresistant prostate cancer. Kojima et al. reported that miR-34a reverses PTX resistance by targeting the downstream genes including SIRT1 and Bcl-2 (145). Yao et al. reported that combination therapy using doxorubicin and miR-34a synergistically enhanced the antitumor property of doxorubicin and inhibited DU145 cell formed tumor growth in vivo (146). Nonetheless, intrinsic challenges associated with oligonucleotide-based miRNA replenishment including off-target effects, poor cellular uptake, and in vivo instability hindered its clinical translation. Even though numerous miRNA delivery systems were developed, most of them were proved less effective or toxic for clinical use (8). Thus, to reverse the aberrant expression of miR-34a by small molecules might
be a potent alternative method for the treatment for PTX-resistant prostate cancer.

Natural and synthetic analogs of chalcones and isoflavones exhibit promising anticancer activity. However, only a few studies have focused on the role of chalcone derivatives on modulation of miRNAs. Rubone(68), isoliquiritigenin(147), and kuwanon V(148) modulate miR-34a, miR-25, miR-9, miR-29a and miR-181a, respectively with potent biological actions. Among these small molecules, Xiao et al. first reported rubone, a chalcone analog, as a miR-34a modulator for the inhibition of hepatocellular carcinoma (HCC) growth(68). In their study, rubone upregulated miR-34a expression in a p53 dependent manner, downregulated the downstream target Bcl-2 and Cyclin D1 expression, and suppressed HCC growth in vivo. However, the antitumor efficacy of rubone as a miR-34a modulator for treating PTX resistant prostate cancer and the underlining mechanisms remains largely unknown. Furthermore, poor aqueous solubility of PTX and rubone (less than 50 mg/L) results in low and variable drug absorption. Thus, novel drug delivery systems for co-delivery of both drugs are required for combination therapy against PTX resistant prostate cancer.

Since the use of solubilizing agents and surfactants may cause organ and systemic toxicity, biodegradable polymers, which can self-assemble into nano-sized micelles, are gaining much attention. Polymeric micelles have spherical structures with a hydrophilic corona and hydrophobic core, which improves the aqueous solubility and stability of hydrophobic drugs(40). The stealth properties of poly (ethylene glycol) (PEG) hydrophilic corona of micelles prevents their recognition by reticuloendothelial system (RES) and therefore minimize their rapid elimination via the EPR effect. In our previous
study, we designed and synthesized poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD), which significantly enhanced the aqueous solubility of embelin, an X-linked inhibitor of apoptosis protein (XIAP) inhibitor(149). Here, we determined the effect of rubone on miR-34a and its target genes and investigated whether it can chemosensitize PTX resistant prostate cancer cells and synergistically inhibit orthotopic prostate tumor in nude mice when administrated intravenously as a micellar formulation with PTX.

3.2. MATERIALS AND METHODS

3.2.1. Cell lines and culture condition

Prostate cancer cell lines LNCaP, C4-2, DU145, and PC3 were purchased from the ATCC and cultured in RPMI1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 37°C incubator supplemented with 5% CO₂. The PTX resistant version of DU145 and PC3 (DU145-TXR and PC3-TXR) were provided by Dr. Evan T. Keller from the University of Michigan. Normal prostate epithelial RWPE-1 cells were provided by Dr. Ming-Fong Lin from UNMC and cultured in complete keratinocyte growth medium, K-SFM (Life Technologies) supplemented with 50 μg/mL of bovine pituitary extract and 5 ng/mL of EGF.

3.2.2. RT-PCR and Western blot analysis

Following the treatment of different concentrations of rubone or PTX and rubone combination therapy, total mRNA was isolated using RNeasy isolation kits (Qiagen, Valencia, CA) and 170 ng total RNA was converted to cDNA using miR-34a
primer before analyzing for miR-34a concentration. To determine protein concentration, cell protein was extracted using RIPA buffer after treatment with rubone at the dose of 5 μM and 10 μM for 48 h. The amount of protein was adjusted to the same concentration, transferred to PVDF membrane, incubated with primary and secondary antibodies, followed by Licor Odyssey system analysis (LI-COR Biotechnology, Lincoln, NE). The primary antibodies used in Western blot and following immunohistochemistry studies are as follows: anti-E-cadherin (Abcam, ab15148), anti-SIRT1 (Santa Cruz, sc-15404), anti-Cyclin D1 (Abcam, ab16663), anti-p53 (Santa Cruz, sc-6243), anti-Bax (Santa Cruz, sc-6236), anti-β-actin (Santa Cruz, sc-1616), anti-TAp73 (Santa Cruz, sc-7957), anti-Elk-1 (Santa Cruz, sc-355).

3.2.3. Cell viability in 2D and 3D models

In 2D model, cell viability was determined by MTT assay after treating the cells with different concentrations of rubone or 5 μM rubone plus different concentrations of PTX. The anti-tumor effect of the combination therapy using PTX and rubone was also determined using 3D tumor model(150) including 3D on top and hanging-drop models. For 3D on top assays in 24 well plate, 200 μL of 100% Matrigel was used as the basement and 2x10^5 single cells were suspended in 300 μL 10% Matrigel in RPMI1640 as the growth medium. After culturing for 48 h, growth medium was exchanged to growth medium containing PTX and rubone at different concentrations, which was replaced with fresh media every two days for 2 weeks before analyzing the therapeutic effect. For the hanging drop model, 40 μL medium containing 4000 cells were added in each well of 3D 96-well hanging drop plate (3D Biomatrix, Ann Arbor, MI) and drug
containing medium was changed every two days for 3 weeks till sphere formation of
the control group.

3.2.4. Cell invasion and migration

The effect of PTX and rubone combination therapy on cell invasion and
migration was determined using Transwell membrane filter inserts (pore size 8 μM) in
6-well culture plates. For invasion assay, 200 μL Matrigel (BD Biosciences, CA) was
added to each transwell insert where RPMI1640 without FBS was used as the cell
culture medium, while RPMI1640 with 10% FBS was added in each well. PTX resistant
DU145-TXR and PC3-TXR cells were cultured for another 72 h after drug treatment.
The number of cells invaded Matrigel was quantified after staining with crystal violet.
For migration assay, 1X10^6 cells were seeded into each transwell insert and cultured
for another 72 h after adding the drugs. The cell number was counted under a
microscope in randomly selected three fields after crystal violet staining for 10 minutes.

3.2.5. Role of CSCs in chemoresistance

We further analyzed CSC population in DU145-TXR and PC3-TXR after
treatment with PTX, rubone and their combination using Aldeflour reagent (Stemcell
Technologies, Vancouver, BC) based flow cytometry. Cells were suspended in
suspension media and stained by Aldeflour reagent, while a negative control
comprising cells treated with ALDH-inhibitor diethylamino-benzaldehyde (DEAB) was
included to gate the unspecific staining.
3.2.6. Polymer synthesis, micelle formulation, and characterization

Poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-dodecanol) (PEG-PCD) was synthesized and characterized by $^1$H NMR as described previously (149). PTX and rubone loaded micelles were prepared by film hydration with 10% theoretical drug loading. Chloroform as solvent was evaporated under vacuum and resulting film was hydrated in 10 mL of phosphate buffered saline (PBS), and sonicated for 10 min using Misonix ultrasonic liquid processor (Farmingdale, NY) with an amplitude of 30, followed by removing the free drug at 5000 rpm centrifugation for 5 min. Blank or drug loaded micelles were characterized by measuring particle size using Malvern Zetasizer (Worcestershire, UK).

To determine the drug loading and encapsulation efficiency, drug loaded micelles were dissolved in 1 mL mobile phase composed of 70:30 V/V of acetonitrile and water. Concentrations of PTX and rubone were measured by reverse phase high performance liquid chromatography (RP-HPLC, Waters Milford, MA) with a UV detector at 228.6 nm for PTX and 324.3 nm for rubone using a reverse phase C18 column (250 mmX4.6 mm, Inertsil ODS). We also compared the drug loading capability of PEG-PCD with commercially available poly (ethylene glycol)-polylactide (PEG-PLA) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol) (DSPE-PEG) of similar molecular weights. PTX and rubone release from PEG-PCD micelles was determined after dialysis (2500-5000 Da cut of) against 50 mL PBS containing 20% ethanol as a co-solvent, which did not dissolve PEG-PCD and break the micellar structure at 37°C in a temperature controlled shaker at the speed of 100
rpm. 1 mL sample was taken at specific time points (1, 2, 3, 6, 12, 24, 48, and 96 h) and replaced with 1 mL PBS containing 20% ethanol. The sample was dissolved with the mobile phase after removing the solvent using a rotary evaporator, followed by determining drug concentration using HPLC.

We further estimated the in vivo stability of PEG-PCD micelles using time-dependent Fluorescence Resonance Energy Transfer (FRET) in the presence of 20% FBS. 50 μg of 3,3'-dioctadecyloxycarbocyanine perchlorate (DiO) as a lipophilic fluorescent energy donor and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) as an acceptor, 1 mg of PTX and rubone were loaded into 10 mg of PEG-PCD. Emission fluorescence spectra ranging from 490 to 590 nm was recorded at an excitation wavelength of 488 nm (donor excitation) and resulted in a strong emission at 565 nm (acceptor emission). We further compared the micelle stability of PEG-PCD with PEG-PLA and DSPE-PEG after drug loading. Cytotoxicity of free drug and drug formulated PEG-PCD micelles were also compared in DU145-TXR and PC3-TXR cells.

3.2.7. In vivo tumor studies

All animal experiments were performed in accordance with the NIH animal use guideline and protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center. To visualize and monitor tumor progression, we developed orthotopic prostate tumor using stably transfected prostate cancer cells with lentivirus encoding GFP and luciferase (LP-hLUC-Lv201-0200,
Genecopoeia, Rockville, MD). A midline incision was made in the lower abdomen of 8 weeks old male nude mice to expose the dorsal prostate lobe, where 30 μL PBS containing 1X10^6 PC3-TXR expressing GFP and luciferase (PC3-TXR-GFP-Luc) was injected. Three weeks after tumor cell injection, mice with orthotopic tumor was randomly divided into four groups of 10 animals per group with blank micelles, PTX (20 mg/kg) loaded micelles, rubone loaded micelles (20 mg/kg), and PTX and rubone (10 mg/kg for each drug) loaded micelles. These formulations were injected intravenously for five doses every other day. The body weight and tumor fluorescence of the mice were recorded once a week. Followed by the last formulation injection, four mice in each group were sacrificed and tumors were excised for determining miR-34a expression by RT-PCR. The therapeutic effect of formulation was further determined by immunohistochemistry (IHC) for Bax, Ki-67, and miR-34a downstream targets including SIRT1, E-cadherin, and Cyclin-D1. The side effects of each formulation were evaluated by hematoxylin and eosin (H&E) staining of the major organs including heart, liver, spleen, lung, and kidney. Other mice were monitored for another two weeks to further evaluate the anticancer efficacy of the formulation.

3.2.8. Statistical Analysis

Results were presented as the mean ± S.E.M. from three experiments for in vitro studies and six experiments for in vivo studies. The statistical difference between the two groups was calculated by unpaired Student’s t-test, and a P < 0.05 was considered to be statistically significant.
3.3. Results

3.3.1. Rubone upregulated miR-34a and reversed the expression of miR-34a downstream targets in PTX-resistant prostate cancer cell lines

Our objective was to determine whether rubone could serve as a miR-34a modulator to reverse the miR-34a downstream tumor-associated gene expression. Thus, we first determined miR-34a expression in different prostate cancer cell lines. miR-34a expression was markedly downregulated in androgen-refractory DU145, PC3, and PTX resistant DU145-TXR and PC3-TXR cells compared to androgen dependent LNCaP as well as normal prostate epithelial RWPE-1 cells (Figure 3-1A), indicating the role of miR-34a in the initiation and progression of prostate cancer (151). Next, we evaluated the cytotoxicity of rubone in these cell lines. As shown in Figure 3-1B, rubone exhibited significantly higher cytotoxicity in DU145-TXR and PC3-TXR cells, suggesting that rubone has stronger anticancer effect in advanced prostate cancer cells, which have lower miR-34a expression (Figure 3-1A). However, rubone did not show obvious toxicity to normal prostate cells (RWPE-1) and hormone sensitive prostate cancer cells (LNCaP) with high miR-34a expression, indicating rubone induced cytotoxicity through miR-34a related pathways. Rubone upregulated miR-34a in PTX-resistant DU145-TXR and PC3-TXR cell lines in a dose dependent manner (Figure 3-1C). After evaluating the anticancer effect of rubone, we determined miR-34a downstream target gene expression after rubone treatment. PTX resistant cell lines showed more chemoresistance related SIRT1 expression (152) and less metastasis related E-cadherin expression (153) (Figure 3-1D and E). Rubone
significantly reversed the expression of miR-34a downstream gene targets of DU145-TXR and PC3-TXR cell lines (Figure 3-1D and E), including E-cadherin, SIRT1, and Cyclin D1, whereas E-cadherin expression was not reversed in DU145-TXR cell line. Furthermore, rubone monotherapy promoted cell apoptosis determined by Bax expression in DU145-TXR and PC3-TXR cell lines. However, rubone showed less effect of reversing miR-34a downstream targets and inducing apoptosis in non-resistant DU145 and PC3 cell lines. These data indicated the downregulation of miR-34a in the progress of prostate cancer and suggest that rubone might work as a specific miR-34a modulator to reverse miR-34a expression for the treatment of androgen-refractory and highly metastatic prostate cancer.

Figure 3-1. Effect of rubone monotherapy on miR-34a expression. A. miR-34a expression in prostate cancer and normal prostate cell lines. B. Cytotoxicity of rubone on prostate cancer and normal prostate cell lines. C. Rubone upregulated miR-34a expression in DU145-TXR and PC3-TXR in a dose dependent manner. Rubone alone reversed the expression of miR-34a downstream proteins in DU145 (D) and PC3 (E) cell lines.
3.3.2. Rubone enhanced the anticancer effect of PTX in PTX-resistant prostate cancer cell lines by reversing the expression of miR-34a downstream targets.

To determine that miR-34a is an anticancer target for reversing chemoresistance of prostate cancer, we evaluated the gene regulation efficiency of miR-34a mimic and miR-34a inhibitor and their effect on PC3-TXR viability. Using lipofectamine 2000 as the transfection reagent, miR-34a mimic and inhibitor could upregulate and suppress miR-34a expression in PC3-TXR cell line, respectively (Data not shown). miR-34a inhibited PC3-TXR cell viability, whereas miR-34a inhibitor promoted cell growth (Data not shown). We further demonstrated that miR-34a can enhance PTX chemotherapy, whereas miR-34a inhibitor promoted cancer cell viability (Figure 3-2A) with PTX treatment in PC3-TXR cell line. Rubone did not enhance the anticancer effect of PTX in chemosensitive DU145 and PC3 cell lines (Figure 3-2B and D), but significantly reversed chemoresistance of DU145-TXR and PC3-TXR cell lines (Figure 3-2C and E). miR-34a inhibitor reversed the effect of rubone on promoting PTX cytotoxicity (Figure 3-2F), indicating rubone promote the effect of PTX through upregulating the expression of miR-34a. To mimic the complexity of in vivo tumor environment, we determined the anticancer effect of PTX and rubone in 3D tumor model. PTX and rubone combination therapy inhibited PC3-TXR cell growth and sphere formation in 3D model, including 3D on top (Figure 3-2G) and hanging drop model (Figure 3-2H). Similar with rubone monotherapy, PTX and rubone combination therapy more effectively reversed the expression of miR-34a downstream gene expression in DU145-TXR and PC3-TXR cell lines compared to non-resistant cell lines.
PTX reduced the expression of E-cadherin in DU145 and PC3 cell lines, whereas PTX and rubone failed to reverse E-cadherin in DU145-TXR cell line. Thus, rubone could work as a miR-34a modulator to reverse the PTX-resistance in prostate cancer and restore the expression of miR-34a targeted genes.

3.3.3. Rubone inhibit cell invasion, migration, and CSC population in a p53-independent pathway

Treatment of prostate cancer always failed due to the metastasis and the presence of CSCs, which is highly chemoresistant. Thus, we further determined
the effect of PTX and rubone combination therapy on the invasion and migration of 
DU145-TXR and PC3-TXR cells. Rubone alone or its combination with PTX 
significantly inhibited DU145-TXR and PC3-TXR invasion (Figure 3-3A and C) and 
migration (Figure 3-3B and D). Furthermore, rubone or its combination with PTX 
significantly downregulated aldehyde activity, which is a CSC marker (Figure 3-3E). 
Collectively, our results demonstrated that combination therapy using PTX and rubone 
significantly reversed chemoresistance, inhibited tumor cell migration and invasion, 
and decreased the CSC population of androgen-refractory prostate cancer cells.

Previously research claimed that miR-34a and p53 axis regulates miR-34a 
expression and tumor suppression(156, 157). However, our results indicated that there 
was no change in p53 expression after rubone treatment, even in PC3-TXR cell line 
(p53 null) (Figure 3-1E and 2J). These results suggest that rubone might upregulate 
miR-34a in p53 independent pathways, including TAp73(90, 158) and Elk-1(159, 160). 
Thus, we determined TAp73 and Elk-1 expression after rubone alone or with PTX. 
Rubone monotherapy or PTX and rubone combination therapy significantly enhanced 
TAp73 and Elk-1 expression (Figure 3-3F and G), suggesting p53 independent 
pathway plays a crucial role in miR-34a upregulation by rubone.
**Figure 3-3. PTX and rubone combination therapy inhibit cell migration, invasion, and CSC population in p53 independent manner.**

The effect of rubone monotherapy or PTX and rubone combination therapy on tumor cell invasion (A, upper line, DU145-TXR; bottom line, PC3-TXR) and migration (B, upper line, DU145-TXR; bottom line, PC3-TXR) were determined by crystal violet staining and quantified (C, invasion; D, migration). E. CSC population were analyzed by Aldefluor staining and quantified by flow cytometry (upper line, DU145-TXR; bottom line, PC3-TXR). PTX and rubone combination therapy reversed miR-34a downstream protein expression of DU145 and PC3 cell lines in p53 independent manner. Rubone alone (F) or with PTX (G) upregulated Tap73 and ELK-1 expression.

### 3.3.4. PEG-PCD micelles are effective drug delivery system for PTX and rubone

Polymeric micelles have been widely used for improving the solubility and enhancing the in vivo stability of hydrophobic drugs. In this study, we used PEG-PCD to form micelles for co-delivery of PTX and rubone. After chemical synthesis as previously described(149), PEG-PCD polymer was characterized by 1H NMR and the particle size distribution of micelles before and after drug loading was measured by dynamic light scattering (Table 3-1). PEG-PCD could form micelles with low polydispersity and drug loading did not affect the particle size. We further compared drug loading and micelle stability of PEG-PCD micelles with two commercially available...
polymers PEG-PLA and DSPE-PEG. After setting up standard methods of HPLC for measuring PTX and rubone concentrations, we determined the drug loading and micelle stability of PEG-PCD, PEG-PLA, and DSPE-PEG. PEG-PCD had the highest drug loading of 9.70 ± 0.10 and 5.34 ± 0.02% for both PTX and rubone (Figure 3-4A and B), respectively compared to PEG-PLA which showed 4.18±0.03%, 1.51±0.02%, and DSPE-PEG with 3.41±0.36% and 3.58±0.27%. We then determined PTX and rubone release from PEG-PCD micelles. Interestingly, PEG-PCD micelles carrying both PTX and rubone had a slower drug release profile compared to single drug loaded micelles (Figure 3-4C and D), probably because hydrophobic and π-π interaction between the two drugs slow down the drug release. We evaluated the cytotoxicity of PTX and rubone formulation on DU145-TXR and PC3-TXR cells. PEG-PCD micelles did not show cytotoxicity to each prostate cancer or normal prostate cell lines (data not shown). Micelle encapsulation decreased cytotoxicity of the combination therapy compared to free drug (Figure 3-4E and F) in DU145-TXR and PC3-TXR cell lines, possibly due to slow drug release from the micelles. To summarize the drug loading and release issues, PEG-PCD micelle might be a potent drug delivery system for in vivo PTX and rubone delivery.

Table 3-2 Characterization of PTX and rubone formulation

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-b-PCD (Blank)</td>
<td>136.50±1.40</td>
<td>0.208±0.010</td>
</tr>
<tr>
<td>PEG-b-PCD (PTX)</td>
<td>132.00±0.72</td>
<td>0.211±0.021</td>
</tr>
<tr>
<td>PEG-b-PCD (Rubone)</td>
<td>150.30±1.43</td>
<td>0.200±0.015</td>
</tr>
</tbody>
</table>
Figure 3-4. Characterization of PEG-PCD micelles.
PTX and rubone loading of DSPE-PEG, PEG-PLA, and PEG-PCD were measured by HPLC using standard curves of PTX and rubone. PTX and rubone loading were summarized in A and B for PTX and rubone, respectively. PTX (C) and rubone (D) release profile in single drug loaded micelles or PTX and rubone loaded micelles were determined by HPLC. The effect of PEG-PCD encapsulation on drug toxicity was determined in DU145-TXR (E) and PC3-TXR (F) cell lines.

3.3.5. PEG-PCD micellar formulation of PTX and rubone suppressed PTX-resistant prostate tumor growth in vivo

To monitor the tumor growth and metastasis in the orthotopic prostate cancer bearing nude mice, we first transduced PC3-TXR cell line with lentivirus expressing both GFP and luciferase. After injecting the cells into dorsal prostate lobe, tumor development was monitored by intraperitoneally injecting luciferin and recording body weight every week. The presence and location of prostate tumor was shown in Figure 3-5A. Tumor fluorescence at 7th week in PTX and rubone combination therapy group was significantly lower than the other three groups (Figure 3-5B). The tumor inhibitory effect was also demonstrated by weekly monitoring the luminescence (Figure 3-5C), which indicating the suppressed tumor growth in combination therapy group. This
orthotopic prostate cancer mouse model is very aggressive so that we observed 20% body weight loss during the treatment, while PTX and rubone formulation therapy had little effect on body weight loss, suggesting the inhibition of tumor growth in combination therapy group (Figure 3-5D). Finally, all the mice were sacrificed to isolate the tumor for measuring the size (Figure 3-5E), which suggest significant tumor suppressing effect of PTX and rubone formulation. miR-34a in tumor was significantly upregulated with rubone alone or combined with PTX treatment (Figure 3-5F). To further demonstrate the anticancer mechanism of rubone in vivo, we isolated the tumor and determined cell proliferation marker and miR-34a downstream targets expression (Figure 3-6). Rubone alone or with PTX significantly reversed E-cadherin, Cyclin D1, and SIRT1 expression. Rubone monotherapy failed to suppress tumor cell proliferation as indicated by Ki-67 staining, whereas PTX and rubone combination therapy significantly suppressed tumor cell growth compared to PTX monotherapy. We further determined TAp73 and Elk-1 expression in tumor tissue. Rubone alone or combination therapy with PTX significantly upregulated TAp73 and Elk-1 expression. These data indicated that rubone upregulated miR-34a expression in p53 independent pathways in vivo. Collectively, our results demonstrated that rubone is a potent small molecule miR-34a modulator to reverse the chemoresistance of advanced androgen-refractory prostate cancer and enhance the therapeutic effect of PTX.
Figure 3-5. Anti-tumor efficacy of PTX and rubone combination therapy.
Orthotopic prostate cancer model was generated using luciferase expressed PC3-TXR cell line. Drug formulation was injected every two days after 3 weeks. A. Location of orthotopic tumor generated from PC3-TXR cell. B. In vivo tumor luminescence was determined by intraperitoneal injection of luciferin (120 mg/kg). C. Body weight of mice in each group. D. miR-34a expression in tumor was determined by RT-PCR. E. Bioluminescence and size of tumors in each group after 7 weeks.

3.4. Discussion

Drug resistance remains the major challenge of cancer chemotherapy even with the discovery of highly efficient anticancer compounds. Furthermore, the skeletal metastasis in advanced prostate cancer patients is the major cause of morbidity and mortality(161). Tumors are composed of bulk cancer cells and small population of CSCs, which are not responsive to most chemotherapeutic agents and result in chemoresistance and tumor recurrence(162). In recent years, miR-34a was found to inhibit CSC growth, metastasis, and chemoresistance by directly repressing the adhesion molecule CD44(87). The downstream targets of miR-34a, including SIRT1(163), LEF1(164), TCF7(165), AR, and Notch-1(166), are crucial factors of proliferation, metastasis, and chemoresistance of advanced androgen-refractory prostate cancer. Furthermore, our data indicated that miR-34a is significantly
downregulated in the progress of prostate cancer, especially in PTX resistant cells (Figure 3-1A). Thus, miR-34a replenishment by systemic delivery using nanoparticles can therefore be developed as a potent therapeutic strategy. However, the off-target effects, in vivo degradation, low efficacy and high cytotoxicity associated with drug delivery systems of miRNA oligonucleotide still need to be overcome for miRNA based clinical therapy.

![Figure 3-6. Mechanism of miR-34a regulation in vivo. E-cadherin, Ki-67, cyclin D1, SIRT-1, Tap73, and Elk-1 expression after PTX and rubone formulation therapy was determined by IHC (Scale bar, 200 μM for backward figure and 50 μM for enlarged figure).](image)

Recently, there are several researches showing small molecular as oncogenic miRNA inhibitor(67, 70) and tumor suppressor miRNA modulator(68) for the inhibition of tumor growth. Among these small molecules, retinoic acid(90), genistein(167), and rubone(68) have been reported to upregulate miR-34a expression in several types of cancer with the mechanism not well characterized. Traditional chemotherapy uses PTX or docetaxel as a monotherapy for inhibiting cancer cell growth, which always fails due to the chemoresistance caused by downregulation of tumor suppressor miRNA. In this study, we present an alternative strategy for fighting PTX resistant prostate cancer through miR-34a upregulation by employing a combination therapy using
rubone as a small molecule miR-34a modulator. Our data suggest that rubone is non-toxic to normal prostate cells, whereas is highly toxic to PTX resistant prostate cancer cells, which have low miR-34a expression (Figure 3-1B). For combination therapy with PTX, rubone can reverse the chemoresistance of prostate cancer at low concentration (5 μM). At this concentration, rubone significantly enhanced the cytotoxicity of PTX in PTX-resistant prostate cancer cell lines, whereas did not influence the anticancer effect of PTX in non-resistant cell lines. Extracellular matrix is key regulator of homeostasis and tissue phenotype to form 3D culture assays(168), which allows the phenotypic discrimination between nonmalignant and malignant mammary cells. Since some crucial signals are lost when cells are cultured in vitro on 2D plastic flasks(150), 3D model could better mimic the in vivo tumor environment and evaluate the anticancer effect of therapeutic agents. Thus, we determined the anti-tumor efficacy of PTX and rubone combination therapy in 3D model (Figure 3-2G and H), where 3D on top allows the tumor to grow on extracellular matrix (Matrigel) and hanging-drop model can help tumor cell form sphere-like structure without Matrigel. PTX and rubone combination therapy inhibited tumor cell growth and disturbed tumor morphology in 3D models. These data indicated that rubone can work as a non-toxic, highly specific miR-34a modulator to enhance the therapeutic effect of PTX.

Previous report claimed that rubone inhibits HCC growth in a p53 dependent manner(68). In that research, rubone has no therapeutic effect in Hep3B cells, which does not express p53. Interestingly, our results showed that rubone significantly reversed miR-34a and its downstream target gene expression in p53-null PC3-TXR
cells (Figure 3-2E and J). Furthermore, rubone enhanced the therapeutic effect of PTX, inhibited the metastasis, and decreased the population of CSC in PC3-TXR cells (Figure 3-3A-E), suggesting that rubone might upregulate miR-34a in a p53 independent pathway. Therefore, we analyzed TAp73(90, 158) and Elk-1(159, 160) expression, which are previously reported to be p53 independent miR-34a regulation pathway. Our data showed that TAp73 and Elk-1 were highly upregulated after rubone monotherapy or PTX and rubone combination therapy (Figure 3-3F and G). This discrepancy may be explained by the extremely low expression of TAp73(169) and Elk-1(170) in Hep3B cells compared to PC3-TXR cells, which means that all known miR-34a regulation pathways are blocked in Hep3B cells. Thus, we conclude that rubone might work as a miR-34a modulator for prostate cancer in a p53 independent manner.

Polymeric micelles can increase aqueous solubility of hydrophobic drugs thereby avoiding the use of toxic solubilizing agents, including DMSO and Cremophor EL. In this study, we synthesized PEG-PCD lipopolymer, which allows the conjugation of multiple lipid chains to a polycarbonate backbone for the optimization of drug loading. The pendant lipid groups in the lipopolymers could increase the interaction of hydrophobic drugs with the core, improve in vivo micelle stability, and prolong circulation half-life. Thus, we compared the drug delivery property of our PEG-PCD with two commercially available polymers PEG-PLA and DSPE-PEG. PEG-PCD has higher PTX and rubone loading compared to PEG-PLA and DSPE-PEG, especially when loading both drugs (Figure 3-4A and B). The decreased drug release was
observed when loading both drugs, indicating drug-drug interaction in the same drug delivery platform could influence the drug delivery property.

![Diagram](image)

**Figure 3-7.** Illustration of rubone working as a miR-34a modulator for combination therapy with PTX.

The anticancer efficiency was evaluated in an orthotopic prostate tumor model to mimic the clinical condition and monitor tumor growth in a non-invasive manner. Tumor growth was significantly suppressed after systemic administration of PTX and rubone formulation compared to other three groups according to the luminescence at each time point (**Figure 3-5C**) and the tumor size at the end of the study (**Figure 3-5E**). Our data also indicated that PTX and rubone combination therapy reversed the downstream target genes of miR-34a through TAp73 and Elk-1 pathways (**Figure 3-6**). However, this orthotopic model using PC3-TXR cell line was very aggressive since we observed severe body weight loss in the progress of tumor (**Figure 3-5D**) and few mice could survive for more than 7 weeks without treatment. Under this severe condition, PTX and rubone combination therapy showed promising therapeutic effect by suppressing the tumor growth and avoid body weight loss.
3.5. CONCLUSION

Based on our results, rubone could be a specific miR-34a regulator to reverse miR-34a and the downstream target gene expression for PTX resistant prostate cancer (Figure 3-7). The replenished miR-34a promoted the anticancer effect of paclitaxel on microtubule disarray, which promotes cell apoptosis and inhibits proliferation. Moreover, this miR-34a replenishment by rubone is in a p53 independent manner in DU145-TXR and PC3-TXR cell lines. PTX and rubone combination therapy formulated by PEG-PCD micelles could significantly suppress PTX resistant tumor growth in vivo. This study illustrated a new therapeutic potent of rubone as a small molecule miR-34a modulator for the treatment of PTX-resistant prostate cancer. Rubone might work with PTX in clinical for chemotherapy to avoid chemoresistance and enhance the therapeutic effects.
CHAPTER 4. MESENCHYMAL STEM CELL AND DERIVED EXOSOMES AS SMALL RNA CARRIER AND IMMUNOMODULATOR TO IMPROVE ISLET TRANSPLANTATION

4.1. INTRODUCTION

Type 1 diabetes, which comprised of 5-10% of the total diabetic population, might be treated by islet transplantation. Despite recent therapeutic success, the wide application of islet transplantation is still limited due to the destruction and dysfunction of transplanted islets caused by immune rejection and loss of islet function, which is characterized as primary non-function (PNF)(106). The major mechanisms behind these two processes are proinflammatory cytokines triggered apoptosis and hypoxia related proteins or miRNAs expression, including Fas, Caspase-3, iNOS, and miR-375(171-174). As a potent approach for inhibiting aberrant protein and miRNA expression, RNA interference (RNAi) by short interfering RNAs (siRNAs) or anti-miRNAs is becoming a promising tool. However, its efficacy is greatly influenced by RNA degradation, poor cellular uptake, and rapid renal clearance after systemic administration(175). Thus, expression vectors are being developed for efficient siRNA delivery or simultaneously silencing multiple genes. Bain et al. first reported the feasibility of islet RNA delivery using adenovirus.(176) Nevertheless, the application of viral vectors is limited due to the potential of insertional mutagenesis and/or severe immune reaction.(177, 178) In contrast, cationic lipids or lipid-like vesicles are relatively safe as small RNA carriers due to their low cytotoxicity, high versatility, and target specificity by surface modification. However, cationic liposomes have low transfection
efficiency in intact human islets, especially when used for delivering plasmid DNA.(109) Therefore, to develop a suitable and effective small RNA delivery system might help transplanted islets survive from immune rejection and PNF process and further improve the outcome of islet transplantation.

Secreted membrane vesicles have attracted much interest because of their potential as biomarkers of diseases, therapeutic agents, and vehicles for drug delivery.(179) Among these vesicles, exosomes (30-100 nm) are natural nano-vesicles secreted by numerous cell types.(116) They have a bi-lipid membrane and cargos including mRNAs, miRNAs, and proteins, which can be transferred and thus affect the protein production of recipient cells.(180) As a novel RNA delivery vehicle, exosomes have high delivery efficacy as they can circumvent endocytosis(181) and escape rapid clearance by the cells of reticuloendothelial system.(182) Previous research indicated that exosomes derived from genetically modified dendritic cells or mesenchymal stem cells (MSCs) can deliver siRNA or miRNA to the mouse brain(116) or cancer cells to inhibit glioma growth.(117) Compared with other cells, hBMSCs might be a more suitable source of exosomes for improving islet transplantation due to their self-renewal potential(183) and immunosuppressive effects(184). Furthermore, some of tissue repair(185) and immunosuppressive properties(186) of MSCs have been reported to be transferred to MSC-derived exosomes. Early studies showed that endothelial progenitor cell-derived microvesicles/exosomes enhanced neoangiogenesis of human islets,(187) while umbilical cord blood MSC-derived microvesicles suppressed peripheral blood mononuclear cell (PBMC) proliferation in
vitro. However, the neoangiogenesis and immunosuppressive effect of hBMSC-derived exosomes to improve islet transplantation and their small RNA delivery potential to human islets remain largely unknown.

In the present study, we utilized hBMSCs and their exosomes to suppress islet apoptosis and PNF at the acute stage (<2 weeks) of islet transplantation by co-delivering siRNA against Fas receptor (siFas) and miR-375 inhibitor (anti-miR-375). The inhibition of the post-transplanted immune reaction is further achieved by intravenously injecting hBMSC and PBMC co-cultured exosomes. Our model enabled reversal of diabetes without a need of insulin injection and proved the clinical potential of hBMSC and its exosome-based small RNA delivery and immunotherapy method to improve the outcome of islet transplantation.

4.2. MATERIAL AND METHODS

4.2.1. Cell Culture and Exosome Isolation.

Rat insulinoma (INS-1E) cells, a kind gift from Professor Claes B. Wolheim (University Medical Center, Geneva, Switzerland) were cultured in RPMI 1640 containing 10% FBS, 1% sodium pyruvate, and 50 μM 2-mercaptoethanol. Human islets were received from Integrated Islet Distribution Program (USA) and cultured in CMRL-1066 medium containing 10% FBS. Primary hBMSCs, which was characterized previously(189), were purchased from Celleng-tech (Coralville, IA) and cultured in HyClone Advanced Stem Cell Medium and exosome-depleted FBS. Plasmid transfected hBMSCs were characterized for adipogenic/osteogenic differentiation
using oil red o/alizarin red staining. In transwell system, 5×10^4 plasmid transfected hBMSCs were adherent at a 0.4-μm transwell cell culture insert, while 2000 islet equivalents (IEQs) were cultured outside in a 6-well plate. hBMSCs in all experiments were cultured for less than 3 passages to avoid differentiation. PBMCs were received from Dr. Howard Gendelman’s lab at the University of Nebraska Medical Center and further cultured in medium composed of RPMI 1640 and 10% FBS. hBMSC-derived exosomes were isolated from 50-70% confluent hBMSC culturing media, while hBMSC and PBMC co-cultured exosomes were isolated from co-cultured media incubating 50-70% confluent hBMSCs and 10^6 PBMCs/mL for 48 h using total exosome isolation reagent (Invitrogen). Co-cultured media was obtained from a T25 flask containing 10^5 hBMSCs and 5×10^6 PBMCs after co-culturing for 2 days.

4.2.2. Cloning, Transfection, RNA Isolation, and Real Time RT-PCR.

shFas and anti-miR-375 sequences were cloned into BbsI/BbsI and Acc65I/HindIII sites of psiRNA-DUO vector (InvivoGen). Lipofectamine 2000 and Xfect transfection reagents (Clontech) were used to transfsect INS-1E cells and hBMSCs, respectively. RNA was isolated from hBMSC-derived exosomes using total exosome protein & RNA isolation kits (Invitrogen). RNeasy and miRNeasy mini kits were used to extract mRNA and miRNA from INS-1E cells and human islets. For exosome RNA, 5 μL RNA extraction products were converted to cDNA. β actin, U6, and total protein concentration were used as internal controls for cellular mRNA, miRNA, and exosome miRNA, respectively.
4.2.3. Exosome and RNA Uptake Study.

hBMSCs were incubated with Dil-C16 (3 μM) for 1 h. Then, hBMSCs were washed three times with 37°C PBS, trypsinized and incubated with 100 islets in the transwell system for 48 h. To determine siRNA transferring, hBMSCs were transfected with Alexa Red conjugated siRNA for 24 h and incubated with human islets for another 48 h. Lipofectamine 2000 and Xfect transfection reagent were used as negative control following the user’s protocol. shFas-Spinach was cloned into Acc65I/HindIII of psiRNA-h7SKzeo vector (InvivoGen) and further transfected to hBMSCs, which was co-cultured with human islets and non-transfected hBMSCs in a transwell system. Before Spinach aptamer imaging, culturing media was replaced with imaging media (DMEM without phenol red or vitamins and contained 25 mM HEPES, 5 mM MgSO4, 50 mM KCl, and 20 μM 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI, Lucerna)) and incubated for 30 min. To determine the endosome uptake of exosome delivered small RNA, early endosome and lysosome were labelled by the lysotracker (Thermo Scientific).

4.2.4. Cell and Islet Viability Study.

After transfection with Lipofectamine/siRNA complexes for 48 h, INS-1E cells were treated with streptozotocin (STZ, 0.2 mM dissolved in 0.1 M citrate buffer) for 6 h, followed by analyzing living cells using fresh medium containing 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT). After co-culturing with plasmid transfected hBMSCs for 72 h, islet/hBMSC co-culture was stimulated with
cytokine cocktail (5 μg/mL IL-1β, 50 ng/mL TNF-α, and 50 ng/mL IFN-γ) for another 4 days, followed by 5 μg/mL calcein AM and 2 μg/mL propidium iodide staining for 30 min. Islet apoptosis was evaluated under fluorescent microscope and quantified by flow cytometry using Alexa Fluor Annexin V/PI staining kit (Invitrogen).

At 48 h after transfection, insulin release of INS-1E cells responding to glucose stimulation was quantified by stimulating INS-1E cells with Krebs-Ringer bicarbonate (KRB) buffer containing basal (2.5 mM) and stimulated (22 mM) glucose for 1 h. To better study the reaction of human islets with glucose stimulation, insulin secretion from human islets was quantified using a dynamic islet perifusion assay. Briefly, 50 handpicked islets were loaded onto a Swinnex 13 chamber (Millipore, Burlington, MA) and perfused with KRB buffer containing basal (1.67 mM) or stimulated (16.7 mM) glucose. The temperature was maintained at 37°C and the flow rate was maintained at 1 mL/min. Islets were first perifused with basal glucose for 60 min and stimulated for 30 min, followed by perifusing with basal glucose till insulin reverse to the basal level. Samples were collected every 2 min and analyzed for insulin concentration by ELISA.

4.2.5. Mixed Lymphocyte Reaction.

PBMCs were first labeled with carboxyfluorescein succinimidyl ester (CFSE) and treated with hBMSC-derived exosomes or hBMSC and PBMC co-cultured exosomes (150 μg/mL protein concentration). To optimize the immunosuppressive effect of hBMSC and PBMC co-cultured exosomes, exosomes were isolated from co-culturing media with the ratio of hBMSC: PBMC=1:5, 1:10, 1:25, and 1:50 with 1×10^5 hBMSCs.
PBMCs were then stimulated with phytohaemagglutinin (PHA) (5 μg/mL) and cultured for another 7 days before flow cytometry analysis. IL-2, IFN-γ, and IL-2sRα in medium and IL-10, PGE-2, TGF-β, VEGF, and HGF in exosomes were determined by ELISA. For analyzing Tregs population, CD4+ cells were first isolated and the population of CD25+FoxP3+ among total CD4+ cells were determined by flow cytometry using human regulatory T cells 3 color kit (R & D systems). miR155, miR-let7b, miR-let7d in medium exosomes were determined by RT-PCR. For in vivo study, NSG mice were humanized by intraperitoneally infusion of PBMCs (5×10⁶/mouse) and co-cultured exosomes (10 mg/kg) were intravenously injected every other day, followed by immunohistochemical (IHC) staining of CD3 and determining blood serum IgG concentration by ELISA.

4.2.6. Islet Transplantation.

NSG mice were purchased from Jackson Laboratory and bred in-house in accordance with a protocol approved by the Institutional Animal Care and Use Committees of the UNMC. STZ (70 mg/kg) was injected intraperitoneally to NSG mice every two weeks until diabetes was induced. Animals were considered diabetic as indicated by two consecutive measurements of blood glucose ≥ 250 mg/dl. Then, 2000 IEQs were co-transplanted with primary hBMSCs, pshFas transfected hBMSCs, panti-miR-375 transfected hBMSCs, and pshFas-anti-miR-375 transfected hBMSCs at the ratio of islet: hBMSC= 1:25 (2000 IEQ and 50000 hBMSCs) under the kidney capsule, followed by PBMCs (5×10⁶/mouse) intraperitoneal infusion four weeks after islet transplantation as described previously.(189, 190) Two weeks after humanization, co-cultured exosomes were injected intravenously in randomly selected 10 mice receiving
islets co-transplanted with pshFas-anti-miR-375 transfected hBMSCs. Three months after islet transplantation, immune rejection and transplanted islets function were evaluated by serum insulin & IgG concentration, followed by intraperitoneal glucose tolerance study. Briefly, overnight fasted mice were subjected to intraperitoneal injection of glucose (2 g/kg). Blood glucose level was determined at 15, 30, 60, 90, 120, and 180 min.

4.2.7. Western Blot Analysis and Immunofluorescence Staining.

INS-1E cells protein was extracted using RIPA buffer after transfection for 48 h. The amount of protein was adjusted to the same concentration and incubates with primary and secondary antibodies, followed by Licor Odyssey system analysis.

To analyze in vivo small RNA delivery efficacy and immunosuppressive effect of hBMSCs and exosomes, mice were sacrificed 1 week or 3 months after islet transplantation. Kidney bearing islets or spleens were isolated, washed with PBS, fixed in 4% paraformaldehyde overnight and embedded in paraffin. The tissue slides were further stained by anti-Fas and anti-Mtpn primary antibodies for kidney and anti-CD3 primary antibody for both kidney and spleen. Protein expression was determined by incubating tissue slides with secondary antibodies. The following primary antibodies were used for western blotting and IHC staining: anti-Fas (Abcam, ab82419), anti-Mtpn (Sigma-Aldrich, HPA019735), anti-CD3 (Abcam, ab16669), anti-insulin (Abcam, ab7842), anti-Beta actin (Santa Cruz, sc-1616).
4.2.8. Statistical Analysis.

Results were presented as mean ± s.e.m. from three experiments for in vitro study and five experiments for in vivo study. The statistical significance of difference between the two groups was calculated by unpaired Student’s t-test, and a P < 0.05 was considered to be statistically significant.

4.3. RESULTS

4.3.1. pshFas-anti-miR-375 Improved Cell Viability and Insulin Release of Rat Insulinoma Cells

Fas/FasL pathway plays an important role in β-cell apoptosis in type 1 diabetes, especially in high glucose condition.(172) miR-375, which also contributes to normal pancreatic islet formation(191), has been reported to downregulate insulin secretion by acting on myotrophin (Mtpn)(108) and PDK1(192). For Fas silencing, two siFas sequences (siRNA 175 and siRNA 480) were selected, of which siRNA 480 showed better Fas silencing, anti-apoptotic and insulin promotion effect (Data not shown). Mtpn, the target protein of miR-375, was upregulated and insulin release was significantly enhanced after anti-miR-375 transfection (Data not shown). Then, siRNA 480 or siFas for human islets and anti-miR-375 sequences were cloned into psiRNA-DUO vector (Figure 4-1A). pshFas-anti-miR-375 transfected INS-1E cells showed better anti-apoptotic effect and insulin release compared to pshFas and panti-miR-375 transfected cells (Figure 4-1B and C).
Figure 4-1. Fas and miR-375 were silenced by hBMSC-derived exosomes delivered siFas and anti-miR-375.

(A) Outline of pshFas-anti-miR-375 construction. (B) pshFas-anti-miR-375 improved INS-1E viability against STZ. (C) pshFas-anti-miR-375 improved insulin release of INS-1E cell line. Exosomes were then isolated from pshFas, panti-miR-375, or pshFas-anti-miR-375 transfected hBMSC media. siFas (D) and anti-miR-375 (E) were highly overexpressed in plasmid transfected hBMSC-derived exosomes determined by RT-PCR. pshFas, panti-miR-375, and pshFas-anti-miR-375 transfected hBMSCs were co-cultured with human islets in a transwell system for 72 h. Fas (F) and miR-375 (G) of human islets were significantly downregulated after co-culturing with plasmids transfected hBMSCs in the transwell system.

4.3.2. pshFas-anti-miR-375 Transfected hBMSC-derived Exosomes Downregulated Expression of Fas and miR-375 in Human Islets.

The adipogenic/osteogenic differentiation of hBMSC was characterized by oil red o/alizarin red staining. 3 passages hBMSCs did not have adipogenic/osteogenic differentiation, whereas hBMSCs over 10 passages might differentiate into adipocyte or osteocyte (Data not shown). To evaluate small RNA delivery potential of hBMSC-derived exosomes to human islets, hBMSCs were first transfected with pshFas, panti-miR-375, pshFas-anti-miR-375, or scramble plasmid using Xfect transfection reagent.
Transfection efficacy was evaluated by GFP expression. Our results showed significantly elevated levels of siFas (Figure 4-1D) and anti-miR-375 (Figure 4-1E) in exosomes after transfection for up to six days. Furthermore, pshFas and pshFas-anti-miR-375 transfected hBMSCs downregulated Fas expression by 53.4% and 46.9% (Figure 4-1F) while miR-375 was downregulated by 69.84% and 55.88% (Figure 4-1G) after co-culturing islets with panti-miR-375 and pshFas-anti-miR-375 transfected hBMSCs, respectively. These results suggest that hBMSCs could deliver small RNAs to human islets by secreting mediators without direct touch.

In previous studies, hBMSCs were proven to deliver siRNA to cancer cells(193) and neuron cells(194) by direct touch and hBMSC-derived exosomes. We first showed that hBMSC-derived exosomes were efficiently taken up by human islets after 48 h (Figure 4-2A). Moreover, our results (Figure 4-2B) proved siRNA can be delivered to human islets from hBMSCs by direct touch and exosomes. Lipofectamine 2000 and Xfect transfection reagent was used as control to evaluate siRNA delivery efficacy of hBMSC to human islets. Our results indicated that hBMSC has higher siRNA delivery efficacy compared to lipid-like polymers (Figure 4-2B). We further linked the Spinach aptamer, which was first reported by Paige et al. as an RNA mimic of GFP and fused to the 3’ end of 5S rRNA to localize this small noncoding RNA,(195) to shFas (Fig. 2F and G) and co-cultured shFas-Spinach plasmid transfected hBMSCs (Figure 4-2C) with human islets for 48 h. Our results (Figure 4-2D) showed that plasmid generated shFas-Spinach RNA aptamer from exosome donating cells (hBMSCs) could be delivered to human islets as recipient cells. Furthermore, endocytic cycling could be
circumvented by exosome delivery (Figure 4-2E). For the feasibility of monitoring the location of Spinach aptamer, we selected hBMSCs rather than human islets as recipient cells. Collectively, our results showed that hBMSC-derived exosomes and contained siRNAs or shRNAs generated from exosome donating cells can be taken up by human islets.

Figure 4-2. Small RNAs delivered by hBMSC-derived exosomes can be efficiently taken up by human islets and circumvent endosome degradation. (A) In a transwell system, hBMSC-derived exosomes can be taken up by human islets after 48 h. (B) siRNAs were delivered from hBMSCs to human islets by direct touch and exosome. shFas-Spinach aptamer can be expressed in hBMSCs (C) and further transferred to human islets in the same transwell system after 48 h (D). Arrows indicate exosome loaded shFas-Spinach aptamer. (E) For the feasibility of identifying the location of shFas-Spinach aptamer, hBMSC was selected as the recipient cell to determine the delivering efficacy. Exosome delivered shFas-Spinach aptamer circumvent endosome process. (F) 2D structure of shFas-Spinach. (G) 3D structure of shFas-Spinach.

4.3.3. pshFas-anti-miR-375 Transfected hBMSC-derived Exosomes Inhibited Islet Apoptosis and Improved Islet Function Against Inflammatory Cytokines.

Recruited T cells at the transplantation site cause the major loss of islet grafts by
stimulating an immune response or secreting inflammatory cytokines, which can upregulate Fas expression on β cells, thus activating Fas signaling.(196, 197) Therefore, we determined the protection effect against inflammatory cytokines from plasmid transfected hBMSC-derived exosomes in the transwell system. After stimulating with cytokine cocktails (5 ng/mL IL-1β, 50 ng/mL TNF-α, and 50 ng/mL IFN-γ) for 4 days, islets co-cultured with pshFas-anti-miR-375 transfected hBMSCs showed better viability compared to other three groups determined by Calcein AM/PI staining and quantified by flow cytometry using Annexin V/PI kit (Figure 4-3A-D). We also indicated that islets co-cultured with pshFas, panti-miR-375, and pshFas-anti-miR-375 transfected hBMSCs all showed improved insulin release in different extension in both basal (1.67 mM) and stimulated (16.7 mM) stage while pshFas-anti-miR-375 showed higher insulin release at each time point and accumulative amount compared to other groups (Figure 4-3E and F). Our results showed that cytokine cocktails challenging suppressed 30.5% insulin release compared to non-treatment group (Figure 4-3F hBMSC vs Fig. 3H hBMSC), which was in accordance with our previous report.(189) We observed 3.88 times accumulative insulin release from Fas and miR-375 silenced islets compared to hBMSCs co-cultured islets (Figure 4-3F). Even though cytokine cocktail treatment suppressed insulin release in each group (Figure 4-3H), pshFas-anti-miR-375 transfected hBMSCs significantly prevented islet impairment (Figure 4-3G), suggesting protective effect from silencing Fas and miR-375.
4.3.4. hBMSC and PBMC Co-cultured Exosomes Suppressed Activation and Proliferation of PBMCs.

Previous reports showed that hBMSC and PBMC co-cultured media exhibited stronger immunosuppressive effect compared to hBMSC media.(198) We first showed that hBMSC and PBMC co-cultured exosomes suppressed PBMC proliferation by CFSE staining (Data not shown). To optimize the immunosuppressive effect of hBMSC and PBMC co-cultured exosomes, we isolated exosome from co-cultured media with the ratio of hBMSC and PBMC 1:5, 1:10, 1:25, and 1:50. The immunosuppressive effect of co-cultured exosomes exhibited a dose-dependent manner with the increased number of PBMC (Data not shown). Co-cultured
exosomes inhibited PBMC activation as indicated by significant decrease of IL-2, IFN-γ, IL-2sRα concentrations in the medium (Data not shown). To work out the immunomodulatory mechanism of exosomes (Figure 4-4A), our results indicated that HGF played an important role in exosome-mediated immunomodulatory effect of hBMSCs (Figure 4-4B), while IL-10, VEGF, PGE-2, and TGF-β showed moderate effect (Data not shown). When PHA stimulated PBMCs were co-cultured with hBMSC and PBMC co-cultured exosomes or hBMSCs, the population of Tregs was increased from 4.9 to 7.0-8.7% (Figure 4-4C). Different from our results, previous research indicated that MSC-derived exosomes increased the population of Tregs(188), because the sources of MSCs in these studies were different. Okoye et al.(199) reported that Tregs suppressed pathogenic T helper 1 cells by regulating miR-155, miR-let7b, and miR-let7d in Tregs derived exosomes. Compared to hBMSC-derived exosomes, our results showed that hBMSC and PBMC co-cultured exosomes more significantly enhanced the expression of miR-let-7b and miR-let-7d (Figure 4-4D), which were reported to suppress cell proliferation and induce apoptosis.(200) Furthermore, co-cultured exosomes downregulated miR-155 expression (Figure 4-4D), which enhances inflammatory T cell development and promotes autoimmune inflammation.(201) It was previously reported that CD14+ cells among PBMCs were responsible for IL-10 related immunosuppression.(189) However, our results indicated that crosstalk between hBMSC and PBMC did not influence IL-10 secretion. Moreover, similar to PGE-2, most IL-10 was directly secreted into medium after PHA stimulation, rather than through exosomes. Thus, IL-10 and PGE-2 might work more specifically in
PBMC activated condition, where both factors were highly secreted in media (Data not shown).

Figure 4-4. Immunosuppressive mechanisms and in vivo immunosuppressive effect of hBMSC and PBMC co-cultured exosomes. (A) The outline of crosstalk between hBMSC and PBMC. (B) HGF concentration in exosome and media after co-culturing PBMC with hBMSC or PHA activation. (C) hBMSC-derived exosomes enhance Tregs population. (D) hBMSC-derived exosomes enhanced Tregs-derived miR-let7b and miR-let7d concentration and downregulated miR-155 concentration.

To evaluate immunosuppressive effect in vivo, our results showed that hBMSC-derived exosomes were accumulated in the liver, spleen, and kidney (Figure 4-5A). hBMSC and PBMC co-cultured exosomes significantly suppressed PBMC activity determined by spleen CD3 staining (Fig. 4-5B-D) and serum IgG concentration (Figure 4-5E), whereas hBMSC-derived exosomes had moderate immunosuppressive effect. In summary, our results indicated that hBMSC and PBMC co-cultured exosomes might be a potent immunosuppressive regimen for inhibiting immune rejection in vitro and in vivo.
4.3.5. pshFas-anti-miR-375 Transfected hBMSCs Combined with hBMSC and PBMC Co-cultured Exosomes Improved Islet Survival in Humanized NSG Mice.

The procedure of our animal surgery was summarized in Figure 4-6A. We evaluated the in vivo gene delivery efficacy by co-transplanting plasmid transfected hBMSCs and human islets in kidney capsule and allow them to settle down for 4 weeks, followed by intraperitoneal injection of PBMC for mimicking the post-transplanted
immune rejection. In a humanized NSG mouse model, non-transfected hBMSCs failed to prevent acute stage apoptosis of islets after transplantation (Figure 4-6B, 30% insulin independence). Both pshFas and panti-miR-375 improved transplanted islets survival (Figure 4-6C and D, 50 and 60% insulin independence, respectively), while pshFas-anti-miR-375 significantly suppressed apoptosis by 90% (Figure 4-6E). After PBMC perfusion, pshFas or panti-miR-375 transfected hBMSCs failed to maintain normal blood glucose level after three months compared to pshFas-anti-miR-375 transfected hBMSCs (10 and 30% vs. 60% insulin independence). However, intravenous injection of hBMSC and PBMC co-cultured exosomes significantly suppressed immune rejection after humanization of the mice receiving islets co-transplanted with pshFas-anti-miR-375 transfected hBMSCs (Figure 4-6F, 100% insulin independence). Our results also showed that insulin content of mice receiving islets co-transplanted with pshFas, panti-miR-375, and pshFas-anti-miR-375 transfected hBMSCs was significantly higher than those received islets co-transplanted with non-transfected hBMSCs (Figure 4-6H and I). pshFas-anti-miR-375 transfected hBMSCs downregulated Fas (Figure 4-6H) and enhanced Mtpn (Figure 4-6I) expression in transplanted islets. In summary (Figure 4-6G), pshFas-anti-miR-375 transfected hBMSCs combined with hBMSC and PBMC co-cultured exosomes induced a totally insulin independence by inhibiting islet apoptosis, promoting islet function, and suppressing immune reaction after humanization.

Three months after islet transplantation, hBMSC and PBMC co-cultured exosomes significantly inhibited PBMC infiltration to transplanted islets and in vivo activity of
PBMCs determined by CD3 staining (Figure 4-7A-C). Almost no detectable islets were observed at the transplantation sites when co-transplanting with non-transfected hBMSCs (Figure 4-7A). Although pshFas-anti-miR-375 transfected hBMSCs help islets survive from acute stage apoptosis, islets were still undergoing apoptosis caused by T cell infiltration (Figure 4-7B). However, transplanted islets were protected from T cell infiltration in the mouse receiving hBMSC and PBMC co-cultured exosomes (Figure 4-7C). In accordance with CD3 staining, serum IgG level was more significantly decreased with increased insulin level after hBMSC and PBMC co-cultured exosome administration compared to other four groups (Figure 4-7D). Our results also showed that islets co-transplanted with pshFas-anti-miR-375 transfected hBMSCs with or without exosome injection exhibited faster and better response to the stimulatory glucose (2 g/kg) (Figure 4-7E). Taken together, these results suggest that hBMSCs and their exosomes might be a potent vehicle for small RNA delivery to human islets. hBMSC and PBMC co-cultured exosomes might work as bio-generated immunosuppressive nanovesicles to inhibit further immune response of transplanted islets. Our model for small RNA delivery and immune suppression might give clinical prospect to improve the outcome of islet transplantation.
Figure 4-6. pshFas-anti-miR-375 transfected hBMSCs combined with hBMSC and PBMC co-cultured exosomes improve the outcome of islet transplantation. (A) Outline of animal study procedure. For islet transplantation, islets were co-transplanted with hBMSC (B), pshFas transfected hBMSC (C), panti-miR-375 transfected hBMSC (D), pshFas-anti-miR-375 transfected hBMSC (E), and pshFas-anti-miR-375 transfected hBMSC with intravenous injection of hBMSC and PBMC co-cultured exosomes two weeks after humanization for four doses (F). Horizontal dotted line indicated insulin independence. Vertical dotted line showed intraperitoneal injection of PBMC to build human immune system. (G) Insulin independence was summarized by the Kaplan-Meier plot. In vivo gene silencing effect of hBMSC and hBMSC-derived exosomes were determined by IHC staining of Fas (H) and Mtpn (I), downstream target of miR-375. Islet location was indicated by insulin staining.

4.4. DISCUSSION

Even though the Edmonton protocol has been developed for more than 20 years, the application of islet transplantation is still limited due to the huge loss of islet mass after transplantation. The immune rejection and PNF of islet grafts remains a challenge
despite the development of new therapeutic regimens, which always induce organ toxicity or severe side effects.\(189\) Compared with small molecules, hBMSCs can be isolated from patient and infused back after in vitro gene modification with reduced side effects impacting other organs and tissues.\(202\) Previous research showed that systemic administration of MSCs prevented the onset of type 1 diabetes by engrafting in injured tissue and differentiate to replace damaged cells.\(203\) However, only less than 1% of transplanted MSCs were observed to reach the injured tissues with most of them being trapped in the liver, lungs, and other organs.\(204\) Even in the case where transplanted MSCs showed tissue repairing effect, engraftment and differentiation of MSCs at the damaged site was low and transient,\(205\) suggesting that MSCs exert most effect through secreted mediators.

Exosomes were first discovered in mature mammalian reticulocytes\(206\) as a role of selectively removing plasma membrane proteins. Recently, two methods were used to load drugs in exosomes: (i) mixing with exosomes and sucrose gradient centrifugation for small molecules;\(207\) (ii) physical disruption\(116\) (electroporation) or gene modification of original cells for nucleic acid. Gene modification is always recommended for delivering nucleic acid due to the stability and aggregation issue in electroporation process.\(208\) In our study, we obtained 672.3 and 407.0 times overexpression of siFas after pshFas and pshFas-anti-miR-375 transfection (Figure 4-1D). Overexpression of anti-miR-375 was 24.3 and 14.2 times higher than control groups after panti-miR-375 and pshFas-anti-miR-375 transfection (Figure 4-1E). Significant discrepancy in expression level of two products might be explained by the
specificity of different primers since the Cp value of siFas in the control group was 36.97 while anti-miR-375 was 26.69. Thus, the specificity of primer might be a crucial issue for calculating the accurate amount of small RNAs inside exosome.

**Figure 4-7.** hBMSC and PBMC co-cultured exosome suppressed immune rejection after islet transplantation.

Islets were co-transplanted with hBMSC (A), pshFas-anti-miR-375 transfected hBMSC (B), and pshFas-anti-miR-375 transfected hBMSC followed by intravenous injection of hBMSC and PBMC co-cultured exosomes two weeks after humanization (C). Three months after islet transplantation, mice were sacrificed and immune activity was determined by CD3 staining, while transplantation site was identified by insulin staining. Immune rejection and transplanted islets function were evaluated by serum insulin & IgG concentration (D) and intraperitoneal glucose tolerance study (E).

For intracellular function of Fas signaling, Schumann et al. reported Fas deficient mice displayed impaired glucose tolerance and decreased insulin release(209) while Feng et al.(210) and Choi et al.(211) claimed that decreased Fas activity reversed the effect of c-Kit inactivation and enhanced insulin release. Similarly, the role of miR-375, which is highly overexpressed in type 1 diabetes patient serum or transplanted islets,(212, 213) is controversial as some groups reported that overexpression of miR-
375 did not affect the development or function of islets,(214) whereas others reported that forced miR-375 expression impaired cell proliferation and insulin secretion.(173) According to our results, Fas gene silencing induced slightly enhanced insulin release. (**Figure 4-3E and F**) Nevertheless, under the treatment of cytokine cocktail, Fas gene silencing showed significant islet protective effect (**Figure 4-3G and H**), probably because Fas/FasL interactions were one of the major mechanisms leading to β cell apoptosis. (215) For miR-375, our results showed that suppressing miR-375 expression significantly enhanced insulin release at each time point (**Figure 4-3E**). Islet apoptosis was significantly inhibited and insulin release was enhanced under inflammatory cytokine treatment after simultaneously silencing Fas and miR-375 (**Figure 4-3G and H**). Therefore, simultaneously silencing Fas and miR-375 might be a potent method for inhibiting islet apoptosis and enhancing islet function under inflammatory challenging.

Previous studies showed that primary hBMSCs failed to prevent the allogenic rejection and maintain normal blood glucose level in the acute stage.(183) To make this situation even worse, high blood glucose from recipient is highly toxic to transplanted islets, which severely promotes their apoptosis.(216) Thus, promoting islet engraftment and insulin release to downregulate blood glucose are the crucial points for the success of islet transplantation. Our results showed that pshFas-anti-miR-375 transfected hBMSCs significantly suppressed islet apoptosis and improved islet function by suppressing Fas expression and overexpressing Mtpn compared to other groups at the acute stage of islet transplantation (**Figure 4-6H and I**).
Furthermore, co-cultured exosomes suppressed immune rejection at the transplantation sites and T cell activity (Figure 4-7). Although 100% insulin independence was obtained, this method should be interpreted with caution because PBMCs consist of T cells, B cells, natural killer cells, and monocytes. The roles of each cell type during the crosstalk between hBMSC and PBMC needs to be fully understood. The only conclusion we could make in this study is that exosomes generated from crosstalk between hBMSC and PBMC significantly suppressed immune reaction. However, we could not fully investigate the cell source of these immunosuppressive exosomes. Furthermore, the dose of each factors with different functions inside co-cultured exosomes needs to be accurately characterized for the safety concern.

Based on our results, we hereby propose a biotherapy and self-therapy model as the following: (i) hBMSCs and PBMCs are isolated from the patient with type 1 diabetes (ii) transfecting hBMSCs with pshFas-anti-miR-375 in vitro (iii) the left hBMSCs are co-cultured with PBMCs, followed by exosomes isolation from the co-cultured media, (iv) patient receives donor islets co-transplanted with pshFas-anti-miR-375 transfected hBMSCs, and (v) co-cultured exosomes are injected to suppress immune rejection. In summary, our studies demonstrate that hBMSCs and co-cultured exosomes might be an efficient small RNA delivery vehicle and immunosuppressive product for human islets and become a promising strategy to improve the outcome of islet transplantation.

4.5. CONCLUSION

Co-delivery of siFas and anti-miR-375 by hBMSCs and derived exosomes
suppressed early apoptosis of transplanted human islets, while further immune activity could be suppressed by intravenously injection of hBMSC and PBMC co-cultured exosomes. This gene & cell therapy by hBMSC and derived exosomes might be a potent method for the improvement of islet transplantation.
CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

5.1. SUMMARY

Prostate cancer is the most pervasive malignancy in man and remains the second leading cause of cancer-related death of men in United States. Since androgen plays important role of tumor growth at the beginning, androgen-related therapy is the first choice for prostate cancer treatment. However, traditional antiandrogen reagent could not inhibit mutated androgen activity. Thus, we developed bicalutamide analog CBDIV17 for better inhibiting the proliferation of prostate cancer cells and tumor growth in vivo. Treatment with paclitaxel as another widely used chemotherapy reagent may also lead to chemoresistance, especially in metastatic tumor cells. We indicated that miR-34a as a tumor suppressor miRNA is downregulated in the process of prostate cancer. Thus, rubone as a recently screened small molecule miR-34a modulator is used for combination therapy with paclitaxel to treat metastatic and PTX-resistant prostate cancer. Using an orthotopic prostate cancer model, we demonstrated that PTX and rubone combination therapy could suppress tumor growth by restoring miR-34a and the downstream gene expression. Since CBDIV17, PTX, and rubone are all highly hydrophobic drugs, we used previously synthesized PEG-PBC and PEG-PCD to form micelles for drug delivery. To enhance the drug delivery efficacy, LHRH was conjugated to PEG-PBC to target prostate cancer cells in vivo. Our results indicated that our formulations could suppress tumor growth in subcutaneous model or PTX-resistant tumor growth in orthotopic model, respectively.
For the treatment of T1D, islet transplantation is still hindered by immune rejection and PNF. To address these two problems, we co-delivered siFas and anti-miR-375 to inhibit islet apoptosis and promote transplanted islet function. Since traditional RNA delivery vehicles including lipid-like polymers and viral vectors have low transfection efficacy or insertional oncogenic potential respectively, we used hBMSCs and their exosomes as novel gene delivery platform. We first construct a plasmid encoding siFas and anti-miR-375 for hBMSC transfection, followed by demonstrating the RNA delivery pathway and evaluating delivery efficacy. To further suppress the post-transplantation immune rejection, we used a humanized NSG mouse model to mimic the activity of immune system and hBMSC & PBMC co-cultured exosomes to suppress the T cell activity. hBMSCs could deliver RNA to human islets by direct touch and exosome, which is demonstrated by using dye labeled siRNA or spinach aptamer linked shRNA. Simultaneously silencing Fas and miR-375 promoted insulin release and suppressed islet apoptosis caused by cytokine cocktail. In a humanized mouse model, pshFas-anti-miR-375 transfected hBMSCs suppressed early apoptosis of transplanted islet and intravenous injection of hBMSC & PBMC co-cultured exosome, which could better suppress T cell activity compared to hBMSC exosomes due to high HGF and immunosuppressive miRNA amount, suppressed immune rejection after islet transplantation. We obtained a 100% insulin independence within 3 months of islet transplantation and suppressed T cell infiltration of transplanted islets with improved function and suppressed apoptosis.
5.2. FUTURE DIRECTIONS

The findings presented in this thesis demonstrated that PEG-PBC or PEG-PCD formed micelles could simultaneously carry hydrophobic drugs or further perform target delivery for different stages of prostate cancer. Systemic administration of these formulations significantly prolonged the half-life and bioavailability of the anti-cancer drugs and more tumor accumulation. The development of analogs from current used drugs might give us new idea for cancer therapy. Furthermore, the newly screened rubone for HCC therapy could be a potent therapeutic agent for treating PTX-resistant prostate cancer. However, the miR-34a regulation mechanism of rubone has not been well studied. In the future, we will further silence Tap73 and Elk-1 to determine if the effect of rubone can be diminished. Except for enhancing Tap73 and Elk-1 expression, rubone might also regulate the protein-protein or protein-miR-34a interaction, including Tap73 and MDM2, or Tap73 and miR-34a precursor. We will also determine this effect in our future study.

In our diabetes project, we used an RNA mimic of GFP to link shRNA for tracking the RNA generation and delivery pathways from hBMSCs to human islets. Actually, this shFas-spinach needs to be further evaluated. For example, we need to evaluate if the Fas silencing efficacy is affected after spinach linking. If not, this kind of spinach-linked shRNA or miRNA could be further used to develop bifunctional oligonucleotide, which could both tracking the location and silencing genes. The only problems are the low fluorescent signal and the compound for monitoring fluorescent is needed, not an automatic fluorescent. On the other hand, the application of exosome for RNA delivery
in T1D is still limited since we could not avoid the usage of hBMSC. We should further optimize exosome isolation method to get enough amount of exosome for RNA delivery to human islet to finally succeed in cell-free therapy.
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