Development of Novel Therapeutic Strategies for Pancreatic Cancer Treatment

Saini Setua

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Abstract
Pancreatic cancer (PanCa) is the third deadliest cancer in the USA due to the late diagnosis and development of chemo-resistance, with a 5-year survival rate of less than 10%. The prognosis of patients with pancreatic ductal adenocarcinoma is extremely poor, and current therapies such as Gemcitabine, 5-FU, Nab-paclitaxel and, FOLFIRINOX alone or in combination, have displayed improved but marginal survival rates for patients. Therefore, research efforts are underway to discover new therapeutic options to treat PanCa and overcome resistance to available therapies.

Mucin, MUC13 is transmembrane glycoprotein, which is aberrantly overexpressed in PanCa and promoting cancer growth. Structural domains of MUC13, lead to oncogenic characteristics during cancer progression. Our lab previously established the role of MUC13 in tumor progression and metastasis by alteration of signaling pathways. Recent observations suggest the role of MUC13 in drug resistance and apoptosis in several cancer types. Therefore, it is of great interest to explore the role of MUC13 in chemoresistance in PanCa. Unlike other cancer types, PanCa is highly resistant to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) that emerges as one of the most-promising cancer therapeutic drugs. It is a death ligand that can selectively induce apoptosis in cancer cells over normal cells. Our recent work has demonstrated that MUC13 expressing cells showed resistance to TRAIL induced cell death and MUC13 knockdown leads to TRAIL sensitivity in cells. We have also observed that MUC13 expression blocks the activation of caspase-8 and Bid in PanCa cells in response to TRAIL treatment. Further investigation showed that alpha and beta domains of MUC13 are indispensable for blocking caspase-8 activation and PARP cleavage, indicating that the MUC13 blocks TRAIL-induced signaling upstream to Bid by inhibiting caspase-8 activation. Current studies revealed a new role of MUC13 in inhibiting TRAIL mediated activation of extrinsic apoptotic pathway in pancreatic cancer.

MicroRNAs (miRNA) have been identified as attractive targets for therapeutic intervention. The functional significance of lost miRNAs have been reported in several human malignancies, including PanCa. Restitution of lost miRNA function can provide a potential therapeutic benefit. Prior work has identified microRNA-145 (miR-145) as a tumor suppressor miRNA in PanCa. The restoration of miR-145 downregulates a number of oncogenes including mucin MUC13 and efficiently inhibits tumor growth in mice. Inhibition of MUC13 using miR-145 restoration resulted in TRAIL mediated increase in apoptotic cell death as evidenced by sub-G0 population and inhibition of MUC13, activation of caspase 8 and, cleavage of PARP-1. MiR-145 replacement can sensitize TRAIL therapy and counteract chemoresistance mechanism in PanCa.

The main challenge for successful translation of miRNAs into clinical practice remains an effective in vivo delivery system. Hence, the focus of this study was to develop and assess the efficacy of a miR-145 based nanoparticle formulation for PanCa treatment. Magnetic nanoparticle (MNP) based nanoformulation of miR-145 (miR-145-MNPF) was developed for the intracellular delivery and sustained release of miR-145. The positively charged polyethyleneimine molecules were used to increase the loading efficiency of miR-145. Treatment of cells with miR-145-MNPF led to efficient intracellular delivery of miR-145 mimics as observed through Prussian blue staining. This led to the simultaneous upregulation of miR-145 levels in cells which resulted in significant downregulation of target oncogenes including MUC13, HER2, pAKT and p53. miR-145-MNPF efficiently restores miR-145 in PanCa cells and inhibits growth and invasion of PanCa. miR-145 restitution using miR-145-MNPF may offer a potential therapeutic strategy for pancreatic cancer.

As discussed earlier that miR-145 restoration sensitized the TRAIL therapy in PanCa cells. Herein, we demonstrate the integration of novel delivery approach to reduce the delivery challenge of TRAIL. We have
engineered unique superparamagnetic nanoparticles (MNPs) for co-delivering miR-145 and plasmid TRAIL for improving TRAIL response in PanCa model. MNP-miR-145-TRAIL nanoparticles were codelivered miR-145 and TRAIL to PanCa cells, which resulted in simultaneous restoration of miR-145 and inhibition of acquired resistance to TRAIL.

The current study demonstrates that acquired resistance to TRAIL in PanCa cells can be minimized with the replenishment of miR-145 expression. Combined actions of miR-145 and TRAIL markedly improve TRAIL-induced apoptotic effects in PanCa cells through the activation of an extrinsic apoptosis pathway as indicated by activation of DR4, FLIP, FADD and enhanced expression of cleaved caspase-8. The co-delivery of miR-145 and TRAIL using MNP nanoparticles inhibited tumorigenic characteristics of PanCa cells. The results were reciprocated and were further confirmed with the inhibition of tumorsphere formation and in vivo tumorigenicity in xenograft mice. Immunohistochemical staining of excised tumor tissues demonstrates an activation of the death receptor pathway and subsequent expression of apoptotic markers.

Pancreatic tumor microenvironment is a complex dynamic space which leads to desmoplasia and involved in metastasis and impediments against intracellular drug delivery. Despite extensive research efforts, there is not considerable progress in cancer therapeutics due to genomic complexity and heterogeneity of pancreatic cancer. Modern tumor therapy must be patient specific and customized for individual patients. It should be tailored for a patient-based response to the specific treatment. Thus, novel delivery vehicles are required that are biocompatible and non-immunogenic. This is possible by utilizing an autologous biological material as delivery vehicles that can be applied as a personalized medicine. Towards this, our lab has optimized an exosome based therapeutic approach, which utilizes exosomes isolated from the cultured tumor adjacent normal (NAT) fibroblast cells. We utilized this scaffold for safe and effective delivery of therapeutic payload. Our results demonstrated that NAT derived exosomal formulation (Exo-ORM) significantly enhanced the efficacy of ormeloxifene to inhibit stroma as indicated by decreased expression of α-SMA, desmin and hyaluronic acid. Exo-ORM formulation effectively inhibit EMT/SHH signaling in PanCa cells and in vivo models. NAT derived exosomes will be a promising therapeutic carrier with preferential size for passive targeting, proficient biophysical characteristics, biocompatible and nonimmunogenic vehicle for PanCa therapy.

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Subhash C. Chauhan, Ph.D.

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University of Tennessee Health Science Center

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Development of Novel Therapeutic Strategies for Pancreatic Cancer Treatment

Author: Saini Setua
Advisor: Subhash C. Chauhan, PhD

A Dissertation Presented for The Graduate Studies Council of The University of Tennessee Health Science Center in Partial Fulfillment of the Requirements for the Doctor of Philosophy degree from The University of Tennessee in Pharmaceutical Sciences: Pharmaceutics College of Graduate Health Sciences

May 2020
DEDICATION

I want to take the opportunity to dedicate the dissertation to my parents Mr. Ranjit Kumar Setua and Mrs. Ranjana Setua, without their unconditional love, support and encouragement, I would be nothing today. My husband Nivesh and my brothers’ immense support and inspiration helped me to achieve this success. Lots of love for my son Vivan.
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I am thankful to all the people who helped me in the successful completion of this dissertation project. I am grateful to University of Tennessee Health Science Center and College of Pharmacy for the facilities, equipment and financial support. I would like to thank all the faculty and staff of the Department of Pharmaceutical Sciences for their support. I would like to thank my family for constant support and encouragement.
ABSTRACT

Pancreatic cancer (PanCa) is the third deadliest cancer in the USA due to the late diagnosis and development of chemo-resistance, with a 5-year survival rate of less than 10%. The prognosis of patients with pancreatic ductal adenocarcinoma is extremely poor, and current therapies such as Gemcitabine, 5-FU, Nab-paclitaxel and, FOLFIRINOX alone or in combination, have displayed improved but marginal survival rates for patients. Therefore, research efforts are underway to discover new therapeutic options to treat PanCa and overcome resistance to available therapies.

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<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μL</td>
<td>Microliters</td>
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<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAF</td>
<td>Cancer associated fibroblasts</td>
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<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>DM</td>
<td>Deletion mutants</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>EB</td>
<td>Ethidium bromide</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EGF</td>
<td>Epidermal growth factors</td>
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<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>EPR</td>
<td>Enhanced permeability and retention</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<td>GEM</td>
<td>Gemcitabine</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>IPMN</td>
<td>Intraductal papillary neoplasm</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibodies</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
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<td>MCN</td>
<td>Mucinous cystic neoplasms</td>
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<tr>
<td>mm</td>
<td>Millimeter</td>
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<td>ml</td>
<td>Milliliters</td>
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<td>mV</td>
<td>Millivolts</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NAT</td>
<td>Normal adjacent tumor</td>
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<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>nm</td>
<td>Nanometers</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>ORM</td>
<td>Ormeloxifene</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PanCa</td>
<td>Pancreatic Cancer</td>
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<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasm</td>
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<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TR</td>
<td>Tandem Repeat</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UPLC</td>
<td>Ultra Performance Liquid Chromatography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</table>
CHAPTER 1. INTRODUCTION

Pancreatic Cancer

Pancreatic adenocarcinoma (PanCa) is one of the deadliest causes of cancer related deaths in the United States (1). The management of PanCa is exceptionally difficult due to the lack of early diagnosis, poor response to available therapeutic modalities and drug resistance (2, 3) and. The five-year survival rate remains poor (5%-6%) (4, 5). It is the third leading cause of cancer related deaths and is expected to become the second most common cause by 2030 (5). As per recent statistics, the estimated number of new cases in 2019 will be 56,770 and the number of expected deaths due to this devastating disease will be 45,750 (1). Current therapeutic strategies available include multimodality therapy inclusive of chemotherapy, radiation and surgery though cancer recurrence remains problematic despite delivery of these modalities. Despite optimal therapy, long term survival remains poor due to chemoresistance and the development of metastatic disease. In addition, advanced stage at diagnosis often precludes any consideration for surgical expiration.

Aggressiveness

Underlying molecular mechanisms associated with the tumor microenvironment is the most interesting research area in PanCa (6). It has been reported that an extensive desmoplastic stroma is one of the major causes of human PanCa and it has a critical role in tumor progression, metastasis and chemoresistance (7). Desmoplastic condition is explained by excessive production of extracellular matrix (ECM) and collagen-I that is directly correlated with stromal cell proliferation and pancreatic stellate cells formation (6). In pancreatic tumor condition, there is an internal crosstalk between epithelia and stroma because of paracrine Hh signaling. These reciprocal interactions play a major role in desmoplasia and create a limitation to penetration of chemotherapeutic drugs (8). And despite extensive research efforts, there is no substantial progress in cancer therapeutics.

Causes of pancreatic cancer: Pathogenesis

PanCa is genomically complex disease due to the mutation of hereditary germline or somatic cells related genes (9). Oncogenes, tumor suppressor gene, cell cycle related genes and apoptotic proteins are also responsible for tumor progression and metastasis (10-13).

Some precursor conditions of this disease are observed during the progression of pancreatic cancer. There are mainly three conditions, which include (1) Pancreatic intraepithelial neoplasm (PanIN), (2) Intraductal papillary neoplasm (IPMN), (3) and Mucinous cystic neoplasms (MCN).
The most well-established precursor lesion in PanCa is PanIN which is non-invasive ductal lesion (14). Microscopic appearance of PanINs are flat or papillary type and generally visualized in intratubular duct of pancreas (9, 15). Prevalence of PanINs is mostly increases with the age and genetic mutations. As a result, invasive carcinoma and chronic pancreatitis takes place. PanINs are diagnosed at the head of the pancreas and they are divided into 3 subgroups based on the condition of epithelial atypia. PanIN-1 (low grade), PanIN-2 (intermediate-grade), PanIN-3 (High-grade) are the major subgroups and further differentiated into smaller subgroups. These subgroups were formed based on the cytological characteristic, architectural atypia and the expression of mucin.

IPMNs are small, asymptomatic tumors of duct epithelium (9, 15). They are very common in patients with a history of smoking and they grow slowly compared to the other conditions (9). Based on their morphology and site of origin they are subdivided into MD-IPMN (main duct type) and BD-IPMN (branch duct type), combination of these two morphologies represent the “mixed duct type” and the “combined duct type” (15-17). IPMNs have been identified as invasive carcinomas with low, intermediate and high grade dysplasia (9). It involves in epithelial proliferation, progresses from main or brunch duct of pancreas and releases mucin (15).

Mucinous cystic neoplasms are most uncommon and asymptomatic precursor lesions of PanCa condition (15). Microscopic morphology divides MCNs into three subgroups: mild, moderate and severe MCN (9). The prevalence of MCNs are most common in women and diagnosed at the head and tail of pancreas (18, 19). As MCNs are also mucinous lesions, they also consist of high CEA and mucinous cytology observed in cyst fluid (20, 21). Noninvasive MCNs have a 100% 5 years survival rate. At the same time invasive MCN patients with surgical resection displayed satisfactory (60%) 5 years survival (22).

**Stages of PanCa**

To understand the criticality of the disease, American Joint Committee on Cancer (AJCC), updated a uniform staging system known as TNM staging (23). Stage I cancer is generally small and confined to the pancreas which can be diagnosed by preoperative imaging and afterwards resection. Stage II cancer is recognized as, noninvasive or metastasized but the primary tumor extended to nearby organs. This stage of cancer is also considered for resectable surgery. The patients with locally advanced tumor are recognized as stage III. This stage of disease could be spread to the arteries. Stage IV cancer is diagnosed with distant metastasis and are virtually never candidates for surgical resection (24).
Epithelial-mesenchymal transition signaling

Metastasis and chemoresistance are critical factors for poor prognosis of pancreatic cancer. Epithelial-mesenchymal transition (EMT) has shown significant contribution in PanCa metastasis and to drug resistance. Cellular plasticity is the leading cause of differentiation of epithelial to mesenchymal transition (25). Cellular cadherins play an important role in EMT progression. E-cadherin and N-cadherin are calcium dependent cell adhesion molecules associated with epithelial and mesenchymal characteristics respectively (26, 27). In the developmental phase, EMT allows the cells to detach and migrate to distant sites, which leads to the downregulation of E-cadherin and over expression of mesenchymal markers, such as N-cadherin, fibronectin and, vimentin. In a variety of cancers, over expression of Snail, Slug and ZEB, TWIST have been identified and they promote chemoresistance and cell proliferation, invasion and, metastasis (25). β-catenin is another key player in the EMT signaling pathway. Regulation of β-catenin controls downstream signaling molecules followed by downregulation of epithelial markers and gain in mesenchymal markers, which makes the cells more invasive and motile (28).

Tumor microenvironment

Pancreatic tumor microenvironment (TME) is a complex and dynamic space around cancer cells which play a predominant role in cancer progression and metastasis (29). This dynamic behavior of the microenvironment assists them to change the surroundings based on the need of the tumor to grow. TME consist of stromal cells, fibroblasts, endothelial cells and stellate cells which collectively lead to the desmoplastic condition. These surrounding cells are involved in biological interaction with the tumor cells and are involved in tumor initiation, progression, and also response to therapy (30). It is clinically proven that PanCa develops resistance against available therapeutic modality especially gemcitabine. There are several research studies demonstrated the pivotal role of desmoplasia in PDAC progression. Extensive desmoplasia enhances tumorigenic behavior and promote chemoresistance in PDAC cells (31). Pathological features of PDAC includes mainly dense stroma which prevents the delivery of chemotherapy from the vasculature to the extracellular compartments and is involved in invasive and metastatic behavior of PanCa. Targeting stroma and the inhibition of tumor metastases are the major challenges in clinical practice because of vast heterogeneity in different types of cancers. Despite the increasing incidence of PanCa over the past several decades, the molecular and biochemical determinants of the disease remain poorly understood and no effective therapeutic regimen exists to significantly improve the clinical course or prognosis of this disease (32). Therefore, development of alternative therapeutic strategies is urgently required.
MUC13: A unique target for pancreatic cancer

Mucins are a family of glycoproteins with an aberrantly glycosylated large extracellular domain (33, 34). Recently, it has been identified that, transmembrane mucin, MUC13 is aberrantly expressed in PDAC but not in the normal pancreas (35). Different domains of MUC13 are responsible for oncogenic characteristics of tumorigenesis. As a characteristic feature, presence of tandem repeat (TR) domain with varying number, length and O-glycosylation is common in any mucin (34, 36). MUC13 consists of an extracellular α-subunit that contain the TR domain, three epidermal growth factors like (EGF-I-III) domains and a sea urchin sperm protein enterokinase arginine (SEA) domain. The intracellular β-subunit consists of the transmembrane domain (TM) and a cytoplasmic tail (CT). Our lab previously reported that MUC13 expression leads to tumor progression and metastasis in PanCa through the alteration of signaling pathways (37-39). Aberrant surface expression and glycosylation of MUC13 is an advantageous factor for antibody guided therapy for PanCa cells over normal cells. Exogenous MUC13 expression in MiaPaca cells increases tumor growth and leads to poor survival of xenograft mice. MUC13 knockdown in HPAF-II cells inhibits the tumor growth and increases survival of xenograft mice (35). It has been reported that MUC13 is progressively expressed from PanINs-I to III and invasive carcinoma in both primary PanCa as well as IPMN, whereas normal pancreas does not show MUC13 expression (40, 41). Thus, overexpression of MUC13 might play a major role in altered molecular mechanisms in the tumor microenvironment which leads to chemoresistance and metastasis (Figure 1-1).

Pancreatic cancer treatment options

Pancreatic cancer patients mainly receive two type of treatments, Local therapy, includes surgery and radiation therapy and systemic therapy includes chemotherapy, immune therapy and targeted therapy. According to National Comprehensive Cancer Network (NCCN) guideline the order of treatments is:

- **Neoadjuvant** (before) treatment is applied to the patient before surgery to shrink the tumor. This therapy can improve the borderline tumor to a resectable tumor.
- **Primary treatment** is considered to be the surgery for PanCa patient. It is the main treatment for resectable cancer.
- **Adjuvant** treatment (after) is used after surgery to kill any part of tumor or cancer cells left behind. This therapy is also useful for recurring condition.
- **First-line treatment** is the first combination treatment given to the patient.
- **Second-line treatment** is applied when first-line treatment didn’t work.

Chemotherapeutic agents

Systemic Chemotherapy is one of the golden standards of treatment options now a days for the majority of cancer patients. There are several drugs available, which are
Figure 1-1. Pancreatic tumor microenvironment and role of MUC13 in cancer progression.
either used alone or in combination to treat solid tumors before or after surgery. All those currently known are majorly utilized for symptomatic relief (42). Continuous infusion of **Fluorouracil** (5-FU) is used as a first line and second line therapy for patients. It is widely used in adjuvant treatment after surgery, locally advanced, metastatic or recurrence. Combination of radiotherapy and 5-FU is preferred therapeutic option. Other combination, such as oxaliplatin with 5-FU and leucovorin is giving for locally advanced cancer.

**Gemcitabine** (2’,2’-difluorodeoxycytidine, dFdC; Gemzar®) is a pyrimidine analog (43) and widely applicable chemotherapeutic agent for several solid tumors such as breast, ovarian, bladder, or non-small-cell lung cancers including PanCa (44). It is considered to be one of the more effective chemotherapeutic agents for PanCa; however, it shows only a marginal survival benefit (6 months) in patients (45). It has been reported that in advanced stage PanCa, GEM exhibited better therapeutic efficacy compared to 5-FU treatment (45). It is used in combination with albumin-bound paclitaxel as neoadjuvant and adjuvant treatment of resectable PDAC, neoadjuvant therapy of borderline PDAC and sometimes used for metastatic tumors. A report suggested that PanCa stem cells are more resistant to chemotherapeutic drugs such as GEM (46). To potentiate the therapeutic efficiency of GEM, eventually it used with combination of other chemotherapeutic drugs such as, capecitabine and cisplatin. For BRCA1, BRCA2, or PALB2 mutations GEM with cisplatin has been used as neoadjuvant therapy for resectable cancer, first and second-line therapy for locally advanced and metastatic cancer.

**FOLFIRINOX** is another well establish therapeutic approach for metastatic PanCa. It is a combination of several chemotherapeutic drugs, such as, fluorouracil, irinotecan, leucovorin, oxaliplatin and folinic acid (42, 43). This drug used as a preferred neo-adjuvant (before) and adjuvant (after) surgery, locally advanced first, second-line and metastatic cancer. Patient with BRCA1, BRCA2, or PALB2 mutations also treated with FOLFIRINOX for resectable cancer. These single-agent drug therapy were not that convincing for solid tumors, however when they were treated in combination, they did not show overlapping toxicity but significant improvement in advanced stage PanCa patients (47-49). In order to treat metastatic PDAC after phase III clinical trial and PDAC patient after resection, FOLFIRINOX therapeutic regimen has been used first time which displayed better therapeutic efficacy with higher survival benefit than gemcitabine alone (50). FOLFIRINOX and combination of nab-paclitaxel/gemcitabine therapy regimens improved survival (51). However, these drugs combination showed more toxicity in patients compared to gemcitabine alone (51).

**Paclitaxel** is Taxol group of anticancer drugs for many cancer therapies, which are approved by US Food, and Drug Administration (US FDA). This drug has been identified as a microtubule stabilizing agent and can induce mitotic arrest, which leads to cell death (52). Combination of paclitaxel and GEM therapy has shown better efficacy in clinical trials. **Nab-paclitaxel** (ABRAXANE) is another chemotherapeutic drug recently introduced for advance and metastatic PDAC treatment. It is albumin bound paclitaxel encapsulated nanoformulation to deliver paclitaxel to tumor site (42, 43). Suitable
biophysical size of the Nab-paclitaxel nanoparticle (130 nm) made them efficient to enhance intracellular delivery of paclitaxel. One of the advantages of these nanosized particles is high tumor accumulation ability due to higher distribution volume and fast clearance (53).

Repurposing of drug for human use has been already approved by federal agencies. For instance, A traditional anti-diabetic molecule, Metformin is now being widely used in various cancer therapies (54-56). In the same regard, we have proposed for the first time that, **Ormeloxifene** (ORM) can be repurposed for PanCa therapy. ORM, is a non-steroidal, non- hormonal anti- estrogen oral contraceptive for human use. Several studies have reported ORM as a potent anticancer agent for breast cancer (57) and head and neck cancers (58). Therefore, identification of a novel therapeutic vehicle that could target TME and inhibit metastasis by overcoming the inaccessibility of drugs to tumor site is urgently required.

**TRAIL** is tumor necrosis factor (TNF)-related apoptosis-inducing ligand (also known as APO2L) emerges one of the most-promising experimental cancer therapeutic drugs. TRAIL has a selective killing ability towards cancer cell but not-toxic to normal cells which makes this therapy more favorable (59). Among the two apoptotic pathway’s TRAIL follows extrinsic pathway where pro-apoptotic ligand TRAIL binds to the cell surface receptors TRAIL-R1 and TRAIL-R2. These receptors are associated with death domains (DD) and eventually TRAIL transmits the apoptotic signals through these receptors to the cells (60).

**MicroRNA in pancreatic cancer**

To improve the therapeutic potential for cancer, gene therapies especially microRNA (miRNA) based therapeutic strategies have become a promising therapeutic modality for several cancers including pancreatic cancer. MiRNA are small non-coding RNA molecules consisting of 18-22 nucleotides, first discovered in the nematode *C. elegans* in 1993 (61). They play a critical role in gene silencing and post-transcriptional regulation of gene expression (62). They can bind imperfectly with 3'UTR region of complementary mRNA sequence (63). They have a pivotal role in biological function during cancer progression, such as, proliferation, apoptosis, cell survival, and metastasis and tumor growth (64). miRNA dysregulation is reported in several cancers, including PanCa, playing a critical role in oncogenic pathway such as tumor growth and metastasis (65). Based on their differential expression, they have been classified as oncogenic and tumor suppressor microRNAs.

Genomic criticality and heterogeneity in nature, dense desmoplasia in PanCa condition are the major causes of unpredicted dysregulation of miRNA in PDAC. Apart from oncogenic dysregulation, miRNA has also associated with tumor suppressor mechanism including chemo sensitization and survival (63). Differential expression of miRNAs during the progression of PDAC such as, transition of PanIN lesions or different grade of PDAC determine the molecular characteristics. It is also associated with the
down regulation of mucin proteins in PanCa, which is a key modulator of cancer progression, stromal abundance and chemo-resistance (63). Modulation of microRNAs can provide a therapeutic benefit by (i) Inhibition of upregulated miRNAs (oncogenic) and (ii) Restoration of lost miRNAs (tumor suppressor).

**Oncogenic microRNAs**

There are several miRNAs that have been screened based on functional studies and miRNA profiling and it has been found that they are associated with tumor progression with precursor lesions. The overexpression of oncomirs promote tumor progression by inhibiting the expression of tumor suppressor microRNAs such as miR17-22, miR125b, and miR-125 (66, 67). The overexpression of miRNA-196b has been identified in PanIN-III (68). Aberrant expression of miR-21, miR-155, and miR-221 are linked to early PanIN lesions (69) and associated with TGF-β expression which leads to tumor progression. Quite a few studies have been performed on miR-21. It has been reported that miR-21 is negative regulator for TGF-β signaling and suppressed SMAD7 expression (70). Consequently, miR-21 will be a potent therapeutic target for several cancers. In PanCa, downregulation of miR-21 leads to the inhibition of desmoplastic condition by regulating the expression of TGF-β (71), which eventually targets the drug resistance in PDAC as overexpression of miR-21 is a major cause of chemo-resistance (72). MiR-155 is identified as a proto-oncogene and it target tumor suppressor TP53INP1 and decreased PDAC patient survival (73).

**Tumor suppressor microRNAs**

Reduced level of MicroRNAs is responsible for tumor progression in various cancers. These miRNAs are known as tumor suppressor microRNAs. In healthy tissues the level of tumor suppressor miRNAs is normal. Restoration of these miRNAs is well documented approach for cancer therapeutics. This can be approached by miRNA mimic or delivering them using a gene vector. There are several reports that indicate that tumor suppressor miRNAs are in preclinical phase of therapy but not successful in clinic to treat cancer. Based on the literature Let-7, miR-34a, miR-96, miR-375, miR-200 family, miR-145, miR-143 are presently most well-established tumor suppressor miRNAs for PanCa therapy. Restitution of lost Let-7 miRNA expression in in-vitro and in-vivo studies showed significant therapeutic efficacy. PanCa cells are sensitized in presence of Let-7, and display reduced cell proliferation and make the cells sensitive towards gemcitabine (74). Another promising onco-suppressive miRNA is miR-34. It can target tumor initiating stem cells in PanCa and inhibit downstream regulators Bcl2 and NOTCH (75). Recently our group has published that miR-145 also functions as a tumor suppressor in PanCa target mucin MUC13 in PanCa cells and reduced tumor growth and invasion (37). The over expression of miR-145 in PanCa cells render them sensitive to GEM (76). So over expression or restoration of tumor suppressor miRNAs is a promising therapeutic modality to diminish PanCa growth. Towards this target there are only a few commonly used approaches, such as restoration of synthetic miRNA mimic, vector mediated delivery of miRNA and nanocarrier based therapy.
**MicroRNA-145 (miR-145)**

MiR-145 is a well-known tumor suppressor in various cancers. It suppresses the functional characteristics of the cell, such as cell proliferation, invasion and migration in different types of cancer conditions. Presence of significant level of miR-145 play a pivotal role in patient survival. Loss of miR-145 in cancer condition is one of the major causes of cancer progression. Pancreatic, gastric, colorectal and lung cancer displayed loss of miR-145 in cancer condition (77-82). It has been identified that in breast cancer, miR-145 has been significantly downregulated in triple negative breast tissue (TNBC) (83-85). There is no detectable level of miR-145 in cervical cancer and in advanced small cell carcinoma of the cervix showed significant level of down regulation of miR-145 (79, 86). Different clinical stages and grades of tumor exhibited varied expression of miR-145 such as hormone receptor in TNBC and lower expression in Lymph node positive non-small cell lung cancer (NSCLC), (83, 87). It has been reported that down regulated expression of miR-145 is not only responsible for tumor progression also leads to chemo resistance (88).

**The role of miR-145 in pancreatic cancer**

Downregulation of miR-145 in PanCa is well documented (89) and k-Ras activation is a one of the major causes for the loss of the mir-143/145 cluster (90). Chemoresistance is a major therapeutic problem for PanCa. Although gemcitabine is a first-line therapeutic option for this lethal disease, GEM resistance is newly identified hindrance in treatment regimen. miR-145 directly targets p70S6K1 and inhibit its expression, which has a pivotal role in drug resistance (76). As a tumor suppressor, miR-145 displayed an effective role in cancer therapeutics to overcome chemoresistance. In PanCa condition, miR-145 can target pluripotency maintenance factors (OCT4, SOX2, NANOG, and KLF4) (91), also inhibit insulin growth factor-1 (92). Enhanced expression of miR-145 regulate the oncogenic factors such as ITGA11, MAGEA4, SET, RPA1, MCM2, ABCC1, SPTBN1 and SPTLC in PanCa progression, growth, stemness (93).

**Delivery of microRNAs: Obstacles**

Although miRNA plays a profound role in cancer biology and therapy, translation into clinical practice is a major challenge for miRNA therapy. Major obstacles in miRNA therapeutics are poor systemic stability, rapid renal clearance, immunogenicity, low stability and lack of efficient delivery at the disease site (94). In the bloodstream, the half-life of any oligonucleotide is measured in minutes, but to improve the half-life there are several methods of modification. During renal clearance, the kidneys accumulate the small RNAs and clear them from the body. At the same time, the liver also plays important role in oligonucleotide clearance. The reticuloendothelial system (RES), is another system which consists of Kupffer cells that help to remove the oligonucleotides from the systemic circulation. Lysosomes consist of phagosomes, responsible for phagocytosis of oligonucleotides, which degrade the nucleotides by nucleases (95). Therefore, the challenge is the delivery of miRNA successfully routed to the target
organs in an active form. Targeted delivery of these therapeutic molecules via nanoparticle is an effective therapeutic modality for cancer (96). Nanoparticle mediated vehicles are able to deliver small RNAs or oligonucleotides to the interstitial spaces of the tumor (96). These nanocarriers can also escape the endosomal degradation and deliver the therapeutic molecule to the cytoplasm of the cells. Appropriate surface modification of these nanoformulation can deliver miRNAs to the target site.

Cationic polymers are most widely used and the oldest method of delivering nucleic acids to disease cells. These polymers are positively charged and can be easily conjugated to the negatively charged nucleic acids. This polymer is suitable for gene delivery due to its low toxicity and immunogenicity as compared to other polymers used in therapeutic delivery (97). There are several naturally and synthetically derived polymers that are used in this field, however, out of all, PEI (Polyethyleneimine) is most suitable and widely used cationic polymer for gene delivery (97). The advantages of PEI in gene delivery aspects are, easy uptake and, release via endocytotic mechanisms (98-101). Enhanced efficacy of gene delivery has been observed when conjugated with iron oxide nanoformulation (101).

One of the most commonly used nucleic acid carriers are liposomes. They are composed of an amphiphilic molecule which form a phospholipid bilayer mimicking the human cell membrane. Utilizing the advantage of their resemblance of cell membranes, liposomes can encapsulate therapeutic payloads and easily pass through the cell membrane followed by the release of the payload (miRNA, siRNA) into the cells (102). Major drawbacks of liposomal delivery systems are low specificity, sensitivity and toxicity (102). Other than liposomes, polymeric micelles, dendrimers and, metal nanoparticles are also used for therapeutic payload for cancer therapy. Surface modification of these formulations could enhance the targetability of the nanoparticulate system (103). Of all the nano carriers iron oxide nanoparticles are most widely used and one of the safest formulations to target cancer cells.

**Nanoparticle-Mediated Delivery**

Efficacy of anticancer drugs is hampered because of systemic degradation, poor bioavailability, unsatisfactory pharmacokinetic profile, drug resistance and side effects. Nanoparticles are capable of targeting tumors efficiently with the help of unique characteristics, such as the “Enhanced Permeation and Retention” (EPR) effect. The nano size of particles is beneficial for increased surface-area-to-volume ratio which leads to

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higher surface attachment capacity (105). Prolonged systemic circulation improves delivery of drug and genetic materials to its intracellular target. Efficient internalization of the nanoparticle leads to reduced cellular toxicity by minimizing the off-target effects. For pancreatic cancer, nanoparticle mediated miRNA delivery has not yet been exploited. Given the scenario miRNA replacement strategy would be a potential therapeutic modality for pancreatic treatment. In Panc-1 xenograft mice model, restoration of miR-34a has been achieved using antibody guided liposomal delivery system (106). Silencing of miR-21 utilizing gold nanoparticles (107) and solid lipid nanoparticles conjugated exogenous miR-375 efficiently target tumor and diminish tumor growth.

Nanotechnology has been efficacious at a commercial scale because of the modernized electronics and energy sector (108). Research in targeted drug delivery using magnetic micro and nanoparticles began 40 years ago (109). Nanotechnology is related to materials and devices in a nanometer scale of 1-100 nm in dimension. Among various types of nanoparticles, magnetite and maghemite are the more promising group of magnetic nanoparticles (MNPs), which are biocompatible (110), biodegradable and easily internalized in cells. Multifunctional nanoparticles exhibit unique characteristics that are highly useful in medical and pharmaceutical applications (108). An appropriate surface engineering of MNPs helps to achieve multifunctional characteristics and successful implementation in diagnosis and drug delivery. Iron oxide based MNPs have been widely used in biomedical applications.

Functionally active iron oxide nanoparticles consist of (1) a magnetic core (2) a biocompatible surface coating for encapsulation (3) a sustained released drug, and (4) a therapeutic biomarker with a targeted linker (111) for effective therapeutic intervention. Based on the demonstrations of recent clinical trials using magnetic nanoformulations (Table 1-1) in different types of cancer, we can expect that magnetic nanoformulations will be a highly beneficial area in therapeutic intervention. The clinical study outcome explains that iron oxide nanoparticles are very effective for thermal therapies, such as hyperthermia, alternative magnetic field application. MR imaging is another field of effective diagnosis and enhanced therapeutic potential.

Magnetic nanoparticles

The paramagnetic property of ultra-small iron oxide nanoparticles (USPIONs) is useful for theranostic strategies (MRI) in biomedical sciences. There are several nanocarriers used for cancer therapeutic applications for drug or gene delivery (Figure 1-2A) but MNPs are advantageous over other nanoformulations because of their multifunctionality (Figure 1-2B). The first nanoparticle made-of iron oxide core stabilized with dextran was approved by the Food and Drug Administration (FDA) for liver imaging. So, MRI is a potential technique to diagnose cancer at early stage utilizing the contrast image method. MRI contrast agents become more acceptable for theranostic applications because of their specific iron oxide core with biocompatible surface coating (111). Considering this feature, a few MNP formulations with different polymer
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<th>Clinical application</th>
<th>Purpose/Outcome</th>
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<td>Clinical hyperthermia of prostate cancer using magnetic nanoparticles: Presentation of a new interstitial technique (112).</td>
<td>This is first clinical trial conducted for interstitial hyperthermia application on locally invasive human prostate cancer.</td>
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<td>Detection and characterization of focal liver lesions: A Japanese phase III, multicenter comparison between gadoxetic acid disodium-enhanced magnetic resonance imaging and contrast-enhanced computed tomography predominantly in patients with hepatocellular carcinoma and chronic liver disease (113).</td>
<td>This clinical application was done on 178 patients with focal hepatic lesions. Combined MR imaging showed significantly higher sensitivity than CT for lesion detection. The sensitivity values were statistically proved (p &lt; 0.05).</td>
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<td>Morbidity and quality of life during thermotherapy using magnetic nanoparticles in locally recurrent prostate cancer: results of a prospective phase trial (114).</td>
<td>This study was performed to inspect the effect of thermotherapy on morbidity and quality of life of patient with locally recurrent prostate cancer. Patient were treated with intraprostatic injection of nanoparticles and alternative magnetic field were applied and a maximum 55°C temperature was reached. This application is achievable, but some modifications are needed for higher magnetic field strength.</td>
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<td>Breast imaging. Preoperative breast cancer staging: comparison of USPIO-enhanced MR imaging and 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging for axillary lymph node staging-initial findings (115).</td>
<td>This clinical trial was performed on 10 patients who were considered for surgery and resection of auxiliary node. Super magnetic iron oxide nanoparticles and 18F-fluorodeoxyglucose positron emission tomography were applied in combination and it is giving 100% sensitivity, specificity.</td>
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<td>Decrease of signal intensity of myometrium and cervical stroma after ultra-small superparamagnetic iron oxide (USPIO) particles administration: an MR finding with potential benefits in T staging of uterine neoplasms (116).</td>
<td>Ultra-small super magnetic iron oxide nanoparticles were used for clinical application on seventeen female patients with uterine malignancy to identify T staging. Intravenous administration of nanoparticles leads to decreases of signal intensity of myometrium which leads to nearly perfect T-staging of neoplastic lesions.</td>
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Figure 1-2. Types of nanoformulations used in cancer therapy.

(A) Liposomes, polymeric micelles, dendrimers, and polymeric nanoparticles.
(B) Multifunctional magnetic nanoparticles (MNPs) and possible theranostic approaches.
coatings have been in clinical reaching the tumor site and accumulating in the tumor tissue (117). An increased retention of magnetic particles in the tumor tissues occurs because of the lack of lymphatic clearance. Specific targeting of the MNPs has been enhanced by active targeting, which is accomplished by using cancer biomarkers HER2, EGFR, CD20, KRAS, BRAF, ER/PR etc. Active targeting can further increase selective cellular uptake and internalization of MNPs in cancer cells (115). This feature promptly routes nanoparticles to the target site independent of the route of administration (118-121). Hyperthermia is another promising technology that uses magnetic MNPs in cancer therapy (122). Under alternating magnetic field (AMF), MNPs can transform the electromagnetic energy into heat. A selective increase of temperature at the tumor site offers radio-/chemo-sensitization and oxygenation in tumor tissue (123).

Physico-chemical characteristics of magnetic nanoparticles

A number of studies suggest that various physio-chemical characteristics determine the pre-clinical and clinical applications of MNPs. The unique stability of MNPs, dictates the physio-chemical properties which can be altered. This is done by choosing a specific method of preparation and opting the suitable layer(s) on the MNP core. This approach can generate an appropriate composition of excipients, size, surface charge, and loading efficiency of therapeutics.

In general, the size of MNPs determine the in vivo behavior, half-life, systemic circulation, receptor binding/cross linking, cell responses and intra-cellular uptake (124-127). Particle size is important for extravasation from bloodstream to tumor tissue (128). Particles smaller than 10 nm are instantaneously removed by renal clearance and particles larger than 200 nm aggregate in the spleen or are phagocytosed by the cells. In both the cases a decreased plasma concentration of MNPs is seen (125, 129, 130). Therefore, an optimized size range of MNPs is required for specific in vivo applications (131-133). It was found that nanoparticles sizing in the range of 10-100 nm is ideal for biological applications. This size range has proven to have longer circulation time easy access to the small capillaries of leaky and irregular tumor vessels (gaps between endothelial cells), enhanced particle accumulation and minimal non-specific uptake by certain type of cells. Yang et al. (134) demonstrated that 10 nm particles were accumulated in the liver while 40 nm particles were found in the spleen.

In addition to size, shape is an important characteristic of MNPs. Cellular uptake, interaction, internalization and in vivo biodistribution mechanisms are directly correlated with shape of magnetic particles (135). Shape control is a major factor to maintain an efficient therapeutic application. Different reaction conditions and chemicals involved in the formation of nanoparticles affect the shape of MNPs. Few research reports confirmed that there are different shapes of particles that can be prepared such as hexagons, cubes, spheres, rods, etc., using different synthesis methods (125, 136). Different surface modifiers or surfactants which contain large size hydrocarbon chains create steric hindrance, which become an obstruction when growing crystals during the particle synthesis process (137). Reports confirm that rod shaped and non-spherical nanoparticles
show higher circulation time than spherical nanoparticles (138). One report explains that particle shapes like nanosphere, nanobead and nanoworm create toxicity in fibroblast cells (139). Therefore, particle shape is another parameter for cellular toxicity.

Nanoparticles (including MNPs) exhibit high surface to volume ratio, making them highly reactive. To protect these MNPs from decomposition (oxidation) they need to be coated with an inert/biocompatible surfactant or polymer. The MNPs are generally stable in very high or low pH, but these conditions are not suitable for *in vivo* delivery. **Table 1-2** illustrates commonly used MNPs with appropriate coating/functionalization and their application. Coated MNPs can enhance targetability and specificity towards several cancer therapeutics. Organic molecules or polymer coating on MNPs can maintain some functional characteristics (129, 136) such as:

- Minimal particle aggregation which leads to enhanced dispersity and colloidal stability
- Increased systemic circulation by minimized clearance by reticuloendothelial system (RES) organs
- Protection from surface oxidation and enhance biocompatibility
- Reduced toxicity of particles by minimizing nonspecific interactions
- Coated particles can be used for drug conjugation, targeting ligands and reporter molecules.

**Magnetic nanoparticles in cancer therapy and imaging**

**Drug delivery**

Chemotherapy is the most common therapeutic intervention in cancer. Adverse systemic reactions like hair loss, nausea and vomiting, effects on the nervous system, liver and kidney toxicity associated with conventional therapy, indicates that an alternative approach is urgently required. As an example, GEM has a substantial role in clinical application for pancreatic cancer. Although the efficacy of GEM is not satisfactory because of short half-life, low cellular uptake and poor bioavailability. To overcome this problem GEM needed higher dose of administration which leads to systemic toxicity (140). This can be accomplished by either reducing the dose of the chemotherapeutic agents required for effective treatment or by increasing the delivery, specificity and activity of the therapeutic agent at the tumor site (141). Therefore, targeted delivery of drug or any other molecule is the new therapeutic strategy which entails specifically targeting tumors while sparing normal cells. Nanotechnology plays a substantial role in targeted delivery in cancer therapy and MNPs act as an appropriate delivery method in a number of biomedical applications (111, 142). Stability, biocompatibility and paramagnetic property of MNPs are the most considerable factor for *in vitro* and *in vivo* delivery (118). The size and surface charge of particles can be modified by different methods of synthesis (111). Hydrodynamic size of particles more
Table 1-2. Surface coating material for magnetic nanoparticles and their applications.

<table>
<thead>
<tr>
<th>Coating material</th>
<th>Target</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural polymer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>Human colorectal Carcinoma by Monoclonal antibody A7</td>
<td>Drug delivery and imaging (143, 144)</td>
</tr>
<tr>
<td>Chitosan</td>
<td>Atrial natriuretic peptide receptors</td>
<td>Nonviral gene/drug therapy, hyperthermia (145, 146)</td>
</tr>
<tr>
<td>Starch</td>
<td>Passive targeting</td>
<td>Imaging and contrasting radiotherapy (147, 148)</td>
</tr>
<tr>
<td>Gelatin</td>
<td></td>
<td>Separation of DNA, drug delivery and targeting (145, 149)</td>
</tr>
<tr>
<td><strong>Synthetic polymer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG</td>
<td>PSMA</td>
<td>Drug delivery to tumors and imaging. (150-152)</td>
</tr>
<tr>
<td>PVA</td>
<td>Passive targeting</td>
<td>Drug delivery especially brain, \textit{in vivo} contrasting and imaging (153, 154)</td>
</tr>
<tr>
<td>PEI</td>
<td>Passive targeting</td>
<td>miR delivery, Nonviral gene delivery, magnetofection (100, 101, 155, 156)</td>
</tr>
<tr>
<td>PVP</td>
<td>Passive targeting</td>
<td>Efficient drug loading, drug delivery and imaging (157)</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>Anti-EGFR antibody</td>
<td>MR imaging (158, 159)</td>
</tr>
<tr>
<td>Lipid</td>
<td>Passive targeting</td>
<td>Immunoassay (160)</td>
</tr>
</tbody>
</table>
than 200 nm may minimize the activity of the formulation (161-163). Multi-layer coating with surfactants or polymers increase the stability of the core and the feasibility of surface modifications (111, 142, 164). β-cyclodextrin (CD) and pluronic polymer (F-127) are well known surface modifier for nanoformulation. Yallapu et al. previously proved that CD and F-127 coated iron oxide cores form smaller sized particles which enhance anticancer drug (curcumin) loading with lower protein binding. This leads to optimal particle uptake into cancer cells without obstructing the magnetization character of nanoparticles (142). These nanoparticles are non-toxic and biocompatible in vitro and in vivo, independent on route of administration (100, 101, 118, 119, 121). The coating of polyethylene glycol (PEG) doubles the residence time in systemic circulation, resulting in reduced particle accumulation in liver and spleen (secondary organs) (165). The first Phase I clinical trial of MNP targeted delivery was performed by Lubbe and co-workers in 1996 (166, 167). The second clinical trial was performed with a magnetic nanoformulation on hepatocellular carcinoma patients in 2002 by the Koda and group (166). In 2004, the third clinical trial was performed with doxorubicin linked magnetic particles on hepatocellular carcinoma patients. In this study the route of delivery of the particles was hepatic artery utilizing the MRI technique (168). Superparamagnetic iron oxide nanoparticles delivered doxorubicin with a 20-fold higher entry of particles and drug release in human PanCa cells (169). PEGylated multiwalled carbon nanotubes modified with superparamagnetic iron oxide which can encapsulate oxaliplatin for colon cancer therapy. The PEGylated particle has shown to have a very effective sustained release property by which it was effective as anticancer therapy similar to a free drug, but had minimal death in the mouse model (170). β-cyclodextrin, polyethyleneglycol (PEG) and polyethyleneimine (PEI) coated Fe₃O₄ nanoparticles were used for 5-Fluorouracil delivery in cancer cells. This formulation showed faster and higher release capability in pH 6.8 than acidic pH (pH 1.2) (171). One of the research explained that natural polymer, chitosan coated MNPs have enhanced anti-cancer efficacy of GEM in breast cancer cells. They have shown uptake of MNPs in cells by Prussian blue method and that an acidic microenvironment is suitable for higher release of GEM. At the same time IC₅₀ of nanoparticle conjugated GEM is significantly lower than free drug (172).

Gene delivery

Magnetic nanoparticles have been used widely for gene delivery. The obstacles in gene delivery include degradation of genetic materials in blood stream, low cellular uptake, and fast renal clearance (173, 174). An ideal delivery system should be stable, biocompatible, biodegradable, and non-immunogenic with good targetability (173). Although magnetic particles are very efficient drug delivery systems, in cancer they need a specific surface modification for effective delivery and enhance stability of DNA or RNA molecules in in vivo systems (109, 111). These biomacromolecules can be attached to the particles through electrostatic interaction or chemical/physical cross linking on the charged surface coating or degradable outer shell (109). In this case particles are coated with polymer matrix like polyvinyl alcohol (PVA), silica, dextran; or encapsulated within the polymer or iron core (175, 176). Different functional groups such as amines, carboxyl, biotin, streptavidin etc. attached to the core or on a matrix can modify the efficacy of the formulation (177). One of the most useful techniques to deliver DNA or
RNA with the particle is to link a positively charged molecule on the surface of the vehicle. This helps to bind the negatively charged nucleic acids with the nanoparticles (109). A widely applicable cationic polymer is PEI, which is the first reported transfection agent to deliver DNA utilizing the secondary amine groups present in the chain length (178). PEI expedites the lysosomal release in cytosol (179). The internalization of particles conjugated with DNA follows clathrin-dependent endocytosis, which may be more efficient when particles are coated with PEI and used in magnetoception (180). When PEI coating conjugated with pluronic F127, complexation and condensation properties of nanoparticles improved remarkably (181). PEI-F127 coated MNPs show stable and high transfection efficiency of microRNA-145 in PanCa cells (101) (Figure 1-3). The delivery can further be increased significantly when an external magnetic field is applied. This technique is known as magnetoception and has a limitation of only being applicable for accessible tumors (182, 183). PEI modified Fe3O4@SiO2 formulation delivers VEGF small hairpin (sh)RNA silencing the VEGF gene with high biocompatibility and low cytotoxicity with other cells (184). It has been reported that superparamagnetic iron oxide nanoparticles coupled with siRNA aimed against Polo-like kinase-1(PLK1), reduce tumor growth and enhance apoptosis in PanCa (185).

**Magnetic resonance imaging**

Magnetic resonance imaging (MRI) is one of the most useful imaging techniques utilized in clinical settings. It gives real-time monitoring and non-invasive functional information with outstanding anatomical details of living organisms (186, 187). MRI is not only used as a diagnostic tool but can be implemented for therapeutic intervention as well. Development of contrast agents for MRI would increase the specificity and clarity of the images of organs (187). These are the two basic principles commonly used in MRI and have been described in several articles (188-190). Spin echo and inversion recovery sequences together with modifications gives developed signal noise ratio, imaging time and contrast resolution (191). These processes are based on the alignment of protons and their precession along with applied magnetic field. Application of transverse radiofrequency on the precessed protons agitated them from the direction of magnetic field. In the subsequent relaxation step the pulse field is removed from the protons to allow them to come back to a ground state. Generation of bright and dark MR image depends on the two independent relaxation process, longitudinal relaxation ($T_1$-recovery) and transverse relaxation ($T_2$-decay) respectively (186). The contrast in magnetic resonance image is the difference of the two relaxation times among various tissues. *In vivo* application of contrast agents can selectively modify the relaxation time (187). MNPs are the most frequently applicable contrasting agent where MNPs accumulate in the tissue, becoming a $T_2$ contrast agent (186). Paramagnetic materials can alter the magnetic field surrounding the tissue which leads to shorten the relaxation times in $T_1$ and $T_2$ (192, 193). Iron oxide nanoparticles are used for MRI in humans which and have the required regulatory approval (194). The efficiency of a particle as an MRI contrast agent, depends on their physicochemical properties such as the size of the particles, their surface charge and chemistry and their bioconjugation property with targeting agents. Large size MNPs indicate higher relaxation times of $T_2$ (195). For example, multifunctional iron
Figure 1-3. Schematic representation illustrating the delivery of miR-145 and its molecular effects in pancreatic ductal adenocarcinoma.

oxide nanoparticles with a surface modified with several layers such as, poly citric acid, poly (ethylene glycol), and folic acid. T2-weighted MRI images of this formulation (Fe₃O₄@PCA-PEG-FA) showed improved resonance signal with increasing concentration of particles (197). Anti HER2 single chain antibody (scFv) conjugated iron oxide nanoparticles (scFv-IONPs) were used for HER2 targeted MRI in, in-vitro and in-vivo tumor models with HER2 low/high expression cells. These studies suggested scFv-IONPs as efficient MR contrast agent in cancer therapeutics (198). Aggregated superparamagnetic iron oxide nanoparticles, surface modified with PEI and PEG polymers, loaded with doxorubicin and folic acid conjugated for cancer specific targeting in in-vivo mouse models. Aggregation of these particles identified by MRI and showed high r₂ relaxation with efficient theranostic approaches (199). Targeted MRI using magnetosome could be an effective detection technique in breast cancer model (200).

**Personalized Medicine Improves Therapeutic Outcome**

Personalized medicine is customizing a new approach of medical treatment to the specific characteristics of individual patient. A unique genetic and molecular profile of a patient makes them receptive to a particular disease. The personalized medicine approach utilizes this scientific advancement to treat a patient. This therapeutic approach is increasing the ability of predict safe and effective therapy for individual patient and designing new clinical treatments. This therapy can discriminate the type of patient has chance to develop a diseased condition in future. For instance, if a theranostic agent applied to both healthy and target individual, it will give a feedback about the positive responses and future risk factor for a patient. This background information will help the medical scient to develop specific therapeutic option for an individual patient. New study reports demonstrated that highly personalized combination treatment can improve therapeutic modality and patient survival who are suffering from resistant cancer.

**Rationale of exosome selection as nano carrier**

Metastasis is a fearful factor in cancer progression which consists of several interconnected activities, such as invasion, circulation, adhesion and proliferation. These series of events eventually establish metastasized tumor in different organs. To control tumor microenvironment novel-tumor targeting strategies are urgently required. Artificial nanoparticles are highly efficient in drug delivery aspect but the major obstacle of these non-self materials, are their stability in the blood stream and the immune responses that they incite. Biocompatibility, immunogenicity and targeting towards specific cell type remain a challenge. More promising recent therapeutic modalities are gene therapy and cell based therapy but still there are adverse issues such as toxicity and host immune responses (201). In tumor microenvironment, extracellular vehicles (EVs) are secreted from tumor cells to communicate with stromal cells (202). EVs consist of exosomes, they are small intracellular membrane bound biological vesicles involved in several biological processes (203). Exosomes are in the size range of 30-150 nm extracellular vesicles, present in all biological fluids (204). They are composed of cell membranes
(phospholipid bilayer) and well tolerated by the host. Because of the physiological characteristics, they can easily fuse with recipient cell membrane and improve the cellular internalization and drug delivery (205). Exosomes can facilitate intracellular communication and naturally deliver the biological cargo which consist of protein, lipid, microRNAs (miRNA) and mRNAs to the recipient cells (202, 206-208). Because of their smaller size, they are being escaped from phagocytosis by mononuclear phagocyte which leads to improved diffusion in tumor tissues by promoting their extravasation (209).

### Exosomes as a personalized therapy

Advance research on extracellular vesicles is a new therapeutic avenue towards personalized medicine (205). To target the multifaced molecular criticality of pancreatic cancer, personalized therapy would be a blessing for PanCa patients. In current therapeutic intervention, it is necessary to customize the medication based on the information about individual patients (204). Concept of personalized therapy is to target to the specific disease characteristics of each patient and responses towards specific treatment (205, 210). Development of diagnostic tools using EVs getting started by several companies such as Exosomics Siena and Exosome Sciences. There are other organizations also concentrating on collection and interpretation of molecular information of EVs to utilize them in personalized therapy (205). Considering the nature of exosomes (EVs), they can play an advantageous role as drug and gene (miRNA) delivery vehicles over nanoparticulate delivery systems such as, liposomes and polymeric nanoparticles. Physical stimulation and autologous source are important factor for using EVs as personalized medicine. The autologous EVs contain the same molecular cargo which is a promising factor for same microenvironment. Customization of autologous EVs by loading drug or nucleic acid give the therapy new hope. Exosomes are present in bodily fluids, and they can protect their cargo from degradation and are stable in systemic circulation. These characteristics of exosomes makes them an attractive tool for diagnostic and therapeutic application that eventually became an efficient candidate for clinical trials (204). Exosomes are not recognized as a foreign body because they are similar as one’s own cellular system. The non-immunogenic nature of exosome makes them interesting to use as a drug delivery vehicle when they are isolated from the autologous human body. This non-immunogenic behavior makes them an attractive molecule for personalized be medicine. In this modern therapeutic intervention, it is suggested that tumor therapy must customized based on available information about individual patients and responses to specific treatment (204). This is possible by utilizing an autologous biological material, which can be applied as a personalized medicine to match the individual circumstances and molecular profile of the patient.

### Summary of Introduction

The focus of the research involves the investigations pertaining to underlying aberrant signaling mechanisms in PanCa and development of novel therapeutic delivery approaches for its treatment. On this regard we have extensively worked on the
identification of molecular markers related to pancreatic cancer, such as oncogenic mucin, MUC13 which is over expressed and tumor suppressor miR-145, significantly downregulated in PanCa condition.

Superoxide iron oxide particles, which have an ability to be used for vast biomedical purposes, such as, hyperthermia and imaging. We have investigated the restoration of miR-145 in PanCa models using iron oxide nanoparticles and how they target MUC13. Here in we have also targeted the chemo-resistance to standard chemotherapeutic agents in pancreatic cancer. PanCa is highly resistant to TRAIL therapy which is clinically approved for other cancers. We were able to delineate the mechanisms to overcome TRAIL resistance and at the same time explain the underlying mechanisms that make these tumors resistant.

The last phase of research focused on the development of personalized medicine for PanCa patients to overcome drug ineffectiveness due to existence of vast heterogeneity. In modern cancer therapeutic strategies, customize treatment is essential based on the information about patients for improved outcomes. In this respect, we have discovered that extracellular vesicles (exosomes) secreted from fibroblasts, isolated from patient derived tumor adjacent normal tissues, can be used as biological nanoplatforms for personalized therapy. These autologous biological materials are biocompatible and non-immunogenic and can utilized as a personalized medicine. Knowing the importance of stroma in hindering drug delivery, we have optimized an exosome based therapeutic delivery approach using a drug, ormeloxifene, which can target both tumor and stroma.

**Hypothesis of the Study**

Oncogenic mucins contribute drug resistance in PanCa and loss of tumor suppressor miRNA also plays a pivotal role in tumor progression. Restoration of miR-145 will lead to overcome therapeutic resistance, inhibition of PanCa growth and progression.

Novel exosomes based therapeutic approach, using exosomes isolated from the patient derived tumor adjacent normal (NAT) fibroblast cells. These autologous vehicles will efficiently deliver therapeutics for personalized medicine to prevent cancer recurrence.

**Specific Aims of the Study**

- **Aim 1**: To develop and assess the efficacy of a miRNA delivery method for the treatment of pancreatic cancer.

- **Aim 2**: To identify the source and molecular mechanisms of TRAIL resistance and counteract TRAIL resistance in PanCa and sensitize tumors towards TRAIL and chemotherapy.
• **Aim 3**: To develop and engineer unique superparamagnetic nanoparticles (MNPs) for co-delivering miR-145 and TRAIL for improving TRAIL response in PanCa.

• **Aim 4**: Isolation and characterization of exosome as a personalized therapeutic carrier for PanCa, and investigate the efficacy of patient derived exosomes in efficient drug delivery approach.
CHAPTER 2. RESTITUTION OF TUMOR SUPPRESSOR MICRORNA-145 USING MAGNETIC NANOFORMULATION FOR PANCREATIC CANCER THERAPY

Introduction

Pancreatic cancer remains a highly lethal human malignancy due to a failure of effective adjuvant therapies (211). MicroRNAs (miRNA) are small noncoding RNAs that regulate multiple biological pathways during cancer development and progression and serve as tumor suppressors or oncogenes. The inhibition of oncogenic microRNAs using anti-miRs and restitution of tumor suppressor microRNAs via miRNA mimics represent a powerful therapeutic strategy in cancer treatment (212) (213). Prior work from our lab and that of others have demonstrated the tumor suppressor role of miR-145 in pancreatic cancer (37), (91, 214, 215). Our study has shown that miR-145 targets a newly identified transmembrane glycoprotein, MUC13 that is aberrantly overexpressed in pancreatic cancer and modulates its associated targets, such as HER2 and p53 (37). Its restoration inhibits MUC13 levels and suppressed tumor growth in pancreatic cancer xenograft mice model (37). Although studies provide compelling evidence for miR-145 involvement in the inhibition of pancreatic cancer, the impact of its sustained release has not been established. Obstacles to the successful and efficient delivery of microRNAs for therapy have yet to be overcome.

Magnetic nanoparticle formulations for drug delivery have previously been generated but due to the high particle size in suspension, loss of magnetization and inefficient internalization in the target cells resulted in poor therapeutic efficacy for cancer treatment (142). Recently, we have engineered a unique magnetic nanoparticle (MNP) based system for gene(s) and drug(s) delivery applications (142, 216-218). We have previously demonstrated that our uniquely engineered MNPs are capable of targeting pancreatic tumors efficiently with the help of the “Enhanced Permeation and Retention” (EPR) effect (216) leading to increased cellular uptake and internalization of MNPs in cancer cells. EPR effect is a unique phenomenon of solid tumors based on their anatomical and pathophysiological differences from normal tissues, molecules of certain sizes are capable of accumulating and retaining in tumor tissues selectively but not in normal tissue. Additionally, these MNPs have shown enhanced MRI properties (compared to conventional MNPs) (218). Polyethylenimines (PEIs) are linear or branched polymers being partially protonated under physiological conditions, that easily form complexes with nucleic acids (219). PEIs have previously been shown to be

excellent delivery vehicles for DNA plasmids and other DNA or RNA molecules including ribozymes and siRNAs (219). The purpose of this study was to generate a miRNA-145 loaded magnetic nanoparticle system utilizing our recently engineered MNPs and assess efficacy of this novel formulation for miR-145 restitution and potential use in pancreatic cancer treatment.

**Materials and Methods**

**Culture of pancreatic cancer cells**

Human pancreatic cancer cells, HPAF-II and AsPC-1 were purchased from American Type Cell Culture (ATCC; Manassas, VA, USA) and cultured using cell specific culture media, F12/DMEM and RPMI (HyClone Laboratories, Inc. South Logan, Utah, USA), respectively, which was supplemented with 10% fetal bovine serum (Atlanta Biologicals) and antibiotic/antimycotic solution at 37 °C in a humidified atmosphere (5% CO2 and 95% air atmosphere). Cells were routinely tested for mycoplasma every six months.

**Chemicals, reagents, microRNA mimics and antibodies**

All chemicals and reagents were purchased from Sigma Aldrich Corporation (St. Louis, MO, USA) and cell culture wares were purchased from Corning life sciences (Tewksbury MA, USA). Non-targeting control (catalog number: AM17111) and miR-145 mimics (catalog number: 4464066), Taqman miR-145 probes (Assay id: 002278), High Capacity cDNA Reverse Transcription kit (catalog number: 4368814) and TRIzol reagent (catalog number: AM 9738) were purchased from Life technologies (Carlsbad, CA, USA). The primary antibodies, anti-HER2 (catalog number: 2165S) and anti-p53 (catalog number: 2527) were purchased from Cell Signaling (Danvers, MA, USA) and anti-β–actin (catalog number: A5316) was purchased from Sigma (St. Louis, MO, USA).

**Preparation of miR-145-MNPF formulation**

Iron oxide nanoparticles were prepared by the co-precipitation using Fe$^{2+}$ (300 mg) and Fe$^{3+}$ (800 mg) ions in the molar ratio ~ 1:2 as described previously (216). Briefly, 200 mg of β-cyclodextrin (β-CD) was added to the solution and stirred for 10 min at 500 rpm. 250 mg of Pluronic F-127 was added to the suspension and stirred overnight followed by washing the particles thrice and re-suspending in water. In this study, we used PEI for the purpose of miR-145 delivery as these polycationic polymers form non-covalent complexes with nucleic acids and aid in efficient gene delivery (220). In order to form a uniform layer, 100 mg PEI was added to the MNP suspension and stirred overnight. These MNP-PEI nanoparticles are stored and loaded with microRNA mimics just prior to treatment of the cells. 100 nM of non-targeting experimental control
(NC) or miR-145 mimics were incubated with 1μg/ml of MNP-PEI nanoparticles in 0.9% NaCl for 30 min to form NC-MNPF (non-targeting control-MNP formulation) and miR-145-MNPF (miR-145-MNP formulation) complex (142).

Particle size and zeta potential

The hydrodynamic nanoparticle size and zeta (ζ) potential of miR-145-MNPF were determined by the dynamic light scattering (DLS) principle using Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK). To measure the particle size 25 μL of 1 mg/ml nanoparticle suspension was added to 3 ml of water and probe sonicated using VirSonic Ultrasonic Cell Disrupter 100 128 (VirTis, Gardiner, NY) for 30 sec. For ζ-potential measurement, the same diluted particle suspension was used.

Transmission electron microscopy

The size and morphology of magnetic miR-145-MNPF were determined by JEOL 200EX transmission electron microscopy (TEM) (JEOL Ltd, Tokyo, Japan) operating at 60 kV. 100 μg/ml nanoparticle suspension was prepared and probe sonicated for 30 sec. 200 mesh formvar-coated copper TEM grid (grid size: 97 μm; Ted Pella Inc, Redding, CA, USA) was used to prepare samples by carefully placing the nanoparticle suspension (20 μl) on the dark side of grid. The excess amount of formulation was removed by filter paper and the grid was allowed to air dry followed by imaging.

Gel retardation assay

The complex formation of MNPs with miR-145 was determined by the gel retardation assay using MNPs and miR-145 at different ratios. 1-10 μg of MNPF was incubated for 30 min with 100 nM of miR-145 mimic. Gel electrophoresis was performed using 2% agarose gel that was allowed to run for 1 hour at different time intervals and photographed the gel to check the particle movement as described earlier (221).

Cellular treatment with MNPF

HPAF-II and AsPC-1 cells were seeded in 24 well plates at 60 - 70% confluency. Cells were treated with experimental controls (MNPs, NC-MNPF) or miR-145-MNPF and incubated up to 48 hrs. After 48 hrs of transfection, cells were used to investigate the functional effects of transfection on proliferation, migration and invasion.
**Cellular uptake**

Prussian blue staining was used to determine the cellular uptake of MNP formulations in the pancreatic cancer cells. Cells were seeded in 24 well plate followed by transfection with NC-MNPF and miR-145-MNPF. After 24 hrs, Prussian blue staining reagent was added to the cells. After 30 min the cells were washed with PBS and the images were captured using phase contrast microscopy to determine the uptake of iron particle inside the cells (217).

**Proliferation assay**

Cell proliferation assay was performed to investigate the effect of miR-145-MNPF by using the Cell Counting Kit-8 (Mayflower Bioscience) and a microplate reader (Cytation 3, BioTek, Winooski, VT, USA), as described earlier (6). Briefly, pancreatic cancer cells (5×10³/ well) were seeded in 96 well plate and allowed overnight followed by treatment with MNPF, NC-MNPF, miR-145-MNPF and incubation for 48 hrs at 37 °C. The anti-proliferative effect of each treatment was calculated as a percentage of cell growth with respect to the control (NC-MNPF) (142).

Additionally, cell viability assay was performed in HPAF-II and AsPC-1 cells after treatment with NC-MNPF and miR-145-MNPF. Following 48 hrs of treatment, cells were trypsinized and counted in triplicates, using cell countess equipment (Invitrogen). Data were plotted as a percentage of cell growth with respect to the control (NC-MNPF).

**qPCR of miR-145**

Following transfection of cells with NC-MNPF and miR-145-MNPF, total RNA was extracted using TRIzol reagent. RNA was quantified using Nanodrop instrument 2000 (Thermo Scientific). 50 ng of RNA reverse transcribed into cDNA using specific RT primers for miR-145 and High Capacity cDNA Reverse Transcription kit. The changes in the expression level of miR-145 was determined by real-time polymerase chain reaction using Taqman PCR master mixture and Taqman probes. RNUB6 was used as an endogenous control for the experiment (37).

**Clonogenicity assays**

Colony formation assay was performed to investigate the effect of miR-145-MNPF on the colony forming ability of pancreatic cancer cells, as described previously (222). Briefly, HPAF-II and AsPC-1 cells were transfected with MNPF, NC-MNPF and miR-145-MNPF. After transfection, cells were trypsinized and re-plated (250×10²/ well) in 12 well plate for 2 weeks. Colonies were fixed, stained with crystal violet and photographed. Colonies were counted manually and plotted as a percent clonogenicity. MNPF colonies were considered as 100%.
**Cell migration and motility assays**

Cell migration was analyzed using scratch assay, as described before (6). Briefly, following transfection with MNPF, NC-MNPF and miR-145-MNPF, HPAF-II and AsPC-1 cells were plated to form a monolayer. Cell monolayer was scraped using a micropipette tip and allowed to incubate and the residual gap length calculated from photomicrographs at the initial time (0hr) and after 24 hrs.

Cellular motility was determined by agarose bead-based cell motility assay. MNP, NC-MNPF and miR-145-MNPF transfected cells were trypsinized and mixed with 0.4% low melting agarose solution. 20 μL of cells-agarose solution was placed onto the fibronectin/ BSA coated plate, each agarose bead containing about 20000 to 30000 cells. Then the plate was placed at 4°C for 7 min to solidify the agarose beads. Complete cell culture media was added to the wells carefully followed by incubation for 3 to 4 days. The plates were photographed for migrated cells using phase contrast microscope at day 0 and 3 (39).

**Cell invasion assay**

Cell Invasion assay was performed to investigate the effect of miR-145-MNPF on the cells using BD Biocoat Matrigel Invasion Chambers (BD Biosciences) (6), as per manufacturer’s protocol. After 48 hrs incubation, the invading cells were fixed with methanol and stained with crystal violet. The invaded cells were counted and plotted as percent invasion of the miR-145 treated cells compared to control (MNPF and NC-MNPF).

**Real time cell proliferation, migration and invasion assays through xCELLigence system**

To further confirm effects of MNPF on cellular growth and motility and invasiveness, real-time migration, invasion and proliferation assays were performed using the xCELLigence system as described earlier (6). xCELLigence system is an electrical impedance-based method that allows for the measurement of cell migration, invasion and proliferation in real-time. Briefly cells (HPAF-II, AsPC-1) were seeded per chamber of cell proliferation (4 × 10³) or invasion and migration (4 × 10⁴) plate and the cells after treatment with miR-145 MNPF or empty MNPF were analyzed in xCELLigence instrument at 37°C, 5% CO₂ for real time cell proliferation, migration and invasion assays.

**Western blotting**

HPAF-II and AsPC-1 cells were transfected with NC-MNPF and miR-MNPF. Whole cell lysate was prepared, and Western blotting was performed as described earlier.
Expression of protein was analyzed by immunoblotting with specific antibodies, anti-MUC13 mAb, anti-HER2, anti-p53 and anti-β–actin.

Hemocompatibility assay

To determine the hemocompatibility of miR-145-MNPF, the blood collected from a healthy donor was used (Biological Specialty Corp; Colmar, PA). The whole blood was centrifuged at 2000 rpm for 10 min, supernatant discarded and Red blood cells (RBCs) were collected for hemolysis assay. The RBCs were re-suspended in 10 ml of PBS and 100 μl of cell suspension was treated with different concentrations of MNPF followed by incubation for 2 hrs at 37 °C. PBS and sodium dodecyl sulfate (SDS) were used as negative and positive experimental controls, respectively. After incubation, the treated cells were centrifuged and degree of hemolysis was analyzed in the collected supernatant using spectrophotometer (Cytation 3, BioTek) at λ_max 570 nm (142).

Statistical analysis

The data were processed using Microsoft Excel and presented as mean±standard error of the mean (SEM). Statistical significance of the data was performed by a Student’s t test. The level of significance was set at *p < 0.05.

Results

Generation of miR-145-MNP nanoformulation

The miR-145-MNPF was formulated as depicted in Figure 2-1A and described earlier(142, 216). PEI was used as a cationic polymer to increase the surface attachment of miR-145. Particle size and charge were identified by dynamic light scattering method. Optimal particle size (163 nm in DLS and 8-10 nm in TEM) and surface charge (30 mV) were determined for miR-145-MNPF (Figure 2-1B through D). Additionally, we observed the complex formation of miR-145 with MNPs as seen in lane 5 and the complexation was further increased in lanes 6-9 with increase in the concentration of MNP (2-10 μg) (Figure 2-1E). This indicated a complete retardation of mobility of the complexes formed, as visualized by reduced band intensity on complexation of miR-145 with MNPF (Figure 2-1E).

miR-145-MNPF is less toxic and hemocompatible

To establish clinical utility of a nanoformulation, evaluation of its general toxicity and hemocompatibility is utmost important. Therefore, we sought to determine the toxicity of our delivery system (MNPF)
Figure 2-1. Generation of miR-145-MNPF and its physicochemical characterization.

A) Schematic representation of the generation of miR-145-MNPF. Co-precipitation of iron oxide nanoparticles followed by β-cyclodextrin and F127 polymer coatings leads to MNP nanoformulation (MNPF). Surface modification of MNPF was done with Polyethylenimine (PEI) and conjugated with miR-145 by incubation in 1mg/ml MNPF in 0.9% NaCl solution. B) Average miR-145-MNPF aggregative size is 163 nm. C) Transmission electron microscopic (TEM) image of miR-145-MNPF showing individual particle size of 8-10 nm. D) Zeta potential of miR-145-MNPF 30.63 mV. E) Gel retardation assay by agarose gel electrophoresis showing the formation of a complex between MNPF and miR-145 through electrostatic interaction. Complex formation is seen in the lane 5 and the complexation further increases in the lanes 6 -9 with the increase in the concentration of MNP (2-10 μg).
and compared it with lipofectamine (Life technologies), the commonly used transfection reagent for nucleic acid delivery in cell culture system. Treatment of human red blood cells with different concentrations of MNPF or lipofectamine indicated less or no toxicity of MNPF in RBCs, even at higher concentrations while lipofectamine was quite toxic as determined by spectroscopic analysis at λ<sub>max</sub> 570 nm (Figure 2-2A, B). Additionally, pancreatic cancer cells were treated with different concentrations of MNPF or lipofectamine for 48 hrs to determine toxicity profile. Spectrophotometric analysis indicated that empty MNPF was not toxic to cancer cells whereas, lipofectamine showed significant toxicity (Figure 2-2C). This data suggests that the MNPF is hemocompatible and PEI conjugation to MNP does not make the formulation toxic confirming that our MNPF is a safe delivery system for pre-clinical and clinical applications.

**miR-145-MNPF effectively internalizes and restores miR-145 in pancreatic cancer cells**

Effective internalization of particle into the cell or intracellular uptake of particle is a key factor to determine the therapeutic efficacy of a nanoparticle formulation. Thus, we investigated the cellular uptake/internalization and delivery efficacy of our miR-145-MNPF into the pancreatic cancer cells. Prussian blue staining of HPAF-II cells that were treated with NC-MNPF and miR-145-MNPF, demonstrate the efficient cellular uptake of the MNP formulations (Figure 2-3A). Cellular internalization was also confirmed through Coumarin-6 dye loaded MNPs through confocal microscopy analysis (Figure 2-3B). Coumarin-6-loaded MNPs efficiently internalized as indicated by high cytosolic staining and strong co-localization to the mitochondrial marker, mitotracker. Less co-localization of MNPs with early endosome and late endosome/lysosome markers indicated their escape from lysosomal degradation. Therefore, increased accumulation of MNPs was observed in mitochondria. This indicates that MNPs are able to escape from late endosome and lysosome compartments and reach into the cytosol/mitochondria for efficient functioning rather than undergoing a lysosome recycling process. These findings are consistent with our previously published studies with drug loaded MNPs (216, 217). The internalization of NC-miR-145 or miR-145-MNPF was almost equal to the MNP and that the miR-145 loading to MNPs did not affect the cellular uptake of these particles into the pancreatic cancer cells.

The expression level of miR-145 was assessed in HPAF-II and AsPC-1 cells that were treated with miR-145-MNPF by quantitative reverse-transcription PCR (qRT-PCR) assay (Figure 2-3C). Our data suggested that miR-145 expression was increased about 19 and 4-fold in AsPC-1 and HPAF-II cells, respectively, as compared to control groups (NC-MNPF). These results suggest that miR-145-MNPF can effectively deliver and reconstitute miR-145 into pancreatic cancer cells.
Figure 2-2. Evaluation of hemocompatibility and toxicity using RBCs and HPAF-II cells.

Hemolysis was performed by incubating MNPF in red blood cells for two hours. A) Images showing the effect of MNPF and lipofectamine on RBCs. B) Cells were centrifuged, and supernatant collected for analysis. PBS and SDS were taken as negative and positive controls, respectively. C) Toxicity of MNPF and lipofectamine was compared in pancreatic cancer cells, HPAF-II using cell proliferation assay.
Figure 2-3. Restitution of miR-145 in pancreatic cancer cells using miR-145-MNPF.

A) Cellular uptake of miR-145-MNPF was determined by Prussian blue staining. Pancreatic cancer cells were seeded and treated with MNPF, NC-MNPF, miR-145-MNPF. After 24 hours, cells were stained with Prussian blue reagent and photographed under phase contrast microscopy (Original Magnifications 100X). B) Determination of fate and subcellular localization of Coumarin-6 dye (green color) labelled MNPF nanoformulation (Original Magnifications 400X). HPAF-II pancreatic cancer cells (1x10⁵/well) were exposed to Coumarin-6 labelled MNPF for 1 hr. Cells were stained with Transferrin from Human Serum, Texas Red® Conjugate, LysoTracker Red and Mito Tracker Red, as markers for endosome, lysosome, and mitochondria, respectively. Green color indicated uptake of Coumarin-6 labelled MNPF and yellow color indicated co-localization of Coumarin-6 labelled MNPF in endosome, lysosome and mitochondria. C) miR-145 restoration in cells was confirmed using Taqman probes by q-PCR. Expression level of miR-145 was observed in cells after 48 hours of transfection. Results represented as fold increase of miR-145 in comparison with NC-MNPF. Bars represent mean ± SEM, n=3, *P<0.05.
miR-145-MNPF inhibits tumorigenic features of pancreatic cancer cells

To investigate the effect of miR-145-MNPF on cell growth we performed cell proliferation and viability assays. Results demonstrated that miR-145-MNPF inhibited cell proliferation both in HPAF-II and AsPC-1 cells as compared to NC-MNPF treated cells (Figure 2-4A). We further determined the effect of miR-145-MNPF on cell viability by counting the cells using Cell countess (Invitrogen) (Figure 2-4B). It was observed that miR-145-MNPF treated cells showed significantly decreased cellular viability (HPAF-II: 72 % and AsPC-1: 26 %) compared to the control (NC-MNPF) treated cells (Figure 2-4B). We also studied the phenotypic changes occurring in pancreatic cancer cells on restoration of miR-145. The cells treated with miR-145-MNPF showed apparent change in morphology compared to control and NC-MNPF (Figure 2-4C). Cells treated with miR-145-MNPF appeared relatively more circular in shape, loosely attached and grew in smaller patches. The effect of MNPF on cell proliferation was also confirmed in real time using xCELLigence system (Figure 2-4D) which showed a similar growth inhibitory pattern. However, we observed slightly lower effect in AsPC-1 cells compared to HPAF-II cells at the same time point. Additionally, the results demonstrate that miR-145-MNPF markedly decreases clonogenicity in HPAF-II and AsPC-1 cells (by 77% and 59%, respectively), compared to NC-MNPF treated cells (Figure 2-4E). Altogether, these results clearly suggest that the miR-145-MNPF successfully delivers miR-145 into the cells which leads to the inhibition of cell proliferation, morphology and colony forming ability in pancreatic cancer cells.

miR-145-MNPF mediated delivery of miR-145 inhibits metastatic phenotypes of pancreatic cancer cells

To determine effects of miR-145-MNPF on metastatic characteristics of pancreatic cancer cells, cell migration and invasion assays were performed using HPFA-II and AsPC-1 cells. Matrigel invasion assay indicated a remarkable inhibitory effect of miR-145-MNPF on the invasiveness of HPAF-II (80 %) and AsPC-1 (76 %) cells (Figure 2-5A, B). Additionally, we investigated the effect of miR-145-MNPF on the cellular motility and migration in pancreatic cancer cells using agarose bead and wound healing assays (Figure 2-6A, B). miR-145-MNPF demonstrated a significant inhibition of the cell migratory potential of HPAF-II and AsPC-1 cells both in agarose bead and wound healing assays as compared to control (NC-MNPF) treated cells. The effect of MNPF on cell migration and cell invasion was also confirmed in real time using xCELLigence system (Figure 2-6C). This assay also showed a similar inhibitory effect on cell migration and cell invasion capability of aforementioned cells (Figure 2-6C).

miR-145-MNPF targets MUC13 and regulates its downstream oncogenic signaling cascade

Considering potent anti-tumorigenic and anti-metastatic potential of miR-145-MNPF in functional assays, we sought to investigate its putative inhibitory molecular
Figure 2-4. miR-145 restoration using miR-145-MNPF inhibits proliferation and clonogenicity in pancreatic cancer cells.

A) Effect of miR-145-MNPF on cell growth and proliferation was performed using cell counting Kit-8 in HPAF-II and AsPC-1 cells. B) Effect of miR-145-MNPF on cell viability by counting the cells using Cell Countess in HPAF-II and AsPC-1 cells. C) Representative images of cells morphology after treated with miR-145-MNPF for 48 h using phase contrast microscopy. D) Effect of miR-145-MNPF treatment on cell proliferation with respect to time (h) was also confirmed by xCELLigence RTCA. E) HPAF-II and AsPC-1 cells were treated with miR-145-MNPF and incubated for 12 days for investigating the colony forming ability of the cells. Colonies were counted and plotted as percent clonogenicity. Percent inhibition of clonogenicity of miR-145-MNPF transfected cells as compared with NC-MNPF. Bars represent mean ± SEM, n=3, *P<0.05.
Figure 2-5. Effect of miR-145-MNPF on cellular invasion in pancreatic cancer cells.

A) Matrigel Invasion Assay. Cells treated with MNPF, NC-MNPF and miR-145-MNPF were plated into the top chamber of the well in serum free media with a Matrigel-coated membrane. 10% FBS was added to the lower chamber as a chemoattractant. After 48 hours of incubation, invaded cells were identified by crystal violet staining followed by imaging. B) Data represented as percent inhibition of invasion of miR-145-MNPF transfected cells as compared with MNPF. C) Effect of miR-145-MNPF on cellular invasion ability was confirmed using xCELLigence system. Bars represent mean ± SEM, n=3, *P<0.05.
Figure 2-6. Effect of miR-145-MNPF on cell migration ability of pancreatic cancer cells.

A) Cell scratch assay. HPAF-II and AsPC-1 cells treated with MNP-NC and miR-145-MNPF were seeded overnight followed by scratch. The scratch was monitored for closure following MNP-NC or miR-145-MNP treatment which was followed by photographing at 24 h. B) Agarose Bead Assay. HPAF-II and AsPC-1 cells were mixed into agarose solution and 20 μl dropped onto fibronectin/bovine serum albumin coated plates. Number of migratory cells (MC) from agarose beads (AB) were photographed (day 0 and day 3) and quantified by counting the MC cells compared to control. C) Effect of miR-145-MNPF on cell migration ability was confirmed using xCELLigence system. Bars represent mean ± SEM, n=3, *P<0.05.
mechanisms. Thus, we evaluated the effect of miR-145-MNPF on the expression of MUC13, HER2, pAKT<sup>Ser473</sup> and p53 (Figure 2-7A, B). Immunoblotting analysis data suggested that miR-145-MNPF effectively suppressed the expression of MUC13, pAKT<sup>Ser473</sup> and HER2 in HPAF-II and AsPC-1 cells. Contrary to this, however, miR-145-MNPF rescued the p53 expression levels. These data further validate the ability of miR-145-MNPF to effectively deliver miR-145 in pancreatic cancer cells accompanying the functional consequences via repression of its direct target MUC13 (Figure 2-7C).

**Discussion**

This study describes engineering of a recently identified tumor suppressor miR-145 (37) loaded unique magnetic nanoparticle formulation (miR-145-MNPF) for efficient delivery of a tumor suppressor miR-145. Our data demonstrated efficient cellular delivery, uptake/internalization capability, restitution of miR-145 and potent anti-cancer effects of this novel miR-145-MNPF in pancreatic cancer cell line models. Successful delivery of microRNAs is governed by several processes that include high cellular uptake of the RNA, escape from lysosomal degradation, non-immunogenicity and stability in the bloodstream. The active routing of the delivered miRNA to the target organ and its entry into the cell is a determining factor for it to reach its intracellular target in an active form. For this purpose, generation of newer and improved delivery systems is highly desirable.

The therapeutic application of miR-145 has been previously evaluated in a xenograft model of pancreatic cancer (37). A strong reduction in tumor growth was observed in mice receiving intra-tumoral miR-145 injection compared to the control treated group, which suggested its therapeutic potential for pancreatic cancer treatment4. We have generated a unique MNP based delivery vehicle for microRNAs that has been used in our previous studies to deliver anti-cancer drugs (142, 216, 217). For the purpose of microRNA delivery, we have further engineered our magnetic nanoparticles by conjugating them with positively charged, branched PEI (MNPF). This enabled the surface attachment of miR-145 to the negatively charged MNPs with reduced toxicity effects of PEI. The use of branched PEI exhibits high transfection efficiency due to the high cationic charge for efficient gene delivery. The MNPF was found to be nontoxic and hemocompatible as it did not show any toxicity in RBCs. The probable reason of the reduced cytotoxicity of the PEI may be due to the presence of a layer of bio-adhesive material, Pluronic F127 that formulates it to render reduced toxic effects. It has previously been observed that Pluronic F127-PEI exhibits better condensation and complexation properties than PEI (181). This strategy seems to provide a promising intervention of as stable, high transfection efficiency nanocarrier for microRNA delivery. Additionally, the use of a Pluronic F127 greatly improved the cellular uptake and reduced aggregation of the particles whereas the layer of β-cyclodextrin serves as a platform for simultaneous drug loading (216) which provides a feasibility to co-deliver a chemotherapy drug along with the miR-145. We investigated the potential of these nanoparticles to deliver microRNAs and our results suggest it to be a promising delivery system to restore miR-145 in pancreatic cancer cells.
Figure 2-7. Effect of miR-145-MNPF on MUC13 and its associated targets.

A) HPAF-II and AsPC-1 was treated with NC-MNPF or miR-145-MNPF. Whole cell lysate were immunoblotted for MUC13, HER2, pAKT<sup>Ser473</sup> and p53 proteins. β-actin was used as a loading control. B) Bars represent the densitometric analysis of the blots by GelQuant. Bars represent mean ± SEM, n=3, *P<0.05. C) Schematic representation illustrating the effects of miR-145 restitution using miR-145-MNPF.
Our studies demonstrating functional effects on proliferation, invasion and migration, as observed in our previous studies (37), are as a consequence of miR-145 restoration, suggesting the efficient delivery of miR-145 due to miR-145-MNPF. The biological functions of a microRNA are dependent on its interaction with its intracellular target which in the case of miR-145 is MUC13 in pancreatic cancer. We observed an effective and active routing of miR-145 into the cell through miR-145-MNPF that was sufficient to successfully deliver it to its intracellular target in an active form as was depicted by decreased MUC13 expression in cells (Figure 2-7). This was accompanied by demonstrating functional inhibitory effects on growth, invasion and motility of cancer cells (Figure 2-4 through 6) via inhibiting MUC13 associated oncogenic protein, HER2, pAKTser473 and restoring p53 levels (Figure 2-7). Our previous studies demonstrating sustained release of therapeutics from these magnetic nanoparticles (142, 216, 217) confirm that miR-145-MNPF will lead to significant and sustained release of miR-145, generating a prolonged effect on its target protein, MUC13 and functional effects on proliferation and invasion. Therefore, this magnetic nanoparticle formulation proves to be an efficient delivery technique for therapeutic intervention.

**Conclusion**

Our miR-145 loaded MNP formulation (miR-145-MNPF) efficiently delivers miR-145 and restores its expression in pancreatic cancer cells. Restitution of miR-145 using miR-MNPF formulation successfully inhibits its target oncogene, MUC13, thereby, suppressing tumorigenic and metastatic phenotypes of pancreatic cancer cells. Therefore, this unique magnetic nanoparticles-based formulation can be efficiently used for microRNA replacement and pancreatic cancer therapy alone or in combination with conventional chemotherapy drugs. Our future goal is to further enhance the specificity of MNPs towards pancreatic cancer cells/tissues via active targeting. This will be achieved by conjugating MUC13 monoclonal antibody to the formulation which will route the formulation specifically to cancer cells having high MUC13 expression. The results from this study offer a new therapeutic strategy for pancreatic cancer treatment that warrants further investigation in future preclinical and clinical studies.
CHAPTER 3. MUC13 IS INVOLVED IN TRAIL RESISTANCE IN PANCREATIC CANCER

Introduction

The management of pancreatic cancer is exceptionally challenging due to the genomic complexity and multidimensional feature of the disease. Overexpression of oncogenes, loss of tumor suppressor miRNAs, lack of early diagnosis, loss of apoptosis, poor response to existing therapeutic modality and chemoresistance are major cause of PanCa progression (2, 3). Alteration of intracellular signaling cascade makes this disease difficult to target. Therefore, understanding molecular mechanisms of cancer progression may offer new therapeutic opportunities of PanCa.

Apoptosis pathway plays a pivotal role in therapeutic perspective because it can attenuate the tumor progression. The developmental process of apoptosis in cells can be persuaded by the intrinsic and the extrinsic pathway, it could start with mitochondria mediated and proceed through the activation of cell surface death receptors (224). TNF-related apoptosis-inducing ligand (TRAIL) is a member of the Tumor Necrosis Factor (TNF) family (60). TRAIL mediated apoptosis in \textit{in vitro} and \textit{in vivo} happened in various tumor cell type without affecting normal cells (60, 225-229). To transmit the apoptotic signal, TRAIL requires binding to the pro-apoptotic ligands such as TRAIL. TRAIL can bind to the all four-membrane bound receptor (TRAIL-R1-R4) and one soluble receptor. For TRAIL mediated apoptosis TRAIL only binds to death receptor TRAIL-R1 (DR4) and -R2 (DR5) which contain a cytoplasmic death domain (DD), those who responsible for caspase activation to facilitate TRAIL-induced apoptosis. Binding of TRAIL to TRAIL-R1/2 leads to recruitment of the adaptor FADD and initiator procaspase-8 and 10 to rapidly form the death-inducing signaling complex (DISC) (230). TRAIL leads to activation of cleaved caspase-8 and 10 which activates effector caspase-3, 6 and 7 committing the cell to apoptosis. The cellular FLICE inhibitory protein (cFLIP), is potent negative regulator of TRAIL signaling pathway. It can control the recruitment and processing of pro-caspase8 in DISC complex (231). Activation of caspase-8 leads to cleavage of Bid and production of truncated tBid, which eventually trigger intrinsic pathway of apoptosis. As per current scenario, TRAIL is in clinical trials for a variety of cancers. Unfortunately, PanCa is highly resistant to TRAIL-induced death as compared to other cancer types.

Mucins play a crucial role in cancer progression, metastasis and resistance. These mucinous proteins are aberrantly expressed in several cancer, including pancreatic cancer. Mucin, MUC13 is a transmembrane glycoprotein, overexpressed in PanCa condition and minimal or no expression in normal cells (35). MUC13 has been involved in PanCa progression (232) and has substantial clinical role as early diagnostic marker (40). In early PDAC precursor lesions (PanIN I), MUC13 expression is subdued but over the course of development from PanIN II/III to late stage (poorly differentiated PDAC) it became aberrantly expressed. Biochemically defined MUC13 structure consist of mainly 2subunits: \(\alpha\)-subunit and \(\beta\)-subunit. The \(\alpha\) subunits comprises TR (Tandem Repeat)
domain, 1 EGF-like domain, and a part of SEA domain. At the same time β-subunit consists TM (transmembrane domain) domain, portion of SEA domain, cytoplasmic trail and 2 EGF like domains (233). These domains of MUC13 are majorly responsible for oncogenic characteristics of pancreatic cancer. It has been reported that, MUC13 is responsible in blocking extrinsic and intrinsic pathway of apoptosis in colorectal cancer (234). There are 4 different studies has mentioned the role of MUC13 in apoptotic signaling pathway in different cancer (235).

In the present study, we show that MUC13 expression attenuate TRAIL induced apoptosis in PanCa cells by blocking activation of caspase-8 and cleavage of PARP-1. Suppression of MUC13 enhances the sensitivity of TRAIL mediated apoptosis in MUC13 expressing cells.

Materials and Methods

Cell culture

All human PanCa cells were purchased from ATCC (American Type Cell Culture) and grown in recommended growth condition. Cell lines were cultured in growth medium (Panc-1: DMEM, HPAH-II: DMEM/Ham's F12, MiaPaca: DMEM and AsPC-1: RPMI, supplemented with 10% FBS, 1% antibiotic and maintained 37°C with 5% CO₂ in a humidified atmosphere. All cell lines were routinely tested for mycoplasma contamination. MUC13 null Panc-1 cells (P-V), stably expressing MUC13 cells (P-M13) (35), also MUC13 expressing (Sh-V) and MUC13 knockdown HPAF-II (Sh-M13) cells were used in this study as described previously (236). These stably transfected cells were maintained with 100 μg/ml of G418 antibiotic or 5 μg/ml puromycin.

Proliferation assay

Cell proliferation was performed to examine the anti-proliferative effect of recombinant TRAIL at different concentration, miR-145 and combination with TRAIL on PanCa cells using MTT assay as described earlier (6). PanCa cells were seeded and treated in 96well plate and after 48 hrs 20 μl of MTT reagent were added to cells. After 2 hrs of incubation, reagent and media were removed and 100 μl DMSO were added to cells and kept in gentle shaking condition for 10 min. Absorbance was recorded at 570 nm and viability of cells determined compared to control.

Cell cycle and apoptosis

To determine the cell cycle arrest and apoptotic population upon treatment with TRAIL we performed the flow cytometric analysis. 70% confluent cells were seeded in 6well plate and synchronized overnight incubating then with serum free media. After the
required treatment time cells were harvested, washed with PBS followed by fixation in ice-cold 70% ethanol in -20ºC. Before data accusation cells were washed with PBS, pretreated with RNase and incubated with propidium iodide (Sigma) to stain DNA. Data acquisition has been done in BD Accuri C6 instrument and analyzed by ModFit software.

**Acridine orange/ethidium bromide (AO/EB) staining: to detect apoptosis**

To examine the nuclear changes and early/late apoptotic body formation Acridine orange/ethidium bromide (AO/EB) staining was performed on PanCa cells based on their MUC13 expressing characteristics. 1 μl of AO/EB solution was added to 25 μl of cell suspension (1.0 × 10^6 cells/ml) just prior to quantification. Cells were imaged under fluorescence microscopy to determine the cellular staining intensity and nuclear morphology.

**Western blot analysis**

PanCa cells were treated with TRAIL at different time points, also treated with miR-145 and combination with TRAIL. Whole cell lysates were prepared and western blotting analysis was performed to evaluate the apoptotic and death receptor pathway proteins as described earlier (6). Samples were run in 4-20% gel and transferred to PVDF membrane followed by blocking with 5% milk for 1 hour and overnight incubation with desired primary antibody at 4ºC. Next day, respective secondary antibody was added to the blots and develop under UVP instrument using ECL chemiluminescence.

**Transfection procedure**

PanCa cells (Panc-1, MiaPaca) were transfected using Lipofectamine 2000 (Invitrogen) as per manufacturer instruction. Cells were seeded in 6well plate in 70% confluence and incubated then FBS free medium for overnight. Further, cells were transfected with α, β, Cyto domains of MUC13, Full length and three MUC13 EGF-like domain deletion mutants (MUC13 ΔEGF 2/3, MUC13 ΔEGF 1/3, and MUC13 ΔEGF 1/2). These deletion mutants contain intact EGF-like binding domains 1, 2 and 3 respectively(232). HPAF-II and AsPC-1 cells transiently transfected with miR-145 mimics (MC11480; Applied Biosystems) and non-targeting control mimic (NC) (catalog number AM17111; Applied Biosystems) for further studies (37).

**Cell invasion and migration assay**

Cell invasion assay was performed in Matrigel Invasion Chambers (BD Biosciences) according to manufacturer protocol (101). Previously transfected cells (35*10^3/chamber) were seeded in serum free medium and treated with recombinant TRAIL. After 24 hrs cells were fixed with cold methanol, followed by removal of
uninvaded cells, and fixed with crystal violet. Phase contrast microscopy images were captured to evaluate cellular invasive behavior upon treatment.

Cell migration assay was carried out in Corning’s 96-well HTS Transwell plate (237). 50*10^3/chamber were seeded and treated with TRAIL. After 24 hrs migrated cells were fixed with 4% paraformaldehyde, stained with crystal violet followed by washing off the unmigrated cells. Phase contrast microscopy image were taken to examine the effect of miR-145 restoration and combination of TRAIL treatment.

Realtime xCELLigence assay: Proliferation, migration and invasion

Real time xCELLigence assay was performed to further confirm the effect miR-145, TRAIL and combination of TRAIL on proliferation, Invasion and migration ability of PanCa cells (HPAF-II & AsPC-1) as describes previously (101). Briefly, pre-transfected PanCa cells were seeded in xCELLigence plates for proliferation (5 × 10^3), invasion and migration (5 × 10^4) respectively followed by recombinant TRAIL treatment. The xCELLigence instrument kept at 37°C, 5% CO₂ to analyze the real time behavior of PanCa cells with treatment with miR-145 and TRAIL compared to control (NC).

Results

MUC13 imparts TRAIL resistance in pancreatic cancer cells: Role of MUC13 in cell survival and apoptosis

Our result demonstrated that MUC13 null cells or knock down of MUC13 in cells showed reduced cell proliferation as compared to MUC13 expressing cells. PanCa cells were treated with recombinant TRAIL at 500 nM and 1 μM. MUC13 null cells (P-V and Sh-M13) displayed 85.83%, 49.66%; 58.79%, 55.97% and MUC13 expressing (P-M13 and Sh-V) cells showed 92.62%, 81.48%; 88.31%, 61.83% proliferation when treated with 500 nM and 1 μM TRAIL (Figure 3-1A). In response to TRAIL treatment MUC13 expressing cells (P-M13, Sh-V) displayed 6.46% and 1.57% of sub G0 population whereas MUC13 null or MUC13 knockdown cells (P-V, Sh-M13) exhibited 35.21% and 46.6% as evidenced by cell cycle analysis which is significantly high apoptotic cell death (Figure 3-1B).

MUC13 induces TRAIL resistance in pancreatic cancer cells

Acridine orange/ethidium bromide (AO/EB) staining represent characteristics of apoptotic population by analyzing visual changes in nucleus of cells which eventually depicted different phases of apoptotic bodies. Fluorescent microscopy images of MUC13 null PANC-1 (P-V) cells displayed more early/late apoptotic population and very minimal live cell population when treated with TRAIL.
Figure 3-1. MUC13 induces TRAIL resistance in PanCa cells.

(A & B) MUC13 expressing cells (P-M13 and Sh-V) show resistance to TRAIL induced cell death and MUC13 knockdown leads to TRAIL sensitivity in cells (P-V and Sh-M13) (B) MUC13 expression promoted survival and knockdown of MUC13 resulted in TRAIL mediated increase in apoptotic cell death as evidenced by sub G0 population (C and D). Bars represent mean ± SEM, n=3, *P<0.05.
At the same time MUC13 expressing Panc-1 cells (P-M13) showed higher number of live cell population and less quantity of cells stated losing membrane potential after treatment with TRAIL (Figure 3-2A). Western blotting analysis data depicted that MUC13 expressing HPAF-II (Sh-V) cells displayed TRAIL resistance in time dependent manner compared to MUC13 knockdown cells (Sh-M13) with same treatment regimen. Bid and Pro-caspase8 expression has been significantly decreased with higher exposure time of TRAIL treatment in Sh-M13 cells, however there was no changes in expression of these apoptotic proteins in Sh-V cells (Figure 3-2B). Therefore, MUC13 expression enhances TRAIL resistance via activation of apoptotic death signaling pathway. To determine if MUC13 affects stability of cFLIP, we used cycloheximide to block the de novo protein synthesis assessed cFLIP expression at different time point after treated with cycloheximide (Figure 3-2C). Immunoblot results indicated that MUC13 stabilizes cFLIP which implied that TRAIL induced apoptosis has been attenuated in MUC13 expressing P-M13 cells and there were almost no changes in expression of cFLIP in MUC13 null cells P-V cells. Recruitment of cFLIP at the DISC were decreased in case of MUC13 null cells (P-V) compared to P-M13 cells.

Which MUC13 domain imparts survival benefit to pancreatic cancer cells?

The preliminary results demonstrated that, MUC13 has been playing a major role in TRAIL resistance in PanCa cells. In this section, we explored the structural domains responsible for resistance factor. Immunoblotting analysis of whole cell lysates obtained from different domains transfection, followed by TRAIL treatment suggested that Alpha (α) and Beta (β) domains are indispensable for blocking caspase-8 activation and PARP cleavage in PanCa cells (MiaPaca, Panc-1) (Figure 3-3A). Previously our lab has generated three deletion mutants of MUC13 which depicted the deletion at EGF domains. It has been reported that these deletion mutants are responsible for tumorigenic characteristics in MUC13 null cells (232). To investigate the deletion mutant responsible for TRAIL resistance, we have transfected Panc-1 cells with vector, full length, MUC13 ΔEGF 2/3, ΔEGF 1/3 and ΔEGF ½ constructs followed by TRAIL treatment. Data suggested that MUC13 ΔEGF 2/3 (DM1) and ΔEGF 1/3 (DM2) are blocking the cleavage of PARP1 and activation of caspase8 however ΔEGF 1/2 showed similar activation like MUC13-FL (Figure 3-3B). Thus, results indicated DM1 and DM2 inhibit TRAIL mediated activation of extrinsic apoptotic pathway.

TRAIL sensitization due to miR-145 restitution inhibits cell proliferation, migration and invasion

As per previous study results, MUC13 can be considered as a key factor of TRAIL resistance in PanCa cells. Our previous publication has revealed that miR-145, tumor suppressor miRNA, an effective therapeutic modality which can target MUC13 in PanCa cells overcome resistance against GEM (37). When miR-145 has been delivered through magnetic nanoformulation, the restoration of miR-145 inhibit the expression of MUC13 and downstream target key proteins (101).
Figure 3-2. MUC13 imparts TRAIL resistance in pancreatic cancer cells.

(A) Effect of TRAIL in MUC13 Null (P-V) and expressing (P-M13) Panc-1 cells using Acridine orange staining. Acridine orange staining shows more apoptotic cell population in MUC13 (Null) Panc-1 cell as compared to Panc-1-M13 (MUC13 expressing) cells (B) Immunoblotting experiment performed following TRAIL treatment in MUC13 expressing (Sh-V) and knockdown (Sh-M13) HPAF-II cells demonstrates that MUC13 inhibits TRAIL mediated activation of apoptotic death signaling pathway.
Figure 3-3. MUC13 domains (alpha and Beta) inhibit TRAIL-mediated activation of extrinsic apoptotic pathway.

(A). Immunoblotting experiment following transfection with different domains of MUC13 to identify the specific domain of MUC13 responsible for TRAIL resistance. MUC13 domains, alpha and Beta are indispensable for blocking TRAIL mediated caspase-8 activation and PARP cleavage. (B) Transfection of MUC13 deletion mutants ΔEGF 2/3 (DM1), ΔEGF 1/3 (DM2) and ΔEGF1/2 (DM3) constructs followed by TRAIL treatment. MUC13 ΔEGF 2/3 (DM1) and ΔEGF 1/3 (DM2) are blocking the cleavage of PARP1 and activation of caspase8.
Our recent study suggested that, restoration of miR-145 in PanCa cells (HPAF-II & AsPC-1) and combination of TRAIL treatment, cells proliferation (Figure 3-4A), invasion and migration has been decreased (Figure 3-4B, C). Further confirmation of the results by real-time xCELLigence data also displayed the same interpretation (Figure 3-4D). Therefore, miR-145 restoration enhanced TRAIL sensitivity in PanCa cells.

Restitution of miR-145 regulates MUC13 expression and enhances TRAIL-induced apoptosis

Restitution of miR-145 in PanCa cells expected to be a therapeutic strategy to overcome TRAIL resistance in pancreatic cancer. Immunoblotting showing the effect of miR-145 restoration on MUC13 inhibition and TRAIL mediated activation of extrinsic apoptosis as depicted by cleavage of PARP and activation of caspase-8 (Figure 3-5A, B). There were almost no changes in caspase-9 expression upon treatment with TRAIL. Therefore miR-145 sensitizes PanCa cells towards TRAIL activating extrinsic apoptotic and inhibiting DNA repair mechanisms pathway. Flow cytometric cell cycle analysis displayed that miR-145 restoration renders the PanCa cells highly responsive TRAIL sensitivity as indicated by increased Sub G0 population farther treatment with TRAIL (Figure 3-5C). Cell cycle analysis data validated that restitution of miR-145 effectively enhanced TRAIL induced apoptotic population in PanCa cells compared to the control treated groups. This triggers the cells towards apoptotic cell death as indicated through activation of caspases, increased sub-G0 population.

Discussion and Conclusion

PanCa is a leading cause of cancer related deaths in US due to late diagnosis and development of chemo-resistance. Mucin, MUC13 is aberrantly overexpressed in PanCa, promoting cancer growth and progression and these effects are abrogated by miR-145 restoration. Unlike other cancer types, PanCa is highly resistant to Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) that emerges as one of the most-promising cancer therapeutic drugs. Herein, we demonstrate the integration of novel approach to overcome chemo-resistance and offer TRAIL-based therapeutic strategies. Results demonstrate that MUC13 expression blocks activation of caspase-8 and death receptor mediated apoptosis in PanCa cells in response to TRAIL treatment as observed through western blotting and flow cytometer. The proliferation results signify that presence of MUC13 expression in cells resist the cells to die. In MUC13 expressing cells the percentage of cell proliferation is always higher than the MUC13 null cells in both the cell lines. It is also evident that even in lower concentration of TRAIL (500nM) Sh-M13 cells showed significantly lower cell proliferation compared to Sh-V cells. Therefore, MUC13 contributes survival advantage in PanCa cells in response to treatment with TRAIL. MUC13 expressing cells show resistance to TRAIL induced cell death. MUC13 knockdown leads to TRAIL sensitivity in cells. The current studies revealed a new role of MUC13 in inhibiting TRAIL mediated activation of extrinsic apoptotic pathway in pancreatic cancer.
Figure 3-4. TRAIL sensitization due to miR-145 restitution inhibits cell proliferation, migration and invasion.

Results demonstrated that restoration of miR-145 sensitized PanCa cells (AsPC-1 and HPAF-II) to inhibit proliferation, invasion and migration (1. A, B, C). Real time xCELLigence results also confirmed the similar effect in both the cells (D). Bars represent mean ± SEM, n=3, *P<0.05.
Figure 3-5. Restitution of miR-145 regulates MUC13 expression and enhances TRAIL-induced apoptosis.

(A & B) miR-145 restoration inhibits MUC13 expression and sensitizes TRAIL in both HPAF-II and AsPC-1 cells. Activation of PARP-1 and cleavage of caspase 8 has been observed in both PanCa cells. Bar graph representing the band intensity of the apoptotic pathway proteins. (C) Cell cycle analysis data validated that restitution of miR-145 effectively enhanced TRAIL-induced apoptotic population in PanCa cells compared to the control treated groups. Bars represent mean ± SEM, n=3, *P<0.05.
The results have provided the first evidence of TRAIL sensitization through miR-145 restitution. Inhibition of MUC13 using miR-145 restoration resulted in TRAIL mediated increase in apoptotic cell death as evidenced by AnnexinV/7AAD and sub G0 population, as well as rendered PanCa cells sensitive to treatment with drugs, such as paclitaxel. Further investigation showed that Alpha and Beta domains of MUC13 is indispensable for blocking caspase 8 activation and PARP cleavage, indicating that the MUC13 domains (alpha and Beta) blocks TRAIL-induced signaling upstream to Bid by inhibiting caspase-8 activation. These observations suggest that MUC13 contributes to the survival advantage in PanCa cells in response to treatment with drugs or death inducing ligands such as TRAIL (Figure 3-6) which can be strategically overcome by miR-145 replenishment. These findings indicate that MUC13 silencing sensitizes PanCa cells towards TRAIL therapy and counteracts chemo-resistance mechanisms in PanCa that may lead to novel combination therapies for PanCa treatment.
Figure 3-6. Schematic representation of possible mechanisms by MUC13 inhibiting effect of TRAIL.
CHAPTER 4. IMPROVING TRAIL THERAPY RESPONSE IN PANCREATIC CANCER BY REPLENISHMENT OF MIR-145

Introduction

Pancreatic cancer is a third leading cause of cancer related deaths in the USA due to late diagnosis and development of chemo-resistance (238). Gem is considered to be one of the most effective chemotherapeutic agents for PanCa; however, it shows only a marginal survival benefit (6 months) in patients and eventually tumors develop chemoresistance (45). Chemoresistance against most effective therapeutic modalities is a major limitation for PanCa treatment (239). Chemoresistance in cancer has been categorized by intrinsic resistance and acquired resistance (240). Intrinsic or innate resistance occurred when patient’s genetic factors do not allow any specific therapeutic option, whereas after exposure of a chemotherapeutic drug for certain time a patient develop acquired resistance. Therefore, overcoming drug resistance using newer delivery approach to target PanCa is an utmost necessity.

TRAIL induced apoptosis is directly related to cell surface death receptors (DR4 and DR5) thus they are the major molecular targets for TRAIL mediated cancer therapeutics (241). Some studies have shown that down regulation of DR4 and DR5 play a major role in TRAIL resistance in cancer, however restoration of TRAIL enhances the expression of TRAIL receptors (DR4 and DR5) (241, 242). Binding of TRAIL to these cell surface rectors and establishment of the protein complex plays a major role as initiator of other signaling events in the cells. Cellular FLICE like inhibitory protein (cFLIP) is a well-established anti-apoptotic regulator which is directly associated with cell death receptors like TRAIL-R1/DR4, TRAIL-R2/DR5, Fas and TNF-R1 complex, known as death-inducing signaling complex (DISC) (243). Overexpression of cFLIP has been identified in multiple of cancers, which act on DISC complex and block the activation of caspase-8/10. Therefore, targeting FLIP and downregulation of its expression, sensitize the cells to TRAIL induced apoptosis (244). TRAIL based antitumor strategies are already in clinical trials in several types of cancers, but very little success has been achieved for clinical trials in PanCa. However, many preclinical studies have been performed targeting several signaling pathways those are responsible for TRAIL resistance (245) (60, 246). There are several researches reported about TRAIL resistance in PanCa (241, 247-250). Unlike other cancer types, PanCa is highly resistant to TRAIL therapy because of overexpression of cFLIP in PanCa cells however minimum or no expression in normal pancreatic ducts was reported (245). Enhanced cFLIP, modulates GSK3β expression through the activation of anti-apoptotic protein AKT, which leads to TRAIL resistance in cells although TRAIL receptors are significantly expressed in PanCa cells (245, 251). It has been reported that expression of DR5 attenuated processing of let-7, interacting through Drosha and DGCR8 in PanCa cells (246). Differential expression of functional and non-functional TRAIL receptors and inflammatory molecule (LPS) has shown to be a major factor for TRAIL resistance in PanCa cells (251). NF-κB signaling pathway is a well-established mechanism for PDAC progression and resistance to the apoptotic pathway. NF-κB has been recently identified as a major resistance regulator of
death receptor pathway using chemokines CX3CL1 which is key mediator for inflammatory cells recruitment during PDAC progression (252). Over-expression of HSP70 is associated with tumor malignancy and poor prognosis. Therefore, HSP70 is one of the responsible factors for TRAIL resistance. Protective autophagy has also played a role in therapy resistance in PanCa (253).

Recently, there are quite a few combinatorial therapeutic approaches also establishing to target TRAIL resistance in PanCa. A natural product chaetospirrolactone (CSL) has been identified as a regulator for of DR4 expression in TRAIL resistance of PanCa cells (254). Activation of the JNK pathway leads to the degradation of cFLIP by quercetin and also sensitizes PanCa cells to towards TRAIL induces apoptosis (250). Metformin has been proposed as an effective combination therapy with TRAIL to sensitize TRAIL therapy in PanCa by the upregulation of DR5 expression via inhibiting the expression of oncogenic microRNA, miR-221 (255). Chloroquine, a well-known autophagy inhibitor showed combination therapeutic potential to TRAIL induced apoptosis by increasing p21 level and G2/M phase arrest in some of the PanCa cells (256).

Loss of microRNA expression is a potential cause of tumor progression and metastasis in several cancers, including PanCa. Prior work has identified miR-145 as a tumor suppressor miRNA in PanCa and restoration of miR-145 provided a potential therapeutic benefit for PanCa therapy (37, 100, 101). For successful translation into clinical practice, recently, we have engineered a unique magnetic nanoparticle (MNP) based system for gene(s) and drug(s) delivery applications (104). In our previous research article, we have surface modified this unique MNP formulation, with PEI to deliver miR-145 to the PanCa cells (101). MNP formulation successfully restituted miR-145 and confirmed antitumor behavior in PanCa cells. Prior work has also identified that, tumor suppressor miR-145 has the ability to sensitize chemotherapeutic drugs (37). Previous research on liver fibrosis indicated that over expression of miR-145 and TRAIL enhanced TRAIL mediated apoptosis by targeting NF-κB pathway in liver fibrosis condition (257).

To overcome the inherent resistance and improve the therapeutic potential of TRAIL for PanCa treatment, co-delivery of TRAIL plasmid and gene therapy (miR-145) are expected to be a novel therapeutic approach. This novel nanoparticle-based platform can co-deliver miR-145, pTRAIL is expected to be a potential strategy to overcome TRAIL resistance by co-delivery of therapeutic agents in combinatorial PanCa therapy. Herein, we demonstrate the integration of novel approach to overcome chemo-resistance and offer TRAIL-based therapeutic strategies.
Materials and Methods

Culture of pancreatic cancer cells and animal

Human PanCa cells (HPAF-II and AsPC-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were maintained at 37°C and 5% CO₂ incubator with 95% air atmosphere and cultured in DMEM/F12 and RPMI-1640 (HyClone Laboratories, Inc. South Logan, Utah, USA) medium, supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA, USA), 1% antibiotic and antifungal solution. Every six months interval cells were tested for mycoplasma. 6 to 8 weeks old female athymic nude/nude mice form Jackson Laboratories used for animal experimentation. All the protocols were approved and reviewed by University of Tennessee Health Science Center Institutional Animal Care and Use Committee (UTHSC-IACUC).

Chemicals, reagents and antibodies

All chemicals and reagents were purchased from Sigma Adrich Corporation (St. Louis, MO, USA) and cell culture wares were purchased from Corning life sciences (Tewksbury MA, USA). Non-targeting control (catalog number: AM17111) and miR-145 mimics (catalog number: 4464066), pEGFP-TRAIL was a gift from Bingliang Fang (Addgene). Taqman miR-145 probes (Assay id: 002278), High Capacity cDNA Reverse Transcription kit (catalog number: 4368814) and TRizol reagent (catalog number: AM 9738) were purchased from Life technologies (Carlsbad, CA, USA). The primary antibodies, anti-HER2 (catalog number: 2165S), pAKTSer473 (catalog number: 9271) and anti-p53 (catalog number: 2527), DR4 (catalog number: sc7863), Caspase-8 (catalog number: 70502), TNF-R1(catalog number: 3736) were purchased from Cell Signaling (Danvers, MA, USA) and anti-β–actin (catalog number: A5316), Flip (catalog number: sc8347), PARP-1 (catalog number: sc8007), were purchased from Sigma (St. Louis, MO, USA). FADD (catalog number: ab24533), KI67 (catalog number: ab833), CK19 (catalog number: ab15463) were purchased from Abcam (Cambridge, United Kingdom).

Preparation of MNP-miR-145-TRAIL formulation

Magnetic nanoparticle formulations were developed and optimized for an efficient delivery of nucleic acids and microRNAs. Briefly, the Fe²⁺ and Fe³⁺ ions were co-precipitated in an aqueous solution under nitrogen atmosphere in the molar ratio of 1:2 (142). 3 ml of ammonium hydroxide was added to the solution to generate uniform magnetic nanoparticles (MNPs). 200 mg of β-cyclodextrin (β-CD) and 250 mg of Pluronic F-127 was added to the particle suspension and stirred for overnight followed by washing of particles as described before (142). In this study, PEI has been used as a polycationic polymer to deliver nucleic acids (miR-145 and plasmid TRAIL). In order to form a uniform layer, 100 mg of PEI was added to the MNP solution and stirred for...
overnight. This MNP-PEI particle were diluted using 0.9% NaCl solution followed by incubation for 30 min with miR-145 and TRAIL plasmid in order to prepare MNP-miR-145, MNP-TRAIL and MNP-miR-145+TRAIL prior to the treatment.

**Particle size and zeta potential**

The hydrodynamic particle size, distribution and zeta (ζ) potential of particles (MNP, MNP-TRAIL, MNP-miR-145-TRAIL) were determined by Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) using the principle of dynamic light scattering (DLS). To measure the particle size, 10 μl of particles diluted in 990 μl of water/PBS and probe sonicated for 30 sec. This particle suspension was used to measure size and zeta potential (101).

**Gel retardation assay**

To determine complexation of MNP particles and pTRAIL, gel retardation assay was carried out at different ration of MNPs and pTRAIL. (1–10 μg) of MNPs were incubated with 1 μg of pTRAIL for 30 min in room temperature. 2% agarose gel was used to perform the assay. Naked pTRAIL and conjugated particles were allowed to run on the gel at 80V for 1hr. To check the particle mobility, we have photographed the gel (UVP gel documentation system) at different time point (221).

**Transfection and treatment with MNP formulation**

PanCa cells (HPAF-II and AsPC-1) were seeded in 24-well plate. When the cells were reached to 60-70% confluency, they starved for overnight and treated with MNPs, MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL and incubated for 48 hrs. After 48 hrs of incubation treated cells were used for several functional assays, such as, proliferation, colony formation, invasion, migration and molecular studies, like western blotting and confocal microscopy.(101).

**RNA isolation and real-time PCR (qRT-PCR)**

RNA was extracted from PanCa cells as well as from mice tumor tissues using TRIzol reagent. All the RNAs (miRNA and mRNA) were quantified using Nanodrop instrument 2000 (Thermo Scientific). The qRT-PCR experiments were performed previously described methods (101) (258). Specific set of primers were used to determine the miR-145 expression level and Caspase-8, PARP-1 and DR4 activity. For miR-145 expression, RNUB6 used as an endogenous control and mRNA was normalized with GAPDH.
Cell proliferation assay

Cell proliferation assay was carried out to determine the effect of the different treatment groups compared to control. After 48 hrs of incubation with different treatment groups, HPAF-II and AsPC-1 cells were seeded in 96-well plate at a density of 5000 cells per well and allowed to attach for overnight. After 48 hrs 20μl of MTT reagent added to each well and incubated in 37°C for 3-4 hrs followed by 100 μl of DMSO was added and incubated for 10 mins and OD was taken at 570 nm using Cytation 3 instrument as described earlier (6). The anti-proliferative effect of MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL were evaluated compared to MNPs treated group.

Colony formation assay

Treated cells were trypsinized and reseeded in 12-well plate at a density of 250 cells per well and let them grow for 12 days. after that cells were fixed with cold methanol and stained with crystal violet and photographed as per previously performed (222). Colonies were counted and represented graphically. This assay was performed to evaluate the clonogenicity of MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL compared to MNPs (control).

Cell invasion assay

Invasion assay was performed to determine the inhibition of number of invaded cells with the effect of MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL compared to MNPs (control) treated cells. Pretreated cells were reseeded (35 × 10³ cells per well) in BD Biocoat Matrigel Invasion Chambers (BD Biosciences). After 48 hrs of incubation the invaded cells were fixed with cold methanol and stained with crystal violet. Uninvaded cells were removed by cotton swab and photographed under phase contrast microscopy (6).

Cell migration assay

Cell migration assay was performed to evaluate the ability to minimize the number of migratory cells while treated with MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL as compared to control cells. Pretreated cells (HPAF-II and AsPC-1) were counted and reseeded equally (50 × 10³ cells per well) in serum free media in the upper chamber of Boyden chamber plate. The lower chamber contained serum-containing medium. After 20-24 hrs cells were fixed with 4% paraformaldehyde and stained with crystal violet. Using cotton swab un-migrated cells on top of the membrane were removed carefully and photographed using phase contrast microscopy as described in our previous study (35). Number of migratory cells were graphically represented as compared to control.
**Hyperthermia**

PanCa cells (AsPC-1 and HPAF-II) were treated with MNP-NC and MNP-miR-145 for 24 hrs. After that cells were exposed to hyperthermia for different time points such as 5, 10,15, 20 and 30 mins (Power 60 and Frequency 450). Temperature increased was monitored and plotted in the graph. Significant reduction of cell viability was observed in 20 and 30 mins of heating and temperature were increased around 42°C to 45°C. After hyperthermia exposure cells were reseeded in 96 wells for cell proliferation study and 6well plate for protein lysates preparation. At this point both the group of cells were treated with recombinant TRAIL. After 48 hrs cells were assessed for % proliferation and whole cell lysates were prepared for molecular study.

**Spheroid assay**

Tumor spheroid assay was performed as described previously (6). This assay mimics the tumor microenvironmental condition and we investigated the effect of MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL as compared to MNPs (control) in spheroid formation ability. PanCa cells (1000/ well) were seeded in 96-well low attachment plate (Corning) in 0.5% serum free medium and allowed them to form small spheroids for 3-5 days. after small spheroids visualized, they treated with previously mentioned treatment groups. After one-week spheroids were photographed and measure their size and plotted compared to control spheroid size.

**Western blotting**

MNP, MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL transfected HPAF-II and AsPC-1 cells were collected and whole cell lysates were prepared using CelLytic (Sigma-Aldrich) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Protein sample were resolved in SDS-PAGE and subsequently transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) (258). Expression of key proteins DR4, TNF-R1, Flip, FADD, HER2, pAKTSer473, p53, and β-actin/GAPDH were investigated by western blotting.

**In vivo xenograft mice model**

Six weeks-old nu/nu mice were purchased from Jackson laboratories and maintained in a pathogen-free environment. HPAFII luciferase cells (3 × 10^6) were suspended in 100 μL PBS (1X) and 100 μL Matrigel (BD Biosciences) and injected subcutaneously in right flack of each mice and waited to grow the tumor volume until 100 mm^3. After 15 days when tumor volume reached around 100 mm^3 then mice were randomly divided in 6 groups (n = 6 per group). At 18th day, mice were treated with MNPs (Control), MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL twice a week for three weeks. The tumor volume and bioluminescence images of mice were measured.
in every 6 days. Finally, the tumor volume (V) were evaluated using length (L), width (W), and height (H) and calculated by V volume \((\text{mm}^3) = 0.5 \times L \times W \times H\). When the control mice tumor volume reached to 1000 mm\(^3\), all the mice were sacrificed and tumors, other organs were harvested. Portion of all tissues will be dissected, fixed with 10% formalin, embedded in paraffin and prepared for further IHC (immunohistochemistry) analyses.

**Bioluminescence imaging study for tumor growth assessment**

Bioluminescence imaging was performed using IVIS Spectrum scanner (PerkinElmer, Waltham, MA) at the UTHSC core facility. After 15 days of tumor cells injection, mice were subjected to bioluminescence imaging. 100 μl of substrate D-luciferin were injected to each mice intraperitonially 5 mins prior to the imaging. Mice were anesthetized during imaging with 2% isoflurane in presence of O\(_2\). Quantification of image was done by Living Image® 4.0 software. Bioluminescence of an organ was quantified by total photon of the area of interest over background intensity which represent by (ROI) photons/second/cm\(^2\)/sr (sr: steradian).

**Confocal immunofluorescence cells**

Immunofluorescence was performed to identify the changes in key protein expression in PanCa cells after treatment with nanoformulation and restoration of miR-145, TRAIL and codelivery of both molecules. This assay was carried out as described previously (37). Using confocal microscopy, we have determined the expression of DR4, FADD, Caspase-8 and Flip in PanCa cells after treatment.

**Immunohistochemistry**

Immunohistochemistry was performed on xenograft tumor tissue sections (5 μm). All the treatment group tissues were deparaffinized, rehydrated followed by heat induced antigen retrieval. Then the slides were stained with TRAIL induced apoptotic pathway key antibodies, such as Caspase-8, PARP1, FADD and epithelial marker antibodies (KI67) (216).

**Results**

**Generation and characterization of MNP-miR-145-TRAIL formulation**

To potentiate the TRAIL based therapy we made a magnetic nanoparticle-based formulation which allow to co-deliver miR-145 and pTRAIL. Previously we have demonstrated about the formulation and delivery of miR-145 using MNPs (101). To
utilize its biocompatible, biodegradable and nontoxic feature, we have developed an MNP formulation to co-deliver miR-145 and TRAIL plasmid in the current study. **Figure 4-1A** is showing the schematic representation of MNP-miR-145-TRAIL formulation. In this study, we have used PEI as a cationic polymeric moiety to enhance the surface attachment through the higher binding efficiency with miR-145 and pTRAIL alone and in combination. In our previously published report, we have engineered tumor suppressor miR-145 loaded MNP formulation for effective delivery and restitution of miR-145 in PanCa (101). In this research project, we are developing a magnetic nanoparticle-based formulation for co-delivery of miR-145 and pTRAIL to PanCa in-vitro and in-vivo model to overcome the TRAIL resistance in PanCa with simultaneous miR-145 restoration. Hydrodynamic particle size and zeta potential of MNP-PEI, MNP-TRAIL and MNP-miR-145-TRAIL were determined. Optimum particle size (MNP-PEI: 202.43 nm, MNP-TRAIL: 228.37 nm and MNP-miR-145-TRAIL: 274.33 nm) (**Figure 4-1B**) and surface charge (MNP-PEI: 16.3 mV, MNP-TRAIL: 0.0733 mV and MNP-miR-145-TRAIL: 0.117667 mV) (**Figure 4-1C**) were observed for efficient delivery to PanCa cells. To achieve an efficient delivery of TRAIL plasmid, the vehicle (MNPs) needs to form a complex with the plasmid (TRAIL) in-vitro. Complex formation of pTRAIL (1 μg) and MNP formulation at different ratios (1 to 8 μg) were observed by complete retardation of MNP-TRAIL formulation in agarose gel. As shown in **Figure 4-1D**, the observed pTRAIL in gel was reduced after the weight ratio 1:1 and completely retarded at 1:2 ratio however naked pTRAIL migrated through the agarose gel freely. It is suggested that, at this particular ratio MNPs could form complexes with pTRAIL and block the migration of pTRAIL in agarose gel electrophoresis.

**MNP-miR-145-TRAIL formulation restitutes miR-145 and TRAIL**

To achieve the TRAIL sensitization, restoration of miR-145 was a crucial step as a therapeutic standpoint. The miR-145 expression level was evaluated in MNP, MNP-miR-145, MNP-TRAIL and MNP-miR-145-TRAIL formulation treated PanCa cells by quantitative PCR (qRT-PCR) assay (**Figure 4-1E**). As compared to control (MNP), miR-145 expression was increased significantly in MNP-miR-145 and MNP-miR-145-TRAIL formulation treated groups. This data confirmed that, delivery and restitution of miR-145 was effectively achieved by MNP formulation in PanCa cells. To examine the sensitization of TRAIL therapy, delivery of TRAIL as therapeutic payload is most important factor. Immunofluorescences result suggested that TRAIL plasmid effectively delivered to the PanCa cells when treated with MNP-TRAIL and MNP-miR-145-TRAIL (**Figure 4-1F**).

**MNP-miR-145-TRAIL formulation inhibits tumorigenic characteristics**

Considering the antitumor efficacy of miR-145 which directly target oncogenic MUC13 (37, 100, 101), we sought to explore the combinational effect of miR-145 and TRAIL to sensitize TRAIL in PanCa cells those who are resistant to TRAIL therapy.
Figure 4-1. Generation and characterization of MNP-miR-145-TRAIL formulation.

(A) Schematic representation of the preparation of the MNP-miR-145-TRAIL. Co-precipitated iron oxide nanoparticles were surface coated with β-cyclodextrin, F127 polymer, PEI and conjugated with miR-145 and TRAIL plasmid. (B, C) Average size and zeta potential of MNP-miR-145-TRAIL were identified using DLS. (D) Agarose gel retardation assay represented the formation of complex between MNP and TRAIL. Complex formation started in the 6th lane at the concentration of 2 μg MNPs and 1 μg TRAIL plasmid. (E) Restoration of miR-145 in PanCa cells was confirmed using Taqman probes by q-PCR. Fold increase of miR-145 was observed at the MNP-miR-145 and MNP-miR145+TRAIL treated groups in comparison with MNP control. (F) Confocal microscopy images confirmed the delivery of TRAIL plasmid in PanCa cells using MNP nanoformulation. Bars represent mean ± SEM, n=3, *P<0.05.
To investigate the cytotoxic effect of miR-145, TRAIL alone and in combination, we performed cell proliferation assay in HPAF-II and AsPC-1 cells. Results demonstrated that co-delivery of miR-145 and TRAIL using MNPs showed better inhibition of cell growth than alone miR-145 or TRAIL in both the cell lines. This result determines that restoration of miR-145 enhances TRAIL sensitization in PanCa cells (Figure 4-2A). Prolonged effect of TRAIL treatment upon restoration of miR-145 were determined by colony formation assay. Result depicted that MNP-miR-145-TRAIL effectively suppress clonogenicity in PanCa cells compared to control as well as TRAIL alone (Figure 4-2B). These finding suggested that simultaneous miR-145 restitution and TRAIL treatment inhibit cell proliferation and clonogenic potential in PanCa cells.

To analyze the invasive behavior upon treatment with MNP-miR-145-TRAIL we performed invasion assay using cell invasion kit (BD Biocoat TM Matrigel) and migration assay using Boyden chamber (corning) in HPAF-II and AsPC-1 cells. MNP-miR-145-TRAIL formulation significantly minimize invasive and metastatic cell (Figure 4-2C, E) number in both the PanCa cells. Results demonstrated that codelivery on miR-145 and TRAIL efficiently enhance the sensitivity TRAIL towards PanCa cells with replenishment of miR-145. Our results describe that MNP-miR-145-TRAIL reduced invasive and migratory cells 78.81%, 78.19% in HPAF-II and 87.07%, 69.11% in AsPC-1 cells (Figure 4-2D, F).

Nanoparticle-mediated miR-145-TRAIL co-delivery leads to TRAIL-mediated apoptosis, which activates death receptor pathway

The immunofluorescence assay was performed to determine the effect of miR-145 and TRAIL on death receptor pathway proteins when they co-delivered using MNP formulation. Confocal microscopy images suggested that combination treatment of TRAIL and miR-145 synergistically enhance the expression of DR4 and FADD, however combination treatment (MNP-miR-145-TRAIL) significantly inhibits the expression cFLIP (Figure 4-3A). As cFLIP serve as anti-apoptotic protein and its expression has decreased with treatment that leads to activation of caspase 8. TRAIL alone did not show any effect on death receptor pathway proteins however when it delivered with miR-145, the combination dose displayed synergistic effect on protein expression on both HPAF-II and AsPC-1 cells. Activation of DR4 and caspase 8 also verified by their expression in mRNA level in both the cells. Both the key factors showed significant upregulated expression upon treatment with MNP-miR-145-TRAIL (Figure 4-3B, C).

Western blotting analysis was carried out to confirm the key proteins expression of death receptor domains. In this study we have treated PanCa cells with MNPs, MNP-TRAIL, MNP-miR-145 and MNP-miR-145-TRAIL. Immunoblotting results demonstrated that MNP-miR-145-TRAIL treatment displayed better activation of DR4 than alone miR-145 and TRAIL treatment. As Fas-associated DD protein (FADD) also a part of this death complex, which subsequently activated upon treatment with MNP-miR-145-TRAIL. Activation of DISC complex play a major role in cFLIP downregulation and proapoptotic complex activation.
Figure 4-2. Therapeutic potential of MNP-miR-145+TRAIL formulation in PanCa cells.

(A) Effect of MNP-miR-145, MNP-TRAIL and MNP-miR145+TRAIL on cell proliferation was observed using MTT assay in both PanCa cells (AsPC-1 & HPAF-II).

(B) Colony forming ability of cells (AsPC-1 & HPAF-II) were determined after treated with MNP-miR-145, MNP-TRAIL and MNP-miR145+TRAIL as compared to control, MNPs. (C, E) Invasion and migration assays. PanCa cells (AsPC-1 & HPAF-II) were treated with MNP, MNP-miR-145, MNP-TRAIL and MNP-miR145+TRAIL and plated in a serum free media into the upper chamber of Matrigel-coated membrane and Boyden chamber plate. 10% FBS was added to the lower chamber as a chemoattractant. After incubation invaded and migrated cells were identified by crystal violet staining followed by imaging. (D, E) Graphs represented as percent inhibition of invasion and migration of MNP-miR145+TRAIL treated cells (AsPC-1 & HPAF-II) as compared to MNP-TRAIL. Bars represent mean ± SEM, n=3, *P<0.05.
Figure 4-3. Codelivery of miR-145 and TRAIL leads to enhanced apoptosis.

(A) Confocal microscopy images determined the expression of death receptor pathway proteins and apoptotic protein (DR4, FADD, FLIP and Cl-caspase 8) in AsPC-1 cells. (B, C) q-PCR results represent activation of death receptor pathway (DR4 and caspase-8) in PanCa cells (HPAF-II & AsPC-1). Bars represent mean ± SEM, n=3, *P<0.05.
downstream key protein’s (HER2, AKT, pAKT, p53) which targeted by miR-145 (Figure 4-4A, B).

A significant reduction in the size of the primary and secondary tumor sphere was observed when treated with MNP-miR-145-TRAIL as compared to control MNPs as well as MNP-TRAIL and MNP-miR-145 alone. The size of these tumor spheres of different treated group depicted that MNP-TRAIL treatment showed negligible effect on sphere formation where as MNP-miR-145 effectively suppressed the growth of the sphere (Figure 4-4C, D). Additionally, MNP-miR-145-TRAIL showed significant size reduction, which suggested that in presence of miR-145, TRAIL is showing better effect in in-vitro tumor sphere formation.

**Hyperthermia promotes TRAIL-induced inhibition of drug resistance-related proteins**

Application of hyperthermia is a major advantage of using MNP particles as therapeutic modality. Scheme represents the workflow of hyperthermia process used to treat the cells and exposed under hyperthermic condition and eventually the cells were die (Figure 4-5A). The Graph representing the temperature rise in cancer cells due to MNP particle treatment under hyperthermic condition. Cells were exposed to hyperthermia at different time points maintaining the conditions (Power 60 and Frequency 450) and 42°C to 45°C temperature raise has been recorded (Figure 4-5B). A significant reduction of cell viability was observed following 20-30 mins of heat exposure followed by incubation for cell proliferation in AsPC1 (Figure 4-5Ci) and HPAF II (Figure 4-5Di) cells. Phase contrast images were taken at time of termination to prove the cell killing due to hyperthermia with MNP treatment (Figure 4-5Cii, Dii).

It has been reported in colon and prostate cancer, hyperthermia has enhanced TRAIL induced apoptotic population (259, 260). In the current study, MNP-miR-145 treated both PanCa (AsPC-I and HPAF-II) cells displayed significant inhibition of cells survival compared to the control (non-targeting) treated groups when they exposed to hyperthermia (Figure 4-6A). It is an important factor to be noticed here that TRAIL alone even under hyperthermic condition did not show any effective cell killing ability while in presence of miR-145 hyperthermia sensitize both PanCa cells similar pattern has been observed in real time xCELLigence study (Figure 4-6B) Western blotting analysis data represented that restoration of miR-145 and hyperthermia effectively modulated the expression of heat shock protein’s (HSP70 and HSP90) and At the same time, inhibited the expression of MDR1 under the hyperthermic condition in presence of miR-145 in HPAF-II and AsPC-1 cells (Figure 4-6C).
Figure 4-4. miR-145 restitution sensitizes TRAIL-mediated death receptor pathway.

(A) AsPC-1 was treated with MNP, MNP-miR145, MNP-TRAIL and MNP-miR145+TRAIL. Whole cell lysate was immunoblotted for DR4, TNFR-1, FADD, FLIP, HER2, AKT, p-AKT and p53 proteins. GAPDH was used as a loading control. (B) Data represent the percent band intensity of key proteins. (C, D) MNP-miR-145+TRAIL effectively suppressed primary and secondary tumor spheroid formation (images: Upper panel; Size quantified bars: lower panel) in PanCa cells (HPAF-II and AsPC-1) (C, D). Bars represent mean ± SEM, *P<0.05.
Figure 4-5. Hyperthermia as a promising therapeutic strategy for cancer treatment.

(A) Schematic flow explains the advantage of using hyperthermia as a selective cancer cells killing ability. (B) Graph represent the temperature raise at different time point after treatment with MNP particles under hyperthermic condition. (C, D) Cell proliferation has been significantly decreased after treatment with MNPs under hyperthermia exposure for 20-30 mins. (E, F) Phase-contrast microscopy images showing cell killing ability of MNP treatment post hyperthermia.

Figure 4-6. Hyperthermia enhances cell killing ability of cancer cells.

(A) After miR-145 (145) restoration, cells were exposed to hyperthermia for 20 mins (42°C) and following treatment with recombinant TRAIL (T) analyzed for MTT assay. (B) Real time xCELLigence data demonstrating similar pattern of cell proliferation. (C) Immunoblotting assay was determined the effect of hyperthermia after treatment with miR-145 and TRAIL on heat shock proteins (HSP70 and HSP90) and resistance involved protein, MDR1.
Potentiation of in vivo TRAIL therapy using miR-145

After investigation of in-vitro therapeutic efficacy, we evaluated in-vivo therapeutic potential of MNP-miR-145-TRAIL along with GEM sensitivity in xenograft mouse model. In this experiment, thirty-six mice were used, and HPAF-II-luciferase cells were injected in right flank of these mice. After 18 days of cell injection, mice were randomly divided in six groups and started treatment intratumorally twice a week, for 3 weeks as per described in material and method section. Tumor growth was monitored by bioluminescence imaging of live mice as well as measured by tumor volume weekly. There was no toxicity observed during the study compared to the control group. The end point bioluminescence images and excised tumor images showed significant reduction in tumor volume in MNP-miR-145-TRAIL (p<0.05) and combination with GEM (p<0.05) (Figure 4-7A, B). MNP-miR-145+TRAIL significantly deliver TRAIL (Figure 4-7C) and miR-145 (Figure 4-7D) in tissues using MNPs. Immunofluorescence images of different treated groups confirmed the internalization of TRAIL in tumor. Also, miR-145 expression was determined in the excised tissues. Significant decrease in tumor volume and weight also observed in MNP-miR-145-TRAIL and MNP-miR-145-TRAIL+GEM group compared to the control group (Figure 4-7F, G). Survival curves also demonstrated that the MNP-miR-145-TRAIL and MNP-miR-145-TRAIL+GEM groups showed prolonged survival compared to the alone MNP-TRAIL, MNP-miR-145, Gem and control groups (Figure 4-7H). H&E staining of different organs depicted no potential toxicity upon treatment with miR-145 and TRAIL (Figure 4-7E). After sacrifice all the mice we have collected the excised tumor tissue samples from all the treatment groups and investigated the TRAIL induced apoptotic key proteins expression in those tissues. We evaluated caspase-8, PARP-1 and FADD activation with MNP-miR-145-TRAIL and MNP-miR-145-TRAIL+GEM treated groups, while KI67 expression were decreased with the treatments (Figure 4-8A). Quantitative PCR also revealed activation of PARP-1, Caspase-8 and enhanced expression of DR4 in MNP-miR-145-TRAIL and MNP-miR-145-TRAIL+GEM treated groups (Figure 4-8B).

Discussion

Recently TRAIL, which is a member of TNF superfamily has opened an influential therapeutic avenue for several cancers, but unfortunately PanCa demonstrated TRAIL resistance in preclinical and clinical level. To overcome TRAIL resistance, combinatorial gene therapy is an effective therapeutic modality. At the same time, successful translation to the clinic by efficient delivery of therapeutic molecules to the target organ is a major challenge. Therefore, this study describes a novel engineered magnetic nanoparticle (MNPs) formulation to co-deliver miR-145 and TRAIL to overcome the TRAIL resistance by restoration of tumor suppressor miR-145 in PanCa model.
Figure 4-7. miR-145-TRAIL therapy inhibits growth and increases mice survival.

(A, B) MNP-miR-145-TRAIL combination with Gem showed effective therapeutic potential in xenograft mouse model. Bioluminescence images and excised tumor represented therapeutic efficacy of miR-145, TRAIL and Gem. MNP-miR-145+TRAIL significantly deliver miR-145 (D) and TRAIL (C) in tissues using MNPs. (E). H&E staining of different organs depicted no potential toxicity upon treatment with miR-145 and TRAIL. Treatments showed significant reduction in tumor growth (F), tumor weight (G) and increased survival efficiency (H). Bars represent mean ± SEM, *P<0.05.
Figure 4-8. miR-145-TRAIL therapy potentiates death receptor pathway in vivo.

Co-delivery of miR-145 and TRAIL potentiates death receptor pathway and enhances Gem sensitivity in mice model. IHC staining of tissue sections (A) and q-PCR of excised tumors (B) represented the activation of apoptotic proteins and inhibition of Ki67. Bars represent mean ± SEM, *P<0.05.
Our first goal was to generate and characterize the formulation (MNP-miR-145+TRAIL). The size of the final formulation is 274.33 nm and zeta potential 0.117 mV, which suggest an ideal delivery vehicle for cancer therapy. In our previous research article, we have used this MNP formulation to deliver miR-145 to PanCa cells, this unique formulation did not show any toxicity to normal cells, cancer cells, RBCs, and they exhibited hemocompatibility. The complexation of TRAIL with MNPs were confirmed by complete retardation in gel. miR-145 restitution and internalization of TRAIL was confirmed by q-PCR and immunofluorescence respectively, suggest efficient delivery of miR-145 and TRAIL using MNP formulation.

Our study demonstrated that the restitution of miR-145 and overexpression of TRAIL effectively sensitize TRAIL mediated antitumor activities and suppressed the tumorigenic features such as, proliferation, clonogenicity, invasion and migration in both PanCa cells. Our results indicate that MNP-miR-145-TRAIL formulation activates TRAIL induced extrinsic apoptosis pathway by activating the death complex which displayed by higher expression of DR4, FADD which leads to downregulation of cFLIP associated with DISC complex showed activation of caspase-8, PARP-1. Our results represent that MNP-miR-145-TRAIL formulation targets miR-145 downstream pathway molecules, that leads to TRAIL induced apoptotic molecular mechanisms. miR-145 restoration also effectively suppressed tumor sphere formation in PanCa cells. So overall TRAIL induced apoptotic pathway has been sensitized with miR-145 restoration.

The in vivo results demonstrated that restitution of miR-145 and combination of Gem enhanced TRAIL mediated therapeutic approach in mice model. It has been observed that MNP-miR-145-TRAIL formulation retained in the tumor and stromal area in mice body by Prussian blue staining. Effective delivery and restoration of miR-145 has been demonstrated by qRT-PCR and delivery of TRAIL by immunofluorescence. Tumor volume was efficiently controlled in MNP-miR-145-TRAIL and combination with GEM treated groups also effectively enhanced the rate of survival in these treated groups. As a result of miR-145 restoration and TRAIL sensitization was evident in excised tumor tissues, demonstrated by significant activation of caspase-8, PARP-1 (IHC & qRT-PCR) and increased expression of FADD (IHC), DR4 (qRT-PCR). Our future goal is to further examine the MUC13 expression in excised tumor tissues to establish the correlation with miR-145 restitution and TRAIL sensitization.

Conclusion

In conclusion, this study offers a novel perception of overcoming TRAIL resistance by restoration of miR-145 in PanCa in vitro system as well as in vivo mice model. This approach has been potentiated by improved and safe intracellular delivery of TRAIL and miR-145 using magnetic nanoformulation (MNP) strategy. Our findings recommend that co-delivery of miR-145 and TRAIL successfully heightened a novel therapeutic modality in pancreatic carcinogenesis.
CHAPTER 5. THERAPEUTIC INTERVENTION FOR PANCREATIC CANCER USING AUTOLOGOUS EXOSOMES

Introduction

Due to paracrine Hh signaling there is an internal crosstalk between epithelia and stroma in pancreatic cancer condition (8). Excessive production of extracellular matrix (ECM) and collagen I has a critical role in stromal cell proliferation and pancreatic stellate cells formation (6). Tumor metastasis and chemoresistance are fearful incidents of desmoplasia during cancer progression (7). To control tumor microenvironment and metastasis, novel-tumor targeting strategies are urgently required. Understanding the molecular mechanism associated with tumor microenvironment and target them as novel therapeutic strategy is the most interesting research area in PanCa (6).

From a therapeutic delivery standpoint, novel delivery vehicles are required that are both biocompatible and non-immunogenic for a patient in order to maximize the chances of cure. Therefore, identification of newer therapeutic approaches that can aid current therapeutic methods are highly desirable. Though the polymeric or artificial nanoparticles are very effective, biocompatibility, immunogenicity and targeting towards specific cell type remain a considerable challenge. Major obstacles in drug delivery aspect are the immune response by non-self material and stability in the blood stream. In a complex and dynamic tumor microenvironment extracellular vesicles (EVs) are key players in intracellular communication. Exosomes are type of EVs, secreted from tumor cells to communicate with the stromal cells (202). They are small extracellular membrane bound biological vesicles involved in several biological processes (262). Exosomes are nanosized, 30-150 nm membrane bound vehicles, present in all body fluids such as blood plasma, saliva, breast milk, urine etc. (263).

Cancer associated exosomes may play a role as carrier in different stages of cancer progression. They are secreted from different cell types and facilitate an intercellular communication by transporting biologically active materials to recipient cells (202, 206). Exosomes contain protein, lipid, microRNAs (miRNA) and mRNAs that are naturally delivered to recipients’ cells (207, 208). Exosomes can protect themselves (cargo) from degradation and because of their non-immunogenic nature, they are stable in systemic circulation. These characteristics of exosomes make them an attractive tool for diagnostic and therapeutic applications which are now also an efficient candidate for clinical trials (204).

Repurposing of drug for human use, already been approved as a new therapeutic modality for cancer. A traditional anti-diabetic molecule, Metformin is now being widely used in various anti-cancer therapies (54-56). In the same regard, we have proposed that ormeloxifene be repurposed for PanCa therapy. ORM, is a non-steroidal, non-hormonal anti-estrogen oral contraceptive for human use. Several studies reported ORM as a potent anticancer agent for breast cancer (57) and head and neck cancers (58). In our previous research articles, ORM has been identified as a stroma inhibitor (6). Identification of a
novel therapeutic vehicle that could target TME and inhibit metastasis by overcoming the inaccessibility of drugs to tumor site is urgently required.

Herein, our lab has optimized an exosome based therapeutic approach, utilizing exosomes isolated from the human pancreatic tumor adjacent normal (NAT) fibroblast cells. We utilized this scaffold for safe and effective delivery of therapeutic payload. The objectives of this study were: (i) Isolation and characterization of exosome as a personalized therapeutic carrier for PanCa therapy. (ii) Investigate the efficacy of patient derived exosomes in efficient drug delivery approach.

**Materials and Methods**

**Procurement of archived and freshly collected human specimens**

Freshly collected pancreatic tumor and matched NAT were procured from Baptist Memorial Health Center, Memphis, TN. Fibroblast cells from tissues (cancer associated fibroblasts; CAF and NAT) were isolated by out-growth method (17) and assessed by cytofilament staining (α-SMA/Hyaluronic acid/Desmin).

**Isolation of exosomes from tissues**

Exosomes were isolated from NAT and CAF using Total Exosome Isolation Reagent while culturing them in exosome depleted FBS media. The isolation of exosomes is an overnight process. Fibroblast cells were cultured in appropriate media for three days with exo-free FBS (Exo-FBSTM Exosome-depleted FBS media, SBI System Biosciences). Cell free conditioned media was prepared by centrifugation followed by syringe filtration using 0.22 μm filter and incubated with 0.5 volumes of the Total Exosome Isolation Reagent (from cell culture media, Thermo fisher scientific) for overnight at 4°C. Then the conditioned media centrifuged at 10000 rpm for 60 mins and collected the exosome pellet. Then exosome pellet was suspended in PBS and protein concentration was determined using Bradford method (Thermo Scientific, Rockford, IL). Exosome suspension was stored at -80 °C for longer shelf life.

**Drug loading into exosomes and characterization**

Exosome formulation of ORM (Exo-ORM) was prepared by incubation method at room temperature. ORM solution were mixed with the isolated exosomes at room temperature for approximately 30 mins. ORM and exosomes dispersion was done in PBS at different concentration of exosome protein (100μg, 400 μg, 500 μg). To remove the free drug from the exosome solution after incubation, centrifugation was done at 10,000 rpm for 10 mins and washed twice with PBS to remove all unbound drugs. The Exo-drug formulation was stored at -80 °C for in-vitro studies.
Particle size and poly dispersity index (PDI) analysis

Exosome and Exo-ORM formulation were diluted in PBS and analyzed for particle size and PDI using a Zetasizer (Malvern Instruments Ltd, Malvern, Worcestershire, UK) by taking the average of 15 measurements per reading.

Drug loading analysis by UPLC

ORM loading in Exo-ORM formulation was determined by UPLC analysis. Briefly, 0.90 ml acetonitrile was added to 100 μl of Exo-ORM to extract the drug and precipitate exosomal proteins. The precipitated proteins were separated by centrifugation at 10,000 x g for 10 mins, and supernatant containing drug analyzed for loading concentration (18). The Waters® H-Class UPLC was used for analytical testing of ORM containing samples. Column used was CORTECS® T3 1.6 μm, solvent A was 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. A two-minute gradient elution was used as follows, (minutes/%A) 0/90, 0-1/10, 1-1.10/90, 1.10-2/90. UV λmax at 220 nm was used for detection using a PDA detector and the retention time for ORM using the above conditions was 1.0 ± 0.1 minute.

Atomic Force Microscopy (AFM) for morphology analysis

The shape and surface morphology of NAT and CAF were determined by AFM. Exosomes and Exo-drug were diluted to 10 μg/ml using deionized water and then 2 μl of the respective samples was placed on a silica wafer and air dried for 30 mins. Atomic force microscopy (Asylum MF-3D, Oxford Instruments, Goleta, CA) in tapping mode and aluminum-coated silicon probes used for imaging. Topographic height, amplitude and phase retraces were imaged concurrently with a fixed force (<1nN) with a scanning rate of 1 Hz. The images were recorded at 256 × 256 pixels and processed using IGOR software.

Identification of sub-cellular localization

To determine the cellular localization of NAT exosomes, PanCa cells (8×10^4) were seeded on 4-well chamber glass slides and treated with exosomes (15μg). Then cells were fixed using 4% paraformaldehyde for 10 mins, permeabilized with 0.1% TritonX-100 in 1X PBS for 10 mins, and washed twice with an additional 1X PBS, followed by a blocking step with 10% donkey serum in PBS for 1 hr. Then cells will be incubated with 30 nM Mito Tracker Red or 75 nM LysoTrackerR Red DND-99 (Life Technologies) to stain as a marker for mitochondria and lysosome, respectively. Internalization of exosomes were identified by CD63 expression. Finally, nuclei were counter stained with DAPI (4’,6-diamidino-2-phenylindole, Life Technologies) and mounted in Vectashield Mounting Medium (Vector Labs, Burlingame, CA). Cells were visualized under confocal
microscope (Carl Zeiss LSM 710, Oberkochen, Germany) under 40X magnification using oil immersion objective.

**Cell proliferation assays**

To determine the in-vitro therapeutic efficacy of the Exo-ORM formulation, cytotoxicity assay was performed using standard MTT 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide) with PanCa cell lines (HPAF-II, AsPC-1). Briefly, PanCa cells were seeded at 5,000 cells/well in 96-well plates. Various concentration of exosomes, Exo-ORM and free ORM (in presence or absence of EGF) were treated to PanCa cells for 48 hrs. After 48 hrs incubation MTT reagent was added and proceed according to previous protocol (19). The absorbance at 570 nm was measured using BioTek™ Cytation™ 3. The percentage of cell proliferation was determined as compared to the treated cells to untreated cells.

**Real-time cell proliferation, migration, and invasion using xCELLigence system**

xCELLigence system is a microelectronic biosensor for cell-based assays. Based on cellular viability, number, morphology and adhesion characteristics, this instrument provides electronic impedance which represent a real time behavior of cells. After performing the conventional methods, the effect of formulation on tumorigenic behavior of PanCa cell were further confirmation by real-time migration, invasion, and proliferation assays using the xCELLigence system. xCELLigence system is an electrical impedance-based method that allows for the measurement of cell migration, invasion, and proliferation in real time. Briefly, cells were seeded in respective plates for cell proliferation, invasion and migration, and treated with formulation and drug loaded formulation. After certain time of incubation at 37 °C, 5 % CO2, results were analyzed in xCELLigence instrument for real-time cell proliferation, migration, and invasion assays.

**Cellular motility and invasion of PanCa cancer cells**

Cell migration assay was performed in a 96 well format HTS transwell plate (Corning). PanCa cells was serum starved for overnight and next day plated at 50,000 cells/well density in upper chambers with serum free culture medium with Exo, ORM and Exo-ORM treatment (in presence or absence of EGF) and allowed to migrate towards FBS gradient to lower chamber for 10-20 hrs. At the end of indicated time, cells were fixed with 4% paraformaldehyde and stained with crystal violet. Then the plate was washed with running water to remove any excess staining. Cells in the upper chamber were completely removed by cotton swab while wet and let them air dried for overnight, then migrated cells were observed/ imaged by using light microscope. For invasion assay, the same protocol was used other than cells will be plated in 24 transwell Matrigel coated serum free culture medium with same treatment applied. Then in similar way invaded cells were fixed and stained to check the motility and invasive characteristics of cells by
phase contrast imaging. We also performed invasion and migration assay through RTCA xCELLigence instrument to demonstrate the real-time changes of cellular behavior.

**Cell cycle analysis and apoptosis**

Cell cycle analysis was performed to determine cell cycle arrest. PanCa cells were plated in 100mm dish at the density of 2x10^6 per plate and allowed to adhere overnight. Next day, cells were exposed to exosome, ORM, Exo-ORM for 24 hrs. After 24 hrs, cells were trypsinized, washed with PBS, fixed with 70% ice cold ETOH and saved at -20°C until further used. At the time of analysis, cells will be incubated with RNase at 37°C for 90 mins and stained with Propidium Iodide (PI) solution (Sigma-Aldrich, St. Louis, MO) for 30 mins at RT in the dark and analyzed by Accuri C6 flow cytometer in FL2 channel and data interpretation was done by ModFit.Lt software. Induction of apoptosis was measured by utilizing Annexin V PE kit (BD Biosciences, San Diego, CA). Annexin V labels the early apoptotic cells by binding to phosphatidylserine which become exposed at the outside of the cell membrane as cells undergo apoptosis considered as an early event of apoptosis. PanCa cells were plated (1x10^6 /plate) treated with above mentioned treatment groups for 24 hrs, both floaters and adherent cells were collected, washed with cold PBS and stained with Annexin V at 5 μL/100 μL of cell suspension concentration for 20 mins in the dark at room temperature. After incubation, cells were analyzed with Accuri C6 flow cytometer in FL2 channel. Data was represented with early and late apoptotic population with graphical representation.

**Western blotting**

Human PanCa cells (HPAF-II and AsPC-1) were treated with ORM and Exo-ORM and cell lysates were prepared to perform western blotting (20) to check the expression of several key proteins associated with EMT and SHH signaling pathway. Tumor tissue proteins also extracted by homogenizing in Tissue Extraction reagent-1 (Life Technologies) and immunoblotting was performed as described earlier (101).

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from PanCa cells (HPAF-II and AsPC-1) treated with exosome as control vehicle and ORM, Exo-ORM using TRIzol reagent. RNA was quantified and integrity was checked by Nanodrop instrument 2000 (Thermo Scientific). The expression level of mRNA was evaluated by qRT-PCR experiment using a specific set of primers and GAPDH was used as endogenous control. This assay was performed as previously explained protocol (216).
Immunofluorescence (cells and tissue)

Immunofluorescence analysis was carried out in PanCa cells and excised tumor tissues as per previously described protocol (37). This assay was performed to determine the changes of expression of key proteins related to stromal markers and EMT pathway. Cells were fixed and tumor tissues sections were deparaffinized, rehydrated followed by heat induced antigen retrieval to stain for specific antibodies (α-SMA, collagen, Vimentin, E-cadherin).

Spheroid assay

Tumor spheroid assay was performed as described earlier (6). We have investigated the effect of exosome, ORM and Exo-ORM as compared to control on spheroid formation and growth which imitate the tumor microenvironment in in-vitro condition. HPAF-II and AsPC-1 cells (1000/ well) were seeded in 96-well low attachment plate (Corning) in 0.5% serum free medium and allowed them to form small primary spheroids for 3-5 days followed by above mentioned treatments. Following incubation of one-week primary spheroids were dissociated into single cells and plated into 6 well low attachment plates at a density of 1×10^4 per well and allowed them to grow another 10 days without adding any treatment. To determine the treatment effect, spheroids were photographed and measure their size and plotted compared to control spheroid size.

In vivo xenograft mice model

Six weeks-old nu/nu mice were purchased from Jackson laboratories and maintained in a pathogen-free environment. HPAFII luciferase cells (3 × 10^6) were suspended in 100 μL PBS (1X) and 100 μL Matrigel (BD Biosciences) and injected subcutaneously in right flack of each mice and waited to grow the tumor volume until 100 mm^3. After 7 days when tumor volume reached around 100 mm^3 then mice were randomly divided into 3 groups (n = 4 per group). At 10th day, mice were treated with PBS (Control), Exosome and Exo-ORM twice a week for five weeks. Finally, the tumor volume (V) were evaluated using length (L), width (W), and height (H) and calculated by V volume (mm^3) = 0.5 × L × W × H. When the control mice tumor volume reached to 1000 mm^3, all the mice were sacrificed and tumors, other organs were harvested. Ex-vivo imaging of tumor and other organs was performed to determine the effect of Exosome and Exo-ORM on metastatic tumors. Portion of all tissues will be dissected and used for whole lysate preparation for immunoblotting and some of the tissues were fixed with 10% formalin, embedded in paraffin and prepared for further IHC (immunohistochemistry) analyses.
**Immunohistochemistry**

Immunohistochemistry was performed as per previously described study (237) on xenograft tumor tissue sectioned into 5 μm thickness. Control and treatment groups tissues were deparaffinized, rehydrated followed by heat induced antigen retrieval. Then the slides were stained with EMT pathway antibodies such as, N-cadherin, β-catenin, tumor microenvironment marker, Hyaluronic acid, and proliferation or epithelial marker Ki67.

**Results**

To design a personalized therapy, autologous exosomes considered to be a promising therapeutic vehicle for PanCa. Freshly collected patient tumor and adjacent normal tissue from Baptist Memorial Health Center, Memphis. Then we have generated NAT and CAF fibroblast from same human patient’s tissues in our laboratory and isolated exosomes from both cell types and utilized them as therapeutic payload to target tumor microenvironment and metastasis in PanCa.

**Isolation of exosomes from patient tumors and their physical characterization**

Patient tissue derived fibroblast cells (NAT and CAF) were investigated by cytofilament staining using α-SMA/Hyaluronic acid/Desmin which implicated true fibroblast has been isolated from the tissues (Figure 5-1A). Isolated exosomes from NAT and CAF conditioned media were evaluated by size, zeta potential and CD63, CD9 marker protein expression (Figure 5-1E). Isolated exosomes from NAT (size: 44.12 ± 0.89; Zeta potential: -14.9 mV) and CAF (size: 70.28 ± 1.47; Zeta potential: -25.35 mV) showed effective size and zeta potential as drug delivery vehicle (Figure 5-1B, C). Atomic Force Microscopy (AFM) image explained the shape and round morphology of the NAT and CAFs (Figure 5-1D) derived exosomes which suggested similar size range identified by DLS. Exosomal marker CD63 expression was also significantly correlated with characteristics of different types of fibroblast derived exosomes. The expression of CD63 marker protein was significantly high in CAF than NAT. Recent study has introduced that CAF is major key player of tumor microenvironment leads to tumor progression, drug resistance and metastasis includes EMT pathway. Exosomes is one of the important components in tumor cells and CAFs interplay (264). It has been reported that CAFs derived exosomes significantly enhances chemoresistance factors which eventually increase drug resistance in PanCa cells and proliferation of pancreatic tumor epithelial cells (265). Referring these oncogenic features of CAF derived exosomes, we selected NAT derived exosomes as a drug delivery vehicle for our study purpose and use these exosomes for further assays.
Figure 5-1. Isolation of exosomes from patient tumors and their physical characterization.

(A) Isolated NAT and CAF fibroblast cells and were assessed by cytofilament staining (Hyaluronic acid/α-SMA/Desmin) using immunofluorescence assay. These fibroblast cells were used to isolate exosomes from respective conditioned media. (B and C) Isolated exosomes from NAT and CAF showing effective size and zeta potential (size: 44.12 ± 0.89; Zeta potential: -14.9 mV) and (size: 70.28 ± 1.47; Zeta potential: -25.35 mV) respectively. (D) Shape and surface morphology of NAT and CAF derived exosomes were identified by Atomic Force Microscopy (AFM) image. (E) Exosomal marker proteins CD63 and CD9 expression was determined by immunoblotting analysis of the both NAT and CAF derived exosomes from two different batches of exosomes.
Patient-derived fibroblasts mediate trafficking of exosomes and exosomal protein/RNA between cells

To determine the delivery and uptake potential of NAT derived exosomes, we have evaluated exosomal RNA and protein expression in PanCa cells by labelled them with specific dye. Exosome uptake was identified by RNA (RFP) and protein (GFP) expression of isolated exosomes in PanCa cells (Figure 5-2A, B). Thus, we hypothesize that NAT derived exosomes will be a potent drug delivery system for PanCa model as it is a natural membrane bound vesicles with non-immunogenic characteristics. Based on this hypothesis, we have delivered the NAT derived exosomes to PanCa cells (HPAF-II and AsPC-1) and data suggested that NAT-Exosomes were efficiently internalized in the PanCa cells which was determined by different tetraspanins, such as CD63, CD81 and MHC. Uptake of exosomes were examined by the confocal microscopy images of CD81 and MHC protein expression in AsPC-1 cells, DAPI was representing the nucleus of cells (Figure 5-2C). We have also treated HPAF-II cells with exosomes at different concentrations (10 μg, 20 μg, 30 μg of exosomal protein) (Figure 5-2D). In each of the concentrations of exosome protein significantly increased level of exo-marker (CD63) expression. Lysotracker and Mitotracker indicated subcellular localization of exosomes in lysosomes and mitochondria respectively (Figure 5-2E, F).

Ormeloxifene loaded exosomes were also characterized by their size, zeta-potential and drug loading efficiency. Results demonstrated that the ratio of exosomal protein and drug loading was always higher in case of NAT derived exosomes than CAF derived exosomes. Size of the Exo-ORM for both types of formulation were identified (NAT: Exo-Orm- 272.25 ± 1.05 & CAF: Exo-ORM- 215.6 ± 1.6) and zeta potential (negatively charged) of the formulations were not much differ from the native exosomes, (NAT: Exo-Orm -20.4 & CAF: Exo-Orm -17.3) (Figure 5-2H). Interestingly, size of the ORM loaded exosomes (NAT and CAF) were completely correlated with the drug loading efficiency of the exosomes. Results demonstrated that, NAT derived exosomes have a higher drug loading capacity in any concentration of exosomal protein than CAF derived exosomes (Figure 5-2Gi, ii) which reflected on the size of different sources of exosomes, this phenomenon suggested therapeutic importance of NAT derived exosomes over CAF exosomes. This further indicates relevance for their utilization in the development of novel therapeutic strategies.

NAT-derived exosomes efficiently deliver drugs to cancer cells with enhanced efficacy to inhibit tumorigenic characteristics

We evaluated the tumorigenic effect of Exo-ORM formulation on PanCa cells HPAF-II and AsPC-1. Results demonstrated that Exo-ORM formulation inhibited proliferation of both the PanCa cells in a dose dependent manner as compared to free ORM after 48 hrs (Figure 5-3A). Interestingly exosome alone did not show any effect on PanCa cells. The effect of Exo-ORM and free ORM on cell proliferation was also evaluated in real time using xCELLigence system, which confirmed similar inhibitory effect on, HPAF-II and AsPC-1 cells (Figure 5-3B).
Figure 5-2. Patient-derived fibroblasts mediate trafficking of exosomes.

(A, B) Exosome (NAT) uptake in PanCa cells (AsPC-1) determined by exosomal RNA and protein visualize by respectively RFP and GFP fluorescence in cells. (C) Delivery of NAT exosome represented by immunofluorescence assay. Exosomal protein marker MHC and CD81 expression showing exosomes in cells and DAPI represent nucleus of PanCa cells when NAT exosomes treated into the cells. (D) Dose dependent uptake of NAT exosomes in HPAF-II cells determined by immunofluorescence. (E, F) Lysotracker and Mitotracker indicated subcellular localization of exosomes (G.i) Drug loading capability of NAT and CAF derived exosomes in different concentration of exosome protein. (G.ii) Graphical representation of drug loading efficiency. (H) Size and Zeta potential of empty and ORM loaded NAT and CAF derived exosomes were determine using DLS.
Figure 5-3. NAT-derived exosomes efficiently deliver drugs to cancer cells with enhanced efficacy to inhibit tumorigenic characteristics.

(A) Pancreatic cancer cells were treated with exosome (NAT), ORM and Exo-ORM in different concentrations. Cell proliferation was significantly decreased in both the cells, as determined by MTT assay. (B) Real time cell proliferation, invasion and migration behavior was demonstrated by xCELLigence assay in both the cell line, showed similar pattern of MTT results. (C, D) Metastatic characteristics of cells (Invasion and Migration) were significantly suppressed with Exo-ORM formulation in AsPC-1 cells, also demonstrated by real time xCELLigence assay. (E) Investigate the effect of exosome, ORM and Exo-ORM formulation on the formation of spheroid and their growth. PanCa cells (HPAF-II and AsPC-1) were treated with Exosome, ORM, Exo-ORM in 96 well low attachment plate for 7 days and followed by another 10 days to form secondary sphere. Phase contrast microscopy images showing the size of the primary and secondary tumor spheroids.
The metastatic behavior of cells was determined by cellular invasion by matrigel invasion assay and migration using Boyden chamber assay. Exo-ORM formulation significantly decrease the invasiveness of PanCa cells compared to the control, exosome and free ORM (Figure 5-3C). The number of migratory cells were also inhibited the Exo-ORM formulation as compared to the controls. The results of the assays were further confirmed by the real time xCELLigence system, which measure the electrical impedance by monitoring the cell behavior such as cell number and morphology in a real time manner (Figure 5-3D). Significant reduction of invasive and migratory cells was identified with the treatment of Exo-ORM after 48 hrs.

Exosomes effectively deliver ORM and suppressed in vitro tumorsphere formation in pancreatic cancer cells

Our results demonstrated that ORM has significantly reduced primary and secondary tumor sphere size in both PanCa cells (HPAF-II & AsPC-1). At the same time when we used exosomes as a delivery vehicle for ORM, similar effect has been observed in PanCa cells (Figure 5-3E). This result indicated that Exo-ORM has a potential to diminish spheroid formation which mimic the in vivo tumor model with PanCa cells.

Exo-ORM formulation induces apoptosis in pancreatic cancer cells

We examined the effect of Exo-ORM on apoptotic index of PanCa cells (Figure 5-4A). Our data demonstrated that ORM and Exo-ORM effectively induced sub-G0 population and early apoptotic population in both the PanCa cells which suggest activation of apoptotic pathway (Figure 5-4Bi, ii). ORM and Exo-ORM treatment led to alter cell cycle phase distribution differently in HPAF-II and AsPC-1 cells. It has been analyzed by flowcytometry that, in HPAF-II cells G0-G1 phase has been arrested and S phase has significantly decreased, alternatively in AsPC-1 cells treatment arrested the S phase and inhibition of G1 phase has been observed (Figure 5-4C, D). We will further investigate the underlying molecular mechanism which alter the cell cycle phases. Real time PCR result has shown anti (Bcl-2) and pro-apoptotic (Bax); gene alteration in both the cell lines. Bax has increased significantly and Bcl-2 expression decreased with treatment of Exo-ORM in HPAF-II and AsPC-1 cells (Figure 5-4Ei, ii).

NAT-derived exosomes enhance the efficacy of ORM to inhibit patient-derived CAFs

We have observed that ORM and Exo-ORM treatment effectively suppressed stromal abundance in patient derived cancer associated fibroblast. Immunofluorescence staining data has revealed that well established stromal marker α-SMA, Desmin and Hyaluronic acid expression were significantly less in CAFs when treated with ORM and Exo-ORM (Figure 5-5).
Figure 5-4. Exo-ORM formulation induces apoptosis in pancreatic cancer cells.

(A, B.i,ii) Pancreatic cancer cells were treated with exosomes, ORM (20µM) and Exo-ORM (20µM) for 24 hrs. ORM and Exo-ORM significantly increase apoptotic population include early and total apoptotic bodies in both AsPC-1 and HPAF-II cells, analyzed by annexin V/7AAD. (C, D) ORM and Exo-ORM alter the cell cycle phases differently in HPAF and AsPC-1 cells, additionally significantly showed sub-G0 population. (E.i,ii) qRT-PCR results demonstrated the apoptotic and antiapoptotic gene Bax/Bcl2 fold expression in both AsPC-1 and HPAF-II cells. Bars represent mean ± SEM, n=3, *P<0.05.
Figure 5-5. NAT-derived exosomes play major role in enhancing the efficacy of ORM on cancer-associated fibroblast cells.

CAF cells were treated with NAT derived exosomes, ORM and Exo-ORM to observe the effect on stromal abundance of cancer microenvironment. Immunofluorescence assay showing stromal marker protein α-SMA, Desmin and Hyaluronic acid expression in CAFs, when treated with exosome, ORM and Exo-ORM and significantly modulate the CAFs characteristics.
Exo-ORM formulation inhibits EMT/SHH signaling in pancreatic cancer cells

Metastasis and chemoresistance are major factors for poor prognosis of pancreatic cancer. Epithelial-mesenchymal transition has shown significant contribution in PanCa metastasis and drug resistance (266). To target EMT pathway in PanCa cells ORM and Exo-ORM were treated and evaluated by immunoblotting (Figure 5-6A through D) and qRT-PCR (Figure 5-6E, F). ORM and Exo-ORM efficiently inhibited the expression of proteins and mRNA in both the PanCa cells. E-cadherin, N-cadherin, vimentin, MMP-2, C-Myc expressions were significantly modulated in protein and mRNA level.

The SHH signaling pathway is well established as a tumor development factor and desmoplastic condition in PDAC progression (267, 268). ORM and Exo-ORM treatment significantly target SHH signaling pathway key proteins and downregulate SHH, Gli-1 and transcription factor, NF-κB expression upon treatment with ORM and Exo-ORM. Additionally, suppressed the expression of anti-apoptotic protein Bcl-2 and increased the expression of Bax (Figure 6A, B, C, D) have been observed. Therefore, results suggested that Exo-ORM treatment effectively downregulate PDAC progression by targeting EMT and SHH pathway via modulation the key and downstream molecules of these pathways.

Ormeloxifene and Exo-ORM inhibit EGF-induced EMT pathway in pancreatic cancer cells

It is already been reported in several research articles, that EGF enhances EMT in PanCa cells (269-271). Preliminary results with 100 nM EGF treatment showed maximum enhanced expression of EMT marker (Vimentin and N cadherin) (Figure 5-7A). MTT assay result demonstrated that EGF treatment effectively enhanced cell proliferation (25%), further ORM (48.64%) and Exo-ORM (52.44%) treatment inhibited cell proliferation even in presence of EGF (Figure 5-7B). It was also been observed that metastatic features such as invasion and migration of PanCa cells also suppressed by ORM and Exo-ORM treatment in EGF induced cells (Figure 5-7C). Following assay results showed that ORM and Exo-ORM reduced EGF induced EMT in PanCa cells (AsPC-1) and regulates all metastasis related factors involved therein. Vimentin, N-cadherin, MMP-2, TWIST, Snail, Slug and Zeb1 expression has reduced after treatment with ORM and Exo-ORM in presence of EGF activation (Figure 5-7D). Although EGF alone has shown significant higher expression in all the metastatic factors. Data suggested that ORM alone and encapsulated with autologous exosomes significantly inhibits EGF induced metastasis in PanCa cells.
Figure 5-6. Exo-ORM formulation induces apoptosis and inhibits EMT/SHH signaling in pancreatic cancer cells.

(A&C) Western blotting analysis of EMT related markers, SHH signaling pathway proteins and apoptotic proteins expression upon treatment with Exosome, ORM and Exo-ORM on AsPC-1 and HPAF-II cells. (B&D) Relative quantification of blots was represented by bar graph. Bar represent mean ± SEM, n=2, *p < 0.05. (E&F) Relative fold change in mRNA of key factors involved in EMT pathway by qRT-PCR.
Figure 5-7. Ormeloxifene and Exo-ORM inhibit EGF-induced EMT pathway in pancreatic cancer cells.

(A) Optimization of EGF concentration by relative expression of N-cadherin and Vimentin in mRNA level. (B) MTT assay representing the effect of EGF and ORM, Exo-ORM (in presence of EGF) in proliferation of AsPC-1 cells. (C) Investigated the metastatic characteristics (Invasion and Migration) of AsPC-1 cells when treated with EGF, ORM-EGF and Exo-ORM-EGF as compared to control. (D) ORM and Exo-ORM significantly modulate the key factors of EMT pathway which responsible for metastasis in pancreatic cancer. Bars represent mean ± SEM, n=3, *P<0.05.
NAT-derived Exo-ORM inhibits tumor in xenograft mouse model

After assessment of efficient in-vitro therapeutic potential of NAT-derived exosomes as a delivery vehicle we evaluated anticancer effect in xenograft mice model. Twelve mice were used in this study and HPAF-II luciferase cells were subcutaneously injected in right flank of these mice. Mice were randomly divided in three groups after 7 days of tumor cells injection. Mice were treated intratumorally with control (PBS), exosomes and Exo-ORM to the mice. Our results suggested that Exo-ORM treated mice successfully demised tumor size as compared to control and exosome treated groups (Figure 5-8A). When mice were treated with Exo-ORM, there was noticeable reduction in tumor volume (Figure 5-8B) and tumor weight (Figure 5-8C). (p <0.05 Ex-vivo images of excised organ displayed organ metastasis in kidney, lungs, spleen in control and exosome treated groups whereas Exo-ORM has shown no metastasis in any of the organs of the mice (Figure 5-8D). NAT exosomes showed specific targeting to the tumor as compared to alone ICG when they injected via intraperitonially (Figure 5-8E). Life expectancy and mice survival has been significantly improved with Exo-ORM treatment (Figure 5-8F). Upon further investigation, we evaluated the EMT marker proteins expression in excised mice tumors of all treated groups. Isolated total protein from tumor tissues indicated that, Exo-ORM treated group showed significant higher expression of E-cadherin and reduced expression of N-cadherin, vimentin, snail expression compared to the control (PBS) treated group through western blotting (Figure 5-8G). At the same time there is slight modulation of protein expression in exosome treated group. Formalin fixed paraffin embedded (FFPE) excised tissue sections showed reduced expression of α-SMA and collagen expression through immunofluorescence (Figure 5-9A). Additionally, we have evaluated that Exo-ORM also target key EMT pathway proteins such as E-cadherin, Vimentin (Figure 5-9B). When NAT exosomes delivers ORM to the tumors immune histochemistry analysis showed, there was reduced expression N-cadherin and Hyaluronic acid, translocation of β-catenin from nucleus to cytoplasm and inhibition of KI67 staining as compared to control and alone exosome treated mice (Figure 5-9C).

Discussion and Conclusion

Pancreatic cancer is characterized by the accumulation of a fibro-inflammatory stroma leading to desmoplasia. We and others have reported the involvement of Sonic hedgehog (SHH) signaling pathway in desmoplasia (272). The stromal cells, which include myeloid, cancer associated fibroblast (CAF) and pancreatic stellate cells have been correlated with tumor promotion and unmasking of anti-tumor immunity (272, 273). Metastasis is another major factor for poor prognosis of pancreatic cancer. EMT has shown significant contribution in PanCa metastasis and to drug resistance. Reports suggested that altered TME, which is influenced by SHH, metastatic and several microRNA pathways, plays crucial roles in PanCa progression, metastasis, and drug resistance. However, strategies for the targeted regulation of TME are not well developed. This study developed a unique autologous exosomal formulation and investigation of its effects on aforementioned signaling pathways is highly innovative (Figure 5-10).
Figure 5-8. NAT exosomes can enhance therapeutic efficacy in xenograft mouse model.

(A) Six weeks-old nu/nu mice were injected with HPAF-II luciferase cells to establish tumor in the right flank. After 7 days mice were treated with PBS as control, NAT exosome and Exo-ORM twice a week for 5 weeks. At 40th day representative whole mice IVIS images and excised tumor images were taken. (B&C) Average tumor volume and tumor weight was represented respectively. Statistical significance was represented by asterisk *. P values as p<0.05 are considered significant. (D) Effect of exosomes and Exo-ORM as compared to control on metastasis was displayed by representative ex vivo images of excised organs from all three groups. (E) Delivery of ICG and Exo-ICG were identified by injecting them intraperitoneally to two group of mice. (F) Survival curve represent the effect of NAT exosome and Exo-ORM on mice survival. (G) Western blotting analysis indicating the effect of NAT exosome and Exo-ORM formulation on EMT marker proteins (E-cadherin, N-cadherin, Vimentin and Snail) of excised tumor lysates (pulled) from all three group of mice. Bars represent mean ± SEM, *P<0.05.
Figure 5-9. Exo-ORM inhibits EMT and desmoplasia in pancreatic xenograft mouse model.

(A) Immunofluorescence staining and confocal microscopy images displayed the effect of exosomes and Exo-ORM as compared to PBS treatment. Exo-ORM inhibits stromal marker protein expression such as α-SMA and collagen as compared to control and exosomes treated mice. Images were captured at 400X. DAPI was used as a counter stain for the nucleus. (B) Confocal microscopy images of control, exosome and Exo-ORM treated mice tissues were indicated the increased expression of E-cadherin and reduced expression of Vimentin when treated with Exo-ORM as compared to control and exosome. (C) Immunohistochemical staining showing the inhibited expression of N-cadherin, β-catenin, Hyaluronic acid and KI67 in tumor mice tissue with Exo-ORM treatment.
Figure 5-10. Schematic representation of NAT-derived exosomes for personalized medicine as a therapeutic intervention in pancreatic cancer treatment.
We have successfully isolated NAT and CAF fibroblast cells followed by purified respective exosomes from the conditioned media and characterized them to utilize as a therapeutic vehicle. Fibroblast cells were well characterized by cytofilament staining of \( \alpha \)-SMA/Hyaluronic acid/Desmin Preferential size and morphology for passive targeting, proficient biophysical characteristics, biocompatibility and non-immunogenic nature makes NAT derived exosomes more efficient than CAF derived exosomes. Efficient delivery of NAT exosomes was identified by their exosomal RNA and protein in PanCa cells. This therapeutic delivery vehicle appears to be a useful personalized approach for PanCa therapy due to its non-immunogenicity and biocompatibility for same patient because they carry the same cargo (proteins, lipids, nucleic acids) of the affected patient.

The main challenge is efficient loading of drug in the exosome without disrupting their physiochemical characteristics. This personalized therapeutic vehicle, NAT exosomes obtained higher drug loading ability in any concentration (100-400 \( \mu \)g) of exosomal protein compared to same concentration of CAF derived exosomes upon maintain proper biophysical appearance. ORM loaded nano sized exosomal vehicles efficiently deliver to PanCa cells and effectively suppressed tumorigenic features such as proliferation, invasion and migration in \textit{in vitro} PanCa model. Those physiological behavior also well confirmed by real time xCELLigence analysis. The enhanced efficacy of ORM has been determined when it delivered through NAT exosomes and efficiently target apoptotic pathway of PanCa cells (HPAF-II and AsPC-1). ORM loaded NAT derived exosomes significantly enhanced apoptotic population in HPAF-II and AsPC-1 which further established by apoptotic protein and RNA (Bax and Bcl-2) expression.

Additionally, it is highly impossible to treat metastasis of pancreatic cancer. Hence, this study was aimed at tackling metastasis behavior in addition to alter tumor microenvironment by using these exosomal formulations. They are expected to reach TME which is difficult for other formulations because of dense stroma in pancreatic cancer. Exo-ORM formulation efficaciously suppress EMT pathway which determined by marker protein and RNA expression (N-cadherin, E-cadherin, Vimentin). Previous report already been established that ORM has a proficient role in targeting SHH pathway in PanCa (6) which again proved that, the Exo-ORM formulation successfully target SHH pathway in PanCa cells. It has been reported that abundance of stroma is a major cause of drug resistance in several cancer type and new treatment modalities are focusing not only solid tumor but also stromal components (274, 275). ORM loaded exosome (Exo-ORM) formulation interestingly reduced stromal abundance of fibroblast cells, demonstrated by fibroblast marker proteins (\( \alpha \)-SMA, Hyaluronic acid, Desmin) expression. Furthermore, Exo-ORM inhibit the secondary tumor sphere formation and growth in PanCa cells. Consequences of these studies confirmed the therapeutic efficacy of Exo-ORM formulation which could target tumor stroma and desmoplastic condition in pancreatic cancer.

There are numerous factors responsible for EGF activation in PanCa which eventually leads to EMT progression (270, 271, 276). In this project we are delivering ORM using patient derived NAT exosomes to inhibit metastasis to hopefully develop a method for personalized medicine. Our prior publication demonstrated that ORM is a
potent stroma inhibitor for pancreatic cancer. In the current studies, we have observed that ORM showed anti-metastatic potential in PanCa in vitro and in vivo models. Exo-ORM formulation effectively suppressed EGF induced EMT in PanCa along with other metastatic key factors in cells.

To determine the in vivo antitumor efficacy of Exo-ORM, we utilized luciferase expressed xenograft mice model. Intratumorally injected Exo-ORM has shown significant reduction of tumor volume as compared to control and exosome treated groups. Ex vivo tumor weight was significantly lower in mice receiving Exo-ORM than other treatment groups. Ex vivo bioluminescence images of different organs demonstrated the inhibition of metastatic potential of Exo-ORM in in vivo system. ICG conjugated exosomes displayed efficient tumor targetability when it compared to ICG alone dye. This result confirms the natural phenomenon of NAT exosomes that they are major player of tumor microenvironment and carries biological cargo to target site (204). This data demonstrated that NAT derived exosomes is an efficient delivery platform for anticancer drug. Immunoblotting, IHC and immunofluorescence analysis of mice tumor protein showed ORM loaded exosomes successfully reduced EMT and stromal abundance in in vivo mice model and significant improvement has been observe in mice survival with this therapeutic modality. In current research project, we have successfully isolated fibroblast cells from patient tissues and extracted exosomes. Efficient loading of ORM in NAT derived exosomes demonstrated antitumor effect in vitro and in vivo, which could develop a novel personalized therapeutic implication for PanCa treatment.

Exosome based drug delivery platform may be a, promising therapeutic modality which is suitable for tumor targeted vehicle. Our observations offer importance of the utilization of NAT derived exosomes for personalized medicine as a potential therapeutic intervention in PanCa treatment.
The management of PanCa is exceptionally difficult due to poor response to available therapeutic regimens, late diagnosis, chemoresistance, metastasis and tremendous genomic alteration. Identification of novel therapeutic approaches with better response and the potential for chemo sensitization is the utmost need to control this complex disease. miRNAs have been identified as attractive targets for therapeutic intervention. The functional significance of lost miRNAs has been reported in several human malignancies, including PanCa. Therefore, restoring lost miRNA function can provide a potential therapeutic benefit. Prior work has identified miR-145 as a tumor suppressor miRNA in PanCa. The restoration of miR-145 downregulates several oncogenes including mucin, MUC13, a glycoprotein that is aberrantly expressed in PanCa, and efficiently inhibits tumor growth in mice.

The specific routing of therapeutic miRNA in active form to the target organs and entry into the cancer cells remains a challenge. In the first chapter, the focus of this study was to develop and assess the efficacy of a miR-145 based nanoparticle formulation for PanCa treatment. We have engineered a unique, magnetic nanoparticle-based formulation for safe and effective delivery of tumor suppressor miR-145 to PanCa cells. Our results have demonstrated the successful delivery of miR-145 in PanCa cells by the efficient cellular uptake of miR-145 evidenced by qPCR and strong anticancer efficacy of miR-145-MNP in PanCa cell line model. This newly developed magnetic nanoformulation was surface modified with F127 and PEI, which made the particles nontoxic and minimized particle aggregation. The MNPF was found to be nontoxic and hemocompatible as it did not show any toxicity in RBCs. These characteristics of the formulation improved the transfection efficiency and therapeutic potential. miR-145 loaded MNP formulation effectively restored miR-145 expression in PanCa cells and diminished the tumorigenic behavior. miR-145-MNP successfully suppressed the expression of oncogenic MUC13 and the expression of other key downstream proteins, which are responsible for tumorigenicity in PanCa.

Recently TRAIL therapy has exhibited promising potential in cancer therapeutics, due to its selective apoptosis inducing capability in tumor cells. TRAIL can bind to death receptors (DR4 and DR5) and eventually activated the DISC complex followed by triggering the apoptotic cascade. Commercially available TRAIL receptor agonists didn’t show convincing anticancer ability in clinical trials in PanCa. In chapter 3 we evaluated one of the molecular bases of inherent or acquired TRAIL resistance in PanCa cells. MUC13 protein has been extensively studied in our lab, and we have identified its predominant role in PanCa development and progression. Role of mucins in drug resistance is reported for other cancers. In this chapter we have investigated that MUC13 expression leads to cell survival and downregulation of MUC13 helps the cells die in PanCa models. Alpha and Beta domains of MUC13 were responsible for blocking caspase 8 activation and cleavage of PARP-1 which encouraged to resist TRAIL induced apoptosis in MUC13 expressing PanCa cells. These results deciphered mechanistic
insight of MUC13 and its involvement in drug resistance that promoted the oncogenicity and metastasis of pancreatic cancer.

Our data has elucidated that, the aberrant expression of MUC13 plays a crucial role in TRAIL resistance in PanCa. We have previously identified that miR-145 is a well-established tumor suppressor in PanCa, which can directly target MUC13 and inhibit oncogenic activity in tumor cells. We have used miR-145, as a therapeutic modality to regulate MUC13 expression in cells and sensitize TRAIL activity. Our results demonstrated that miR-145 restoration strategically improves TRAIL therapy by suppressing tumorigenic activities and enhancing the apoptotic population in PanCa cells. Systemic delivery using recombinant TRAIL is challenging. In chapter 4 we have delivered plasmid TRAIL as a therapeutic molecule. Herein, we have used MNP formulation to co-deliver miR-145 and plasmid TRAIL to overcome TRAIL resistance in PanCa models. Our results demonstrated that, the formulation significantly restores miR-145 into the cells and enhances TRAIL sensitivity in PanCa cells. MNP-miR-145-TRAIL formulation significantly enhanced DR4 receptor expression, activates the death domain and downregulates cFLIP expression. This molecular alteration leads to TRAIL induced apoptosis by activating caspase 8 and cleavage of PARP-1. This unique co-delivered formulation successfully triggered TRAIL sensitivity in in vivo mouse model. MNP-miR-145-TRAIL formulation actively delivered miR-145 and TRAIL to the tumor and effective restoration of miR-145 strategically forces the tumor cells into undergoing TRAIL induced apoptosis. Moreover, we have provided an innovative and promising TRAIL based therapy for PanCa model.

Pancreatic tumor microenvironment is a complex organization and difficult to manage. TME consists of fibroblast cells, stromal cells, stellate cells etc. and they are responsible for desmoplastic condition in PDAC. Several studies have mentioned the role of SHH pathways, activation of NF-κB and EMT pathways in PanCa progression, metastasis and drug resistance. To ameliorate this dreadful oncogenicity, it is very important to identify patient specific therapeutic approach by considering responses against particular therapy. In our 5th chapter we demonstrated the personalized therapeutic approach to target pancreatic cancer. To ameliorate this oncogenic we demonstrated a personalized therapeutic approach, considering a specific patient’s responses towards a particular therapeutic approach. Autologous exosomes are extra cellular vesicles and have been selected as a personalized therapeutic vehicle to deliver the anticancer drug, ormeloxifene. ORM is a potent anticancer drug which targets TME and it could significantly inhibit desmplasia in PanCa. Patient derived tumor adjacent normal (NAT) exosomes were loaded with ORM and significantly suppressed tumorigenic behavior in PanCa cells and stromal abundance in CAFs. They have significantly repressed SHH and EMT pathways in in vitro and in vivo models in PanCa.

In the future, we will elucidate the combinatorial effects of miR-145 and ORM by co-delivering them to PanCa mice models. We will utilize the patient derived exosomes as a personalized delivery vehicle to deliver the therapeutic molecules and will investigate the effect on tumor microenvironment. We expect that, MUC13 has a role in drug resistance which can be controlled by miR-145 restitution and ORM can target the
desmoplasia and metastasis in PDAC. We will also take the advantage of aberrant expression of MUC13 in PanCa cells and will develop a targeted therapeutic approach by conjugating anti-MUC13 monoclonal antibody to the nanoformulation. This new therapeutic intervention will be a persuasive approach for pancreatic cancer.
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