Pancreatic Ductal-derived Mesenchymal Stem Cells: their distribution, characterization and cytotoxic effect on pancreatic cancer cells

by

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Abstract

Mesenchymal stem cells (MSCs) have attracted significant attention in cancer research as a result of their accessibility, tumor-oriented homing capacity, and the feasibility of autotransplantation. This study detected the sensitivity of pancreatic cancer cell lines (PCCs) to pancreatic-derived, engineered MSCs under different culture conditions. Pancreatic ductal tissue was extracted from adult human pancreas. MSCs were derived and expanded ex-vivo and verified to fulfil criteria for human MSCs according to the guidelines of the International Society for Cellular Therapy. MSCs were analyzed for distribution and migratory capacity to the site of pancreas and PCCs in in vivo and in vitro models, and found to have homing capacity to the pancreas and towards PCCs (MSCs were attracted to all PCCs compared to normal human A1F8 cells and they displayed significant attraction to the media obtained from cancer cells compared to normal media (p<0.05)). PCCs (BXPC3, ASPC1, Panc-1, TRM6 and HP62) were analyzed by FACS for TNF-α Related Apoptosis Inducing Ligand (TRAIL) receptors. MSCs engineered with non-secreting TRAIL (MSC\textsuperscript{nsTRAIL}) and secreting TRAIL (MSC\textsuperscript{stTRAIL}) and PTEN (MSC\textsuperscript{PTEN}) were used for both direct and indirect co-cultures. TRAIL/PTEN expression was assessed by both ELISA and western blot analysis; higher molecular weight was observed in the MSC\textsuperscript{nsTRAIL} (56kDa) compared with MSC\textsuperscript{stTRAIL} (26kDa). The TRAIL content of supernanatats from MSC\textsuperscript{stTRAIL} was significantly higher than MSC\textsuperscript{nsTRAIL} (p<0.05). PTEN-RFP fusion protein showed a higher molecular weight of 74 kDa in comparison with endogenous PTEN (47 kDa). A real time detection of MSCs cytotoxicity on PCCs displayed proportional cancer cell death to the ratio of conditioned media used from MSC\textsuperscript{nsTRAIL}, MSC\textsuperscript{stTRAIL}, and MSC\textsuperscript{PTEN}. Naive MSCs exhibit intrinsic cytotoxic effect on pancreatic cancer cells and this effect was potentiated by TRAIL/PTEN-engineering. This study provides a practical platform for the development of
MSC-based therapy for pancreatic cancer.
Preface

I have contributed to this research as an MSc student and all of the following work has been accomplished by me with the help of Dr. Dai and our lab volunteers/work study students more specifically Mr. Jarrett Rayat, Ms. Ada Young, Ms. Kelsey Reinheimer, and Mr. Adam Mah.

1. **TRAIL-engineered pancreas-derived mesenchymal stem cells: characterization and cytotoxic effects on pancreatic cancer cells**

2. **Potential implications of mesenchymal stem cells in cancer therapy.**

3. **MSC(TRAIL)-mediated HepG2 cell death in direct and indirect co-cultures.**

The introduction and concluding chapters consist primarily of a combination of all published/in press material. The section 1.3 of the introduction is mainly from the review (#2) which I participated as a second author with some modifications and reprinted with permission of Cancer Letters Journal. The works in Chapter 2 and parts of Chapter 3 have not been published and requires further experimentation for completion. Chapter 3 consists of the paper identified in (#1) which experiments were completed by myself with the help in writing of the introduction and parts of conclusion from our collaborator and Dr. Dai, was used with some modifications and is reprinted with permission from Cancer Gene Therapy journal. (#3) on the list is a collaborative work in which, sections of the methodology such as initial screening, cell count, and preparation of MSCs were completed by me and Dr. Dai, I also assisted in proof-reading the manuscript and providing comments to it. Any references to these publications are cited accordingly.
Table of Contents

Abstract........................................................................................................... ii
Preface ........................................................................................................... iv
Table of Content .......................................................................................... v
List of Tables ................................................................................................ vi
List of Figures ............................................................................................... vii
List of Acronyms ......................................................................................... x
Acknowledgments ......................................................................................... x
Dedication ....................................................................................................... xv

Chapter 1: The Challenge of Pancreatic Cancer & Novel Treatment Strategies 1
  Section 1.1: Pancreatic Cancer & Treatment shortfall ........................................ 1
  Section 1.2: Molecular Pathways and Pancreatic Cancer .................................... 4
  Section 1.3: Mesenchymal Stem Cells ............................................................... 13
  Section 1.4: Summary and Specific Aims .......................................................... 29

Chapter 2: Pancreatic Ductal MSCs: isolation, propagation, and characterization 30
  Section 2.1: Introduction .................................................................................. 30
  Section 2.2: Materials and Methods ................................................................. 32
  Section 2.3: Results ....................................................................................... 42
  Section 2.4: Discussion ................................................................................... 50

Chapter 3: TRAIL/PTEN Engineered MSCs & Their Cytotoxic Effect on Pancreatic Cancer Cells 54
  Section 3.1: Introduction ................................................................................ 54
  Section 3.2: Materials and Methods ................................................................ 55
  Section 3.3: Results ....................................................................................... 63
  Section 3.4: Discussion ................................................................................... 88
  Section 3.5: Summary & Conclusion ................................................................. 94

Future Directions .............................................................................................. 95
References ....................................................................................................... 97
List of Tables

Table 1.1 Genetic profiling of pancreatic carcinoma ........................................... 4

Table 1.2 Basic criteria for defining human MSCs ................................................. 16

Table 1.3 MSC-derived factors and immunosuppression .................................... 21
**List of Figures**

Figure 1.1 Molecular pathways involved in pathogenesis and progression of pancreatic cancer .................................................. 6

Figure 1.2 Summarizes pros and cons of MSCs within tumor environment .............. 26

Figure 1.3 Putative personalized treatment of cancer with engineered MSCs ............ 28

Figure 2.1 Experimental design: Pancreatic ductal MSC isolation, propagation, and characterization .............................................. 31

Figure 2.2 MSCs distribution post-injection via tail vein in NSG mice model .............. 32

Figure 2.3 Real time cell analyzer system: various plates and impedance technology ...... 38

Figure 2.4 Isolation and propagation of MSCs ........................................... 42

Figure 2.5 Surface biomarker determinations .............................................. 43

Figure 2.6 Differentiation capacity of MSC to adipogenic and osteogenic tissue ....... 44

Figure 2.7 Cell index (CI) readings of titration and adherence properties of MSCs .... 45

Figure 2.8 Serum induced migration of MSCs ........................................... 46

Figure 2.9 MSC migration towards pancreas, liver, lung, and kidney tissue ........... 47

Figure 2.10 Dynamic distributions of intravenously transplanted CFSE-MSCs in peripheral blood if NSG mice ...................................... 48

Figure 2.11 Dynamic distributions of intravenously transplanted MSCs in different tissue using the genomic DNA .................. 49

Figure 3.1 Experimental design: TRAIL/PTEN-engineered MSCs & their pro-apoptotic properties on pancreatic cancer cells ................................. 55

Figure 3.2 The structure of PTEN-RFP expression vector ................................ 59

Figure 3.3 FACS analysis of the TRAIL receptors across all cancer/non-cancerous/stem cells .................................................. 63

Figure 3.4 FACS analysis of the TRAIL receptors on the pancreatic cancer cells ....... 65
Figure 3.5 ELISA & immunoblotting analysis of TRAIL and PTEN engineered MSC culture media

Figure 3.6 Demonstration of PTEN-RFP trafficking between MSC and Panc-1 cells

Figure 3.7 Direct co-culture of MSC, MSC{sub}TRAIL, and MSC{sup}TRAIL at varying concentrations

Figure 3.8 Cell viability assessment on HP62 cells in indirect co-culture

Figure 3.9 Live/Dead Assay: indirect co-culture of MSC{sup}PTEN conditioned media with HP62 cells

Figure 3.10 Live and Dead Assay: indirect co-culture of MSC{sup}PTEN conditioned media with ASPC1 cells

Figure 3.11 Live and Dead Assay: indirect co-culture of MSC{sup}PTEN conditioned media with HP62 cells

Figure 3.12 Live and Dead Assay Analysis: cell count, percent live and dead cells

Figure 3.13 Titration of the cancer cells using the RTCA system

Figure 3.14 A close up look at the 20,000 cells/ well titration of the cancer cells

Figure 3.15 Migration kinetics of MSCs to cancer cells

Figure 3.16 The chemo attractant effects of cancer cells conditioning media (CM) on MSC migration

Figure 3.17 Real-time monitoring of conditioned media-induced cytotoxicity in pancreatic cancer cells

Figure 3.18 Area under the curve analysis of RTCA cytotoxicity data

Figure 3.19 Preliminary dynamic real time indirect co-culture of MSC{sup}PTEN conditioning media with Cancer cells

Figure 3.20 Area under the curve analysis of RTCA cytotoxicity data for MSC{sup}PTEN

Figure 3.21 Dynamic real time cytotoxic assay: direct co-culture of naive MSC with Cancer cells
Figure 3.22 Area under the curve analysis of direct naive MSCs culture with cancer cell lines ____________________________83

Figure 3.23 Preliminary dynamic real time cytotoxic assay: direct co-culture of MSC^{PTEN} with cancer cells______________________________84

Figure 3.24 Area under the curve analysis of MSC^{PTEN} direct culture with cancer cell lines ____________________________85

Figure 3.25 PE-PTEN intracellular staining using FACS ____________________________86
### List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>RAC-beta serine/threonine-protein kinase</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein kinase b</td>
</tr>
<tr>
<td>Ang1</td>
<td>Angiopoietins -1</td>
</tr>
<tr>
<td>APAF1</td>
<td>Apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>AR</td>
<td>Alizarian red</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BAX</td>
<td>Bcl-2-associated X protein</td>
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<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
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<tr>
<td>BID</td>
<td>BH3 interacting-domain death agonist</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>C.N</td>
<td>Catalogue number</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine-dependent <strong>aspartate-directed proteases</strong></td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine receptor type 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony-forming unit fibroblast</td>
</tr>
<tr>
<td>CI</td>
<td>Cell index</td>
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<tr>
<td>CIM-plates</td>
<td>Cell Invasion, Migration plates</td>
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<tr>
<td>c-Kit</td>
<td>C-kit protein</td>
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<td>CM</td>
<td>Conditioning media</td>
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<td>c-Met</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DCR</td>
<td>Decoy receptor</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ddH2O</td>
<td>Double-distilled water</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pi</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPC4</td>
<td>Decapentaplegic homolog 4</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptors</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EMT</td>
<td>Epithelial–mesenchymal transition</td>
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<td>E-Plate 16</td>
<td>Electrode- covered plates</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factors</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen.</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
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<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL2</td>
<td>Interleukin 2</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric-oxide synthase</td>
</tr>
<tr>
<td>ISCT</td>
<td>International Society for Cellular Therapy</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid enhancer-binding factor</td>
</tr>
<tr>
<td>LSM1</td>
<td>Sm-like protein</td>
</tr>
<tr>
<td>MADM</td>
<td>Msc adipogenic differentiation medium</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAPK/Erk kinase kinase</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium media</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>M KK</td>
<td>MAP kinase kinase</td>
</tr>
<tr>
<td>MMAC</td>
<td>Mutated in multiple advanced cancers</td>
</tr>
<tr>
<td>MMP</td>
<td>Metrix metalloproteinase</td>
</tr>
<tr>
<td>MODM</td>
<td>Msc osteogenic differentiation medium</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSCs</td>
<td>Human pancreatic mesenchymal stem cells</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NK Cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>Notch</td>
<td>Neurogenic locus notch homolog protein</td>
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<tr>
<td>NSG</td>
<td>Nod scid gamma</td>
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<tr>
<td>nsTRAIL</td>
<td>Non-secreting TRAIL</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil red o</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PCCs</td>
<td>Pancreatic cancer cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase-1</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-triphosphate</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-l-lysine</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycosylation</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>RM</td>
<td>Regular media</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTCA-DP</td>
<td>Real-time cell analyzer dual plate</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell facto</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
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<tr>
<td>SFM</td>
<td>Serum free media</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>sHLA-G5</td>
<td>Soluble HLA-G5</td>
</tr>
<tr>
<td>SMAD4</td>
<td>Mothers against decapentaplegic homolog 4</td>
</tr>
<tr>
<td>stTRAIL</td>
<td>Secreting TRAIL</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TAT</td>
<td>Trans-activator of transcription</td>
</tr>
<tr>
<td>TCF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>T\text{eff}</td>
<td>Effector T lymphocytes</td>
</tr>
<tr>
<td>TGF-\text{β}</td>
<td>Transforming growth factor-\text{β}</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>Tissue inhibitor of metalloproteinases 3</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-\text{α} Related Apoptosis Inducing Ligand</td>
</tr>
<tr>
<td>T\text{regs}</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless type</td>
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</table>
Acknowledgements

This work was supported by the grants from: Juvenile Diabetes Research Foundation, the VGH & UBC Hospital Foundation and the Guangxi Ministry of Science and Technology. I am thankful for both the Canadian Institutes of Health Research (CIHR) Master’s Award: Banting and Best Canada Graduate Scholarship and CIHR Transplantation Training Program Award that have supported my work as a graduate student. Furthermore, I would like to extend my appreciation to the Experimental Medicine program at UBC who have been a great source of support. I owe particular thanks to Dr. Garth Warnock, who continually supported me and provided me with many opportunities to expand my horizons which enabled me to learn many valuable skills. I am indebted to Dr. Long-Jun Dai for enlarging my vision of science and providing coherent answers to my endless questions. A special thanks to my supervisory committee members Drs. Bruce Verchere, Francis Lynn, and Alice Mui whose penetrating questions taught me to question more deeply. Also a special thanks to Dr. Marzban who supported me from the first day I started this journey and provided me with the foundation to learn more and excel in my work. I offer my enduring gratitude to the faculty and staff at UBC’s Department of Surgery and VCHRI. I appreciate the generosity of Vancouver Prostate Center for providing us access to the RTCA system, more specifically Dr. Eric LeBlanc and Ms. Kate Frewin. I am grateful to Mr. Derek Dai (animal unit), Dr. Lisa Xu (FACS machine) and Dr. Ingrid Barta (histology) for their generosity in sharing their technical expertise with me, which greatly assisted in this research. I also would like to extend my gratitude to The Immunity and Infection Research Centre which has been a tremendous resource throughout the course of my work, more specifically Mr. Jeffrey Helm. I am obliged for the assistance of my volunteers and work-study students Mr. Jarrett Rayat, Mr. Hong Lu, Ms. Ada Young, and Ms. Kelsey Reinheimer. I am grateful for Mrs. Crystal Robertson’s assistance in administrative chores.
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I would like to dedicate this work to my late uncle and aunt who passed away from cancer and my father who has now survived his cancer diagnosis.
Chapter 1: The Challenge of Pancreatic Cancer & Novel Treatment Strategies

1.1 Pancreatic Cancer & Treatment shortfall

According to 2012 cancer statistics, in the United States pancreatic cancer is ranked 10th in terms of new cancer cases and 4th in cancer deaths, with an overall 5-year survival rate of 4% after diagnosis. This poor prognosis can largely be attributed to the tendency of this cancer to rapidly spread to the lymphatic system and distant organs. Similarly, Canadian cancer statistics (2012) report pancreatic cancer affecting approximately 4600 Canadians each year and with dismal cure rates at about 2-3%. The number of newly diagnosed patients nearly equals that of the deceased; no current treatment, whether surgical, chemotherapy or a combination thereof, results in an effective clinical response in more than 20% of patients and no treatment therapies have been found that extend survival beyond a few months. Clinically, pancreatic cancer presents in up to 50% at a locally advanced stage and, in up to 40% with metastatic disease, dramatically reducing the chance of curative resection. Factors such as age, smoking, past medical history, and other conditions such as diabetes and pancreatitis have been associated risk factors of pancreatic cancer.

With the pancreas being both an exocrine and an endocrine organ it has two distinct functions in the body and thus has different functioning compartmental units. The exocrine component consists of acinar cells, secretory units, and a network of large ducts that drain secretions into the small intestine. The acinar cells form the majority of the exocrine tissue and...
generate digestive enzymes. Ductal cells secrete bicarbonate and mucous. The endocrine pancreas consists of clusters of cells commonly known as islets; they are neighboring to the exocrine pancreas and are categorized by their secretory function: β-cells producing insulin, α-cells producing glucagon, δ-cells producing somatostatin, PP cells producing pancreatic polypeptide, and finally ε-cells producing ghrelin. Both exocrine and endocrine tumors can develop from different pancreatic cell phenotypes. Endocrine tumors originate from islet cells and account for 2-4% of newly recognized neoplasms whereas exocrine tumors originating from the ductal tissue are by far the most common form of malignant pancreatic cancer accounting for 95% of newly identified cases. Despite the fact that surgical options for pancreatic cancer are now associated with acceptable outcomes, they often prove ineffective in controlling the disease with reported recurrence rates approaching almost 80% (both locally and distant) and a 5-year survival rate of only 10% to 24% for cases involving total resection. Lack of distant metastasis and local vascular invasion are the two criteria that must be present to qualify for a curative resection. The most common type of a such resection is known as the Whipple procedure in which the tumor-bearing region of the pancreas along with a portion of stomach, duodenum, gallbladder and part of bile duct are removed and the remaining regions are reattached to support digestive capabilities of the patient. In the case of advanced disease, palliative surgery can be performed to lessen symptoms and pain.

Other management options range from systemic chemotherapy alone to combined forms of treatment with chemo-radiation and chemotherapy. Chemotherapy treatments can be categorized as adjuvant (treatment after surgery), neo-adjuvant (treatment prior to surgery), and palliative. The most common chemotherapy drugs used to treat pancreatic cancer are: gemcitabine (Gemzar®), 5-fluouracil (5FU), capecitabine (Xeloda®), cisplatin, and oxaliplatin (Eloxatin®). These drugs function on the basis of cross-linking mechanisms in which their
reactive region interacts with the cell’s DNA or RNA nucleotides, thus disrupting the cell cycle progression leading to cancer cell apoptosis \(^{18,19,20}\). Additionally, due to the inability of chemotherapy drugs in distinguishing between normal and cancerous cells, the side effects of these drugs are also detrimental to the normal rapidly dividing cells of the body such as: the cells of the hair follicle and the lining of gut.

These drugs are usually administrated individually but can also be used in combination depending on the type and extent of the cancer. However, the survival benefit is limited and many patients with advanced disease are deemed for palliative care. Moreover, these drugs have adverse side effects that cause the patients considerable discomfort while reducing quality of life. Chemoradiotherapy is an efficient therapeutic approach for pancreatic cancer patients; however, there are no data to support a radiotherapy alone treatment option \(^4\). Finally, given the lack of a unifying chemotherapeutic approach and the numerous debilitating side effects of these drugs/treatments, significant attention has been directed to the tumor microenvironment to identify potential yielding therapeutic targets.

Due to these poor treatment options for pancreatic cancer patients, new therapeutic strategies are being developed using anti-cancer gene therapy agents and treatments as an alternative solution or in combination to the existing treatments. These strategies can be categorized as either using RNA interference or antisense oligonucleotides which inhibits the activated oncogenes (KRAS, LSM1, Akt, Wnt, etc.) or approaches to restore function of tumor suppressor genes (p53, p16/CDKN2A, DPC4/SMAD4, etc.)\(^1,4\). Table 1.1 summarizes the frequency of abnormalities of most common oncogenes and tumor suppressor genes in pancreatic cancer.
Two recent very encouraging reports have demonstrated the use of oncolytic poxvirus and lentiviral vectors to successfully treat cancer patients\textsuperscript{21,22}. Monahan \textit{et. al} (2000) describes some of the limitations of using a virus as a vector in a clinical setting. These limitations are summarized as: 1. packaging space limitations; 2. increased risk of insertional mutagenesis; 3. the body’s immune system rejection, and 4. limitations of producing high titer virus for clinical use\textsuperscript{23}. Below I will briefly discuss some molecular mechanisms involved in pancreatic cancer. Our proposed strategy uses mesenchymal stem cells (MSCs) as vehicles, and regular plasmids as vectors, thereby avoiding virus-related concerns. In the current study we focus on combining gene therapy to engineer MSCs with anticancer genes in order to target pancreatic cancer cell lines. Engineered MSCs could serve as an ideal delivery vehicle for anticancer compounds, by homing to cancer cells on the microscopic level.

### 1.2 Molecular Pathways and Pancreatic Cancer

Understanding the molecular mechanisms involved in pancreatic cancer will enable researchers to integrate new solutions for the treatment of this devastating disease. Hanahan \textit{et. al} (2000 & 2011) describes the progression of cancer from neoplasia to metastasis in 6 major steps\textsuperscript{24,25}. These steps include: autonomous production of growth factors, lack of response to anti-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oncogene</th>
<th>KRAS</th>
<th>LSM1</th>
<th>AKT</th>
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<td>Tumor suppressor genes</td>
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<td>P53</td>
<td>50-75</td>
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<tr>
<td></td>
<td>DPC4/SMAD4</td>
<td>55</td>
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**Table 1.1 Genetic Profiling of Pancreatic Carcinoma.** Modified from Bhattacharyya (2006)\textsuperscript{1} acronyms are summarized on pg 108.
growth signals, escaping apoptosis, boundless replicative capability, continuous angiogenesis, and finally tissue invasion/metastasis. There is no particular order for such events to occur and depending on type of cancer these events could happen individually, or in combination. It is now widely accepted that amongst causes for the progression of this disease, the activation of oncogenes and the inhibition of tumor suppressor genes are the key pivotal elements. Thus, much effort has been dedicated to providing greater understanding about these molecular mechanisms and their role in the progression of the disease. Implications of some of the molecular pathways and anti-cancer genes that are involved in pancreatic cancer for the current study are discussed in the sections that follow. These pathways were chosen in accordance with the current knowledge on mesenchymal stem cell’s anti-tumorigenic properties and the anticancer genes used in the current study. Pathways of interest for this study are depicted in Figure 1.1 and explained in depth in the sections that follow.
Figure 1.1 Molecular pathways involved in pathogenesis and progression of pancreatic cancer. From left to right: with the binding of Wnt to Frizzled receptors, dishevelled cascade is activated that in turn activates gene expression that leads to cell proliferation. With the binding of growth factors to the receptor tyrosine kinase, RAS pathway is activated that leads to a series of other molecules activation that can regulate gene expression in the nucleus and further lead to cell proliferation and growth. Similarly, growth factors and survival factors binding to the corresponding receptor can lead to modification of PIP2 and activation of PIP3. This pathway leads to cell proliferation via negative feedback to the mitochondria and activation of gene regulation in nucleus. PTEN can actively inhibit this pathway. Lastly, with the binding of TRAIL to its death or decoy receptors cells can regulate apoptosis via the activation of BAX and BAD mitochondria proteins. TRAIL (TNF-related apoptosis-inducing ligand), FADD (Fas-Associated protein with Death Domain), DR (Death Receptor), DCR (Decoy Receptor), Caspase (cysteine-dependent aspartate-directed proteases), BID (BH3 interacting-domain death agonist), BCL2 (B-cell lymphoma 2), BAX (Bcl-2-associated X protein) DIABLO (direct IAP binding protein with low pI), APAF1 (Apoptotic protease activating factor 1), PIP2 (Phosphatidylinositol 4,5-bisphosphate), PI3K (Phosphatidylinositol 3-kinases), PIP3 (Phosphatidylinositol (3,4,5)-triphosphate), PTEN (Phosphatase and tensin homolog), PDK1 (Phosphoinositide-dependent kinase-1), ILK (Integrin-linked kinase), AKT/PKB (Protein Kinase B), mTOR (mammalian target of rapamycin), RTK (Receptor Tyrosine Kinase), RAS (Rat sarcoma), RAF (Rapidly Accelerated Fibrosarcoma), MEK (MAPK/Erk kinase), MAPK (Mitogen-activated protein kinase), MKK (MAP kinase kinase), MEKK (MAPK/Erk kinase kinase), Wnt (wingless type), and TCF (T-cell factors).

1.2.1 KRAS oncogene and pancreatic cancer

Point mutation and gene amplification are two mechanisms by which oncogenes can become activated. Almoguera et al. (1998) reported that more than 90% of human pancreatic adenocarcinomas possess an activation of Kirsten rat sarcoma (KRAS) viral oncogene homolog. This is the highest rate of KRAS point mutation observed in any known cancer; however, KRAS mutations are also seen across other models of thyroid (55%), colorectal (35%), and lung (35%) carcinomas. KRAS, a 21kDa protein of Ras family, is known to be associated with cell proliferation and growth. This guanine nucleotide binding protein has the ability to automatically inactivate signal transducers. The wild type form of this protein has the ability to
switch between the inactive (GDP-bound) and the active (GTP-bound) form of this protein via cell surface receptor initiations\textsuperscript{30}. However, in the mutated form of KRAS, the oncogenes contain a point mutation substitution of codon 12, 13, or 61\textsuperscript{4,29}. This mutation leads to the inability of the protein to use its intrinsic down-regulation capacity and thus it remains in the active form continuously leading to uncontrolled cell growth. GTP-bound KRAS activates mitogen-activated protein kinase (MAPK) pathway and stimulating gene regulation and cell proliferation. A simplified depiction of this pathway can be observed in Figure 1.1. The wide scale expression of the KRAS gene in pancreatic carcinoma samples and its role in the cell survival/ proliferation mechanisms makes it a very significant target for the treatment of pancreatic cancer.

\textit{1.2.2 PI3K/AKT/mTOR Pathway and Pancreatic Cancer}

Activation of the phosphatidylinositol 3′-Kinase (PI3K-Akt) pathway has been observed in many malignancies. Furthermore, the Akt2 gene has been observed to be amplified in a number of studies including different pancreatic cancer specimens and cell lines\textsuperscript{31,32}. Akt regulates apoptosis and cell proliferation through multiple mechanisms\textsuperscript{33-35}. One of the key players in Ras protein cascade is class 1 PI3K\textsuperscript{36}. PI3K can phosphorylate negatively charged phospholipids on cell membranes (phosphatidylinositol) that signal the recruitment of pleckstrin homology (PH) domain-containing proteins to the cell membrane\textsuperscript{33}. This recruitment signals the attachment of phosphoinositide-dependent kinase-1 (PDK1) and its target serine/threonine-specific protein kinase (Akt) to the PH domains, which results in the activation of Akt that is known to be associated with cellular survival pathway\textsuperscript{33}. On the other hand, the Akt pathway is down regulated by MMAC/PTEN (mutated in multiple advanced cancers/phosphatase on chromosome ten phosphatase) that counteracts to PI3K mechanism by selectively dephosphorylating the negatively-charged phospholipid on the cell membrane\textsuperscript{37}. Inactivation or
deletion of the MMAC/PTEN gene has been observed in many tumor models including pancreatic carcinomas\textsuperscript{38}. It is important to note that many Ras and Akt activators are receptor tyrosine kinases that are also overexpressed in human pancreatic cancer models. These include (but are not limited to) epidermal growth factor receptors, insulin-like growth factor 1 receptors, platelet-derived growth factor receptors, and fibroblast growth factor receptors\textsuperscript{37,39-43}. Additionally, one of Akt’s main targets is the mammalian target of rapamycin (mTOR), which regulates G1 to S phase in cell cycle progression upon the availability of nutrients\textsuperscript{44,45}. Phosphorylation of the tuberous sclerosis complex (TSC) via Akt, activates mTOR\textsuperscript{46}. Therefore, by activation of PI3K/Akt pathway and availability of nutrients, mTOR becomes activated and could stimulate the translation of proteins needed in the cell cycle progression. Finally, these observations support the proposed importance of the PI3k/Akt/mTOR pathway as a junction point in survival/death cascades of pancreatic cancer model and their interactions with MSCs (which are known to down-regulate the Akt pathway, discussed in section 1.3) which makes understanding the mechanisms involved in this pathway a critical one.

\textit{1.2.3 Wnt/β-catenin Pathway and Pancreatic Cancer}

Wnt (Wingless and INT-1 (integration 1)) pathway is known to be associated with the proliferation, morphogenesis and differentiation of several organs\textsuperscript{47}. Furthermore, Wnt is known to be associated with pancreas development\textsuperscript{48,49}. The role of β-catenin has been reviewed by MacDonald \textit{et.al} (2009)\textsuperscript{50}. There are nineteen different Wnt ligands identified in mammals that are associated with the activation of canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) pathways\textsuperscript{51}. Normally β-catenin will become phosphorylated which targets it for degradation. With the binding of Wnt ligand to its receptors of the frizzled\textsuperscript{52} family of proteins, it enables the activation of an intracellular signaling pathway which transports the β-catenin to the nucleus\textsuperscript{51}. In the nucleus, β-catenin interacts with transcriptional factors such as T-
cell factors (TCFs) and lymphoid enhancer factor (LEF) and by this interaction it can control specific gene expression within the cell (as shown in Figure 1.1). Any mutation leading to the up-regulation of Wnt or down-regulation of its inhibitors will enhance the function of β-catenin, thus leading to the cells’ proliferation, carcinogenesis, and tumor growth. Zeng et al. (2006) demonstrated that such activation has been observed in over 65% of pancreatic cancers. Moreover, the non-canonical Wnts (Wnt-signaling that is independent of β-catenin function) have been shown to be deregulated in pancreatic adenocarcinoma samples and could play a significant role in the progression pancreatic cancer. Finally, in a pancreatic cancer model, the importance of this pathway combined with its interactions with MSCs (discussed in section 1.3) demonstrates that the Wnt pathway is a critical point of study for MSC related pancreatic cancer therapy.

### 1.2.4 TRAIL and PTEN Pathways and Pancreatic Cancer

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) is a member of the TNF super-family. TRAIL signaling induces a signal which works through the extrinsic apoptotic pathways in the cell. TRAIL was initially identified and cloned based on its sequence of homology to the extracellular domain of CD95 ligand (CD95L) and TNF. TRAIL is an anticancer protein which selectively causes apoptosis of transformed or tumor cells through the activation of death receptors (DR), with no effects on healthy cells. However, gain of TRAIL resistance have been observed in different cancer cells over the years. TRAIL mRNA is also widely expressed in many tissues including peripheral blood lymphocytes, spleen, thymus, prostate, ovary, small intestine, colon and placenta that is in contrary to other members of TNF super family of proteins. Under normal conditions, TRAIL can contribute to the activation-induced cell death of T cells; and the expression of membrane bound TRAIL in
different cells of immune system such as natural killer cells, B cells, monocytes, and dendritic cells post cytokine stimulation can further strengthen their response to tumor tissue\textsuperscript{63-67}. In humans, TRAIL ligand can bind to five different receptors: TRAIL receptor 1 (DR4), 2 (DR5), 3 (decoy receptor 1, DcR1), 4 (DcR2) and a soluble receptor, osteoprotegerin (OPG)\textsuperscript{68}. The death receptors (leading to cell apoptosis) and the decoy receptors that contain a non-functioning truncated death domain play a critical role in cell death homeostasis. Additionally, the ligand can bind the OPG receptors but in much lower affinity\textsuperscript{69}. Mechanisms of TRAIL function are reviewed in depth by Johnstone \textit{et al} (2008)\textsuperscript{58}. Briefly, following the binding of the ligand to the death receptors, oligomerization of the receptors occur which in turn recruits FADD (Fas associated death domains). FADD and caspase 8 combine to produce a functional complex known as death-inducing signalling complex (DISC). Next, a cleavage of caspase 8 results in its activation which in turn activates BH3-only protein (BID) and caspase 3 downstream\textsuperscript{70,71}. BID’s activation leads to a leaky mitochondrial membrane through BAX and BAD (pro-apoptotic molecules). An apoptosome is formed via an active combination (ATP dependent) of caspase 9, apoptotic protease-activating factor1 (APAF1) and cytochrome c complex\textsuperscript{58}. The full mechanism of TRAIL apoptosis pathway is also depicted in Figure 1.1. Through multiple positive and negative loops, cells would undergo apoptosis and display the morphological features associated with it.

The intrinsic apoptotic pathway plays a critical role in controlling apoptosis in cancer cells. PTEN (phosphatase and tensin homolog) serves as the main negative regulator of PI3K-AKT-mTOR. Being one of the most commonly activated pathways found in human cancers, PI3K-AKT-mTOR signaling pathway leads to cell growth, survival and proliferation of cancerous tissue. PTEN dephosphorylates PIP3 to PIP2 thereby directly opposing the activity of
PI3K. The mechanisms by which PTEN contributes to apoptosis, loss of cell cycle controls, and genomic mutations that occurs in tumorigenesis are summarized in a review by Liu et. al (2008) and depicted in Figure 1.1. In addition, PTEN plays a vital role in regulating apoptotic stimuli and the response to the chemotherapeutic agents. PTEN is one of the tumor suppressor genes most frequently inactivated in human cancers. This loss in function is contributed mainly to mutations, deletions or methylations that occur in the genome of metastatic and de novo human cancers. Somatic mutations occur in a large percentage of human cancers, with the highest numbers found in cancerous endometrium (38%), central nervous system (20%), skin (17%), and prostate (14%). Genetic mutations in PTEN occur at much lower rates in human pancreatic cancer (~1%) ; but evidence suggests that mechanisms such as promoter methylation, amplified expression of AKT2 genes, or lower mRNA levels could lead to the loss of PTEN function or its corresponding signaling pathways. Different PTEN expressions have been observed in our preliminary analysis of pancreatic cancer cells and thus overexpressing of this protein could result in pancreatic cancer cell death via the PTEN/Akt pathway. Moreover, other signaling pathways that are linked to pancreatic cancer such as Ras and growth factors TGF-β and insulin-like growth factor-1, are known to down-regulate the expression of PTEN and thus lead to cell proliferation and reduced apoptosis. Compared with other classical tumor suppressor genes, PTEN is haploinsufficient, thus a single copy is unable to prevent cancer. Improving PTEN function in cancer cells would bring a halt to PTEN mutation-dependent cancer cell growth and provide great promise for a cancer therapy option. Pappas et. al (2007) and Jin et. al (2008) demonstrated that wild-type PTEN can be introduced into cells by using either viral or non-viral vectors. Another direct strategy is to deliver cell permeable recombinant wild-type PTEN into cells via fusion PTEN with a cell permeable protein transduction domain such as TAT (trans-activator of transcription). In the present work, PTEN-engineered MSCs will be
examined together with TRAIL to investigate a benefit of a dual-targeted therapeutic strategy for treating pancreatic cancer.

In summary, there are numerous genes associated with the pathogenesis of pancreatic cancer. Some of those reviewed here were chosen as anticancer genes in combination with MSCs as a means to develop a novel gene therapy strategy for pancreatic cancer. The aim is to induce pancreatic cancer cell apoptosis via targeting both the extrinsic (by activation of pro-apoptotic receptors or ligands associated with them) and the intrinsic (initiated via mitochondrial pathway as illustrated in Figure 1.1) apoptotic pathways.

1.3 Mesenchymal Stem Cells

Friedenstein and colleagues first identified a group of adult stem cells sourced from human bone marrow, now commonly known as mesenchymal stem cells (MSCs). MSCs originate from the mesodermal germ layer and are capable of differentiation to tissue of muscle, vascular system, and connective tissue. Bone marrow-derived MSCs’ self-renewal and differentiation capacity allow them to be categorized as stem cells, and their multi-lineage potential is of immense biomedical interest. Due to their easy extraction from tissue, fast turnover ex vivo, and viability after auto- and allotransplantation, MSCs became the first type of stem cells developed for tissue engineering and regenerative medicine. Aside from their differentiation capacity, these cells could secrete a number of growth factors and cytokines, thus modifying the response of immune system. Le Blanc et.al (2005) summarized that MSCs have an intrinsic capacity to provide important cellular cues in survival of damaged tissues with

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† A version of this work has been published in Cancer letters: Dai et.al Cancer Lett. 2011 Jun 1;305(1):8-20. Reprinted with permission from Cancer letters
or without direct contribution in long-term tissue repair. Furthermore, Loebinger and Janes (2010) described the use of stem cells as an ideal anticancer gene delivery vehicle in various pre-clinical models due to their tumor migration and incorporation capacity. In the current study, we aim to use engineered MSCs together with anti-cancer genes as a delivery vector to treat pancreatic cancer cells.

1.3.1 The distribution of MSCs

Due to inadequate current understanding of the exact nature and localization of MSCs in vivo, much attention has been devoted to defining the exact nature of their distribution. Aside from bone marrow, MSCs and MSC-like cells are shown to be present in adult and fetal tissues such as pancreas, circulating blood, heart, adipose tissue, skeletal muscle tissue, placenta and amniotic fluid. Vaananen (2005) suggested that almost all organs that contain connective tissue also contain MSCs.

The existence of MSCs can be verified using methods such as: the use of protein surface biomarkers in vitro and post-infusion distribution analysis of MSCs in vivo. These methods, although considered to be sensitive, have disadvantages. Using markers could be difficult as they could face the nonspecific engraftment of MSCs in different locations. As an alternative approach, a comparison between characteristics of bone-marrow derived MSCs and MSCs that are isolated from different tissues could increase our understanding in this area. Da Silva Meirelles et al. (2006) analyzed and characterized MSCs from different tissues and organs of adult mice from the C57Bl/6 and BALB/c mice strains and MSCs were isolated from either perfused or non-perfused animal tissues. They concluded that MSCs can be found throughout all organs and tissues. Although MSCs have ubiquitous distribution, bone marrow-derived MSCs are most frequently used in therapeutic research. Bone marrow-derived MSCs appear as 0.1-5×10⁵ cells (rodents) and 1-20×10⁵ cells (humans) in bone marrow suspensions and are
considered a rare population of cells within the bone marrow compartment\textsuperscript{113,114}. Additionally, Caplan (2009) describes that the number of MSCs in bone marrow decreases by 20-fold from infancy to the adulthood\textsuperscript{115}.

1.3.2 The characterization of MSCs

MSCs were first characterized by their clonogenic potential determined by the capacity to configure themselves into colony-forming unit-fibroblast (CFU-F). Different tissue-derived MSCs may have differences in their differentiation capacity even if cultured in identical microenvironments. In a comparative study conducted by Sakaguchi \textit{et al} (2005)\textsuperscript{116}, isolated cells from bone marrow, synovium, periosteum, skeletal muscle, and adipose tissue were studied for their colony forming capacity and differentiation into osteogenic and adipogenic cell phenotypes under defined conditions. It was reported that highest rates of osteogenesis were observed in bone marrow-, synovium-, and periosteum-derived MSCs, accordingly. On the other hand, adipogenesis-positive cells were observed at the highest rate in the synovium- and adipose-derived MSCs. The characteristics of MSCs from the same origin may also vary with the methods of isolation and expansion in different laboratories under different conditions. Much effort has been focused on the direct identification of MSCs from bone marrow and other tissue sources\textsuperscript{117-119}. Ucelli \textit{et.al} (2008) reported on variation of specific markers seen on MSCs \textit{in vitro}. Negative expression for hematopoietic markers such as CD14, CD34 and CD45 or costimulatory molecules of CD80, CD86 and CD40 were observed\textsuperscript{120}. On the other hand, variable expression levels of CD44, CD71 (transferrin receptor), CD73 (ecto-5′-nucleotidase), CD90 (THY1), CD105 (endoglin), and the ganglioside GD2 and CD271 (low-affinity nerve growth factor receptor) were reported\textsuperscript{120}. This variability in expression levels may result from species differences, tissue sources, and culture conditions\textsuperscript{120}. Current acceptable methods for identifying MSCs include a thorough study on the combination of their physical, phenotypic and functional
properties\textsuperscript{121}. The basic criteria that are now widely accepted amongst scientists for identification of MSCs are summarized by Dominici \textit{et al} (2006)\textsuperscript{122} and depicted in Table 1.2.

<table>
<thead>
<tr>
<th>Category</th>
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<td>Cultivation</td>
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<td>Positive expression (≥95%)</td>
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<td>Negative expression (≤2%)</td>
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<td>CD 14 or CD 11b</td>
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<td>CD 19 or CD 79α</td>
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<td>CD 45</td>
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<tr>
<td></td>
<td>HLA-DR</td>
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<tr>
<td>In vitro differentiation</td>
<td>Under specific stimulus cells should differentiate into osteoblasts, adipocytes and chondroblasts</td>
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</tbody>
</table>

Table 1.2 Basic criteria for defining human MSCs. Reprinted with permission from Cancer letters: Dai \textit{et al} Cancer Lett. 2011 Jun 1;305(1):8-20.

\textbf{1.3.3 The therapeutic potential of MSCs}

MSCs have strong capacity to be differentiated into different tissue types\textsuperscript{94,123}. Properties of MSCs that have made them attractive cell therapeutic agents include: easy and fast acquisition \textit{ex vivo}; ease of transfection; and their immune-privileged properties arising from low major histocompatibility complex I (MHC I) and no MHC II expression. However, it is worth mentioning that the expression of MHC I and MHC II in undifferentiated MSCs could be proportionally increased with the differentiation extent of MSCs\textsuperscript{124}. The therapeutic benefit of MSC transplantation
in various disorders characterized by cell injury or cell loss are shown in some recent studies such as myocardial infarction\textsuperscript{125,126}, traumatic brain injury\textsuperscript{127-129}, Parkinson’s disease\textsuperscript{130}, type 1 diabetes\textsuperscript{95,131,132} and liver disease\textsuperscript{133}. Lately, with the discovery of MSCs’ tumor tropism, much interest is dedicated to determining the role MSCs could play in cancer therapy.

1.3.4 The interactions of MSC and cancer

Malignant tumor cells live within a complex microenvironment better known as tumor ‘stroma’\textsuperscript{134}. Some of the complex building blocks found in solid tumors are the supporting cells which include: fibroblasts, endothelium, pericytes, lymphatics and a mononuclear infiltrate\textsuperscript{135}. These stromal elements play a critical role in tumor survival, structural support, and vascularization. Thus, any therapy targeting both the stromal components and the malignant cells could lead to an efficient anti-cancer therapeutic approach. Next, we will take a closer look at the properties that MSCs possess which could make them an ideal anti-cancer agent.

1.3.5 Tumor-directed migration and incorporation of MSCs

Advancing tumors continuously produce cytokines, chemokines and other inflammatory mediators\textsuperscript{136}. Such signals are capable of attracting respondent cell types such as MSCs. Transwell culture studies and animal models \textit{in vitro} and \textit{in vivo} were used respectively to determine the tumor tropism capabilities of MSCs. Various studies confirm the capacity of MSCs for tumor tropism and incorporation in almost all human cancer cell lines, such as malignant glioma\textsuperscript{137-139}, breast cancer\textsuperscript{140,141}, Kaposi’s sarcomas\textsuperscript{142}, lung cancer\textsuperscript{143}, colon carcinoma\textsuperscript{144}, pancreatic cancer\textsuperscript{99,135,144}, melanoma\textsuperscript{145} and ovarian cancer\textsuperscript{141}. Furthermore, higher frequency of migration and incorporation was seen \textit{in vitro} co-cultures and \textit{in vivo} xenograft tumors respectively.
The mechanism underlying MSC’s tumor tropism is still not fully understood. Production of chemo-attractant molecules from tumors and the expression of corresponding receptors on the MSCs are the required criteria for the migration and tumor tropism of MSCs. Dwyer et al. (2007) showed a collection of chemokines and cytokines that could interact with MSCs receptors\(^{146}\). As reviewed by Sasportas et al. (2009), a number of cytokine-receptor pairs have been found to be associated with the capacity of MSCs to migrate towards tumor tissue\(^{137}\). These include but are not limited to: SDF-1/CXCR4, SCF/c-Kit, HGF/c-Met, VEGF/VEGF receptor, MCP/CCR2, and HMGB1/RAGE\(^{147-149}\) and adhesion molecules, \(\beta1\)-and \(\beta2\)-integrins, and L-selectin\(^{147-149}\). Furthermore, factors such as IL-8, neurotrophin-3, TGF-\(\beta\), IL-1\(\beta\), TNF-\(\alpha\), PDGF, and EGF have also been shown to enhance MSCs tumor tropism capabilities\(^{150,151}\). Additionally, the presence of chemokine receptors such as CCR(1,4,7,9,10), CXCR(4,5,6), CX3CR1, and c-Met on MSCs might be contributing factors to the tumor tropism properties of MSCs\(^{147,152}\). MSCs migratory capacities have been likened to leukocyte movement toward sites of inflammation; some possible pathways and mechanisms have been discussed in recent reviews\(^98,153\).

Homing of MSCs is more complex and not yet as clearly understood as their tumor tropism. Homing capacity of MSCs can be defined as the cell’s tendency to return to their site of extraction. Several reports indicated that variations in protocols used for isolation and expansion of MSCs \textit{ex vivo} affects their homing capacity. Kemp et al. (2005) suggested that both the phenotype and homing capacity of MSCs can be altered by the duration of passaging time\(^{154}\). Furthermore, progressive passaging of the cells is associated with a decrease or loss of chemokine receptors (i.e. CXCR4), adhesion molecule expression, and lack of further chemotactic response\(^{147,155}\). Moreover, the confluence rate has been shown by De Becker \textit{et al.} (2007) to be directly proportional to the production of the tissue inhibitor of metalloproteinase-3
(TIMP-3) and inhibiting trans-endothelial migration\textsuperscript{156}. Although there is no question with regards to the migration and integration capacity of MSCs towards tumor tissue, their exact function inside the tumor remains ambiguous. With a better understanding of MSC-tumor interactions, one can deduce more thorough conclusions in regards to the role they play in tumor development or destruction. Next we aim to address both the pro-tumorigenic and anti-tumorigenic properties of MSCs.

\subsection*{1.3.6 Pro-tumorigenic effects of MSCs}

The anatomy of a tumor consists mainly of two separate but interlinked compartments: the parenchyma and the stroma. The parenchyma compartment is better known as the neoplastic compartment (malignant cells) and the stroma is the non-malignant supportive tissues that includes: extracellular matrix (ECM), blood vessels, immune and inflammatory cells, connective tissues, and MSCs\textsuperscript{157}. The stroma plays a critical role in tumor growth as it acts as an intermediate structure between the tumor microenvironment and host tissues. It is believed that change in either of the two compartments could contribute to accelerated tumor growth. Studies indicating the pro-tumorigenic capacity of MSCs have shown that MSC-induced immunosuppression capabilities, MSC-mediated angiogenesis, epithelial-mesenchymal transition (EMT)-mediated enhancement of tumor stroma and potential malignant transformation could play a vital role in tumor growth and formation.

\section{1. MSC-mediated immunosuppression}

Influence of MSCs on both the innate and cellular immune pathways has been widely observed \textit{in vitro}. Almand \textit{et.al} (2001) have shown that in peripheral blood obtained from cancer patients, a higher frequency of MSCs with suppressive functions were observed\textsuperscript{158}. For MSCs to be incorporated into a tumor, a capacity to down-regulate the immune system is necessary\textsuperscript{159,160}. Interferon-gamma (IFN-\(\gamma\)) alone or with the aid of TNF-\(\alpha\), IL-1\(\alpha\) or IL-1\(\beta\) is thought to activate
MSCs\textsuperscript{161}. This MSC activation is observed in the IFN-\(\gamma\) knockout mouse model of graft versus host disease (GVHD) and shows that in the absence of IFN receptors, MSCs will not become activated\textsuperscript{162}. Additionally, inhibition of lymphocyte proliferation and suppression of the immune function of effector T lymphocytes (T\textsubscript{eff}), such as: CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, B cells, and natural killer cells (NK cells) have been observed by MSCs\textsuperscript{132,163}. The interaction and influence of MSCs on the immune system has proven to be more complex than previously thought and it appears that this could be attributed to direct cell interactions between MSCs and other soluble factors that are released from MSCs.

**a) Induction of lymphocyte inhibition**

The molecular mechanisms involved in the inhibition of T lymphocytes via MSCs are not fully understood. Different mechanisms have been proposed: such as cell cycle arrest \textsuperscript{164,165}, immunomodulatory activity of growth factors and other molecules secreted by MSCs \textsuperscript{166-168}, and influence of the maturation of dendritic cells (DC) \textsuperscript{168}. MSC-derived soluble factors and their possible functions are listed in Table 1.3.

**b) Induction of regulatory T cells (T\textsubscript{reg})**

The role of T\textsubscript{reg} in the immune system response has been studied extensively. It is widely accepted that T\textsubscript{reg} are capable of down-regulating proliferation of other T cells through a cell-cell contact mechanism, IL10 and TGF-\(\beta\) secretion. Recent in vitro experiments demonstrate induced generation of T\textsubscript{reg} via human MSCs \textsuperscript{140,168,169}. This differentiation of T\textsubscript{reg} can be explained by the release of TGF-\(\beta\) and sHLA-G5 by MSCs (summarized in Table 1.3). Patel et.al (2010) compared levels of T\textsubscript{reg} populations before and after co-cultures of breast cancer cells with MSCs and observed a nearly 2-fold increase of T\textsubscript{reg} in the co-cultures compared with controls\textsuperscript{140}. They further concluded that TGF-\(\beta\)1 secreted by MSCs is largely responsible for this
increase in population. Research indicates the importance of T\textsubscript{reg} in the microenvironment of human tumors.

<table>
<thead>
<tr>
<th>Soluble Factors</th>
<th>Targets</th>
<th>Possible Functions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO</td>
<td>CD4\textsuperscript{+} T, NK</td>
<td>Proliferation inhibition through depleting tryptophan from local environment.</td>
<td>167, 170</td>
</tr>
<tr>
<td>PGE\textsubscript{2}</td>
<td>CD4\textsuperscript{+} T, NK, DC</td>
<td>Proliferation inhibition and cytotoxicity.</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>Monocyte, pDC</td>
<td>Direct stimulation of cell activation and indirect DC inhibition and T\textsubscript{reg} stimulation.</td>
<td>168</td>
</tr>
<tr>
<td>sHLA-G5</td>
<td>CD8\textsuperscript{+} T, NK</td>
<td>Proliferation inhibition and cytotoxicity.</td>
<td>172, 173</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{reg}</td>
<td>Promotion of generation.</td>
<td>174</td>
</tr>
<tr>
<td>TGF-\beta</td>
<td>CD4\textsuperscript{+} T</td>
<td>Proliferation inhibition and cytotoxicity.</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{reg}</td>
<td>Promotion of generation.</td>
<td></td>
</tr>
<tr>
<td>HGF</td>
<td>CD4\textsuperscript{+} T</td>
<td>Proliferation inhibition and cytotoxicity.</td>
<td>175</td>
</tr>
<tr>
<td>iNOS</td>
<td>CD4\textsuperscript{+} T</td>
<td>Inhibition of cell activation through the production of nitric oxide.</td>
<td>161</td>
</tr>
<tr>
<td>MMP-2, MMP-9</td>
<td>CD4\textsuperscript{+} T</td>
<td>Inhibition of cell activation through cleaving CD25 from T lymphocytes.</td>
<td>176</td>
</tr>
</tbody>
</table>

A subset of CD4⁺CD25^{high} Foxp3⁺ cells (T_{regs}) are expressed more significantly in tumor microenvironments than the frequency observed in the peripheral circulation of patients with cancer\(^{177-179}\). To this end, more follow-up studies of MSC-induced T_{regs} function are still needed to fully understand the mechanism of how MSCs function to regulate T_{regs}.

2. MSC-mediated tumor vascular formation

Optimal tumor growth is highly dependent on the angiogenesis capacity of the tumor site which could have a profound impact in metastasis of the tumor itself. Frequently, tumor necrosis is seen in the inner layers of solid tumors where vascularization occurs at a much lower density due to an inability to match the speed of the tumor cell growth. Thus, any therapy involved with anti-angiogenesis is considered of vital importance in the field of cancer therapy. Activation of endothelial cells has been reported through soluble factors and MSC’s cell to cell contacts\(^ {180}\). The expression of proangiogenic factors such as: angiopoietins-1 (Ang1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factors: FGF-2 and FGF-7 have been observed in MSCs\(^ {155,181}\). The expression of receptors such as IL-8 on the MSCs encourages recruitment of endothelial progenitors\(^ {155}\) and these factors are known to induce angiogenesis and rapid vascularization. Chopp et.al (2002) demonstrated that MSCs or their supernatant derived from culture induce significant angiogenesis in cornea treatments\(^ {128}\). They further concluded that the supernatant is even more effective than direct use of growth factors such as vascular endothelial growth factor (VEGF). Generation of vessel-like tubular structures has been observed upon transplantation of MSCs in matrigel plugs \textit{in vivo}\(^ {182}\). Overall, the role of MSCs in angiogenesis in a tumor microenvironment still remains very complex and more in-depth studies are needed to reach definitive conclusions.
3. MSC-mediated Reinforcement of Epithelial-Mesenchymal Transition (EMT)

EMT is best characterized as a stage in which cells are modified in their adhesion capabilities, polarity, cytoskeletal system, expression levels of intermediate filaments, motility and resistance to anoikis\(^\text{183,184}\). EMT is associated with the epithelial derived cancers, representing nearly 90% of human cancers\(^\text{184}\). This characteristic is especially important during organogenesis of the embryonic stage and wound healing. It can be deduced that if EMT occurs in an uncontrolled manner it could facilitate the progression and metastasis of cancer. Iwatsuki et.al (2010) describes that cancer cells undergoing this transition become more aggressive and progressive in nature leading to metastasis\(^\text{185}\). In a recent study on breast cancer, Martin et.al (2010) verified that significant up-regulation of EMT occurs upon co-culture of MSCs with cancer cells\(^\text{186}\). The observed changes were due to cell contact mediation and via MSCs.

4. Potential Malignant Transformation of MSCs

Fear of malignant transformation of stem cells has been a significant concern in cell-based therapies. Similarly, the potential of malignant transformation of MSCs has been a major concern. Human MSCs were identified to differ from their murine counterparts as they did not go through spontaneous transformation\(^\text{187}\). Bernardo et.al (2007) attributes this finding to the loss of replicatively senescent human MSCs during long-term culture, and concludes that using human MSCs are therapeutically safe\(^\text{188}\). Alternatively, other studies done by Wang et.al (2005) and Rubio et.al (2008) have shown opposite findings\(^\text{189,190}\). Houghton et.al (2004) reported that bone marrow-derived MSCs could lead to gastric cancer\(^\text{191}\). Though there are different findings associated with the malignant transformation of the MSCs, there is a consensus to avoid unnecessary manipulation and prolonged passaging for MSCs used for therapy\(^\text{129,132}\). Next we
will consider some of the mechanisms by which MSCs possess anti-cancer effect on tumor tissue.

1.3.7 Anti-tumorigenic effects of MSCs

The anti-tumorigenic properties of MSCs have been attracting immense scientific attention while different animal models have indicated the inhibition of tumor growth via MSCs. Maestroni et.al (1999) initially reported inhibition of tumor growth and metastasis by co-injection of murine MSCs and tumor cells in lung cancer and melanoma mice models. By using a subcutaneous co-transplantation model of colon cancer cells and MSCs in rats, Ohlsson et.al (2003) demonstrated the tumor inhibition capabilities of MSCs. Recently, studies have shown the intrinsic capability of MSCs to produce anti-tumorigenic effects in pancreatic cancer, hepatic cancer and Kaposi’s sarcoma. Next we will describe three possible mechanisms by which MSCs impose their anti-tumor effects: down-regulation of Wnt and Akt pathways, and the intrinsic antitumor capability of MSCs.

1. MSC-mediated down-regulation of Wnt signaling pathway

Although cancer and stem cells have very unique and different origins, they share many biological characteristics. Many signaling pathways that regulate differentiation, cell survival, and cell death including Wnt, Notch, Shh and BMP are shared by both cell types. The Wnt pathway is extensively involved in stem cell self-renewal and differentiation. While abnormalities in this pathway have been related to human tumor progression and expansion; this pathway also plays a critical role in pancreatic cancer development as discussed in section 1.2.3. In a study done by Qiao et.al (2008) MSC-mediated down-regulation of Wnt signaling pathway was observed using a hepatoma animal model. Through their findings they showed that during co-injection of human hepatoma cells (H7420) and human MSCs, the rate of proliferation in H7420 cells decreased dramatically, with an increased rate of apoptosis and
down-regulation of all targets of the Wnt pathway (Bcl-2, cMyc, PCNA and survivin) most likely via producing inhibitors of Wnt. Additionally, they concluded that this observation is correlated with the paracrine function of MSCs as when they used the conditioned media, similar results were observed. Further studies are required to understand the mechanisms of Wnt pathway induced apoptosis via MSCs in tumor microenvironment.

2. MSC-mediated down-regulation of Akt pathway

Opposite to what was observed in the Wnt signaling pathway study in hepatoma models, the MSC-mediated down-regulation of Akt pathways required direct cell contact in a Kaposi’s sarcoma model. As discussed in section 1.2.2, amplification of the Akt pathway is a repeated theme in pancreatic as well as other cancer models. This molecule has been the hallmark of human Kaposi’s sarcoma. Khakoo et.al (2006) demonstrated that intravenous injection of MSCs resulted in their homing to the site of the tumor as early as 48h that effectively inhibited the tumor growth. In this in vitro study, an inhibition of the activation of Akt protein kinase via MSCs was observed. They demonstrated that for this inhibition to occur, direct cell contact is required and they were able to reverse the inhibition by anti-antibody against E-cadherin molecules. In their in vivo model, inhibition of Akt activation was observed in areas neighboring MSC infiltration. These findings confirm the anti-tumorigenic properties of MSCs via the Akt pathway and also showed that deregulated Akt may be a specific target of MSCs in the tumor microenvironment.

3. Intrinsic antitumor properties of MSCs

The intrinsic antitumor properties of MSCs have been observed in a number of studies. Lu et.al (2008) displayed cell cycle arrest in response to MSCs in lymphoma, hepatoma, and insulinoma cells which was followed with an increased rate of apoptosis in the respective tumor cells and, reduction in malignant ascites in vivo and in vitro. This observation has also been
seen in other cancer models using MSCs. In pancreatic cancer, the intra-peritoneal injection of MSCs reduced the tumor growth rate and improved longevity in a SCID mice model\textsuperscript{194}. In a breast cancer model, both the intratumoural and IV injections of naive MSCs lead to reduced tumor growth and increase cancer cell apoptosis\textsuperscript{203}. The actual mechanisms stimulating these intrinsic antitumor properties in MSCs remains complex and further research is required to discern what influences are activating these results, while down-regulation of Akt, Wnt and NFkB pathways have been reported to be of great importance in the explanation of these intrinsic MSC properties\textsuperscript{204}. Figure 1.2 summarizes both the pro-tumorigenic and anti-tumorigenic effects of MSCs in a tumor microenvironment.

Figure 1.2 Summarizes pros and cons of MSCs within tumor environment. The related mediators are indicated in the corresponded processes. The anti-tumorigenic effects of MSCs are exhibited in the right panel. The middle part of the figure illustrates the change of tumor size. Reprinted with permission from Cancer Letters: Dai \textit{et.al} Cancer Lett. 2011 Jun 1;305(1):8-20
Results of such investigations may vary due to influences of primary tumor origin, location and subtype, as well as the MSC isolation protocol, time of propagation and the route of administration. It is evident that conflicting data in regards to the role of MSCs in tumor microenvironments indicate that this field of research is still in its early phases and more conclusive work is necessary to substantiate and solidify our knowledge in this area.

1.3.8 Putative personalized medicine with anticancer-engineered MSCs

As science advances and knowledge expands about how each patient is unique in their individual reaction to a therapy, more attention is transferred to the theme of personalized medicine. MSCs have an immense potential to be used in either the auto- or allotransplantation discipline of cancer or regenerative medicine. Our goal remains to expand on the antitumor properties of MSCs while incorporating their other potential benefits and developing them as a vehicle for delivery of anti-cancer genes to cancer targets. MSCs-engineered with anticancer genes are capable of specifically attacking tumor cells through multiple mechanisms: 1) tumor-directed migration and incorporation; 2) anticancer agent delivery, and 3) organ-specific vector construction. The work in this study focuses on the first two of these mechanisms. With the extensive variations that exist amongst patients, we aim to use multiple anticancer genes in an effort to determine which provide a more customized approach to yielding maximal killing of the targeted cancer site. For translation of this research into clinical settings, personalized therapy for each patient is critical. This is summarized in Figure 1.3 which illustrates a general scheme of putative personalized treatment with anticancer gene-engineered MSCs.
Figure 1.3 Putative personalized treatment of cancer with engineered MSCs: Most patients with advanced cancer are treated with surgical therapy, which provides direct access to tumor tissue for cell identification making these specifically schemed protocols an ideal clinical model. Clinical ward, operating room (upper panel) and clinical laboratory (lower panel) are utilized in this procedure. 1) MSCs are isolated from the patient. 2) and 3) MSCs are isolated and expanded in vitro. 4) and 5) they are then transfected via different anticancer genes and plated in transwell plating systems. 6), 7) and 8) primary cancer cells are isolated from the patient and plated against different engineered MSCs in the transwell plates. 9) and 10) most efficient engineered MSCs are recognized via apoptotic assays and they are then engineered in required numbers and transplanted back in to the patient. Reprinted with permission from Cancer Letters: Dai et.al Cancer Lett. 2011 Jun 1;305(1):8-20
1.4 Summary and Specific Aims

Pancreatic cancer remains one of the most challenging cancer types to treat. Current conventional therapies for pancreatic cancer are passive and symptomatic in nature. With the pressing demand on scientists to find new breakthrough therapeutic strategies, using MSCs combined with anticancer agents hold great promise.

We hypothesize that (1) MSCs engineered with specific anticancer genes can effectively act on the site of tumor tissue due to their tumor tropism and homing capacity; and (2) by using TRAIL and PTEN anticancer genes that induce apoptosis through two different mechanisms, we can detect the sensitivity of pancreatic cancer cell lines to engineered MSCs under different culture conditions.

Objective 1: To isolate and characterize MSCs from the ductal tissue of human pancreas donors.

Aim 1.1. Isolate and expand ductal pancreatic human MSCs ex vivo.

Aim 1.2. Evaluation of the isolated cells by using the basic criteria provided by ISCT (for their cultivation, phenotype, and differentiation capacity) to confirm their mesenchymal stem cells origin and to perform further evaluation of MSC’s adherence properties in real time.

Aim 1.3. Further characterization of isolated MSCs in vivo and in vitro for their homing capacity.

Objective 2: To determine the effects of MSC_{TRAIL} and MSC_{PTEN} induced cell death or cytotoxicity on cells of human pancreatic cancer models.

Aim 2.1. Examining the cytotoxic effects of engineered-MSCs on pancreatic cancer cell lines using trans-well and real-time monitoring technology

Aim 2.2. To test the migration and tumor tropism properties of MSCs during co-culture with different pancreatic cancer cells.
Chapter 2: Pancreatic Ductal MSCs: isolation, propagation, and characterization

2.1 Introduction

MSCs can be obtained from many different organs. One source that is widely studied in the field has been the bone marrow-derived MSCs. Though the characteristics between MSCs across other organs are very similar, the individual mechanistic differences between MSCs derived from different organs in *in vivo* or *in vitro* models remains a mystery. Properties of bone marrow-derived versus pancreatic-derived MSCs could be custom-designed to exert maximal impact on treatments for pancreas diseases. Although previous investigations have shown the homing capacity of bone marrow-derived MSCs in different animal models, the promise of pancreatic-derived MSCs is that they could show a stronger homing capacity to target neoplasms of pancreatic tissue origin.

Pancreatic ductal carcinoma with survival rates of 2-3% is deserving of attention for novel therapeutic strategies. Our aim is to isolate, propagate, and characterize human ductal-derived MSCs. Specific aims of this section of study are summarized in Figure 2.1. Ductal tissue of human pancreas cadaver donors was used to isolate and expand *ex vivo* pancreatic MSCs. Further, verification of the phenotype and characterization of these cells was performed according to the criteria defining human MSCs. To further our understanding of their characteristics we have designed an experiment to examine tissue distribution, homing capacity and migratory patterns in blood and tissues of an animal model.
Figure 2.1 Experimental Design: Pancreatic ductal MSC isolation, propagation, and characterization. Isolated pancreatic ductal-derived MSCs were tested for the following characteristics: surface biomarkers, differentiation capacity, adherent properties, migration and homing capacity.
2.2 Materials and Methods

2.2.1 Animals

NOD.scid gamma (NSG) mice (NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ) were purchased from The Jackson Laboratory and housed in the Child & Family Research Institute under specific pathogen free conditions. All mice were cared for according to the guidelines of the Canadian Council of Animal Care and regulations of the University of British Columbia in consultation with veterinarians. These mice are severely immunocompromised leading to a better engraftment of human tissue/cells. They lack mature T cells, B cells, or functional NK cells which enables for a much better engraftment of human stem cells [195]. The mice were fed regular chow and had free access to water and enrichment in the cage.

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**Figure 2.2** MSCs distribution post-injection via tail vein in NSG mice model. 1,2 MSC-CFSE were prepared *in vitro* and were injected through the tail vein (3). Following 1 h, 1 day, 4 days or 7 days post-injection the animals were sacrificed and samples of organs and blood were taken for analysis (4, 5, & 6).

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\* A version of this work has been published in Cancer Gene Therapy (CGT): Moniri *et al.* 2012 (in press). Reprinted with permission from CGT
At age 12 weeks, injections of labeled MSCs were performed via the tail vein. MSCs (2 x 10^6) pre-labeled with Carboxyfluorescein diacetate succinimidy ester (CFSE) were injected through the tail vein. Mice were sacrificed at 1hr, 1day, 4 and 7 days, tissue and blood were collected accordingly.

2.2.2 Cells and culture conditions

Human pancreatic MSCs were isolated from ductal tissue of human organ donor pancreas and ex vivo expanded as previously described. Briefly, human pancreas were obtained (with consent) from adult heart-beating cadaver organ donor (young male donor) through the organ procurement program of British Columbia Transplant (BCT, Vancouver, Canada). Pancreatic ductal tissue taken from the collagenase digestion chambers during islet isolation was chopped into fragments of 1-3mm. Primary culture was initiated by seeding chopped tissue onto 100 mm culture dishes (CellBind, Corning, Acton, MA, USA). Subculture was performed once newly grown cells reached sub-confluence. The MSCs were cultured in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin-streptomycin solution (all from Invitrogen, CA, USA) and incubated at 37°C in a humidified, CO₂ atmosphere. The cells used in this study were limited within seven passages numbers upon thawing from the frozen stock. Cells were frozen upon initial harvest and propagation and were stored in at -80°C in 1.5 ml cryopreservation vials. They were frozen in 1 ml total volume containing: 70% cell suspension +20% FBS+ 10% dimethyl sulfoxide (DMSO) in -80°C freezer for one week and moved into liquid N₂ for prolonged storage. When thawing the cells, care was taken to transfer cells from the vial to fresh media as soon as they thawed in 37°C water bath under aseptic conditions. After one wash using fresh media, cell suspension was seeded onto a 25cm² culture flasks and incubated at 37°C and 5% CO₂ and 95% air.
2.2.3 Differentiation culture conditions

MSCs were seeded at a density of $1 \times 10^5$ cells/well in 6 well culture plates that were pre-coated with poly-l-lysine (PLL) purchased from ScienCell Research Laboratories (CA, USA) at a concentration of $2 \mu g/cm^2$ for one day prior to the seeding. This solution was prepared from the stock concentration of 1mg/ml. 150 µl of PLL was diluted in 10 ml of cell culture grade water. After thorough mixing, the wells were coated and left at 37°C over night. Next day the wells were washed twice using sterile PBS and cells were seeded. The cells were then seeded and left inside the incubator (37°C and 5% CO$_2$ and 95% air) until they reached 95% confluency with regular media changes every 2 days total of 4 days since seeding. The media was then replaced with either MSC Adipogenic Differentiation Medium (MADM) or the MSC Osteogenic Differentiation Medium (MODM) kit at 2 ml per well purchased from ScienCell Research Laboratories (CA, USA). The media was changed every 3 days for the period of 18 days total and on the last day staining was performed using Oil Red O (ORO) and Alizarian Red (AR) solutions as described below.

2.2.4 ORO and AR staining

Stock solution (0.5%) of ORO (C.N. 26125) was combined with a solution of isopropanol (99.94%). We then mixed three parts of the stock solution to 2 parts PBS to produce the working solution, and incubated for 30 minutes at room temperature (RT), then filtered using a 0.45 µm syringe filter. After an additional 20 min, the reagent was applied to cells using a 0.2 µm syringe filter. The cells were washed twice using the same PBS used in the working solution. To detect osteogenesis, we used 1% AR Solution (C.N. 58005) into double distilled water (ddH$_2$O) and the pH was adjusted to 4.21 with 0.1% ammonium hydroxide. The solution was then filtered into an amber bottle (light protected) with a Whatman #1 filter purchased from VWR (Edmonton, Alberta, Canada). Finally, it was injected on onto the cells using a 0.2µm syringe filter. Cells
were washed before and after staining with ddH2O. Staining was followed by 20 min incubation at room temperature and 3 washes in PBS (ORO) or ddH2O (AR) accordingly. The cells were covered by PBS or ddH2O for analysis using a light microscope.

### 2.2.5 Flow Cytometry (FACS) analysis

The cluster of differentiation (CD) marker expression of phycoerythrin (PE-CD44, CD90, CD105, and CD34) and allophycocyanin (APC -CD73) conjugated antibodies and their corresponding isotypes were purchased from eBiosciences (San Diego, USA) and used per the manufacturer’s recommended protocol. Sub-confluent cells were detached with 0.25% trypsin-EDTA and washed with PBS. A total of 1x 10\(^6\) cells were resuspended in 100 µl PBS for each reaction, and then 5 µl of antibody solution at the concentration of CD44 (0.625 µl/ml), CD90 (0.25 µl/ml), CD105 (1 µl/ml), CD34 (0.5 µl/ml), CD73 (0.125 µl/ml) was added to each Eppendorf tube and incubated at 4°C for 30 min. The cells were washed twice with PBS and re-suspended in 500 µl FACS buffer (PBS+5%FBS) which was followed by FACS analysis.

CFSE-labelled MSCs were injected via the tail vein to NSG mice and samples of blood obtained by cardiac puncture under anesthesia and other organs were taken at different time intervals (1hr, 1 day, 4 days, and 1 week post injection). CFSE tracing dye was purchased from Invitrogen (Burlington, ON) was used in accordance with the manufacturer’s protocol with some modifications. Briefly, cells were digested and centrifuged to obtain pellet and aspirate the supernatant. 6 µM of CFSE dye was prepared from the stock solution in PBS. Cells were resuspended gently in pre-warmed (37°C) CFSE staining solution and then incubated for 10 min at 37°C. Cells were Re-pelleted by centrifugation washed 2 times with PBS and resuspended in saline solution prior to injection into the animals. In each FACS experiment, 15,000 events were recorded and the mean fluorescence intensity of each receptor was assessed on the live cell population. The reference gating location was determined by both isotype IgG and secondary
antibody controls with the aid of Flowjo software purchased from Tree Star, Inc (OR, USA).

2.2.6 DNA gel analysis

Genomic DNA primers were designed to assess distribution of MSCs post injection. Briefly, each sample’s DNA was extracted using the QIAGEN - DNeasy Blood & Tissue Kit. The DNA samples were quantified using a nanodrop-2000 (Fisher Scientific Company, Ottawa, Canada) and normalized with DNase, RNase, protease free water purchased from Sigma (Oakville, Ontario, Canada) to 100ng/µl of concentration. Fifty microliters of PCR reaction material were used for each polymerase chain reaction (PCR) reaction using an Eppendorf thermal cycler. PCR was performed using the following parameters: 1. 94°C for 3:00min, 2. 94°C for 0:30sec, 3. 60°C for 0:30 sec, 4. 72°C for 1:00min, to step 2 and 35 cycles, 72°C for 5:00min, and lastly the samples were held at 4°C overnight. Primers were then diluted to make 10 µM master mix out of which 2 µl of primers were used. PCR Taq Mastermix (G013, Applied Biological Materials Inc., Richmond, BC, Canada) were used according to manufacturer’s protocols and each tissue sample was tested with both primers and the PCR products were run on an agarose gel to identify bands. The PCR products were run on a 1% agarose gel using safeview dye (NBS Biologicals Ltd., UK). These primers were designed in such way to have high specificity for mouse or human as needed. This aimed to distinguish any human DNA in mice tissue. Mouse specific forward sequence of GGACAACGACGGACAGCCGG and reverse TCTCTGGCACGGCCTGCAAG and human specific forward sequence of CCTTCCCACAGACCACAGTT and reverse sequence of ACTGGCCCTGATGATCTGAC were designed with the help of Dr. Ali Moeen and purchased from Invitrogen (Burlington, ON). The mean pixel intensity of bands subtracted from the background was measured using UN-SCAN-IT ver. 6.1 software purchased from (Silk Scientific Inc, Utah, USA). Furthermore, the dimensions of the square in which the pixels were calculated were kept the same across all of the
gels.

2.2.7 Real time assessment of cell viability & migration capacity

A novel technique that uses real time cell monitoring can be used to detect real time migration, cytotoxicity, and adherence/proliferation of cells during direct and indirect co-cultures with MSCs. RTCA xCelligence system was developed by the partnership of Roche Applied Science (Quebec, Canada) and ACEA Biosciences Inc. (CA, USA). This new technology, allows for uninterrupted, label free and real time analysis the cells. This technology is now widely used by researchers across the world in many different areas and the full list of references to the device is available via Roche’s website.\textsuperscript{207}

The RTCA is equipped with 3 plates designed for different assays: cell proliferation/cytotoxicity, migration/invasion, and co-culture studies. The functional mechanistic of this instrument is summarized in the previous reviews.\textsuperscript{208,209} Briefly, this machine functions on the basis of electronic impedance reading from the gold plated sensor electrodes that are placed at the bottom of the plates (in adhesion and cytotoxicity plates) and the lower face of migration plates. Electronic readings change as cells attach or detach from the surface electrodes, thus producing a change in impedance that is calculated via complex algorithms and plotted as cell index (CI) values; the more cells attaching leads to higher CI readings on the machine and vice versa. This impedance reading could be affected by the quality of cell interactions and adherent properties between each cell and the electrodes. Figure 2.3 (a,b,c) illustrates the differences seen within each plate system and in (d) the correlation between the CI readings with cell adherence to the plate is demonstrated. As the number of attached cells increases a higher value of CI is recorded until the plate is 100% confluence at the zenith of the curves and as the cells go through cell death the CI value is decreased accordingly.

This technology creates a label free environment for the cells and can closely represent
influences upon cells during an experiment without the use of toxic assays or end point assays that can lead to termination of the experiment. The use of this technology enabled us to expand our knowledge for cell mediated cytotoxicity, migration capacity, and proliferation.

Figure 2.3 Real time cell analyzer system: various plates and impedance technology. a) depicts the cytotoxicity/proliferation plate b) displays the migration/invasion plate with their upper and lower chambers c) shows the insert used in conjunctions with the plate in “a” that is used for co-culture studies. d) Demonstrates the relationship between cell adherence to the gold plated microelectrode and the CI readings by the machine.

a. Cell Adherence Property

In the present study, the Real Time Cell Analyzer Dual Plate (RTCA-DP) instrument was used to assess the adherent property of MSCs. An initial titration of different cell densities
(100,000 cells per well to 781 cells/well) was performed for MSCs. Briefly, cells were trypsinized and counted using the trypan blue exclusion and a haemocytometer; they were then resuspended in culture medium. Background measurements were taken from the wells by adding 100 µl of the same medium to the culture plates. Subsequently, RTCA Software Package 1.2 (Roche, Canada) was used to calibrate the plates. A volume of 100 µl of cell suspension was added to the wells to make a final volume of 200 µl. All tested cells were allowed to settle at the bottom of the wells, at room temperature for 15 min, before incubating at 37°C and 5% CO₂ on the RTCA cradle. The impedance signals were recorded every 5 min for the first 25 scans (2h) and every 20 min until the end of the experiment (up to 72h).

b. Cell migration assessment: Serum induced, homogenized tissue, and 0.5 g fragmented tissue

The RTCA system was used to monitor the MSCs’ migration capacity by using migration specific plates. These plates contain an upper chamber (where MSCs were placed) which contains fenestrated membranes with interdigitated gold microelectrodes and a lower chamber (where the target tissue/agent is incubated). For all migration assays, 40,000 MSCs were prepared in serum-free media (SFM) and plated into the upper chamber of the migration plates in a total volume of 160 µl. Background reading control for each well along with SFM in the bottom chamber control were measured for each plate. Data were recorded for 25 scans, every 5 minutes (first 2h), followed by every 10 min until the end of the experiment (12h or 24hr).

i) Serum induced migration: MSCs were plated on the top chamber and in SFM conditions and serum containing media alone was left on the lower chamber for the total volume of 160 µl per well. MSC in SFM (upper chamber) and SFM alone (lower chamber) was used as a control and normalization purposes.

ii) Homogenized tissue migration assay:
For organ specific migration assay, organs of pancreas, liver, lung, kidney, spleen, brain, and heart were excised from the experimental animal post-mortem. Briefly, for homogenized mice tissue, organs of interest were excised from the NSG mice and each was weight while maintaining aseptic conditions. They were then normalized by the smallest weight (spleen tissue 0.1 g) and suspended in SFM which was kept constant across all samples. This was followed by tissue homogenization. Samples were kept on ice at all times before the incubation period. Samples were suspended in SFM for the total volume of 160 µl and they were then seeded in the lower chamber of the plates and left to settle for 30 minutes on ice under aseptic conditions.

iii) Fragmented tissue migration assay:
For tissue fragment samples, similar methods were used as above except that once organs were excised they were chopped finely under aseptic conditions into fragments. This was followed by weighing 0.5g from each fragment sample and suspending it with 160 µl SFM and for subsequent seeding in the corresponding lower chamber of the migration plates. Samples were left at room temperature (RT) for 30 min until they settled. The upper chamber was then assembled and filled with 25 µl of corresponding media to each well. This was then left to equilibrate for 1h inside the incubator at 37°C and 5% CO₂. After this incubation time, the background reading was measured for each of the wells. After this stage, corresponding number of MSCs were seeded into the upper chamber to make the total volume of 160 µl.

2.2.8. Statistical analysis
Cell index calculations for real-time dynamic migration assessments were performed automatically by the RTCA Software Package 1.2 of the RTCA system (Roche, Canada). Normalizations were performed using the RTCA Software Package 1.2. Numerical data were expressed as mean ± standard deviation. Statistical differences between the means for the
different groups were evaluated with Prism 5.0 (GraphPad software, La Jolla, CA, USA) using Student’s t-test with the level of significance at p<0.05 unless otherwise stated.
2.3 Results

2.3.1 Pancreatic ductal MSCs expansion and characterization

MSCs were verified by their phenotype, membrane biomarkers, and their differentiation capacity. Cells adhered to the culture flask and possessed phenotypic characteristics as shown in Figure 2.4.

![Figure 2.4 Isolation and Propagation of MSCs.](image)

MSCs grew out of the fragmented tissue sections on day 3 and became sub-confluent on day 7. After removal of the tissue fragments using a sterile surgical scalpel, MSCs were passaged and became confluent within 3 days (Figure 2.4 c). We acknowledge that there might be some periductal tissue involved during this isolation.

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In freshly isolated cells, surface biomarkers showed positive expression of CD44+ (4.23% negative and %95.8 positive), CD73+ (%0.257 negative and %99.6 positive), CD90+ (%0.059 negative and %97.3 positive), CD105+ (%314 negative and %98.5 positive) and negative expression of CD34- (%97.8 negative and %2.2 positive) as shown in Figure 2.5.

**Figure 2.5 Surface biomarker determinations.** Using FACS analysis from left to right on the top row CD105+,CD73+,CD90+. Bottom row from left to right CD34-,CD44+ expressions were detected.
Compared with controls, after 18 days of incubation under adipogenic and osteogenic differentiation media, positive staining of ORO and AR reagents were observed respectively (Figure 2.6).

Figure 2.6 Differentiation capacity of MSC to adipogenic and osteogenic tissue. The left 3 panels show oil red O staining for the adipogenic tissue. The right 3 panels show alizarin red staining for osteogenic tissue.
2.3.2 Pancreatic ductal MSCs Titration and binding characteristics

![Graph](image)

**Figure 2.7 Cell index (CI) readings of titration and adherence properties of MSCs.**

Titration ranges from 100,000 cells per well to 781 cells/well. Panel a) shows 25,000-100,000 cells per well. Panel b) depicts 781-12,500 cells/well. Each line indicated mean ± s.d. (N = 3).

The unique adhering property of MSCs is shown in the titration studies in Figure 2.7 (a&b). With a higher number of cells/well there is a bigger initial `burst` of attachment of these cells to the culture flasks (increased in CI reading) during the first 10h. This is followed by a detachment phase as shown in 6,250-100,000 cells/well as indicated by the lower CI readings and finally a growing/adherence phase.
2.3.3 Distribution and migration capacity of MSCs in vitro and in vivo

MSCs demonstrated a significant serum-directed migration capacity enabling them to move to where the serum source is located (Figure 2.8).

![Graph showing serum induced migration of MSCs](image)

**Figure 2.8 Serum induced migration of MSCs.** Serum free media (SFM) conditions were set as a baseline and the values were normalized for the regular media (RM) containing FBS. Each line indicated mean ± s.d. (N = 3)

After cells were seeded into the top chamber in SFM conditions and regular media was placed in the lower chamber this migration pattern was observed. MSCs migrated through the top plate to the site of serum containing media in the lower chamber as demonstrated. The chart was normalized against the SFM control.
Figure 2.9 (a&b) identifies the migration capability of MSCs that were exposed to either homogenized whole organ tissue samples of NSG mice or 0.5 g fragmented samples of the same tissue, all of which are performed under SFM conditions.

MSCs migration capacity was observed in tissues of pancreas, liver, lung and kidney. Migration pattern was observed greatest in the liver>lung>kidney>pancreas in the homogenized tissue assay. In the fragmented tissue assay, migration patterns of MSCs was greatest in lung>kidney>liver>pancreas, with pancreas producing migration capacity during the first 2h.
**In vivo analysis:** CFSE-MSCs that were injected into NSG mice appeared temporally in phases at 1hr and 4 days post injection in the blood samples as shown in Figure 2.10.

![Figure 2.10 Dynamic distributions of intravenously transplanted CFSE-MSCs in peripheral blood if NSG mice. CFSE-MSCs were observed in the blood sample at 0.42% 1hr post injection, on day 1 only %0.02 of CFSE was observed in the blood sample. On day 4 the CFSE-MSCs reappeared at 0.74% and on day 7 the detection became lower at 0.23%](image)

1hr post injection, CFSE-MSCs were detected in the blood in 1h post-injection, day 1 post-injection there was no detection, they are detected again on day 4, and on day 7 no detection was observed.
Dynamic analysis of distribution of MSCs in different organs of NSG mice post-injection time intervals of (1hr=time 0, 1, 4, and 7 days) are shown in Figure 2.11.

Human ductal pancreatic MSCs demonstrated homing capacity to the site of pancreas during 1hr, 1 day and 4 days post injection. Other organs such as heart, lung, and liver also displayed migratory patterns of MSCs.
2.4 Discussion

MSCs have attracted extensive interest in the clinical setting due to their accessibility, tumor-oriented homing capacity and the feasibility of autotransplantation. Ankrum and Karp (2010) described 21 different clinical trials dedicated to studying the effects of MSCs in different diseases. In this chapter, we report results of studies to isolate and characterize human pancreatic ductal-derived MSCs with the goal to further use them in a pancreatic cancer gene therapy model. Based on the criteria for defining human MSCs established by the International Society of Cellular Therapy (ISCT), we have successfully isolated and characterized the pancreatic ductal MSCs. Pancreatic-derived MSCs have shown the capacity to adhere to the surface of culture flasks (Figure 2.4). Surface biomarkers have been in accordance with the minimal criteria of human mesenchymal stem cells as depicted in Table 1.1 and Figure 2.5. Furthermore, differentiation capacity of MSCs to osteogenic and adipogenic tissue has been verified with corresponding positive staining of AR and ORO (Figure 2.6). This capacity to differentiate is known to be important for the self-healing and tissue healing capabilities of MSCs as shown in bone marrow-derived MSC repair studies of large bone defects, cartilage defects and genetic diseases such as osteogenesis imperfect. Further studies are needed to show the implications of such ability in ductal-derived MSCs of pancreas. MSCs were kept in culture to 7 passages for reasons discussed in section 1.3.6(4) in order to eliminate MSCs capacity for malignant transformation.

The homing capacity of MSCs has been a subject of debate since discovery. The potential of MSCs to migrate to site of origin gives the scientists a tool for specific organ/tissue targeting related therapies. Studies done on baboons and nude mice have indicated that bone marrow-derived MSCs will home to bone marrow post injection. Furthermore, Sorti et al.
has shown that bone marrow-derived MSCs do possess chemokine receptors of CXC, CC, and CX3C family might promote migration to the pancreatic islet if activated in a murine model\textsuperscript{216}. MSCs are known to be cells with low levels of MHC I and no MHC II, thereby making them attractive for use in allotransplant models, because they possess a mechanism by which they reduce T cell and B cell proliferation\textsuperscript{166,217,218}. These mechanisms were found to be associated with the release of soluble factors by MSCs and cell-cell contact but did not include T-cell apoptosis. Migration capacity experiments in our studies strengthen the hypothesis that pancreatic-derived MSCs could have the potential to home back to the site of pancreas. Further experimentation using different tissue-derived MSCs is needed to confirm such conclusions.

Our preliminary \textit{in vivo} animal studies (Figure 2.11) suggest that the ductal derived MSCs have homing capacity to the pancreas of NSG mice; due to low number of animals this cannot be fully verified. More experiments are needed to verify these observations. Alternatively, using real time PCR primers could lead us to quantify the results observed to determine the likelihood of such observation. In other studies, human islets are shown to produce a set of chemokines more specifically: CCL2, CXCL1, CXCL8, and CXCL12, and less of CX3CL1, CCL3, and CCL20\textsuperscript{216}. Additionally, bone marrow-derived MSCs possess the receptors for CXCL12 and CX3CL1\textsuperscript{216}. The same receptors might be responsible for the observations in these studies. Further studies of the chemokine receptors present on the ductal-derived MSCs could lead to more conclusive data to confirm such hypothesis.

We have uniquely identified a double-waved appearance (appeared in two time phases) of MSCs in the blood post injection in our animal model as shown. A potential explanation for this observation is the unique characteristics of binding kinetics that these cells possess. MSCs seem to adhere for a short period of time and then detach themselves from the culture well followed by a slower period of attachment. This phenomenon, if confirmed, might have
important implications in the clinical setting and targeting of cancerous tissue as MSCs could traffic their way to find the “right” target, pancreas, once injected into the patients. This observation has been recently described and it is attributed to cells ‘tendency to first spread and then contract transiently’ to make room for neighbouring cells. Furthermore, Ruster et al. (2006) demonstrated the unique characteristics of bone marrow-derived MSCs motion and adhesion capabilities when interacting with endothelial cells. They summarized their observation as a coordinated rolling and rapid adhesion to the endothelial cells. These findings show another layer of tissue specificity that MSCs might have which would be of great interest clinically.

Our *in vitro* real time studies suggest that MSCs have a capacity to migrate to the site of serum-containing media with SFM conditions (Figure 2.8). During migration/invasion studies either in RTCA or using the conventional method of Boyden chamber, this can be of great importance. In such studies this knowledge will enable us to determine the correct migration gradient require for setting controls and suspension media used for cells in either upper or lower chambers. The RTCA system has an advantage over the conventional Boyden chamber that enables a label free and real time platform for the cells to simulate *in vivo* conditions. Knowing this property of MSCs will enable researchers to determine the basis of their migration gradient with much needed consideration as MSCs are innately attracted to migrate towards serum containing media thereby avoiding false positive responses from their migration assays.

Further *in vitro* studies, demonstrated the migration capacity of MSCs to other organs in the body. The proteins of the homogenized and fragmented tissues of different organs show that during the first 3-5h of the experiment the MSCs do travel to the site of pancreas more aggressively than any other tissue (Figure 2.9 a&b) for the first 5-7 h and a decline is seen in CI thereafter. Moreover, a decline in the migration rate of MSCs to the pancreas after 5h may be
explained by the proteolytic enzymes secreted, or released from pancreas exocrine tissue which interferes with the binding capacity of MSCs to the bottom surface of the pores in the migration plates. To investigate this, further experiments could include studies where the enzymatic effects of pancreas are blocked using protease inhibitors and the effect of the migration is studied. It is important to note that overall, high migration capacity were observed towards the lung tissue in fragmented samples and kidney fragments also displayed high migration capacity of MSCs in homogenized samples. Analysing factors or surface markers on both these tissues types could yield a better understanding for such observation. With the preliminary results we have thus far, it can be deduced that our ductal derived MSCs have homing capacity to the site of the pancreas as well as other tissues tested. The data we have gathered here compare favorably with similar data that have been shown in studies of bone marrow-derived MSCs in murine and baboon experiments$^{223,224}$. 

To conclude, these studies confirm the feasibility for acquisition and expansion of pancreatic MSCs, along with their capacity to home back to the site of pancreas that could lead to a novel therapeutic approach in pancreatic cancer treatment.
Chapter 3: TRAIL/PTEN Engineered MSCs & Their Cytotoxic Effect on Pancreatic Cancer Cells

3.1 Introduction

Based on the studies described in Chapter 2, we next sought to investigate the effectiveness of TRAIL and PTEN engineered pancreatic MSCs on pancreatic cancer cell lines. Pancreatic cancer therapy remains one of the toughest challenges in medicine. As a result of late diagnosis and the aggressive nature of this disease, a more specific therapy is essential. Mesenchymal stem cells (MSCs) have been known to have tumor-directed migration and homing capacities. Tumors are in a state of continuous production of cytokines and chemokines; MSCs appear to be attracted to such signals and thus migrate towards the site of tumors. Mechanisms involved in this property were discussed in section 1.3. We aim to investigate the attraction between isolated pancreatic ductal MSCs and various pancreatic cancer cells and seek evidence for tumor tropism properties as an anti-cancer gene delivery system. Our specific aims include: testing MSCs’ innate anti-tumorigenic capacity, migratory capacity towards pancreatic cancer cells, and efficiency in delivering anti-cancer genes (TRAIL and PTEN) to the pancreatic cancer cells.

The experimental design for this part of study is summarized in Figure 3.1. Here we aim to engineer MSCs with TRAIL and PTEN and use them as a vehicle to deliver this anti-cancer gene to the site of pancreatic cancer cells. This work is accomplished through the application of

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direct and indirect culture techniques as explained in the sections that follow. Furthermore, we seek evidence for the tumor-directed migration of MSCs.

Figure 3.1 Experimental Design: TRAIL/PTEN-engineered MSCs & their pro-apoptotic properties on pancreatic cancer cells. MSCs were engineered with anti-cancer genes and cancer cell lines were tested for the expression of TRAIL receptors. Direct and indirect co-culture studies were performed. Further, tumor directed migration of MSCs were examined.

3.2 Materials and Methods

3.2.1 Cells and culture conditions

Human pancreatic cancer cell lines (BXPC3, ASPC1 and Panc1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and the transformed human islet cell lines, HP62 and TRM6 were the gifts from Dr. Ou (University of British Columbia,
Vancouver, Canada). A collection of 11 other cancer cell lines were also collected before the start of this experiment including those derived from: pancreas (PANC1, HP62, ASPC1, TRM6, and BXPC3), prostate (CH3, LNGαP, and PC3), Nervous system (M17 and Kelly), liver cell (HepG2), leukemia cell (CEM), ovarian cell (SKOV), and Mast cell (MC9c). For the purpose of this study we have focused our attention only on the pancreatic cell lines. Cell lines were maintained in culture condition consistent with the MSCs as described in section 2.2.2 (cultured in minimum essential medium (MEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin- streptomycin solution and incubated at 37°C in a humidified, 5% CO2 incubator). Direct culture in this study implies the direct incubation of naive or engineered MSCs with the cancer cell lines and the indirect culture is by using the conditioned media (CM) obtained from the naive or engineered MSCs. The human glioblastoma cell line DBTRG that is deficient in chromosome 10 was also used in the PTEN studies.

3.2.2 Flow cytometry (FACS) analysis

Expression of TRAIL receptors on the pancreatic cancer cells was detected using FACS. Sub-confluent cells were detached with 0.25% trypsin-EDTA and washed with PBS. A total of 1-5 x 10^5 cells were resuspended in 200 μl PBS for each reaction, and then 10 μl of primary antibody solution (25 μg/ml) (antibodies to human TRAIL death receptors and decoy receptors DR4, DR5, DcR1, DcR2 or isotype IgG, R&D Systems, Minneapolis, MN, USA) was added to each Eppendorf tube and incubated at 4°C for 30 min. The cells were washed three times with PBS and re-suspended in 200 μl PBS. Then, phycoerythrin-conjugated secondary antibody (R&D Systems) was added to each reaction. The cells were incubated for 30 min at 4°C in the absence of light. After being washed 3 times with PBS, cells were resuspended with 500 μl PBS in FACS analysis tubes for analysis (FACSCalibur, Vancouver General Hospital and Child and Family Research Institute). PTEN Intracellular staining was performed via using human PTEN
(PE conjugated) antibody (BD biosciences, Mississauga, Ontario, Canada). Briefly, a total of 1x 10^6 cells/ Eppendorf tube were washed using 1× perm wash (BD biosciences). The cells were thoroughly resuspend in 100 µl of Cytofix/Cytoperm solution (BD biosciences) and incubated for 25 min at 4°C after which they were washed once with perm wash. This followed by 30 min of antibody incubation (5 µl/ 50 µl of perm wash solution). Cells were then washed once again using perm wash and resuspended in 250µl of FACS staining buffer for analysis. For all FACS experiments the same experimental conditions were followed as explained in section 2.2.5.

### 3.2.3 TRAIL- or PTEN- bearing vector transfection of MSCs

TRAIL-bearing expression plasmids were used for the transfection. As described in the our previous report 206, two types of plasmids were used, non-secreting TRAIL (nsTRAIL, Addgene) and secreting TRAIL (stTRAIL, Advanced Protein Technologies, Richmond, Canada). The transfections were performed with TransIT-2020 (Mirus, Madison, WI, USA) as suggested by the manufacturer. Briefly, MSCs were plated at 6 x 10^5 cells per well in six-well plates in 3 ml of MEM medium and dated as day 0. On day 1, the cells were transfected with 2.5 µg of nsTRAIL or stTRAIL plasmid DNA (each well) respectively. Control cells were treated with TransIT-2020 reagent without plasmid DNA. Transfected MSCs were named as MSC_{nsTRAIL} and MSC_{stTRAIL} respectively with a transfection efficiency of ~ 90% although it was not formally quantified. For the direct co-culture experiments, the transfected MSCs were harvested with 0.25% trypsin-EDTA (Invitrogen) on day 2. For the purpose of indirect co-culture and the assessment of TRAIL expression, conditioned media (CM) and transfected MSCs were collected on day 3. Immunoblotting analysis was used to detect the cellular expression of TRAIL in the MSCs, and the soluble TRAIL protein in the culture supernatants was measured using enzyme-linked immunosorbent assay (ELISA).
3.2.3 PTEN-bearing vector transfection of MSCs

PTEN vector was designed with the help of Dr. Lu at the UBC Brain Research Center. Mammalian expression plasmid pDsRed1-N1 was used as the primary structure. As shown in Figure 3.2, an 18 amino acid leading sequence and an 11 amino acid trans-acting activator of transcription (TAT) sequence were inserted at the multiple cloning site followed by human PTEN (403 amino acids). A TAT-PTEN-RFP fusion protein was designated and the predicted molecular weight was around 74kd. Construction of the PTEN vector was verified by DNA sequencing, microscopy, and preliminary immunoblotting analysis of PTEN in MSC cell lysate. Similar transfection protocol was used for PTEN plasmid with a difference of 48hr incubation post transfection.
Cell viability was detected using a live/dead Viability/Cytotoxicity Assay Kit (Invitrogen) as per the manufacturer’s instruction with a slight modification. A total of $1 \times 10^5$ cells were plated onto 24-well plates in 500 μl of MEM medium on day 0. For the indirect co-cultures the media were replaced with 25%, 50% or 100% conditioned media on day 1. On day 3 for the direct and day 4 for the indirect co-cultures, the cultures were washed twice with PBS. Freshly prepared working solution (250 μl per well on the 24-well plates, containing 1 μM calcein AM and 2 μM EthD-1) was then added directly to the cultures and incubated at room temperature for 10 min in the dark.

Half of the concentration used for TRAIL study was used in our preliminary studies of PTEN to avoid losing cells during the washes (250 μl per well on the 24-well plates, containing 0.5 μM calcein AM and 1 μM EthD-1). In the PTEN study, similar data were collected as with
the TRAIL study but the exception of BXPC3, TRM6, PANC1 cell lines. Additionally, pre-labelled CFSE-PANC1 cells and MSC-PTEN-RFP cells were co-cultured together to demonstrate PTEN migratory ability from MSCs to the cancer cell line. Images were taken using a fluorescence microscope (IX71, Olympus, Markham, Ontario, Canada) and the related analysis was performed through ImageJ (provided online by the National Institute of Health of USA).

3.2.5. Automated assessment of cell viability and migration in real time

a) Titration of cancer cells

An initial titration of different cell densities (20,000, 10,000, 5,000, 2,500 cells/well) was performed for each cell line in order to find the “optimal” cell density in this experiment. The ‘optimal’ density was determined by observing the time point for the zenith of the cell proliferation profile. Cells were trypsinized and counted using the trypan blue exclusion method and haemacytometer and then were re-suspended in culture medium. Background measurements were taken from the wells by adding 100 µl of the same medium to the culture plates. A volume of 100 µl of cell suspension was then added to the wells to make a final volume of 200 µl. All cells were allowed to settle at the bottom of the wells at RT for 15 min, and then were incubated at 37°C and 5% CO₂. The impedance signals were recorded every 5 min for the first 25 scans (2h) and every 20 min until the end of the experiment (up to 70h for cell titrations).

b) Cytotoxicity assessment: indirect co-culture

Following 20h as described above, different volumes (50, 100 or 190 µl of 25%, 50% or 100% CM respectively) were removed from each well and replaced with the appropriate volume of CM, and the experiment was run for 72h. The impedance signals were recorded using the
same time intervals for up to 20h. The culture medium was then replaced with CM and a recording performed every 5 min until the end of the experiment (up to 72h).

c) Cytotoxicity assessment: direct co-culture

The procedure prior to 20h remains the same as explained above with the addition of the preparation for the direct culture insert that needs into be prepared a day in advance as well. Cells for the insert were trypsin-digested (Naive MSC or MSC-PTEN cells). They were then suspended in 2 mL of fresh media followed by a cell count. 10 µl of cell suspension at the concentration of $3 \times 10^4$ cells/ per well were seeded in the corresponding wells and 80 µl of cell culture media was added to each well. Controls consisted of wells with no cells seeded on the inserts. After ~24h incubation at 37°C and 5% CO2, inserts were taken to the RTCA unit, and wells were prepared with 130 µl of fresh media before the inserts were slowly lowered to corresponding wells. The recording proceeded on the machine for 72h.

d) Migration capacity of MSCs

The RTCA system was also applied to monitor cell migration using migration plates. Cancer cells were prepared in serum free media (SFM) in a total volume of 160 µl. Cells were then seeded in the lower chamber of plates and left to settle for 30 min at room temperature (RT) in sterile conditions. The upper chamber was then mounted and 25 µl of corresponding media was added to each well and left to equilibrate the RTCA system for 1 h at 37°C and 5% CO2. After incubation, a background reading was taken for each well. As per the manufacturer’s protocol, 40,000 MSCs were prepared in SFM and plated into the upper chamber of the CIM-plates to a total volume of 160 µl. Readings were recorded initially at 25 scans every 5 min and then followed every 10 min until the end of the experiment (up to 24 h).
3.2.6. Statistical analysis

Cell index for real-time dynamic cytotoxicity assessment (N= 3) and slope calculations for the migration assessments were calculated automatically by the RTCA Software Package 1.2. Area under curve (AUC) was calculated using the Trapezoid Rule. Normalizations were performed using the RTCA Software Package 1.2. Numerical data were expressed as mean ± standard deviation. Statistical differences between the means for the different groups were evaluated with Prism 5.0 (GraphPad software, La Jolla, CA, USA) using Student’s t-test with the level of significance at p<0.05 was used in vector design studies. One way ANOVA with Bonferroni correction; p <0.05 was taken as statistically significant.
3.3 Results

3.3.1 TRAIL receptor expression in all collected cells

Four TRAIL receptors were detected by FACS analysis for all screened cell lines and data is shown in Figure 3.3.

Figure 3.3 FACS analysis of the TRAIL receptors across all cancer, transformed, primary and stem cell lines. The FACS analysis is shown for the 4 receptors in the different cell lines. % expression of these receptors: DR4, DR5, DCR1, and DCR2 are displayed above. From left: cells of pancreas (PANC1, HP62, ASPC1, TRM6, and BXPC3), prostate (CH3, LNGαP, and PC3), stem cells (bone marrow MSC, pancreatic ductal MSCs, and HEK), Nervous system (M17 and Kelly), liver cell (HepG2), leukemia cell (CEM), ovarian cell (SKOV), and Mast cell (MC9c)

The following receptors: DR4, DR5, DCR1, and DCR2 are displayed. From left: cells of

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pancreas (PANC1, HP62, ASPC1, TRM6, and BXPC3), prostate (CH3, LNGαP, and PC3), stem cells (bone marrow MSC, pancreatic ductal MSCs, and HEK), Nervous system (M17 and Kelly), liver cell (HepG2), leukemia cell (CEM), ovarian cell (SKOV), and Mast cell (MC9c).

Throughout all the cell lines DR5 appears to have the most profound expression. Ductal-derived MSCs had the highest combine decoy receptor expression compared to the rest of cells. MC9c, SKOV, and M17 displayed the most substantial expression of DR5 compared to other TRAIL receptors they possess. The highest amount of DR4 expression was seen in prostate cancer cell lines with the highest in LNGαP cell line.
3.3.2 TRAIL receptor expression in pancreatic cancer cells

The percentage expression of positive cell population for each individual receptor of pancreatic cell lines is summarized in Figure 3.4.

![FACS analysis of the TRAIL receptors on the pancreatic cancer cells](image)

**Figure 3.4** FACS analysis of the TRAIL receptors on the pancreatic cancer cells. DR4, DR5, DCR1, and DCR2 are shown above. a) The FACS analysis is shown for the 4 receptors in the different cell lines. Y-axis represents FL1H, and x-axis represents FL2-PE-(DR4, DR5, DCR1, DCR2). b) percent expression of these receptors are displayed. The red lines on the left figure represents reference gating determined by the isotype control and live cell population. From left to right the percentages of expression of DR4, DR5, DCR1 and DCR2 (BXPC3: 16.69, 67.25, 6.295, 23.5] TRM6: 0.821, 75.2, 12, 18.6] ASPC1: 3.67, 82.1, 14.5, 4.86] HP62: 4.45, 86.3, 1.21, 49.8] PANC1: 1.48, 1.90, 0.73, 0.80). Reprinted with permission of CGT, Moniri et al. 2012 (in press)

Fig.3.4a represents the expression of TRAIL receptors seen in pancreatic cancer cell lines. Fig. 3.4b demonstrates the TRAIL receptor expression in percentage (from left to right: DR4, DR5, DCR1 and DCR2 (BXPC3: 16.69, 67.25, 6.295, 23.5] TRM6: 0.821, 75.2, 12, 18.6] ASPC1: 3.67, 82.1, 14.5, 4.86] HP62: 4.45, 86.3, 1.21, 49.8] PANC1: 1.48, 1.90, 0.73, 0.80). HP62 had the highest DR5 and DCR2 expressions. BXPC3 and TRM6 displayed the highest and
the lowest DR4 expression accordingly. ASPC1 demonstrated the highest DCR2 expression amongst all 5 cell lines. PANC1 cells displayed the lowest expression for all 4 receptors amongst pancreatic cell lines.

3.3. 3 TRAIL and PTEN vectors

Fig. 3.5 displays the ELISA and immunoblotting analysis of the TRAIL and PTEN vectors.

**Figure 3.5 ELISA & immunoblotting analysis of TRAIL and PTEN engineered MSC culture media.** This work has been published Anticancer. **a)** Cells were seeded on 6-well plates (6 x 10^5 cells/well) on day 0. transfection with different TRAIL-bearing plasmids on day 1. The conditioned media was recovered on day 3 and tested for four independent experiments. **b)** TRAIL analysis in MSC cell lysates. MSCs were harvested on day 3. The left panel showed signals with anti-TRAIL antibody and the right panel displayed signals with anti-GFP antibody on the same blotting membrane. Anticancer Res. 2011 Nov;31(11):3705-12 **c) preliminary Immunoblotting analysis of PTEN in MSC cell lysates.** Cell lysates were harvested from naive MSCs (control), MSC^{PTEN-RFP} (PTEN-RFP) and the same RFP vector-transfected MSCs (RFP). The blotting membrane was hybridized with anti-PTEN antibody. The blue arrow indicates endogenous expressions of PTEN in MSC cells and red arrow points the additional expression of transfected PTEN-RFP fusion protein.

Figure 3.5 a displays the ELISA analysis of the presence of soluble TRAIL in culture supernatants. The TRAIL content in MSC^{stTRAIL} showed more significant expression compared...
with MSC\textsuperscript{nsTRAIL}. Figure 3.5b demonstrates the TRAIL expression in the cell lysate by immunoblotting, the TRAIL-GFP displayed a higher molecular weight due to the fusion of GFP with the TRAIL protein. Figure 3.5c represents the preliminary results of immunoblotting for the MSC-PTEN vector design. The blue arrow indicates endogenous expressions of PTEN in MSC cells and red arrow points the additional expression of transfected PTEN-RFP fusion protein at a higher molecular weight. The fusion protein showed a higher molecular weight of 74kDa in comparison with endogenous PTEN (47kDa).

### 3.3.4 Demonstration of PTEN Trafficking

To simulate the \textit{in vivo} conditions, cancer cells (PANC1) pre-labeled with CFSE were co-cultured with MSC\textsuperscript{PTEN} as seen in Figure 3.6

![Figure 3.6](image)

**Fig.3.6 Demonstration of PTEN-RFP trafficking between MSC and Panc-1 cells.** Panc-1 cells were plated on day 1 and MSCs were seeded into the same plate on day 2. The images were taken on day 3. M: MSC cells transfected with PTEN-RFP (RFP, red fluorescence protein); M: MSC cell transfected with PTEN-RFP but not expressed yet; P: Pancreatic cancer cell (Panc-1) pre-labeled with CFSE (green). The yellow arrows point two cancer cells which contain PTEN-RFP. (Original magnification, 400 x)

RFP labeled PTEN appears to travel into the PANC1 cells as depicted by the 3\textsuperscript{rd} and 4\textsuperscript{th} panel in the figure.
3.3.5 MSC^{TRAIL}-mediated HP62 and PANC1 cell death in direct and indirect co-cultures

Fig. 3.7 displays the MSC-mediated cell death in direct mixed co-culture under different conditions.

![Image showing MSC mediated cell death in mixed co-culture](image.png)

**Figure 3.7 Direct co-culture of MSC, MSC^{nsTRAIL}, and MSC^{STRAIL} at varying concentrations.** This is shown in 2 different MSC/HP62 and MSC/PANC1 ratios. Total of 1 x 10^5 cells/well were seeded into 24-well plates on day 0 and assessed on day 3 with LIVE/DEAD assay. a) HP62 cells co-cultured with naïve MSCs, MSC^{nsTRAIL} and MSC^{STRAIL}. In all cases as the ratio of MSC increased more apoptotic HP62 cells were observed. b) PANC1 cells co-cultured with naïve MSCs, MSC^{nsTRAIL} and MSC^{STRAIL}, in opposite to HP62 cells, in direct combination of naïve MSCs, PANC1 cells displayed a significant rate of apoptotic cells. The treatment with engineered TRAIL did not display a more significant population of dead cells.

Figure 3.7 a) the number of dead cells were much higher in the high MSC/HP62 ratio co-cultures than in the low MSC/HP62 ratio co-cultures for all three types of MSCs, MSC^{STRAIL}, and MSC^{nsTRAIL}. Additional cell death was only observed in the high MSC/HP62 ratio co-cultures and in 75% MSC^{STRAIL}:25%HP62 b) in contrast, PANC1 cells display the highest rate of dead cells in the direct culture with normal MSCs compared to the other conditions. In higher ratio of MSC: PANC1 only MSCs were observed in the field.
The representative images of indirect co-cultures of HP62 and PANC1 cells with conditioned media were shown in Fig. 3.8. HP62 cell death was proportionally related to CM from secreting and non-secreting TRAIL transfected MSCs. Marked cell death was not detected with CM from naive MSCs under the current experimental conditions (top row a & b). On the other hand, in PANC1 cells dead cells were detected from the CM of naive MSCs. There were also faint signals for dead cells from the engineered MSCs.

Figure 3.8 Cell viability assessment on HP62 cells in indirect co-culture. a) As the ratio of MSCsTRAIL was increased the number apoptotic HP62 cells increased accordingly. b) Similar increase was seen in PANC1 cells as the ratio of conditioning media was increased. Important to note that PANC1 cells also displayed apoptotic cells under normal MSC conditioning media.
3.3.6 MSC<sup>PTEN</sup>-mediated cell death in indirect co-cultures

Figures 3.9-11 represent indirect culture of MSC<sup>PTEN</sup> conditioned media at 3 different ratios of 25%, 50%, and 100% CM and the corresponding regular and MSC media controls. HP62 cells had detectable cell death in all 3 different ratios. For ASPC1 cells, it was clear that as the ratio of CM increased, more cell death were observed. It is important to note that some apoptotic cells were also observed under the naive MSC media conditions. Similarly, for DBRTG cells, a significant increase of dead cells was observed in the 100% CM ratio. No significant differences were observed between the 25% and 50% CM ratios of DBTRG cells. It is worth noting that with increase of CM concentration; less attached cells were also observed in all 3 cell lines.

Figure 3.9 Live/Dead Assay: indirect co-culture of MSC<sup>PTEN</sup> conditioned media with HP62 cells. From top to bottom each well of cells were treated using: 100% CM, 50% CM, 25% CM, 100% regular media, and 100% MSC media. Apoptotic cells were seen in all 3 ratios of CM (%25, %50, and %100). Controls did not display significant dead population of cells. Two controls of normal media and naive MSCs CM were also tested. Arrows indicate the red staining of the dead cells. 100× total magnification.
Figure 3.10 Live and Dead Assay: indirect co-culture of MSC\textsuperscript{PTEN} conditioned media with ASPC1 cells. From top to bottom each well of cells were treated using: 100% CM, 50% CM, 25% CM, 100% regular media, and 100% MSC media. As the ratio of MSC\textsuperscript{PTEN} CM %25<%50<%100 was increased more apoptotic cells were observed. There were also some dead cells in the wells corresponding to naive MSC’s conditioned media. Two controls of normal media and naive MSCs CM were also tested. Arrows indicate the red staining of the dead cells. 100× total magnification.
Figure 3.11 Live and Dead Assay: indirect co-culture of MSC\textsuperscript{PTEN} conditioning media with DBRTG cells. From top to bottom each well of cells were treated using: 100% CM, 50% CM, 25% CM, 100% regular media, and 100% MSC media. As the ratio of MSC\textsuperscript{PTEN} CM was increased %25<%50<%100 more apoptotic cells were observed. There were also few dead cells in the wells corresponding to regular media. Two controls of normal media and naive MSCs CM were also tested. Arrows indicate the red staining of the dead cells. 100× total magnification.

Figure 3.12 display the preliminary analysis of figures 3.9-3.11. Top panel represents the total number of cells counted in each field for HP62, ASPC1, and DBRTG cells which were kept above 250 cells per field. DBRTG displayed the highest percent dead cells of 20% that was followed by 8% in HP62 cells and 2% in ASPC1 cells when treated by 100% CM. As the ratio of CM decreased, the percent dead cells detected were also decreased.
3.3.7 Real time detection of cellular adherence and titration

Four densities of each pancreatic cell type were titrated and shown in Figure 3.13. Based
on the results of the titration experiment, the applied cell density was defined as 20,000 cells/well, and 20h was determined as the time point for the cytotoxic treatment. As the ratio of the cells/well was increased, the resulting graph depicted corresponding lines with increased CI values. Both HP62 and PANC1 cells reached the zenith of the graphs at 10-20,000 cells/well.

Figure 3.13 Titration of the cancer cells using the RTCA system. From left to right, top row: HP62 cells, BXPC3 cells, middle row: PANC1 cells and TRM6 cells. The bottom figure is ASPC1 cells. All cells were titrated from 20,000 cells to 2500 by a dilution factor of 2 and are the average of 2 repeated trials. The bars indicate the standard deviation.
Figure 3.14 summarizes the 20,000 cells per well density of each of the cancer cell lines. HP62 cells zenith in CI value at ~48hr, PANC1 cells at ~60hr, and the BXPC3, TRM6, were not determined during the 72hr incubation time.

3.3.8 Dynamic observation of MSC migration toward pancreatic cancer cells

Figure 3.15 displays the migration induced kinetics of MSCs toward each of the pancreatic cancer cell lines.
Figure 3.15 Migration kinetics of MSCs to cancer cells. MSCs (40,000/well) were seeded into insert wells on CIM-Plate 16 and pancreatic cancer cells (20,000/well) were plated at the bottom of the transwell. Results shown are mean ± s.d. (N = 3) were compared with human fibroblast cells (A1F8) as a reference of normal human cells. A dramatic increase in CI was indicating migration capacity of MSCs to the cancer cells in comparison to the normal human cells. Reprinted with permission of CGT, Moniri et al. 2012 (in press)

Figure 3.15 displays the migration induced kinetics of MSCs toward each of the pancreatic cancer cell lines. All pancreatic cancer cells induced MSC migration compared with normal fibroblast human cell controls. In the indirect use of cancer cell’s CM, the most active cell migration was observed during the first 3 hours.

The chemotactic effects on MSCs of conditioned media from each cancer cell are shown in Figure 3.16. Data presented as slope (1/hr) for the first 3 hours shows that conditioned media from all tested pancreatic cancer cells induced significantly higher rates of MSC migration compared with regular media control. Conditioned media from BXPC3 displayed the most significant migratory rate in comparison to the CM of other cancer cells.
3.3.9 Real time detection of CM-induced cytotoxicity using TRAIL engineered MSCs

Based on the results of titration test, the applied cell density was set at 20,000 cells/well, and 20 h was determined as the time point for the treatment. Figure 3.17 presents the real-time dynamic changes of Cell Index (CI) with the application of various CM concentrations for all studied cell lines. The corresponding calculation of area under curve for these types of cells was summarized in Figure 3.18 where it is shown that ASPC1, TRM6, and HP62 cells displayed significant reduction in cell viability of CM treated groups compared to controls. There was no significant inhibition of BXPC3 cell viability during the observation period. However, these cells responded at a later time beginning at approximately 50h post application of CM (data not shown).
Figure 3.17 Real-time monitoring of conditioned media-induced cytotoxicity in pancreatic cancer cells. Cells (20,000/well) were seeded in E-Plate 16 with normal MEM medium. Cell toxicity was monitored using the RTCA instrument. After ~20h, culture medium was replaced with CM from MSC_{TRAIL} or CM from MSC_{nTRAIL}. Arrows indicate the time points of CM replacement and the density of the colors represents the different CM concentrations, i.e. 100%, 50% and 25%. Top to bottom and left to right panels: BXPC3, HP62, ASPC1, PANC1, and TRM6 cell lines are shown. All cells except PANC1, displayed a decrease in CI value when treated at higher doses of CM from MSC_{TRAIL}. Graph displays the indirect cytotoxic effects of CM from engineered and naive MSCs on the cancer cells. Reprinted with permission of CGT, Moniri et al. 2012 (in press)
Figure 3.18 Area under the curve (AUC) analysis of RTCA cytotoxicity data. The controls show the values obtained from the treatment of CM from naive HPMSCs and normalized with the values from regular normal medium. * p<0.05, ** p<0.01, *** p<0.001, One way Anova, Bonferroni's correction. N=3 ± s.d. Black lines represents the comparisons of regular MSC CM with MSC_{stTRAIL} or MSC_{nsTRAIL} CM. Red lines represent the comparison of the same concentration of CM between the MSC_{stTRAIL} or MSC_{nsTRAIL}. Reprinted with permission of CGT, Moniri et al. 2012 (in press)
3.3.10 Real time detection of CM-induced cytotoxicity using PTEN engineered MSCs

Figure 3.19 displays preliminary RTCA results of indirect co-cultures using CM of MSC\textsuperscript{PTEN} on pancreatic cancer cell lines: BXPC3, PANC1, HP62, and ASPC1.

Figure 3.19 Preliminary dynamic real time indirect co-culture of MSC\textsuperscript{PTEN} conditioning media with Cancer cells. Cells (20,000/well) were seeded in E-Plate 16 with normal MEM medium. Cell toxicity was monitored using the RTCA instrument. After ~20h, culture medium was replaced with CM from MSC\textsuperscript{PTEN}. Arrows indicate the time points of CM replacement and the density of the colors represents the different CM concentrations, i.e. 100%, 50% and 25%. Top to bottom and left to right panels: BXPC3, ASPC1, PANC1, and HP62 cell lines are shown. Each line represents an average of N=2±s.d (shown as dotted lines).

BXPC3 cells display the cytotoxic effects of the CM at higher concentration (100%CM) and at much later time during incubation ~59hr. ASPC1 however displays the first sign of being affected via CM at ~35hr. PANC1 cells also display CI reduction at ~32hr and HP62 cells display the reduction in CI at ~42h. Cytotoxic effects of the CM is directly proportional to the
ratio of CM used on all of the cells as depicted by reduction of the CI value.

Figure 3.20 shows the area under the curve calculated from the results of Figure 3.19. ASPC1 and HP62 cells displayed a reduction in AUC compared to the control. PANC1 cells displayed a gradual decrease in AUC as the CM was increased. BXPC3 cells didn’t show any difference between CM treated runs and the controls.

Figure 3.20 Area under the curve (AUC) analysis of RTCA cytotoxicity data for MSC\textsuperscript{PTEN}. From left to right, the AUC for BXPC3, ASPC1, PANC1, and HP62 cells is calculated. All cells except BXPC3 displayed reduction in AUC when treated with the MSC\textsuperscript{PTEN} CMs. N=2 ± SEM.

3.3.11 Real time detection of cytotoxicity of direct co-culture of naive MSC with cancer cells

Figure 3.21 displays the preliminary RTCA results of direct co-culture of naive MSCs on pancreatic cancer cells (BXPC3, PANC1, HP62, and ASPC1) and DBRTG control.
Figure 3.21 Preliminary dynamic real time cytotoxic assay: direct co-culture of naive MSCs with cancer cells. Cells (20,000/well) were seeded in E-Plate 16 with normal MEM medium. Naive MSC (30,000/well) were seeded in specialized inserts. After ~20 h, culture medium was replaced with inserts containing naive MSC. Arrows indicate the time points of insert addition. Top to bottom and left to right panels: BXPC3, ASPC1, HP62, PANC1 and DBTRG cell lines are shown. Each line represents an average of N=3± s.d (shown as dotted lines). Control wells represent no cells plated on the insert.
BXPC3 cells displayed a higher CI value from naive MSC treated wells compared to the controls. No differences in CI values were observed in the ASPC1 cells. HP62 cells displayed a minor shift to the left in resulted the apex to form at ~40h compared with the control at ~42hr indicating some cytotoxic effect from naive MSCs. PANC1 cells displayed the most significant decrease in CI value when in directed culture with naive MSCs compared to the controls. No significant difference was observed in DBTRG cells between control and naive MSCs treatment. The decrease in CI value is analyzed via AUC of all cells in figure 3.22. This analysis yielded significant reduction in AUC of BXPC3, HP62, and PANC1 cells when in direct culture with naive MSCs compared to the control.

**Figure 3.22 Area under the curve (AUC) analysis of Naive MSC direct culture with cancer cell lines.** From left to right, the AUC for BXPC3, ASPC1, HP62, PANC1, and DBTRG cells is calculated. BXPC3, HP62, and PANC1 cells displayed significant reduction in AUC of cells cultured with naive MSCs compared to the control. ASPC1 and DBRTG cells didn’t show any significant difference between the two groups. N=3 (PANC1 N=2) ± SEM. Student’s t-test p <0.05.
3.3.12 Real time detection of cytotoxicity of direct co-culture of MSC<sup>PTEN</sup> with cancer cells

Figure 3.23 displays the preliminary RTCA results of direct co-culture of MSC<sup>PTEN</sup> on pancreatic cancer cells (BXPC3, PANC1, HP62, and ASPC1) and DBRTG control.

**Figure 3.23 Preliminary dynamic real time cytotoxic assay: direct co-culture of MSC<sup>PTEN</sup> with Cancer cells.** Cells (20,000/well) were seeded in E-Plate 16 with normal MEM medium. MSC<sup>PTEN</sup> (30,000/well) were seeded in specialized inserts. After ~20h, culture medium was replaced with inserts containing MSC<sup>PTEN</sup>. Arrows indicate the time points of insert addition. Top to bottom and left to right panels: BXPC3, ASPC1, HP62, PANC1 and DBTRG cell lines are shown. Each line represents an average of N=3±s.d (shown as dotted lines). Control wells represent no cells plated on the insert.
All of the cells displayed reduced CI values except ASPC1 cells. BXPC3 displayed reduction in CI values at ~35hr. HP62 cells also had a shift of apex to the left and cytotoxic effects were seen in them at ~55hr. PANC1 cells had the most dramatic reduction in CI values starting at ~20hr. DBRTG was then next with what it appears to be the second most significant drop in CI values in comparison with the control wells at ~40hr. Figure 3.24 summarizes the analysis of AUC for figure 3.23. BXPC3, HP62, PANC1, and DBRTG displayed a reduction in AUC calculated. However, only PANC1 and HP62 displayed significant difference when compared to the controls.

Figure 3.24 Area under the curve (AUC) analysis of MSC<sup>PTEN</sup> direct culture with cancer cell lines. From left to right, the AUC for BXPC3, ASPC1, HP62, PANC1, and DBTRG cells is calculated. Both HP62 and PANC1 cells displayed significant reduction in AUC of cells cultured with MSC<sup>PTEN</sup> compared to the control. BXPC3 and DBRTG cells displayed reduction in AUC but no significant difference was detected between the two groups. ASPC1 cells displayed small increase in AUC when in culture with MSC<sup>PTEN</sup> compared to the control. N=3 ± SEM. Student’s t-test p <0.05.
3.3.13 FACS intracellular staining of PTEN for cancer cells and MSCs:

Figure 3.25 displays the intracellular staining for PTEN protein across the tested cell lines of HP62, PANC1, BXPC, MSC, and DBRTG.

![Figure 3.25 PE-PTEN intracellular staining using FACS. From top to bottom: HP62, PANC1, BXPC3, MSC, and DBRTG cells were tested. Positive expression was recorded at 15000 events per test for 19% in PANC1, 5.6% in MSCs, 27.9% in BXPC3, 1.38% in HP62 cells when compared to the unstained and isotype controls.](image-url)
BXPC3 cells displayed the strongest basal PTEN expression compared to all cells. This was followed by PANC1, MSCs, and HP62 cells. No positive detection was observed in DBRTG cells.
3.4 Discussion

Sadly, pancreatic cancer often presents with locally advanced stage (up to 50%), and metastatic disease (up to 40%) at the time of diagnosis, thus dramatically reducing the chance of curative resection. Conventional therapies such as surgical resection, chemo- and radiotherapy have some efficacy; however, they result in a high recurrence rate and reduce quality of life. Much attention is deserved to forming a new strategy in dealing with this disease. With the discoveries of new anticancer genes and the identification of tumor tropism of MSCs, a new therapeutic strategy is promising. Our proposed therapy combines these modalities, with use of MSCs as vehicles, and regular plasmids as vectors, to deliver anti-cancer agents to the site of cancer cells.

Two anti-cancer genes of choice examined in this study are TRAIL and PTEN. TRAIL and its receptors are important components of the extrinsic pathway of apoptosis. DR4 and DR5 possess death domains and thus with binding of the TRAIL ligand they could make the cell undergo apoptosis through the activation of downstream caspase cascades. Decoy receptors DcR1, DcR2 and OPG receptors do not possess an internal death domain, although they could compete with the death receptors for binding with TRAIL ligand and/or producing homeostasis in the cellular microenvironment\textsuperscript{225}. Sheridan et.al (1997) explained that overexpression of the decoy receptors could prevent apoptosis induced via TRAIL in some cells\textsuperscript{226}. Death receptors (DR4 and DR5) are frequently expressed in a variety of cancer and transformed cells\textsuperscript{59,227}. On the other hand, PTEN functions via the intrinsic pathway and regulates cellular capacity to proliferate through the PI3K-AKT-mTOR pathway. As a main regulatory molecule in this pathway, PTEN’s down-regulation or activation could play a vital role in carcinogenesis and cell

\textsuperscript{7} A version of this work has been published in Cancer Gene Therapy (CGT): Moniri et.al 2012(in press). Reprinted with permission from CGT
death accordingly.

High levels of DR5 were identified in human pancreatic adenocarcinoma (BXPC3 and ASPC1), insulinoma (HP62), and SV40 viral DNA-transformed human islet cell line (TRM6). However, contrary to an earlier report by Stadel et al. (2010) human pancreatic epithelioid carcinoma cell line (PANC1) showed remarkably low expression of death receptors\textsuperscript{227}. This could be due to different sources of the cell line, culture media, or antibody used between these studies. Different patterns of TRAIL receptor expression could help us in identifying the effectiveness of our therapeutic strategy. For instance, given a low expression of TRAIL receptor, we can hypothesize a lower response to our proposed gene therapy. Other studies by Jacob et al. (2005) and Lemke et al. (2010) have confirmed TRAIL-induced apoptosis in pancreatic cancer cells\textsuperscript{228,229}. Mohr et al. (2010) demonstrated the use of MSCs as an anti-cancer gene delivery system on pancreatic neoplasms\textsuperscript{230}. Although cells undergo apoptosis through the TRAIL pathway by engaging death receptors on their surface\textsuperscript{58}, the presence of such receptors does not guarantee response to MSC-TRAIL mediated targeting. More in depth analysis is required in order to fully elucidate this therapeutic strategy. Factors such as: transfected anticancer genes, number and ratio of MSCs used, type of co-culture, and the intrinsic capacity of MSCs affects the interactions between MSC and tumor microenvironment. For example, these studies show that naive MSC-induced low cell death was observed at low ratio of MSC/HP62 co-cultures, while remarkable cell death was exhibited in the high MSC/HP62 ratio co-cultures. This effect was potentiated in PANC1 cells (Figure 3.7). The results indicated that MSCs alone play an important role in this MSC-mediated HP62 cell and PANC1 cell death, although the cell type of the dead cells could not be identified in the current study. In our direct co-culture of cancer cells and naive MSCs using RTCA, intrinsic tumorigenic effects of MSCs were observed, with PANC1 cells responding the most to such
treatment. Similar observations were seen in DBRTG, BXPC3, and HP62 cell lines but in less strength as described earlier (Figure 3.21 and Figure 3.22). This intrinsic capacity of MSCs to kill tumors was formerly identified in studies with experimental Kaposi’s sarcoma\textsuperscript{142}, hepatocellular carcinoma\textsuperscript{195}, and colorectal carcinoma\textsuperscript{231}. Recently, we have published similar observations using hematoma cancer model (HepG2 cells)\textsuperscript{114,206}. Additional MSC\textsuperscript{TRAIL}–induced cell death was observed in areas adjacent to the MSCs which was also consistent with our recent report on liver cancer\textsuperscript{206}. This observation could be the result of MSC tumor tropism. Cancer cell-induced chemo-atraction on MSCs was discussed earlier and the role of the cell-to-cell interactions along with other soluble factors that resulted in MSC’s tumor direct migration were discussed.

MSC\textsuperscript{stTRAIL} appeared to have a more profound deleterious impact on cancer cells than the MSC\textsuperscript{nsTRAIL}. This was further demonstrated in our co-culture studies in real time (Figure 3.17) or via live and dead assays (Figure 3.7 & 3.8). Similar observation was also seen in our recent work on liver cancer model\textsuperscript{206}. Although live and dead assay appeared to be a functional assay, the drawback of using this assay is that it only applies to the cells that stay on the culture surface during the staining. The detached cells, most of which may be dead cells, are not included in the assessment unless further FACS optimization is performed. An alternative approach that eliminates those pitfalls is to use the label-free assessment of cells via the impedance-based RTCA system. In our real time study CM-induced cytotoxic effect of engineered MSCs (Figure 3.17 & 3.18) were observed on ASPC1, TRM6 and HP62 cells during the period of observation. As the concentration of CM of MSCs\textsuperscript{stTRAIL} was increased, a more potent effect on the cancer cells was observed recorded by a reduction in CI values compared with controls and the CM derived from MSC\textsuperscript{nsTRAIL}. BXCP3 cells were less affected during the same time course, but cytotoxic effects of engineered MSCs can be observed from 80-95h (data not shown, in order to
keep consistent with other cell’s time lines). This observation could be the result of the role of expression of decoy receptors in this cell line. Accounting for the low TRAIL expression receptors seen in PANC1 cells, there may be little difference between the two CM-induced cytotoxicities. However, these cells demonstrated the most sensitivity to naive MSC treatment as discussed earlier. *In vivo* studies are required to investigate the therapeutic potential of engineered MSCs in a real microenvironment of pancreatic cancer.

In conclusion, induction of TRAIL anticancer gene in MSCs can play a critical role in TRAIL-mediated killing of cancer cells that express death receptors. To validate this therapeutic approach in a clinical setting, TRAIL receptor expression profiles should be quantified to determine feasibility of therapy using TRAIL anticancer gene. Moreover, studies suggest that a number of cancer cells of malignant nature are resistant or have gained resistance to apoptosis induced via TRAIL. Mueller *et.al* (2011) recently published data on colorectal carcinoma which demonstrated that TRAIL-transfected MSCs overcome TRAIL resistance in selected cell lines when treated with TRAIL-engineered MSCs through direct intercellular interactions.

In summary the anticancer properties of MSCs demonstrated an intrinsic anticancer effect and TRAIL-engineered MSCs induced specific tumor cell apoptosis. Engineered MSCs combined with the use of RTCA, can most effectively and efficiently identify anticancer gene suitable for treatment of specific cancers. As hypothesized in our recent reviews, the present study could provide a platform towards the development of personalized cancer therapy for pancreatic cancer patients.

With the final goal of using multiple anticancer genes as an optimized therapeutic approach for treating pancreatic cancer patients, we have also examined applications for PTEN. Since some cancer cells do not respond to TRAIL or develop resistance to TRAIL therapy,
preliminary studies using MSC\textsuperscript{PTEN} as an alternative to MSC\textsuperscript{TRAIL} have been performed. PTEN is known to be largely down-regulated in most human cancer cells; mechanistic workings of PTEN inside of cells were described in section 1.2. For instance, the expression of PTEN that is observed in KRAS murine model of pancreatic cancer, demonstrated that PTEN loss to induction and acceleration of pancreatic cancer development\textsuperscript{87}. Our preliminary work suggests that MSC\textsuperscript{PTEN} might be used to potentiate the MSC\textsuperscript{TRAIL} effects to induce apoptosis in cells that do not respond to our TRAIL alone therapy.

In anticipation of a TAT-PTEN vector, it is necessary to establish a method for the PTEN protein to be able to reach inside the cell where its actions are observed. Interestingly, our PTEN results in direct and indirect co-culture studies (Figure 3.19, 3.20, 3.23, 3.24) demonstrated that PANC1 cells which were not sensitive to TRAIL have shown a significant decrease in CI values in either that demonstrates the cytotoxic potential for MSC\textsuperscript{PTEN}–induced cell apoptosis. To draw a more comprehensive conclusion these data have been analyzed further via area under the curve calculations which yielded a better understanding of our current results. Our studies using the live and dead assay demonstrated a weak signal for the dyes which can be explained by the lower dose of staining used as compared with MSC\textsuperscript{TRAIL} study. DBTRG cells which lack chromosome 10 that contains the genes for PTEN have demonstrated tremendous response in higher ratios of CM from MSC\textsuperscript{PTEN} (Figure 3.11 and Figure 3.12) and also early response in our direct co-culture study. Surprisingly, ASPC1 cells only showed response in our indirect study of using CM from MSC\textsuperscript{PTEN} both in real time and microscopy analysis and no response was recorded in our direct co-culture in real time analysis. Similar patterns were seen in BXPC3 cells as in our TRAIL study which showed responses at a much later time than other cancer cells, which could indicate the cell density dependant nature of treatment in these cells. HP62 cells did not display a greater response in the PTEN study compared to the TRAIL study. Leading to conclusion that HP62
cells appear to be more responsive to TRAIL than PTEN therapy. Preliminary basal screening of cancer cells for PTEN expression (Figure 3.25) further suggests that cells such as HP62 and DBRTG which had low intrinsic PTEN expression level do respond to our proposed strategic therapy; Further, PANC1 cells which have a relatively higher levels of basal PTEN expressions also displayed significant response to our proposed therapeutic strategy of using PTEN as an anti-cancer agent in comparison to the TRAIL study. Although BXPC3 cells have high basal expression levels of PTEN, they didn’t displayed a significant response in our study leading to a conclusion that there might be other mechanisms involved in these cells that prevent them from going through apoptosis via the PI3K/AKT/mTOR pathway. To further our understanding on this topic, a future experiment detection the PTEN levels pre- and post-treatment should be performed to solidify our understand on the uptake rate of PTEN from each cell during the therapy.

In conclusion, we have designed a unique PTEN vector using trans-activator of transcription together with transfected MSCs. We have demonstrated that MSCs have the potential to produce PTEN and deliver it locally to our cells (Figure 3.6). This was further emphasized in our preliminary results of co-cultures of MSC\textsuperscript{PTEN} and its CM with various cancer cells. Although further analysis and more in-depth investigations are required to expand our understanding as to the extent of this therapeutic approach, the initial results are promising. This could provide a platform for multi-gene therapy of pancreatic cancer using MSCs as a vehicle of delivery.
3.5 Summary & Conclusions

We have uniquely isolated and characterized human ductal pancreatic MSCs. We have provided some evidence of the homing capacity of MSCs to the site of pancreas and their capacity for tumor-directed migration. Furthermore, the MSCs’ tumor migration and homing capacity may enable us to deliver specific anti-cancer genes locally and constantly. The impedance-based RTCA system was used in the present study to continuously evaluate cell viability and cell migration in real time. We have successfully used these MSCs as a vehicle to deliver TRAIL and PTEN to pancreatic cancer cell lines and induce cytotoxic effects on them. MSC-mediated cell death is potentiated in these studies by TRAIL gene transfection on death receptor-expressing pancreatic cancer cells and by PTEN on most cells plus PANC1 cells that was not able to undergo cell death via TRAIL therapy. The intrinsic properties of mesenchymal stem cells could play an important role in the induction of pancreatic cancer cell death under co-culture conditions. This study provides a practical platform for the development of MSC-based and personalized treatment of pancreatic cancer. Furthermore, the ultimate goal of this project is to engineer multiple anti-cancer genes onto MSC carriers thus potentiating a more potent injury to the cancer cells. These studies demonstrate a promising influence of MSCs on pancreatic tumor tissues, and merit further investigation of the properties and characteristics of these strategies to control pancreatic cancer.
Future Directions

Some future directions that should be pursued are summarized below. In our homing and distribution studies of MSCs, we aim to quantify human DNA found in different organs in our tail injection MSC distribution model using real time PCR analysis. Specimens collected post-injection of MSCs from different organs of NSG mice needs to be labelled via human MSC specific antibody for further quantification. An increase in sample size and using the real time PCR primers could help us quantify our observations more and to confirm our current understanding and solidify our results.

Future studies for chapter 3 should include the completion of the assessment of MSC^{PTEN} in culture, under different models of direct and indirect methods using the cancer cell lines and analysis of the current data. Our next goal would be to attempt to combining two anti-cancer genes (PTEN and TRAIL) into a single vector that could function both intrinsically and extrinsically in potentiating apoptosis in pancreatic cancer cells. Characterization of the conditioned media from the cancer cells by mass spectroscopy can determine the constituents of their media to further our understanding of MSC’s tumor tropism properties. A direct co-culture study of MSC^{TRAIL} with pancreatic cancer cells using RTCA can help us understand the role of MSC^{TRAIL} in direct culture conditions. We aim to determine the viability of dual vector engineered MSCs and their transfection efficiency. Given the opportunity we have for acquiring primary human pancreatic cancer tissue from the new facility at Vancouver General Hospital’s pathology department, we would propose to test the effect of our engineered MSCs on samples of human pancreatic cancer tissues in real time. Lastly, the effects of both TRAIL and PTEN on MSC migration capacity should be tested using the RTCA machine.

Additionally, we have planned to focus our attention on expanding our in vivo models
that could contribute immensely to our understanding of our proposed therapeutic approach for the treatment of pancreatic cancer. Initially, we would like to examine the effect of injection of both naive and engineered MSCs in our NSG mice subcutaneous cancer model using both adenocarcinoma and insulinoma cells. Furthermore, we will expand our experiments with two *in vivo* models of spontaneous cancer forming of both insulinoma (Rip Tag 2) and adenocarcinoma (KRAS) to further test the effectiveness of our MSC-based therapy. To conclude, given the successful and positive results by the end of such studies the ultimate goal would be to translate this research into a clinical trial.
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117