January 2013

A Three Dimensional Scaffold for Anticancer Drug Development

Yvonne Girard
University of South Florida, girard.yvonne@yahoo.com

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the Medicine and Health Sciences Commons

Scholar Commons Citation

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
A Three Dimensional Scaffold for Anticancer Drug Development

by

Yvonne Girard

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Department of Molecular Medicine College of Medicine University of South Florida

Major Professor: Subhra Mohapatra, Ph.D.
Robert Deschenes Ph.D.
Burt Anderson Ph.D.
Srinivas Bharadwaj Ph.D.

Date of Approval:
October 16, 2013

Keywords: Biopsy, chemosensitivity, electrospinning, epithelial mesenchymal transition, inhibitors

Copyright © 2013, Yvonne Girard
Dedication

I dedicate this work to my beloved family. Their love, support and understanding have made this journey possible. To my husband Chris Girard for his sense of humor, endless patience and unwavering support. Thank you for being my biggest fan, voice of reason counselor and a steadfast shoulder during the challenging times. To my sons Angelo and Joel whose dedication to serving their country and humanity continues to inspire me. To my daughters Danya, Andrea and Renee for their strength, courage and devotion to God and family, and to my grandchildren Leila, Jayden and Zion who have brought great joy and happiness into our lives.
Acknowledgments

This dissertation reflects the generosity and kindness of many people I have met during my graduate studies. It is with immense gratitude that I acknowledge the support and encouragement of my major Professor Dr. Subhra Mohapatra whose rigorous scholarship and challenging mentorship have made this work possible. I am appreciative of my committee members Dr. Robert Deschenes, Dr. Burt Anderson and Dr. Srinivas Bharadwaj for their encouraging words, thoughtful criticisms and for taking the time out from their busy schedules to guide me. Special thanks to Dr. Shyam Mohapatra and Dr. Gary Hellermann for their valuable guidance and advice over the years. Thanks to Dr. Shekhar Bhansali for encouraging me to take the engineering courses that would later contribute to the development of this project, Dr. Arun Kumar for introducing me to nanotechnology and Mr. Bernard Batson for his mentorship. Most importantly I would like to thank my lab family for the laughter and camaraderie and for their support and contribution to this project. Special appreciation to Dr. Chunyan Wang for her assistance in the chemical optimization of the scaffold. Her expertise and knowledge contributed significantly to this project. Thanks to Dr. Jaya Mallela and Sowdharya Ravi for their assistance with immunofluorescence staining and animal work, Mark Howell for his assistance in the construction of the scaffold, Mahmood Alibrahim for assisting with the construction of the films and Ryan Green for assistance with the biopsy experiments. They are the best group of people I have ever worked with. Thanks to my supportive, forgiving and generous friends, Dr. Shara Pantry and Dr.
Ellissa Parker-Athill for always being there when I needed them. I would also like to thank the staff of the Lisa Muma Weitz Microscopy Core Lab. To Amanda Garces for teaching me everything I know about electron microscopy and Dr. Byeong Cha for teaching me confocal microscopy. Thanks to Carl Adams in the Department of Electrical Engineering at USF for assisting in the construction of the electrospinner, and to IGERT and the Sloan foundation for their financial support.
Table of Contents

List of Figures ........................................................................................................................................ vi

List of Tables .......................................................................................................................................... vii

Abbreviations ......................................................................................................................................... ix

Abstract .................................................................................................................................................. xi

Chapter One .......................................................................................................................................... 1
  1.1 Overview of the Drug Discovery Program ..................................................................................... 1
  1.2 The cost of drug development ........................................................................................................ 2
  1.3 2D versus 3D culture systems ......................................................................................................... 3
  1.4 Types of 3D cultures in cancer research ......................................................................................... 5
      Fibrous scaffolds ............................................................................................................................... 6
      Hydrogels ......................................................................................................................................... 7
  1.5 Tumor spheroids ............................................................................................................................ 8
  1.6 Methods for spheroid production ................................................................................................. 10
      Forced Floating method .................................................................................................................. 10
      Hanging drop method .................................................................................................................... 10
      Spinner flasks method .................................................................................................................... 11
      Microfluidic cell culture platforms ................................................................................................ 11
      Commercial systems ...................................................................................................................... 12
  1.7 Limitations of spheroid systems ..................................................................................................... 12
Chapter Two: Design and construction of the novel 3D scaffold ................. 14

2.1 Introduction ............................................................................................................. 14

2.2 Electrospinning ...................................................................................................... 17

Polymers used in electrospinning ............................................................................. 20
Uses of electrospun fibers ......................................................................................... 21
Synthetic polymers used to make the scaffolds ......................................................... 22
  Poly(lactic-co-glycolic acid) PLGA ........................................................................... 22
  Poly(L-lactic acid) (PLA) .......................................................................................... 23
  Poly-ethylene glycol (PEG) ....................................................................................... 24
  Chitosan .................................................................................................................... 25

2.3 Methods .................................................................................................................. 26

Construction of the PLGA scaffold ....................................................................... 26
Scanning electron microscopy (SEM) ...................................................................... 26
Determination of pore size ....................................................................................... 27
Cell culture .................................................................................................................. 27
Immunofluorescence staining ................................................................................... 28
Celltracker staining ................................................................................................... 28
Synthesis of methoxy PEG-PLA copolymer ........................................................... 29
Fourier transformed infrared spectroscopy (FTIR) .................................................. 30
Proton nuclear magnetic resonance spectroscopy (1H NMR) .................................. 30
Construction of the scaffolds ................................................................................... 31
Construction of chitosan composite and PLGA/PEG scaffolds ................................ 31
Construction of films ................................................................................................. 32
Migration assay ................................................................. 32
Tumouroid diameter and number estimation .......................... 32
Statistical analysis .............................................................. 33

2.4 Results ......................................................................... 33
Growth and proliferation of cells on PLGA scaffold ................. 33
Characterization of the mPEG-PLA polymer ......................... 35
The scaffolds ...................................................................... 37
Growth of tumouroids on the 3P scaffold ............................... 38
Tumouroid sizes and numbers .............................................. 40
Long term tumouroid culture ................................................ 41
Parameters for tumouroid formation ..................................... 45
Effects of topography on tumouroid formation ....................... 47
Effects of charge on tumouroid formation ............................. 47
Cell migration from tumouroids onto tissue culture plate ......... 51

Chapter Three: Tumouroids grown of 3P scaffolds mimic in vivo tumorigenesis .......................... 52

3.1 Introduction .................................................................... 52
Epithelial mesenchymal transition (EMT) ............................... 54
Loss of E-cadherin expression is the hallmark of EMT .......... 54
EMT and the tumor stroma .................................................. 55
EMT and stem cells ............................................................. 57

3.2 Methods ....................................................................... 57
Immunofluorescence staining .............................................. 57
3.3 Results ........................................................................................................................................ 58
Tumoroid formation induce EMT ........................................................................................................ 58
Timeline of EMT ................................................................................................................................ 60
EMT in other tumoroids ...................................................................................................................... 60

Chapter Four: The 3P scaffold can be used as a platform to assess chemotherapeutic efficacy and for the growth of tumor biopsies .................. 64
4.1 Introduction .................................................................................................................................. 64
Antiproliferative drugs LY294002 and U0126 ................................................................................. 66
4.2 Methods ....................................................................................................................................... 67
Inhibition of tumoroid formation and IC50 determination ................................................................. 67
Treatment of established tumors with inhibitors ............................................................................... 67
CellTracker staining ............................................................................................................................ 68
Culturing fine needle aspirates of implanted mouse tumors ........................................................... 68
Immunofluorescence staining ............................................................................................................ 69
Approval declaration for animal studies ......................................................................................... 69
Statistical analysis ............................................................................................................................... 70
4.3 Results .......................................................................................................................................... 70
Prevention of tumoroid formation by inhibitors .............................................................................. 70
IC-50 determination ............................................................................................................................ 70
Treatment with LY294002 inhibitor ............................................................................................... 71
Treatment with U0126 inhibitor ........................................................................................................ 73
Diffusion of doxorubicin into 3P tumoroids ...................................................................................... 74
Treatment with inhibitors abrogate EMT .......................................................................................... 77
Growth of biopsy tumoroids ................................................................. 78
Presence of tumor stroma in biopsy tumoroids .................................... 78
Chemosensitivity of tumor biopsy grown on 3P scaffolds ......................... 78

Chapter Five: Discussion and conclusion ............................................. 83

Chapter Six: Future Work ..................................................................... 90

References .......................................................................................... 92

Appendix: Copyright permission ..........................................................114

About The Author .................................................................................. End Page
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The Electrospinning equipment</td>
<td>18</td>
</tr>
<tr>
<td>2.2</td>
<td>Schematic of the electrospinning process</td>
<td>19</td>
</tr>
<tr>
<td>2.3</td>
<td>Poly(lactic-co-glycolic acid)</td>
<td>23</td>
</tr>
<tr>
<td>2.4</td>
<td>Poly(lactic acid)</td>
<td>23</td>
</tr>
<tr>
<td>2.5</td>
<td>Polyethylene glycol</td>
<td>24</td>
</tr>
<tr>
<td>2.6</td>
<td>Chitosan</td>
<td>25</td>
</tr>
<tr>
<td>2.7</td>
<td>Schematic of the synthesis of mPEG-PLA copolymer</td>
<td>30</td>
</tr>
<tr>
<td>2.8</td>
<td>PLGA scaffolds</td>
<td>34</td>
</tr>
<tr>
<td>2.9</td>
<td>Growth of mesenchymal stem cells on PLGA scaffolds</td>
<td>35</td>
</tr>
<tr>
<td>2.10</td>
<td>Growth of PC3 and NIH3T3 cells on PLGA scaffolds</td>
<td>36</td>
</tr>
<tr>
<td>2.11</td>
<td>Coculture of PC3 and WPMY-1 stromal cells on PLGA scaffolds</td>
<td>37</td>
</tr>
<tr>
<td>2.12</td>
<td>FTIR of mPEG-PLA polymer</td>
<td>38</td>
</tr>
<tr>
<td>2.13</td>
<td>$^1$H NMR of mPEG-PLA polymer</td>
<td>39</td>
</tr>
<tr>
<td>2.14</td>
<td>Growth of cells on MN scaffolds</td>
<td>40</td>
</tr>
<tr>
<td>2.15</td>
<td>SEM of tumoroids on the 3P scaffold</td>
<td>42</td>
</tr>
<tr>
<td>2.16</td>
<td>Growth of tumoroids on scaffolds</td>
<td>43</td>
</tr>
<tr>
<td>2.17</td>
<td>Composite images of tumoroids on a 3P scaffold</td>
<td>44</td>
</tr>
<tr>
<td>2.18</td>
<td>Tumoroid numbers and sizes</td>
<td>44</td>
</tr>
<tr>
<td>2.19</td>
<td>Day 10 to day 20 tumoroids</td>
<td>45</td>
</tr>
<tr>
<td>2.20</td>
<td>Seeding density and tumoroid formation</td>
<td>46</td>
</tr>
<tr>
<td>2.21</td>
<td>Other cell line tumoroids</td>
<td>48</td>
</tr>
<tr>
<td>2.22</td>
<td>Absence of tumoroid formation on scaffolds</td>
<td>48</td>
</tr>
<tr>
<td>2.23</td>
<td>Effects of topography on tumoroid formation</td>
<td>49</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.24</td>
<td>Effects of charge on tumoroid formation</td>
<td>50</td>
</tr>
<tr>
<td>2.25</td>
<td>Tumoroids cultured on tissue culture plates</td>
<td>51</td>
</tr>
<tr>
<td>3.1</td>
<td>EMT in LLC-1 tumoroids</td>
<td>59</td>
</tr>
<tr>
<td>3.2</td>
<td>Timeline of EMT</td>
<td>61</td>
</tr>
<tr>
<td>3.3</td>
<td>EMT in other tumoroids</td>
<td>62</td>
</tr>
<tr>
<td>3.4</td>
<td>Absence of EMT in 3P/Chitosan tumoroids</td>
<td>63</td>
</tr>
<tr>
<td>4.1</td>
<td>Inhibition of tumoroid formation</td>
<td>72</td>
</tr>
<tr>
<td>4.2</td>
<td>Inhibition of tumoroid formation abrogates EMT expression</td>
<td>72</td>
</tr>
<tr>
<td>4.3</td>
<td>Cytotoxicity response of MCF-7 cells to inhibitors</td>
<td>73</td>
</tr>
<tr>
<td>4.4</td>
<td>LY294002 induced cell death in LLC-1 tumoroids</td>
<td>75</td>
</tr>
<tr>
<td>4.5</td>
<td>U0126 induced cell death in LLC-1 tumoroids</td>
<td>76</td>
</tr>
<tr>
<td>4.6</td>
<td>Diffusion of doxorubicin in to MCF-7 tumoroids</td>
<td>77</td>
</tr>
<tr>
<td>4.7</td>
<td>EMT in tumors treated with inhibitors</td>
<td>79</td>
</tr>
<tr>
<td>4.8</td>
<td>Biopsy tumoroids on 3P scaffold</td>
<td>80</td>
</tr>
<tr>
<td>4.9</td>
<td>Tumor stroma in tumor biopsies</td>
<td>81</td>
</tr>
<tr>
<td>4.10</td>
<td>Chemosensitivity of biopsy tumoroids</td>
<td>82</td>
</tr>
</tbody>
</table>
List of Tables

Table 1.1 Research spending per new drug ..........................................................3
Table 1.2 The Key Strengths and Weaknesses of 2D and 3D Models ....................8
Table 2.1 Characteristics of the Scaffolds..............................................................41
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>3P</td>
<td>PLGA/mPEG-PLA</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>calcein acetomethoxy</td>
</tr>
<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
</tr>
<tr>
<td>CSC</td>
<td>cancer stem cells</td>
</tr>
<tr>
<td>CXCR 4</td>
<td>chemokine receptor 4</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenyindole</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>EthD-1</td>
<td>ethydium homodimer-1</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FNA</td>
<td>fine needle aspirate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transformed infrared spectroscopy</td>
</tr>
<tr>
<td>GA</td>
<td>glycolide</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HTS</td>
<td>high throughput screening</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LA</td>
<td>lactide</td>
</tr>
<tr>
<td>LLC-1</td>
<td>Lewis lung carcinoma-1</td>
</tr>
<tr>
<td>LY29402</td>
<td>PI3K inhibitor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MN</td>
<td>multilayered nanofiber</td>
</tr>
<tr>
<td>mPEG</td>
<td>methoxy polyethylene glycol</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PI3K/AKT</td>
<td>phosphatidylinositide 3-kinases/protein kinase B pathway</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic-acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>Rho</td>
<td>Rho family of GTPases</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
</tr>
<tr>
<td>SMAD</td>
<td>mothers against decanpentalplegic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>U0126</td>
<td>MAPK inhibitor</td>
</tr>
</tbody>
</table>
Abstract

Attrition rates for anticancer drugs are much higher than any other therapeutic area. Only 5% of the agents that demonstrate anticancer activity in the preclinical stages of development demonstrate clinical efficacy in phase III trials. This high attrition rate becomes alarming when we consider that the cost of research and development can amount to 1 billion dollars. To exacerbate this problem, many new cancer drugs are being discontinued, withdrawn or suspended. The reasons for this high attrition rate are complex and may be partly attributed to suboptimal preclinical strategies such as the use of two-dimensional (2D) cell culture systems to evaluate new agents during the development and testing stages. Cancer cells cultured in 2D do not mimic the complexity of the three-dimensional (3D) milieu of tumors in vivo. There is overwhelming evidence that in vitro 3D culture systems more accurately reflect the tumor microenvironment and present better predictive value for assessing the efficacy of new chemotherapeutic agents. The development of 3D culture systems for anticancer drug development remains an unmet need. Despite progress, a simple, rapid, scalable and inexpensive 3D-tumor model that recapitulates in vivo tumorigenesis is lacking. Herein, we report on the development and characterization of a 3D nanofibrous scaffold produced by electrospinning a mixture of poly(lactic-co-glycolic acid) (PLGA) and a block copolymer of polylactic acid (PLA) and mono-methoxy polyethylene glycol (mPEG) designated as 3P. Cancer cells cultured on the 3P scaffold...
formed tight aggregates similar to *in vivo* tumors, referred to as tumoroids that depended on the topography and net charge of the scaffold. 3P scaffolds induced tumor cells to undergo the epithelial-to-mesenchymal transition (EMT) as demonstrated by up-regulation of vimentin and loss of E-cadherin expression. 3P tumoroids showed higher resistance to anticancer drugs than the same tumor cells grown as monolayers. Inhibition of ERK and PI3K signal pathways prevented EMT conversion and reduced tumoroid formation, diameter and number. Fine needle aspirates, collected from tumor cells implanted in mice when cultured on 3P scaffolds formed tumoroids, but showed decreased sensitivity to anticancer drugs, compared to tumoroids formed by direct seeding. These results show that 3P scaffolds provide an excellent platform for producing tumoroids from tumor cell lines and from biopsies and that the platform can be used to culture patient biopsies, test for anticancer compounds and tailor a personalized cancer treatment.
Chapter One

1.1 Overview of the drug discovery program

Advances in genomics and proteomics have rapidly increased the number of molecular targets for therapeutic intervention to treat cancer; however the number of drugs that advance through clinical trials in the drug discovery program is a paltry 5%. The development of all new chemotherapeutic drugs to treat cancer follows a similar progression. A potential drug target is identified and lead compounds are designed, developed and screened from small molecule libraries. Preclinical testing using both in vitro cell culture techniques and in vivo animal models, is performed to determine pharmacokinetic and toxicological efficacy. The final step is clinical testing of the most promising compounds in human subjects. Toxicity and lack of clinical efficacy are the main causes of failure usually at phase 111 of clinical trials. Most pharmaceutical companies use a set of specific high-throughput screening (HTS) assays in the preclinical stages utilizing innovative approaches in robotics and high speed computer technology. The common approach is to screen identified small molecule libraries using 2D monolayer cell-based assays in multiplate formats. The assays can be used to determine empirical and mechanistic responses such as viability and cytotoxicity, signal

---

1 A portion of this chapter have been previously published by the author in an article entitled “A 3D Fibrous Scaffold inducing Tumoroids: A Platform for Anticancer Drug Development”, PlosOne, October 16 2013, Vol.8, Issue 10 and have been reproduced with permission from the publishers.
transduction pathways such as kinase activation or cellular responses at the transcriptional and translational level. Although these cell-based assays are established in the drug discovery process their value in predicting clinical response to new agents are limited. This lack of predictability is attributable to the fact that cells in 2D cultures do not mimic the response of cells in the 3D environment of an organ, tissue or tumor. Today’s strategies for drug discovery require HTS systems that can mimic the human tissue environment to optimize preclinical and preanimal selection, thus dismissing compounds that are ineffective and toxic while prioritizing promising candidates early in the developmental stages. It is now recognized that 3D cell culture systems better reflect the in vivo pathophysiological environment observed in human tissues and may soon replace 2D cell culture systems.

1.2 The cost of drug development

It is often said that the main expense in the drug discovery program is failure. In 2011, nearly 900 anticancer drugs were in clinical trial or under FDA review, yet only 12 drugs were approved. It takes 10-15 years to develop a new drug from the time it was discovered to treating patients and these numbers represents hundreds of drugs that will not make it to market. Behind these failures are hundreds of millions of dollars spent in research and development (R&D) and clinical trial costs. For example, from 1997 to 2011 Pfizer spent $108 million dollars and only had 14 drugs approved for market while Novartis spent $83 million and had 21 drugs approved. R&D expenditures will top $136 billion in 2013, up from $134 billion the previous year and are on track to exceed $149 billion by 2018, according to the analysts.
Table 1.1 Research spending per new drug.

<table>
<thead>
<tr>
<th>Company</th>
<th>No. of drugs approved</th>
<th>R&amp;D spending per drug ($millions)</th>
<th>Total R&amp;D spending 1997-2011 ($millions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AstraZeneca</td>
<td>5</td>
<td>11,791</td>
<td>58,955</td>
</tr>
<tr>
<td>GlaxoSmithkline</td>
<td>10</td>
<td>8,171</td>
<td>81,708</td>
</tr>
<tr>
<td>Roche</td>
<td>11</td>
<td>7,804</td>
<td>85,841</td>
</tr>
<tr>
<td>Pfizer</td>
<td>14</td>
<td>7,727</td>
<td>108,178</td>
</tr>
<tr>
<td>Johnson&amp;Johnson</td>
<td>15</td>
<td>5,886</td>
<td>88,285</td>
</tr>
<tr>
<td>Merck &amp; Co. Inc</td>
<td>16</td>
<td>4,210</td>
<td>67,360</td>
</tr>
<tr>
<td>NovartisAG</td>
<td>21</td>
<td>3,983</td>
<td>83,646</td>
</tr>
</tbody>
</table>

Adapted from: Inno Center for Research in Biomedical Innovation; Thompson Reuters Fundamentals via FactSet Research Systems, 2011.

1.3 2D versus 3D cell culture systems

The culture of cells on 2D surfaces has provided important insights into tumorigenesis and cancer biology, however most pathophysiological parameters of tumors are minimized or lost under these conditions. Cells in 2D assume a flat and spread morphology and can adhere freely along the horizontal plane during culture, but lack support for extending upwards on the vertical plane [1]. Consequently, cells assume a forced apical to basal polarity that has been shown to modulate sensitivity to apoptosis, migration and cell signaling [2,3]. In addition, there are limited contacts with neighboring cells confined to the flat edges that they share, in contrast to \textit{in vivo} conditions where cells closely interact with each other and the extracellular matrix at various orientations [4]. In 2D cultures, mass transport of gases, growth factors, cytokines, hormones, or drugs are rapidly and uniformly diffused over a short distance and equilibrate rapidly into cells. In contrast, the three dimensional architecture of tissues, create physiological gradients that facilitate slow diffusion across multiple layers. These gradients are
essential for the regulation of cell signaling, homing, angiogenic sprouting, development and the uptake of drugs [5-7]. Cell migration in 2D is confined to a restraint free plane and encounters little or no resistance to migration from the surrounding extracellular matrix (ECM) while in a 3D setting such as that observed during cancer metastasis, migration is regulated by mechanical interactions with the microenvironment and cells must often negotiate or cleave the physical matrix to extend or squeeze through. Research have consistently demonstrated that cancer cells cultured in three dimensional (3D) cultures exhibit behavior and expression profiles that better reflect cancer cells in their native in vivo environment [8,9]. Observable differences between 3D and 2D are seen in cell morphology, proliferation, viability, gene and protein expression, sensitivity to chemotherapeutic drugs, response to stimuli, and overall in vivo relevance [10]. For example, the use of hepatoblastoma cell line (HepG2) cultured in 2D monolayer culture is considered the gold standard in early screening of drug candidates for hepatotoxicity. However cells in 2D culture express low levels or the absence of drug metabolizing enzymes such as cytochrome P450s [11] in contrast with hepatocytes in 3D collagen cultures that show responses similar to in vivo conditions. Cells in 3D culture tend to grow slower than cells in 2D culture reflecting the Gompertz equation, an algorithm used to quantitatively evaluate the in vivo neoplastic growth rate [12,13]. Cells in 3D cultures show decreased sensitivity to apoptosis and display chemotherapeutic differences in responses to drugs that may desensitize cancer cells similar to those observed in vivo [14]. For example, MCF-7 breast cancer cells cultured on chitosan-based scaffolds, displayed more tumor-like lactate production and drug resistance to tamoxifen than cells cultured in 2D [15]. EMT-6 mammary cells fully
recapitulated the cell adhesion mediated drug resistance induced *in vivo* but was lacking in cultured monolayer of the same cells [16]. It is evident that that the microenvironment within which cancer cells reside contribute to the spatial and temporal signaling domains that direct cell fate. The differences in cell behavior between 2D and 3D cultures suggest that perturbations in gene and protein expression may stem from how cells sense and respond to microenvironmental cues that are differently expressed in a 2D or 3D context. 3D systems have yet to be incorporated into drug development programs because challenges exist to satisfying the criteria for HTS mainly automation, scalability, low cost and wide applicability. However it is believed by many in the field that it is just a matter of time. A summary of the strengths and weaknesses of 2D and 3D models are found in Table 1.2.

### 1.4 Types of 3D cultures in cancer research

The most common 3D models utilize matrices or scaffolds, derived from biological, synthetic, or natural sources to form fibers, sponges, hydrogels, beads or microspheres [17]. The biologically-derived matrices are composed of common ECM proteins such as collagen [18], hyaluronic acid [19], gelatin [20] and chondroitin sulphate [21], that themselves can initiate signaling cascades by acting as ligands for integrins and receptors involved in gene expression [22]. Collagen 1, for example, targets genes involved in motility, invasion, and migration of cancer cells by downregulating epithelial marker expression and upregulating integrin receptor expression [23,24]. Commercially available biological matrices such as Matrigel contain common ECM molecules found in basement membranes such as laminin, collagen 1V, perlecan, and nidocan that activate
signal transduction pathways and angiogenesis [25,26]. Synthetic matrices are usually derived from bio-degradable biocompatible polymers such as the linear aliphatic polyesters, poly(lactic-co-glycolic acid) (PLGA) [27], poly(L-lactic acid) (PLA) [28], and poly(e-caprolactone)(PCL) [29] and are approved by the FDA for uses in tissue engineering and regenerative medicine applications. Examples of naturally occurring matrices are those made from chitosan derived from crab shells [30], alginate from seaweed [31], cellulose [32], and silk [33]. Although they lack the signaling molecules inherent in biologically derived matrices, synthetic and natural matrices are chemically pure and do not present the possibility of contamination from eukaryotic sources. In addition, they impart mechanical, physical, and optical features to the scaffold and better control over material properties. Hybrid composites containing different matrix types are common depending on the cell and culture conditions. They may be comprised of two or more matrices that impart mechanical or biological properties that are lacking in the individual constructs [34,35]. A few of the most common 3D systems are discussed below.

**Fibrous scaffolds**

These scaffolds mimic the filamentous ECM and possess the inherent advantages of high surface to volume ratio, controlled porous architecture, selective adsorption of ECM proteins and allows cells to grow at various orientations [36]. There are several approaches to fabrication of nanofibers namely phase separation, self assembly, template synthesis, melt blowing and electrospinning. The most widely used of these techniques is electrospinning that can produce scaffolds consisting of random or aligned
nanofibers. The process is simple and versatile and almost any soluble polymer can be processed into fibers.

**Hydrogels**

Hydrogels are formed by the crosslinking or self-assembly of a variety of biological, natural or synthetic materials to produce structures that contain over 90% water. A wide and diverse range of polymer compositions and techniques have been used to fabricate hydrogels. Depending on the type, extent of crosslinking and molecular weight between crosslinks, the gel can be described as physical, chemical or ionotropic. Polymers and crosslinkers can be cationic such as chitosan [29], anionic such as alginate [30], amphipathic such as collagen or neutral such as agar; examples of synthetic polymers are PEG and PVA. Hydrogels have limited pore size for free diffusion of water and metabolites and exchange of nutrients and wastes, and strictly confine the overall migration or leakage of individual cells. Thermoreversible hydrogels are used extensively in therapeutic drug delivery because of their ability to undergo sol-gel-sol transitions at different temperatures. To improve cell attachment and spreading for 3D cancer cell culture applications, hydrogels are linked to adhesion ligands such as the ubiquitous RGD (arginine-glycine-aspartate) peptides that bind to integrins on cell surfaces. Hydrogels are increasingly used for encapsulating tumor cells within microfluidic cell culture systems as well as for constructing nanostructures within these devices that mimic defined topology of the native ECM.
Table 1.2. The Key Strengths and Weaknesses of 2D and 3D Models.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>2D</th>
<th>3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Unnatural spreading</td>
<td>Clustered round morphology; lose polarity</td>
</tr>
<tr>
<td></td>
<td>morphology</td>
<td></td>
</tr>
<tr>
<td>Value in predicting clinical</td>
<td>Limited</td>
<td>High</td>
</tr>
<tr>
<td>efficacy of drugs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viability</td>
<td>Lower</td>
<td>Higher</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Can be made to differentiate, rarely or occasionally</td>
<td>Differentiate naturally as \textit{in vivo}</td>
</tr>
<tr>
<td>Gene and protein expression</td>
<td>Changes occur in gene and protein profiles</td>
<td>Significantly more changes occur, which mimic \textit{in vivo}</td>
</tr>
<tr>
<td>Drug metabolism</td>
<td>Metabolism is artificial</td>
<td>Metabolism reflects in vivo physiological context</td>
</tr>
<tr>
<td>Response to stimuli</td>
<td>Occasionally reflects physiologic context</td>
<td>Mimics \textit{in vivo}</td>
</tr>
<tr>
<td>\textit{In vivo} relevance</td>
<td>None or low</td>
<td>High</td>
</tr>
</tbody>
</table>


1.5 Tumor spheroids

The most validated 3D model in cancer research is the multicellular tumor spheroid. They have being used for a variety of experimental studies in chemotherapy and radiotherapy and are being pursued in high-throughput screening (HTS) programs for drug development. Spheroids appear as spherical aggregates of cells that resemble micrometastasis and intravascular regions of tumors. Similarities are observed with respect to histomorphological features, growth kinetics and micro-milieu [37]. Spheroids display functional and mass transport properties attributed to differentiation patterns that allows the appropriate cell to cell and cell to matrix interactions and appropriate ECM assembly. Observed is the establishment of pathophysiological gradients to oxygen,
nutrients and catabolites and the subsequent development of a central necrotic core consisting of quiescent, hypoxic, anoxic and necrotic cell sub-populations surrounded by a concentric arrangement of proliferating heterogeneous cells [38,39]. The metabolic adaptation of central cells to the microenvironmental stresses created by diffusion gradients causes the innermost cells to exit the cell cycle and enter the G0 state [40] followed by changes in ploidy [41] extracellular pH and ECM constituents [7]. This reflects the situation observed in vivo of proliferating tumor cells adjacent to capillaries while innermost cells become quiescent and die by necrosis or apoptosis. The tumor microenvironment plays a critical role in the dissemination of cancer and spheroids have emerged as powerful tools that can model the cellular heterogeneity found in tumors. Spheroids are amenable to coculture of different cell types such as endothelial cells, immune cells and fibroblasts. The stroma is well known to support and respond to tumorigenesis directly influencing the tumor process. For example it has been shown that the human colon carcinoma cell line, HRT-18 when co-cultured with tumor associated macrophages, stimulate tumor cell migration and inhibit tumor cell proliferation [42]. Studies have shown that spheroids are inherently more resistant to virtually all anticancer cytotoxic drugs than conventional monolayer cultures. For example endometrial cancer spheroids (RL95-2 and KLE cell lines) showed more resistance when treated with doxorubicin than cells cultured on monolayer [43]. Potential mechanisms implicate the pathophysiological gradients and the concentric arrangement of heterogeneous cell populations within the spheroids that may affect drug diffusion kinetics, alter expression profiles of signaling pathways, and modulate of DNA repair mechanisms and cell cycle distribution. Several comparative transcriptomic
studies have shown numerous genes associated with cell survival, apoptosis and drug resistance are differentially expressed in cells grown as spheroids than in monolayer culture [44,45].

1.6 Methods for spheroid production

A broad range of simple and complex technologies are presently being used to produce spheroids utilizing biological, natural and synthetic materials. The most common materials utilize biologically derived matrices such as matrigel [46], natural matrices such as alginate and agarose [37,47] and synthetic polymers such as polyethylene glycol (PEG) [48,49] and poly-hydroxyethyl ethacrylate (poly-HEMA). Tumor spheroids are produced by a variety of methods that share the common feature of promoting cell–cell attachment by resisting cell–surface connections.

Forced floating method
This method is one of the simplest methods used to generate spheroids. Modification of the vessel surface with polyHEMA or agarose will prevent cells from attaching thereby promoting cell–cell contacts which in turn, promote spheroid formation [50]. This method is simple, inexpensive, reproducible and compatible with HTS. The disadvantages of this system are that it is time consuming and labor intensive.

Hanging drop method
This method is based on the principle that surface tension at the liquid air interface between cells, induce spheroid formation. This method is relatively simple allows for defined sizes and has been reported to have high reproducibility producing one 3D spheroid per drop, for numerous cell lines [51]. The disadvantages are that it is labor
intensive, require regular media changes and changing culture medium without compromising spheroid integrity is difficult [52].

**Spinner flasks systems**

These systems operate on the principle that cell suspensions kept in constant motion either stirred or rotated, prevent cells from adhering to the container walls, and promote cell–cell interactions and spheroid formation. Such systems are suitable for mass production and long term culture. The drawback of using a spinner flask is that the sheer force experienced by cells, can adversely affect the cellular physiology. Rotating bioreactors, function by similar means as the spinner flask bioreactor but, instead of using a stirring bar, the culture container itself is rotated. The rotating-wall vessel developed by NASA in 1992 was designed to mimic microgravity and exert a low sheer force on cells in culture. This low sheer force is one of the main advantages of using this system. Other advantages are that it is simple, enables large-scale production, long-term culture and ease in changing culture medium. The limitation of using the NASA bioreactor is that it requires specialized equipment [53].

**Microfluidic cell culture platforms**

Advanced spheroid methods utilize nano and micro technologies such as microfluidic devices for the production of spheroids. Various groups have developed microfluidic devices to culture spheroids in micro wells, micro chambers or on micro textured surfaces. These sophisticated systems are complicated and require specially trained personnel to fabricate and operate the devices. Many of these devices suffer from material compatibility issues with hydrophobic drugs which may limit their applicability.
Other problems include the inability to retrieve and characterize the spheroids and the need to use specialized equipment that are expensive [54]. Microfluidic devices can be adapted for HTS and can enable qualitative and quantitative analysis, continuous perfusion and the minimal use of reagent volume.

**Commercial systems**

During the last few years, methods for the formation of spheroids have become commercially available. They are mostly based on diverse gel matrix scaffolds such as AlgiMatrix (Invitrogen), QGel and HydroMatrix (Sigma-Aldrich), or natural heteropolysaccharides that form rigid, brittle, agar-like gels (GELRITE, Sigma-Aldrich) and NanoCulture plates from Scivax designed for high-content analysis and high through put screening [55].

### 1.7 Limitations of spheroid systems

Spheroids fail to mimic the biomechanical characteristics of *in vivo* conditions. The system is static compared to *in vivo* conditions where exchange of fluids and wastes is a continuous process [56]. Animal-derived materials used to make spheroids risk the potential for transmission of diseases and may present batch-to-batch variability [57]. Applications to HTS is hampered by the lack of standardized and rapid analytical methods such as imaging and spectroscopic tools that can address typical problems such as autofluorescence and methods to gently and rapidly recover cells for example in hydrogels for mRNA retrieval. Non-adherent cells such as hematopoietic cell lines are difficult to maintain in culture and may require co-culture with adherent cells for analysis. Cell manipulations on some 3D polystyrene or polycaprolactone supports
require trypsinization that causes cell stress for [58]. To address these limitations we constructed a novel 3D scaffold made from biodegradable biocompatible materials that can be easily and rapidly produced. Our central hypothesis is that the scaffold can mimic in vivo conditions and can be used to recapitulate tumorigenesis, assess chemotherapeutic efficacy and serve as a platform for the growth of tumor biopsy. Towards these ends we will utilize the following aims. 1) Design and construct the 3D scaffold. 2) Show that the scaffold can recapitulate in vivo tumorigenesis observed as the induction of EMT in tumoroids. 3) Demonstrate that the tumoroids can be used to assess drug efficacy. 4) Show that the scaffolds support the growth of tumor and stromal cells from the tumor biopsy.
Chapter Two

Design and construction of the 3D scaffold

2.1 Introduction

Synthetic 3D substrate topography has been shown to influence cell migration, differentiation, and gene expression similar to that observed in vivo [59,60]. During tumor progression, the extracellular matrix modulates the phenotype of cells by generating tensional forces within the matrix. Cells respond to these geometric cues by restructuring their cytoskeleton which in turn translates to biochemical signals within the cells thereby altering gene and protein expression [61]. One possible mode of this signal transmission is mechanotransduction where physical cues are transduced via mechanical forces to signaling pathways involved in detecting and responding to the ECM [62-64]. This interaction is initiated at the cell-matrix interface via integrins, communicated to focal adhesion complexes which in turn activate downstream signaling pathways. The protein tyrosine kinase, focal adhesion kinase (FAK), responds to both substrate rigidity and tension via the actin cytoskeleton and is an important regulator for cells to detect and sense the topographical features in the ECM [65] [66] [67] [68,69] [70]. In particular, the ERK/MAPK signaling cascade is activated by focal adhesion elongation and acts as a mediator of cellular differentiation. ERK1/2 translocates to the

2 A portion of this chapter have been previously published by the author in an article entitled “A 3D Fibrous Scaffold inducing Tumoroids: A Platform for Anticancer Drug Development”, PlosOne, October 16 2013, Vol.8, Issue 10 and have been reproduced with permission from the publishers.
nucleus in cells cultured on different topographical features and affects the expression of transcription factors and cellular differentiation [71,72]. Effects are observed on the nucleus such as nuclear reshaping, redefinition of chromosomal territories and force-induced alterations in nuclear pore diameter. At the transcriptional and post transcriptional levels there may be altered physical accessibility of genes to transcription factors and on mRNA splicing, editing or transport. At the translational level, alterations in the activity state of proteins, conformational changes in structure, composition of protein assemblies, and exposure of cryptic binding sites on receptors [73-75]. These findings indicate that the architecture of the niches where cancer cells reside may be critical for their tumorigenic behavior and therefore, drugs targeted at impairing specific architectural features of the microenvironment should be looked at as possible novel therapies to inhibit invasive progression. The goal of our study is to create a 3D scaffold that would recapitulate in vivo conditions. We hypothesize that this novel 3D scaffold would exhibit structural geometry similar to the native tumor ECM that allows the assembly of tumors cancer cells into irregular tumor-like structures called tumoroids that recapitulate the micrometastasis of in vivo tumors. Current 3D culture models lack the important topographical features that define the native tumor microenvironment. We chose a nanoscale fiber scaffold as the platform because of the ease and reproducibility of production. Electrospinning is an excellent method for generating fibrous scaffolds of specific composition, fiber diameter and pores from synthetic polymers such as poly(lactic-co-glycolic acid) (PLGA) and poly(e-caprolactone). The scaffold mimics the ECM in providing support and physical attachments. The nano- and micro-topographic and mechanotransductive cues of electrospun polymeric scaffolds
[76-82] have been reported to stimulate migration, differentiation and gene expression of cancer cells [83-85]. The electrospinning conditions can be adjusted to produce fibrous scaffolds tailored for specific cell culture needs [82,86]. Cancer cells have also been induced to form spheroids on electrospun galactosylated fibers [87] and 3D scaffolds [88]. However, these tumor spheroids have been poorly characterized and it is not known whether they resemble in vivo tumors. In our preliminary study we prepared a variety of scaffolds in order to identify optimum conditions for assembly and construction. This required the design and construction of an in-house electrospinner that satisfied the appropriate criteria for high energy voltage supply, a continuous flow pump system and a contained grounded receiver. The electrospinner was easily constructed from available materials and requires minimal maintenance and space.

The initial scaffold helped to define the parameters for electrospinning the appropriate concentrations of polymer. We used poly(lactic-co-glycolic acid) (PLGA) because it is an FDA approved biocompatible, biodegradable polymer. Numerous tumor cell types grew on the PLGA scaffold as well as cocultures with stromal cells. Although this model satisfied a simplistic approach, by providing a spatial 3D environment for cells to grow in it did not provide the appropriate environment for the formation of in vitro tumors. We optimized the PLGA scaffold by adding a diblock polymer made from a combination of methoxy polyethylene glycol (mPEG) and poly(lactic acid) (PLA). Different ratios of the polymers were used to construct the scaffolds. Most of the combinations failed except for one which we designated 3P. The 3P scaffold mimicked the topographical and mechanical features of the ECM and induced the self-assembly of various tumor cells into tumoroids. To explore the effects of topography and chemistry on tumoroid
formation, we cultured Lewis lung carcinoma (LLC-1) cells on films and scaffolds containing various combinations of the polymers used to construct the 3P scaffold. To determine the effects of charge, we constructed a chitosan (positively charged) 3P scaffold and observed that tumoroid formation was inhibited or curtailed on these constructs while the 3P scaffold formed and maintained tumoroids. Transfer of tumoroids from the 3P scaffold to tissue culture plates resulted in complete cellular disassociation and eventual confluency on the plates. These results underscore the unique combination of topographical and chemical cues required to produce tumoroids on the 3P scaffold.

2.2 Electrospinning

Electrospinning is a technique that produces scaffolds of random or aligned fibers and is based on the principle that strong electrical forces overcome the weaker forces of surface tension in a charged polymer liquid. At a certain threshold voltage, a charged jet is ejected from the tip of needle. The jet moves in the direction of an external electric field, elongates according to external and internal electrical forces, and experiences instability. The jet segments are then deposited as a nonwoven mat of fibers on a grounded receiver [82,86,89,90]. A typical electrospinning setup consists of a syringe through which the liquid to be electrospun is forced, a high voltage source with positive or negative polarity, which injects charge into the liquid, and a grounded collector (Fig.2.1). A syringe pump is typically used to force the liquid through a needle forming a pendant drop at the tip. An electrode from the high voltage source is directly attached to
the metal needle. The voltage source is then turned on and charge is injected into the polymer solution.

Figure 2.1. The Electrospinning equipment. The device consists of an enclosure box, a high voltage supply unit, a syringe and a syringe pump. The enclosure box was built from plastic and acts as an insulator to ensure safety and to minimize the effects of environmental factors such as humidity. The syringe is attached to the pump and to the polypropylene tubing that has a steel needle attached. The tubing and the needle are fed through a tubing hole into the enclosure box and clamped at 20 cm from the collector plate. The needle is connected to the power unit by attaching the positive lead cable from the unit to a point near the needle tip using a clip.

Increasing the electric field strength causes the repulsive interactions between like charges in the liquid and the attractive forces between the oppositely charged liquid and collector to begin to exert tensile forces on the liquid, elongating the pendant drop at the tip of the capillary. As the electric field strength is increased further, a point will be reached at which the electrostatic forces balance out the surface tension of the liquid
leading to the development of the Taylor cone. If the applied voltage is increased beyond this point a fiber jet will be ejected from the apex of the cone and be accelerated toward the grounded collector. The electrospinning process is governed by many parameters. Solution parameters including viscosity and molecular weight, process parameters such as flow rate, electric field and distance of the tip to the collector and ambient parameters consider the temperature and humidity of the environment. These factors play significant roles in determining the morphology and diameter of the fibers.

Figure 2.2. Schematic of the electrospinning process. The liquid polymer is placed in the syringe that is attached to the syringe pump. The polymer is pumped through the syringe towards the steel needle. The high voltage supply unit is switched on as soon as a droplet of the solution is formed at the tip of the needle. The droplet becomes charged and electrostatic repulsion counteracts the surface tension of the liquid, causing the droplet to elongate at a critical point leading to the formation of the Taylor cone. A fiber jet ejects from the cone, elongates by a whipping process and accelerates towards the collector plate where the fibers are deposited.
Fiber orientation can be controlled to produce aligned fiber assemblies that mimic those seen, for example, in muscle and tendons and for targeted drug delivery, by introducing various fabrication principles such as coaxial electrospinning that allows for two polymer solutions to be electrospun simultaneously [91,92]. Electrospinning is an inexpensive process that can be easily scaled up. It has been used extensively to produce scaffolds for cell culture and tissue engineering applications. Considerations on the mechanical properties of electrospun scaffolds such as topography and geometry have been shown to influence cell behavior, gene expression, signaling, adhesion and migration [93-98].

**Polymers used in electrospinning**

A wide range of polymers are presently being used in electrospinning to form fibers ranging from the micron to the nanometer scale. Numerous biological, natural and synthetic materials are presently being used for electrospinning. Examples of natural matrices are chitosan and silk fibroin, [99,100] and biological matrices are collagen, fibrinogen, hyaluronic acid and gelatin [101-104]. Materials from natural and biological sources are advantageous because of their intrinsic properties of biological recognition, including presentation of receptor-binding ligands and susceptibility to cell-triggered proteolytic degradation and remolding [105,106]. However, electrospinning of these natural biopolymers is often very difficult compared to synthetic polymers because they are difficult to process and demonstrate low mechanical strength [107]. Materials derived from synthetic sources are commonly used and are mainly biodegradable, biocompatible polymers such as linear aliphatic polyesters such as poly(e-caprolactone, polystyrene, polyvinyl alcohol, poly(lactic-co-glycolic acid) and poly(lactic acid) that are
approved by the FDA for use in tissue engineering and regenerative medicine applications [89,108-111]. Electrospinning with copolymers offers enhancement of polymeric materials that can improve on mechanical strength, thermal stability and barrier properties. The use of copolymers allows for the design of new materials of desirable properties that can enhance the characteristics of the copolymers over the homopolymers. Biodegradable hydrophobic polymers usually possess excellent mechanical properties but tend to lack important cell affinity properties important for cell culture applications. Incorporation of appropriate hydrophilic copolymers can enhance, cell affinity, morphology, pore size and other physical properties [112].

**Uses of electrospun fibers**

Electrospun scaffolds are presently being used in tissue engineering and biomedical applications. For tissue engineering applications, electrospun scaffolds are generally used as temporary templates for cell seeding, invasion, proliferation, and differentiation prior to the regeneration of biologically functional tissues or natural extracellular matrix. It has been shown that these scaffolds promote cell–matrix and cell–cell interactions with the cells having a normal phenotypic shape and gene expression and electrospun fibers mimic those found in the extracellular matrix and has demonstrated effectiveness as a substrate for cell growth [113]. Electrospinning is the most extensively used fabrication method for preparation of these nanometer to micron-sized fibers. Natural or biological polymers such as collagen, alginate, silk protein, hyaluronic acid, fibrinogen, chitosan and starch are often used for preparing scaffolds because of their biocompatibility and signaling motifs. Electrospun scaffolds have been used to engineer
tissues such as bone [114], heart [115], nerves [116] and tendons [117]. Electrospun mats have been used as drug carriers as drug delivery systems because the large surface area allows for fast and efficient solvent evaporation, that favors easy dispersion of the drug [118]. Depending on the polymer carrier, the release of pharmaceutical dosage can be designed as rapid, immediate, delayed, or modified dissolution. Drugs have been encapsulated within electrospun fibers by mixing the drugs in the polymer solution before electrospinning [119]. A variety of drugs have been incorporated into electrospun fibers for delivery such as the antitumor drug hydroxycamptothecin [119], the antibiotic tetracycline [120] and the anti-inflammatory drug Ibuprofen[121].

Synthetic polymers used to make the scaffolds

Poly(lactic-co-glycolic acid) (PLGA)

PLGA is synthesized by means of random ring-opening co-polymerization of glycolide (GA) and lactide (LA). The structure of PLGA is shown in Fig.2.3. The rate of degradation of the copolymer can be decreased by increasing the GA content or increased by increasing the LA content. PLGA undergoes degradation by hydrolysis in vivo to produce lactic acid and glycolic acid that enters the Krebs cycle and is excreted as carbon dioxide and water. A part of the glycolic acid is also excreted in the urine. Because these two monomers are endogenous and easily metabolized a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications. The polymers are commercially available with different molecular weights and copolymer compositions [26].
Figure 2.3. Poly(lactic-co-glycolic acid)

\[ \text{x= number of units of lactide ; y= number of units of glycolide.} \]

**Poly (L-lactic acid) (PLA)**

PLA is an aliphatic polyester derived from natural plants such as tapioca, sugar cane and corn. The structure of PLA is shown in Fig.2.4. It consists of lactic acid monomers that undergoes similar degradation by hydrolysis *in vivo* and attributes its hydrophobic properties to the presence of methyl groups on the alpha carbon of the monomers. PLA occurs naturally as the pure enantiomeric poly (L-lactic acid) with a semi-crystalline structure. However, most types of PLA used for biological applications exist in the racemic D and L forms and are amorphous polymers [27].

Figure 2.4. Poly (lactic acid)

\[ \text{n= number of lactic acid units.} \]

PLGA and PLA are well known for their ability to undergo modifications, good degradation behavior, scalable mechanical properties, oxygen and water vapor permeability and high aspect ratio. Limited availability of hydrophilic functionality
combined with surface hydrophobicity can compromise their interactions with proteins and cells [122]. However, electrospinning counteract these hydrophobic effects, by conferring topographical and mechanical cues such as nano to micrometer size fibers that mimic the filamentous extracellular matrix.

**Poly-ethylene glycol (PEG)**

PEG is a hydrophilic biocompatible, biodegradable synthetic polymer otherwise known as poly (oxyethylene) or poly (ethylene oxide) at high molecular weights. The structure of PEG is shown in Fig.2.5. PEG is synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring [123]. Studies of PEG have shown that PEG typically binds 2–3 water molecules per ethylene oxide unit. Due to both the high flexibility of the backbone chain and the binding of water molecules, the PEG molecule acts as if it were five to 10 times as large as a soluble protein of comparable molecular weight. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins and repel cells [124,125]. PEG have been combined with PLGA and PLA to form electrospun composite scaffolds for tissue engineering and drug delivery applications[126-128].

![Figure 2.5. Polyethylene glycol](image)

\[ n = \text{number of poly (ethylene oxide) units.} \]
Chitosan

Chitosan is a linear polysaccharide composed of randomly distributed $\beta$-(1, 4-linked D-glucosamine (deacytelated unit) and $N$-acetyl-D-glucosamine (acetylated unit). The structure of chitosan is shown in Fig.2.6. It is a biocompatible, biodegradable, nontoxic, anti-microbial and hydrating agent. Chitosan is produced commercially from chitin found in the exoskeleton of crabs and shrimp and the cell walls of fungi. The presence of primary aliphatic amines in the chemical structure of chitosan makes this polymer distinct from other commonly available polysaccharides. The primary aliphatic amines of chitosan can be protonated under acidic conditions that makes them cationic polyelectrolytes. Chitosan with high degrees of deacetylation support the attachment of different cell lines and this has been attributed to its tendency to form polyelectrolyte complexes with glycosaminoglycans and the cellular attachment protein heparin [99].

Figure 2.6. Chitosan

$n=\text{no. of chitosan oligosaccharides.}$
2.3 Methods

Construction of the PLGA scaffold

The PLGA scaffold was made from 3 gm of PLGA (Sigma-Aldrich) dissolved in 10 mls of a solution of dichloromethane and chloroform (80/20 v/v). The solution was placed in a 20 ml glass syringe attached to a polypropylene tube fitted with a 23 gauge steel needle that was clamped at 20 cm from a copper plate covered with aluminum foil. The syringe was attached to an automatic pump (KD Scientific) that was set a flow rate of 0.2 ml/hr. An electrode from the high voltage source (Gamma High Voltage) was directly attached to the metal needle. The voltage was applied as soon as the polymer begins to leave the needle. The solution was electrospun at a constant positive voltage of 20kV [110]. The electrospun scaffolds were collected on the aluminum covered plate. The scaffolds were gently removed then cut to approximately 7x7mm² and placed in 96 well plates, sterilized in isopropyl alcohol, washed three times in PBS then additionally sterilized by exposure to high intensity UV light for one hour.

Scanning Electron microscopy (SEM)

Cells were fixed in a 50:50 (v/v) solution of 2.5% glutaraldehyde in 0.2M cacodylate buffer (pH 7.1) for 24 hrs. The scaffolds were washed in 1% osmium tetroxide in cacodylate buffer at 40°C for one hour. The scaffolds were washed in cacodylate buffer then further dehydrated in an ascending series of ethanol at concentrations 10%, 35%, 50%, 70%, 95% and 100% for ten minutes. Final dehydration was done in hexamethyldisilazane for 10 minutes. Samples were air dried, then sputter-coated with
gold at 19.32aM for 30 seconds under argon gas [129]. Scaffolds were viewed on a Jeol JSM 6490 scanning electron microscope.

**Determination of pore size**

For measurement of pore size, SEM images were taken randomly from scaffolds (n=3) and pore sizes were measured per image (16 pores/scaffold; total=48)). To compensate for variation in diameter, three measurements representing the shortest and longest distances were drawn through the center of the pores and the final diameter was calculated as the average of the value.

**Cell culture**

Cancer cells used in this experiment were LLC-1, BG-1 (human ovarian cancer cells), PC3 (human prostate cancer cells), B16 (mouse melanoma cells), MCF-7, MDA-MB-231 and MCF-10A (human breast cancer cells) and WPMY-1 (human myofibroblast stromal cells); cells were purchased from ATCC [130]. Mesenchymal stem cells (MSCs) were derived from transgenic mice expressing green fluorescent protein (GFP) that were purchased from Jackson Labs. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 1% (v/v) penicillin and streptomycin (GIBCO). Cells were maintained in a 37°C humidified incubator with an atmosphere of 5% carbon dioxide/95% air. Single-cell suspensions were prepared by treatment with trypsin-EDTA (GIBCO) and resuspended in complete medium before monolayer or tumoroid culture was set up. Cells from a single-cell suspension were added to scaffolds in 96 well plates in a total volume of 100 µl.
Immunofluorescence staining

Cells were washed three times with phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, followed by permeabilization in 1.0% Triton X-100 for 15 minutes. They were then incubated with 1% bovine serum albumin (BSA) for 30 mins at room temperature then incubated with the primary antibody overnight at 4°C. They were washed three times with PBS then incubated with the secondary antibody for one hour at room temperature in the dark. Primary antibody to Ki-67 nuclear antigen (mouse anti-human) (Dako) was diluted at 1:100 in 1% BSA. Secondary antibody was goat anti-mouse conjugated to Alexa 488 (Invitrogen) diluted at 1:100 in 1%BSA. Phalloidin conjugated to Alexa 555 (Invitrogen) was diluted at 1:40 in 1% BSA. Scaffolds were washed twice with PBS before being viewed under a microscope. For calcein acetomethoxy/ ethidium homodimer-1 (calcein AM/EthD-1 )staining, cells were washed three times with PBS. 2µl of calcein AM (2µM) and 2µl of EthD-1 (4 µM) were added to 1ml of PBS and gently mixed. 100 µl of calcein AM/EthD-1 solution was added directly to cells and incubated for 20 minutes in the dark, then washed twice with PBS before being viewed under a fluorescence microscope (Olympus BX51) or a confocal microscope (Leica TCS).

CellTracker staining

For coculture experiments cells grown to confluency were washed with PBS twice and single-cell suspensions were prepared by treatment with trypsin-EDTA; 1x10^4 cells were resuspended in 1 ml of pre warmed serum free DMEM media. This was added to 1.0 ml of pre warmed Celltracker Green (0.3125µM) or Celltracker Orange (2.5µM) in serum free media and incubated in a 37°C humidified incubator with an atmosphere of 5%
carbon dioxide/95% air for 45 mins. The solution was gently spun at 1200 rpm for 3 minutes then the supernatant was removed and the cells were incubated in 1 ml serum free media for 30 mins at 37°C . The media was removed and replaced with 1 ml DMEM media containing 5% FBS and 1% (v/v) penicillin and streptomycin. Equal concentrations of cancer cells and stromal cells (5x10^3) were added to a culture chamber and maintained in a 37°C humidified incubator with an atmosphere of 5% carbon dioxide/95% air for 24 hrs. Cells were washed three times then fixed with 4% paraformaldehyde for live viewing using a wet lens under a confocal microscope [131].

**Synthesis of methoxy PEG-PLA copolymer**

Methoxy PEG-PLA block copolymer was prepared by ring-opening polymerization. Briefly, 3, 6-dimethyl-1, 4-dioxane-2, 5-dione (LA) was dried in a vacuum oven at 40°C overnight. 1.0 gm of mono-methoxy poly (ethylene glycol) (mPEG) was placed in a flame dried 100 ml three-necked round-bottom flask and stirred at 80°C under vacuum overnight. Various amounts of dried LA polymer (1gm, 2gm, 4gm and 10gm) and 0.5 µl of 0.2 wt% stannous octoate (Sn (Oct)_2) were added to the flask under the protection of argon gas. The mixture was dissolved in 20 ml anhydrous toluene and heated at 140°C under argon gas for 5 hours. Solid products of the diblock copolymers were obtained by adding the sample solutions to 30 mls ice cold diethyl ether. The products were dissolved in 2 mls dichloromethane and precipitated in 30 mls cold diethyl ether twice, for purification. The final copolymer was dried in a vacuum oven at 50°C for 48 hours [126].
Figure 2.7. Schematic of the synthesis of the mPEG-PLA copolymer

\[ \text{mPEG} + \text{Lactide} \xrightarrow{\text{stannous 2-ethyl-hexanoate}} \text{mPEG/PLA} \]

\[ \text{CH}_3\text{O}[\text{CH}_2\text{CH}_2\text{O}]_m + \overset{\text{O}}{\overset{\text{O}}{\overset{\text{O}}{\text{CH}_2\text{CH}_2\text{O}}} \xrightarrow{\text{Toluene, } 140^\circ\text{C, } 5\text{ hours}} \text{CH}_3\text{O}[\text{CH}_2\text{CH}_2\text{O}]_m \]

\( m = \text{number of poly(ethylene oxide) units; } n = \text{number of lactide units.} \)

**Fourier Transformed Infrared spectroscopy (FTIR)**

FTIR was used to determine if the mPEG/PLA copolymer was successfully synthesized and to determine the structure of the polymer. 2 gm of the mPEG-PLA polymers were dissolved in 1 ml chloroform and 0.5 mls were added to the surface of the polyethylene IR card. The chloroform was allowed to evaporate in a vacuum oven (50°C) overnight before putting the card into a Thermo Scientific IR200 spectrometer for analysis. Fourier transform infrared spectra represented an average of 64 scans in the mid-IR wavenumber range 400–4000 cm\(^{-1}\) with a spectral resolution of 4 cm\(^{-1}\). Background scans were obtained from a blank IR card [132].

**Proton Nuclear Magnetic Resonance Spectroscopy (\(^1\text{H NMR}\))**

\(^1\text{H NMR}\) was used to determine the molecular weight and structure of the mPEG/PLA copolymer. 0.5gm of the mPEG-PLA polymer was dissolved in 1 ml deuterated chloroform (CDCl\(_3\)) in a 3ml tube then transferred to a 5mm NMR tube. All spectra were measured by using a Bruker AVANCE DRX 500 instrument operating at 500.13 MHz. The Bruker XWIN-NMR software version 3.5pl5 running on a standard PC was used for acquisition of all spectra.
Construction of the scaffolds

For construction, 3 gm of mPEG-PLA (mPEG: PLA 1:1, 1:2, 1:4 and 1:10) were mixed with 1.2 gm of PLGA and dissolved in 5mls of a solution of dichloromethane and chloroform (80/20 v/v). The solution was placed in a 20 ml glass syringe attached to a polypropylene tube fitted with a 23 gauge steel needle that was clamped at 20 cm from a copper plate covered with aluminum foil. The syringe was attached to an automatic pump that was set a flow rate of 0.2 ml/hr. An electrode from the high voltage source was directly attached to the metal needle. The voltage was applied as soon as the polymer begins to leave the tip of the needle [110]. The electrospun scaffolds were collected on the aluminum covered plate. The scaffolds were gently removed from the plate then cut to approximately 7x7mm$^2$ and placed in 96 well plates, sterilized in isopropyl alcohol, washed three times in PBS then additionally sterilized by exposure to high intensity UV light for one hour.

Construction of chitosan composite and PLGA/PEG scaffolds

The chitosan coated scaffolds were constructed by immersion of scaffolds in 1wt% chitosan (deacetylation>95%) in 1% acetic acid solution for four days. The chitosan solution was removed and the scaffolds were washed with PBS three times then sterilized under UV light for one hour prior to cell culture. The PLGA/PEG scaffolds were constructed by immersion of scaffolds in 1% wt% PEG in dH$_2$O for 3 days. The solution was removed washed with PBS three times then sterilized under UV light for one hour prior to cell culture.
Construction of Films

For the PLGA /mPEG-PLA film 3 gm of mPEG-PLA was mixed with 1.2 gm of PLGA and dissolved in 5mls of a solution of dichloromethane and chloroform (80/20 v/v). 100µl of the solution was delivered onto 22x30 mm glass coverslips and the solvent was allowed to evaporate for 24hours then sterilized under UV light for one hour prior to cell culture. For the mPEG-PLA film 60 mg of the polymer was dissolved in 1ml of a solution of dichloromethane and chloroform (80/20 v/v) and 100µl of the solutions were delivered onto 22x30 mm glass coverslips and the solvent was allowed to evaporate for 24 hours then sterilized under UV light for one hour prior to cell culture.

Migration assay

For the migration assay, day 10 tumoroids were transferred to a tissue culture plate containing complete DMEM medium and the migration was monitored from day 2 to day 4 by direct viewing under a light microscope or stained with calcein AM /EthD-1 stain and viewed under a fluorescent microscope. Tumoroids were also transferred to new 3P scaffold and similarly stained and observed.

Tumoroid diameter and number estimation

For measurement of tumoroid diameter, at least 4 scaffolds/time points and 5 tumoroids/ scaffold were examined. Five fluorescent images were taken randomly per scaffold with at least one spheroid captured per image. To compensate for variation in radii, two diameters representing the shortest and longest diameters were drawn through the center of the tumoroid and the final diameter was calculated as the average of both values using Image J software. Tumoroid numbers were counted from images
taken systematically across each scaffold. Aggregates of size ≥50 µm were counted as tumoroids. For longer term culture greater than eight days, scaffolds were transferred to larger wells to accommodate demanding nutrient requirements. Larger tumoroids can be easily detached from the scaffold using gentle pipetting and transferred to new scaffolds. Tumoroids and monolayer cells were stained with calcein AM/EthD-1 stain. The scaffolds were placed on glass slides, covered with glass coverslips then viewed under a microscope.

Statistical analysis

Quantitative data were expressed as mean +/- standard deviation. Student’s t-test was used to analyze the statistical significance of the data between groups. Values with p<0.05 were considered statistically significant. All experiments were performed in triplicate.

2.4 Results

Growth and proliferation of cells on the PLGA scaffold

The PLGA scaffolds provided structural support and shape to cells, allowed cell attachment, provided good spatial interconnectivity, a high surface-to-volume ratio and good porosity for fluid transport. Confocal image (Fig.2.8A) and SEM image (Fig.2.8B) of the PLGA scaffold showed randomly aligned fibers that combine to form a highly porous mesh. A 3D confocal image of PC3 prostate cancer cells cultured on the scaffold showed that cells grew in all orientations (Fig.2.8C). The parameters that affected the pore size, diameter and thickness of the scaffold included voltage, distance from needle tip to the surface of the collecting sheet and concentration of the polymers in the
solvent. The diameter of the fibers and pore size were calculated from the SEM images and ranged from 0.61 to 4.95 µm with pores of mainly subcellular sizes (<10 µm).

Figure 2.8. PLGA scaffolds. (A) Confocal image of a PLGA scaffold showing a random mat of fibers forming a highly porous mesh. The scaffold was imaged green using the green filter of the confocal microscope and indicates the auto-fluorescence of the scaffold. (B) SEM images of PLGA scaffolds were used to determine the diameter of the fibers (0.61 to 4.95 µm) and pore size (<10 µm). (C) Confocal image showing the 3D orientation of PC3 prostate cancer cells growing in all directions and were not limited to surface areas alone. PC3 cells were cultured for 48 hrs. The scaffold fibers appear blue using the blue filter of the confocal microscope. Actin filaments (red) were stained with phalloidin conjugated to Alexa 555.

Various cell types grew and proliferated on the scaffold. This included mesenchymal stem cells (MSCs) (Fig.2.9), PC3 prostate cancer cells and 3T3 fibroblast cells (Fig.2.10). Recognizing the role of the tumor stroma in cancer growth and differentiation, we wanted to determine if the scaffold could accommodate the growth of cocultured cells. We cocultured PC3 cells with WPMY-1 prostrate stromal cells on the PLGA scaffold and on monolayer. Results from images of cells stained with Celltracker dyes that allowed for live cell imaging and immunostaining for the nuclear antigen Ki-67 present in proliferating cells, showed that cocultured cells grew and proliferated on the
scaffold (Fig. 2.11). Ki-67 is a cell marker for proliferating cells, and is strictly observed in cells undergoing active phases of the cell cycle, but is absent in resting cells. The PLGA scaffold provided the appropriate 3D environment for cells to grow and supplied the template for optimizing the next generation of scaffolds.

**Figure 2.9. Growth of mesenchymal stem cells on PLGA scaffolds.** Bone marrow derived mesenchymal stem cells were obtained from transgenic GFP mice. (A) SEM of monolayer of cells that were cultured for 48hrs. (B) SEM of MSCs on scaffold that were cultured for 48 hrs. (C) Confocal image of MSCs (green) on scaffold that were cultured for 48 hrs. Actin filaments (red) were stained with phalloidin conjugated to Alexa 555. The scaffold appears light green using the green filter on the confocal microscope.

**Characterization of the mPEG-PLA polymer**

To optimize, we chemically modified the PLGA scaffold with the addition of mPEG-PLA polymer. The synthesis of mPEG/PLA was confirmed by FTIR and $^1$H NMR. In the FTIR spectra the major peaks assigned to the copolymer mPEG-PLA were 2850 – 2950 cm$^{-1}$ (C-H stretching of CH$_3$), 1760 cm$^{-1}$ (ester C=O stretching) and 1087–1184 cm$^{-1}$ (O-CH$_2$ stretching) (Fig. 2.12). The molecular structure of the mPEG-PLA copolymer was characterized by $^1$H NMR (Fig. 2.13). The molecular weight of the PLA block of the
mPEG-PLA copolymer was determined to be 23.1kDa using the intensity of the terminal methoxy proton signal at 3.39ppm as the internal standard.

Figure 2.10. Growth of PC3 and NIH 3T3 cells on PLGA scaffolds. PC3 cells were cultured for 48hrs on monolayer or on PLGA scaffold. 3T3 fibroblast cells were cultured for 48hrs on monolayer or on the PLGA scaffold. SEM of both cell types shows the morphological differences of cells on 2D monolayer versus 3D scaffold. Cells on the monolayer appear to have a spread morphology compared to those on the scaffold.
Figure 2.11. Coculture of PC3 and WPMY-1 stromal cells on PLGA scaffolds. (A) 2D monolayer coculture of PC3 cells (green) and WPMY-1 stromal cells (red). Cells were stained with Celltracker dyes and cocultured for 24 hrs before viewing using a wet lens on the confocal microscope. (B) Coculture of PC3 cells and WPMY-1 stromal cells on PLGA scaffold. (C) Enhanced confocal image of PC3 cells interacting with WPMY-1 stromal cells on the PLGA scaffold. (D) Proliferating PC3 and WPMY-1 stromal cells (green) on monolayer. (E) Proliferating PC3 and WPMY-1 stromal cells (green) on PLGA scaffolds. Cells were immunostained for the nuclear antigen Ki-67 (green) which is expressed in proliferating cells, and counterstained with phalloidin conjugated to Alexa 555 (red). The scaffold is imaged blue (B, E) using the blue filter of the confocal microscope.

The scaffolds

Scaffolds were constructed from a mixture of various mPEG-PLA copolymers and PLGA. The scaffolds were designated multilayered nanofiber (MN) scaffolds. Table 2.1 highlights the characteristics of the MN scaffolds. The MN 1:1 and MN1:2 polymers were not amenable to electrospinning while the MN1:4 and MN 1:10 readily
electrospun. The MN 1:10 polymer either did not form tumoroids or formed loose aggregates that did not resemble tumoroids while the MN1:4 polymer formed tumoroids with LLC-1 and PC3 cells (Fig. 2.14). Henceforth the MN1: 4 scaffold is referred to as the 3P scaffold.

![FTIR spectrum of mPEG-PLA polymer](image)

**Figure 2.12. FTIR of mPEG-PLA polymer.** FTIR shows strong absorption at 1760 cm\(^{-1}\) assigned to the –C=O stretch of PLA. The stretch of the C-O-C band of the mPEG and PLA is shown at 1087 and 1184 cm\(^{-1}\), respectively. The peaks at 2850 and 2950 represent –CH\(_2\) stretching of the mPEG, confirming that the mPEG-PLA polymer was successfully synthesized.

**Growth of tumoroids on the 3P scaffold**

SEM of the 3P scaffold showed the diameter of the fibers ranged from 0.69 to 4.18 µm with pores of mainly sub cellular sizes (<10 µm) (Fig. 2.15A). There were no differences observed in the pore size or diameter of the 3P scaffolds versus the PLGA scaffolds.
The SEM images of LLC-1 (Fig.2.15B) and MCF-7 (Fig.2.15C-D) tumoroids showed typical morphology with a smooth surface, tight cell junctions and indistinguishable cellular boundaries. SEM image of MCF-7 cells show intertwining of the fibers into and around the tumoroids that allows for anchoring and stabilization (Fig.2.15C). We compared the growth of LLC-1 cells on the 3P scaffold with cells cultured on monolayer and on PLGA and observed that cells did not form tumoroids on monolayer or on PLGA scaffolds, but cells cultured on the 3P scaffold formed tumoroids on day 3 that increased in size on day 5 (Fig.2.16). Images (100x) were taken systematically across each scaffold and a composite of these images show tumoroid distribution on day 3 (Fig.2.17).

Figure 2.13. $^1$H NMR of mPEG-PLA polymer. The molecular structure of the mPEG-PLA polymer is shown. Peak ‘A’ is assigned to the methyl group and (-CH$_3$) and ‘C’ to the methylene protons (-CH-) in the PLA units. The peak at ‘B’ is attributed to the methylene protons of PEG oxyethylene units. The small peak at ‘D’ is attributed to the methylene protons of -O-CH$_2$-CH$_2$- in the PEG end block that linked to PLA.
Tumoroid size and numbers

The average diameter of LLC-1 tumoroids grown on a 3P scaffold at initial density of 5x10^3 per ml for days 2, 3, 4, 5, and 8 were 93 µm, 200 µm, 211 µm, 279 µm, and 325 µm, respectively. The average tumoroid number/ scaffold were 20, 55, 58, 59 and 66 respectively (Fig.2.18). The minimum size of 50 µm was used as the cutoff point because at this size tumoroids have begun to develop 3D cell-cell interactions over cell-matrix interactions.

Figure 2.14. Growth of cells on the MN scaffolds. LLC-1 cells (5x10^3) were cultured for three days on the MN1:4 and MN1:10 scaffolds. Tumoroids formed on the MN1:4 scaffold but not on the MN1:10 scaffold. Scale bar=100µm. PC3 cells (7x10^3) formed tumoroids at day5 on the MN1:4 scaffold while cells on the MN1:10 scaffold formed loose aggregates that did not resemble tumoroids. Cells were stained with calcein AM/EthD-1 that stains live cells green and dead cells red. Scale bar=50 µm.
Table 2.1. Characteristics of the Scaffolds.

<table>
<thead>
<tr>
<th>Name</th>
<th>Electrospinning potential</th>
<th>Cell growth</th>
<th>Tumoroid formation</th>
<th>Fiber diameter</th>
<th>Pore size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>0.61-4.95µm</td>
<td>&lt;10µm</td>
</tr>
<tr>
<td>MN1:1</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MN1:2</td>
<td>No</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MN1:4</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>0.69-4.18µm</td>
<td>&lt;10µm</td>
</tr>
<tr>
<td>MN1:10</td>
<td>Yes</td>
<td>Yes</td>
<td>Variable</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

NA= Not applicable
The MN1:4 scaffold was renamed the 3P scaffold.

Long term tumoroid culture

For long term culture, scaffolds were transferred to larger wells to accommodate demanding nutrient requirements. Larger tumoroids were detached from the scaffold using gentle pipetting and transferred to new scaffolds. We followed tumoroid growth on the scaffold on days 10, 14, 18 and 20 and observed that tumoroid diameter increased over time. Typically, tumoroids exhibit a spherical proliferative geometry and beyond the diffusion capacity of oxygen and fresh growth medium the innermost cells become quiescent and die resulting in the development of a necrotic core. This was observed in day 20 tumoroids that had attained diameters >500 µm (Fig.2.19).
Figure 2.15. SEM of tumoroids on the 3P scaffold. (A) SEM of 3P scaffold. Fiber distribution in the 3P scaffold shows a random mat of fibers interspersed with pores. SEM was used to determine fiber diameter as 0.69-4.18µm and pore size <10 µm. (B) LLC-1 tumoroid growing on the 3P scaffold that was cultured for 3 days. (C-D) SEM images of an MCF-7 tumoroid cultured on the scaffold for 7 days. (C) This image shows the intertwining of the fibers through the tumoroid anchoring and stabilizing it to the scaffold. (D) A representative image of the entire tumoroid.
Figure 2.16. Growth of tumoroids on scaffolds: LLC-1 lung cancer cells (5x10^3) were cultured on monolayer, PLGA and 3P scaffolds from day 1 to day 5. Cells cultured in monolayer and PLGA scaffold grew but did not form tumoroids for all days observed. Cells cultured on 3P scaffold, formed tumoroids on day 3 that increased in size on day 5. Cells were stained with calcein AM/EthD-1 that stained live cells green and dead cells red. Scale bar = 50µm.
Figure 2.17. Composite image of tumoroids on a 3P scaffold: A representative composite image of LLC-1 tumoroids on a 3P scaffold (day 3) viewed under fluorescence microscope (100x). This image represents the distribution of tumoroids on a single scaffold. Tumoroids were stained with calcein AM/EthD-1 stain. Scale bar =500µm.

Figure 2.18. Tumoroid numbers and sizes. The average tumoroid number per scaffold from day 2 to day 8 went from 20 to 67. Tumoroid numbers were counted from images taken systematically across each scaffold (n=4 scaffolds/time point). The average size of tumoroids for day 2 to day 8 ranged from 93 to 325 µm, respectively. The data is presented as mean +/-SD; n= 4 scaffolds/time points and 5 tumoroids/ scaffold.
Figure 2.19. Day10 to day 20 tumoroids. Representative images of day 10 to day 18 tumoroids taken with a light microscope. Tumoroids were stained with calcein AM/EthD-1 that stained live cells green and dead cells red. The day 20 tumoroid was taken with a confocal microscope and Z stack imaging shows the central area of necrosis that is typical of tumoroids that have diameters greater than 500 µm. Scale bar = 500µm.

Parameters for tumoroid formation

The parameters essential for tumoroid formation depended on concentration of cells, the type of cells and the time from initial seeding of the cells. Density studies of LLC-1 cells cultured at concentrations $3 \times 10^3 - 1 \times 10^4$ at day 1- day 5 showed that cells formed tumoroids at different time points and concentrations and that the higher the concentrations of cells, the faster the tumoroids were formed with subsequent increase in tumoroid diameter over time. Growth kinetics showing the relationship between the growth rate of the tumoroids and the concentration of cells is seen in Fig.2.20. All cell types cultured on the scaffold formed tumoroids. This included B-16 melanoma, BG-1 ovarian cancer cells, MCF-7, MDA-MB-231 and MCF-10A breast cancer cells(Fig. 2.21). Irrespective of tumor types, these cell lines grew tumoroids at different times. For breast cancer cell lines, the highly invasive MDA-MB-231 grew tumoroids faster than the less invasive MCF-7 and MCF-10A cells. However, MCF-7 and MCF-10A cells grew larger tumoroids than MDA-MB-231 cells.
Seeding density and tumoroid formation. LLC-1 cells were cultured at concentrations $3 \times 10^3$ to $1 \times 10^4$/scaffold from day 1 to day 7. Cells were stained with calcein AM/EthD-1 to detect live (green) and dead (red) cells. It was observed that the higher the concentrations of cells, the faster the tumoroids were formed with subsequent increase in tumoroid diameter over time. Scale bar =50µm.
Effects of topography on tumoroid formation

Physico-chemical cues may be responsible for tumoroid formation on the 3P scaffold. Deconstructing the mechanical and chemical components of the scaffold may aid in understanding these mechanisms. We cultured cells on an mPEG-PLA scaffold and compared growth with cells on PLGA scaffolds and on monolayer. Cells grew on PLGA/PEG scaffolds, PLGA scaffolds and on monolayer, but did not form tumoroids (Fig.2.22). To explore the effects of topography on tumoroid formation, we cultured LLC-1 cells on 3P films made from polymers used to construct the 3P scaffold and on mPEG-PLA films. Cells on the 3P film formed tumoroids that easily dissociated when the substrate separated from the slide on day 5. Cells on the mPEG-PLA films grew into large disorganized aggregates that lacked the defined shape and structure of a tumoroid and also dissociated by day 5 (Fig.2.23). This underscores the contribution of topography and chemistry in the maintenance of tumoroid integrity.

Effects of charge on tumoroid formation

To elucidate the effects of charge on tumoroid formation we constructed a composite 3P/Chitosan scaffold. We used chitosan because it is a naturally occurring polysaccharide with a net positive charge at physiological pH which would increase the hydrophilic properties of the scaffold, impart a positive charge to the neutral charge of the scaffold, and would enhance cell adhesion to the surface. A comparison of cells grown on 3P vs. 3P/chitosan composite scaffolds showed that LLC-1 cells proliferated but did not form tumoroids on 3P/Chitosan scaffold while cells cultured on the 3P scaffold formed tumoroids at day 3 and continued to day 5. (Fig.2.24).
Figure 2.21. Growth of other cancer cells as tumoroids on 3P scaffold. All of the cancer types cultured on the 3P scaffold grew tumoroids. Breast cancer cells, MCF-10A (5x10^3 cells/scaffold) at day 5, MDA-MB-231 (5x10^3 cells/scaffold) at day 4, MCF-7 (7x10^3 cells/scaffold) at day 5, B16 melanoma (5x10^3 cells/scaffold) at day 5 and BG-1 ovarian cancer (5x10^3 cells/scaffold) at day 5 grew tumoroids. Cells were stained with calcein AM/EthD-1. Scale bar =50µm.

Figure 2.22. Absence of tumoroid formation on scaffolds. LLC-1 cells (5x10^3/scaffold) proliferated on PLGA/mPEG scaffolds but did not form tumoroids similar to that observed on PLGA scaffolds, while tumoroids formed on the 3P scaffold (day 4). Cells were stained with calcein AM/EthD-1 to detect live (green) and dead (red) cells. Scale bar =50µm.
Figure 2.23. Effects of topography on tumoroid formation: LLC-1 cells were cultured on 3P and mPEG/PLA films ($5 \times 10^4$) and on 3P scaffolds ($5 \times 10^3$) from day 3 to day 5. Cells were stained with calcein AM/EthD-1 to detect live (green) and dead (red) cells. Cells on the 3P film formed tumoroids that easily dissociated along with the film on day 5. Cells on the mPEG/PLA film grew into large disorganized aggregates that lacked the shape and structure of a tumoroid and also dissociated by day 5. Cells on the 3P scaffold formed tumoroids on day 3 and continued to day 5. Scale bar = 500 µm.
Figure 2.24. **Effects of charge on tumoroid formation.** LLC-1 cells were cultured from day 2 to day 4 on the 3P/chitosan composite scaffold. Chitosan imparted a net positive charge to the neutral scaffold at physiological pH. Cells grew on the 3P/chitosan composite scaffold but did not form tumoroids demonstrating better cell-scaffold over cell-cell interactions. Cells cultured on the 3P scaffold formed tumoroids on day 3 and day 4. Cells were viewed by SEM and calcein AM/EthD-1 staining. Scale bar =50µm.
Cell migration from tumoroids onto tissue culture plate

LLC-1 tumoroids that were transferred from the 3P scaffold to a regular tissue culture plate adhered to the plate and migrated out from the tumoroid underscoring the importance of 3P topography in tumoroid formation. LLC-1 cells gradually migrated away from the tumoroid on day 2 and ultimately formed a confluent monolayer by day 4 (Fig. 2.25). Tumoroids transferred to new scaffolds however maintained their morphology and shape over the same time period.

Figure 2.25. Tumoroids cultured on tissue culture plate. Tumoroids (day 10) were transferred from the 3P scaffold onto tissue culture plate and cultured for two days. Cells grew out from the tumoroid onto the plate starting at day 2. Top panel: phase contrast image; bottom panel: cells stained with calcein AM/EthD-1 to detect live (green) and dead (red) cells; side panel: close up view of cells migrating from the tumoroid to the plate (day 2).
Chapter Three

Tumoroids grown on 3P scaffolds mimic in vivo tumorigenesis

3.1 Introduction

The extracellular matrix is essential in directing numerous cellular processes during cancer. Cells adhere and interact with their underlying ECM via integrins linked to focal adhesions in their cytoskeleton. This represents the primary mechanotransductive axis of the cell and enables cells to apply forces to the ECM as well as sense mechanical perturbations emanating from the environment \[133\]. These interactions are similar when cells are cultured on various 3D artificial scaffolds \[134\] - \[135\]. Epithelial mesenchymal transition (EMT) is a highly orchestrated and complex event involving the integration of biochemical signals from integrins acting as ECM receptors, and growth factor receptors such as transforming growth factor-β (TGFβ) and receptor tyrosine kinases that activate Rho family of small GTPases \[136-138\]. TGF-β is the major inducer of EMT and signals in a canonical manner to phosphorylate cytoplasmic SMAD proteins that translocate to the nucleus and stimulate the EMT transcriptional program or requires the activities of signaling pathways such as phosphatidylinositide 3-kinases/protein kinase B pathway (PI3K/AKT) and the mitogen activated protein kinase pathway (MAPK), indirectly through transactivation in a non-canonical manner \[139-141\]. PI3K signaling can be activated by integrins and members of the Rho GTPases.
that control cytoskeleton re-modeling, a major necessity during the morphogenic process of EMT [142,143]. In response to changes in their environment, cells will attempt to match their internal stress to the external environment. Increased cell contractibility and conformational changes in response to stiffness can influence biochemical signaling cascade via integrin clustering that induce the binding of RGD sequences on the latent TGFβ receptor resulting in activation. The consequences lead to the activation of EMT signaling pathways, the downregulation of E-cadherin and the upregulation of mesenchymal genes [134,144,145]. These interactions suggest that EMT can no longer be examined as a purely cellular event, but one that is affected by the physical status of the microenvironment. At the biomaterial/cellular interface both biochemical and mechanical cues from in vitro 3D scaffolds play a role in activating the EMT program. It has been shown that breast cancer cells induce EMT when grown on aligned electrospun fibers than on scaffolds consisting of random fibers [146]. The formation of spheroids in 3D matrices further introduces pathophysiological cues that may modify cancer cell fate towards a metastatic differentiated pathway. Considering that 3P scaffolds induced tumor cells to form tumoroids that resemble micrometastatic tumors, we determined whether LLC-1 tumoroids formed on 3P scaffolds could undergo the EMT and compared these results with cells grown on PLGA scaffolds and in 2D monolayer culture. We hypothesize that the novel 3P scaffold allows for the formation of tumoroids that mimic in vivo tumorigenesis, observed by the induction of EMT. This was shown by the expression of vimentin and the loss of expression of E-cadherin suggesting that tumoroid formation correlate with enhanced invasive potential and tumorigenicity, characteristics that define the EMT and that appear to be manifested
within the context of the 3P environment. EMT expression coincided with tumoroids formation on the scaffold.

**Epithelial mesenchymal transition (EMT)**

EMT is a developmental program that regulates embryonic morphogenesis and involves major phenotypic and molecular changes in cells. Experimental models reveal that EMT is re-utilized during the re-epithelialization of wound healing and tissue fibrosis as well as during tumorigenesis \[147,148\]. EMT occurs in oncogenically transformed cells that house a variety of genetic and epigenetic abnormalities that conspire with the molecular cascade that underlies EMT to elicit metastatic dissemination. As cancer evolves towards metastasis, EMT imparts malignant features such as motility, invasion, resistance to apoptosis and therapeutic drugs and the acquisition of stem-like properties to cells \[149-151\]. Invasion is characterized by the loss of cell to cell contact, loss of basolateral polarity and adherens junction, cytoskeleton rearrangement and changes in the interactions of tumor cells with the extracellular matrix. This is as result of the disruption and delocalization of tight junction complexes for example claudins and zona occludins-1, and formation of actin stress fibers. This is succeeded by the loss of E-cadherin expression and activity that results in the nuclear accumulation of β-catenin.

**Loss of E-cadherin expression is the hallmark of EMT**

The hallmark of EMT is the transcriptional repression of E-cadherin. It is a calcium dependent transmembrane glycoprotein responsible for cell-cell adhesion, and cytoskeleton organization. The loss of function has been associated with the
progression of metastasis and poor prognosis in several cancers including prostate, breast and lung [152,153]. The repression of E-cadherin is a consequence of the association of transcriptional repressors with E-box sequences within the E-cadherin promoter. Known transcription factors such as the snail family of zinc finger proteins Zeb-1, Snail 1 and 2, and the basic helix-loop-helix protein Twist, form repressive complexes in a sequential manner [154-157]. In various human carcinomas, functional loss of E-cadherin results from the production of a defective protein, which may be a result of gene mutation, abnormal post-translational modification (phosphorylation or glycosylation) or increased proteolysis [158-160]. Besides its regulation by the transcriptional repressors the E-cadherin gene expression can also be regulated at the transcriptional level by silencing through promoter hypermethylation. In fact, E-cadherin has been one of the first genes with promoter hypermethylation identified at very high frequencies in human cancer specimen [161]. Concomitant with the loss of E-cadherin is the expression of mesenchymal markers such as vimentin that is ubiquitously expressed in mesenchymal cells as well as other markers such as smooth muscle actin (SMA) and fibronectin.

**EMT and the tumor stroma**

During the progression into malignancy, EMT may be induced by a variety of stimuli, including growth factor signal transduction pathways to the loss of E-cadherin functional gene or protein degradation and mutation. Signal transduction pathways involving TGF-β, wingless-related integration site (WNT), PI3K/AKT, MAPK and Notch pathways cooperate by crosstalk via growth factors and cytokines to effect repressor functions.
In many cancers, the tumor-stroma produces a variety of growth factors, such as epithelial growth factor [165], platelet derived growth factor (PDGF) [166] and fibroblast growth factor [167] and the most important inducer of EMT, TGF-β [168] that are able to induce the transcriptional repressors of E-cadherin. For example, hepatocyte growth factor (HGF) stimulates the EMT process by activating MAPK signaling resulting in the activation of the transcription factor Egr-1 and the subsequent expression of Snail-1 leading to the downregulation of E-cadherin gene expression [169]. In addition to MAPK signaling, PI3K signaling plays a key role in inducing and maintaining EMT. Cells expressing a constitutively active form of AKT, the most important downstream effector of PI3K signaling, induced expression of Snail-1, which in turn repressed E-cadherin gene transcription and induced EMT [170]. Furthermore, autocrine PDGF receptor signaling in the presence of oncogenic Ras hyper activated PI3K signaling which is required for cell survival during EMT [171]. TGF exercises two opposing functions during cancer. In the early stages it functions as a tumor suppressor by inhibiting proliferation and inducing apoptosis. However, in cells that have escaped its pro-apoptotic effects, TGF enhances cell invasion, migration and evasion of immune surveillance [172]. TGF-β is the major inducer of EMT and signals in a canonical manner via two serine/threonine receptors to phosphorylate and form complexes with cytoplasmic mothers against decanpentaplegic (SMAD) proteins that translocate to the nucleus and stimulate the EMT transcriptional program. Upregulation of mesenchymal genes such as Vimentin, Fibronectin, and matrix metalloproteinase are important operands in bestowing migratory and invasive properties and are targeted by TGF-β. The Notch pathway, crosstalks significantly with the TGF-β pathway via SMADs that
upregulate the Notch receptor ligand Jagged 1, and induces cell cycle arrest. WNT signaling elicits sequestration of glycogen synthase kinase that promotes the stabilization of Snail further cooperating with other transcription factors to promote EMT.

**EMT and stem cells**

Empirical evidence connecting EMT to the emergence of stem cells has recently been reported [173]. Pathophysiological conditions such as tumorigenesis can trigger differentiated cells to acquire a multipotent stem-cell-like phenotype through EMT induction [174]. Studies also indicate that metastatic cancer cells, which have presumably undergone EMT, may exhibit a cancer stem cell (CSC) phenotype. For instance, disseminated breast cancer cells found in pleural effusions are enriched for a CD44\textsuperscript{high} and CD24\textsuperscript{low} CSC-like population [175]. In pancreatic tumors, CD133+ mesenchymal like cells that also express the chemokine receptor 4 (CXCR4) are found predominantly at the invasive front of tumors where they may be primed for metastatic spread [176]. A four-week period of exposure of immortalized human bronchial epithelial cells to tobacco carcinogens has been shown to induce a persistent, irreversible, and multifaceted dedifferentiation program marked by EMT and the emergence of stem cell-like properties [177].

**3.2 Methods**

**Immunofluorescence staining**

Cells were washed three times with phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature, followed by
permeabilization in 1.0% Triton X-100 for 15 minutes. They were then incubated with 1% bovine serum albumin (BSA) for 30mins at room temperature then incubated with the primary antibody overnight at 4°C. They were washed three times with PBS then incubated with the secondary antibody for one hour at room temperature in the dark. The primary antibodies against vimentin (rabbit polyclonal) (Cell Signaling) were diluted at 1:200 and E-cadherin (mouse monoclonal) (Santa Cruz Biotechnology) was diluted at 1:200. Secondary antibodies were goat anti-rabbit conjugated to Alexa 594 and goat anti-mouse conjugated to Alexa 488 (Invitrogen). All antibodies were diluted in 1% BSA [178]. Cells were washed three times with PBS then two drops of the mounting media containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) were added prior to covering with a glass coverslip and viewing on a fluorescent microscope.

3.3 Results

Tumoroids formation induce EMT

LLC-1 cells were cultured as monolayer or on PLGA and 3P scaffolds and examined for EMT marker expression. Cells on the 3P scaffold formed tumoroids by day 3 that expressed vimentin while none was observed in the monolayer culture or in cells cultured on PLGA scaffolds. Additionally tumoroids exhibited a loss of E-cadherin expression on 3P scaffolds and EMT. However cells cultured on monolayer or on PLGA scaffolds retained E-cadherin expression and an absence of vimentin and no EMT (Fig.3.1).
Figure 3.1. EMT in LLC-1 tumoroids. LLC-1 cells (5x10^3) were cultured as monolayer, or on PLGA or 3P scaffolds for three days, then fixed and immunostained with anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue) for staining nuclei. (A) LLC-1 cells on PLGA scaffolds and on monolayer showed E-cadherin expression while cells cultured on the 3P scaffolds that formed tumoroids by day 3 did not express E-cadherin. (B) Cells on PLGA scaffolds and on monolayer showed no vimentin expression while tumoroids on the 3P scaffold showed vimentin expression. Cells cultured on monolayer and PLGA scaffold showed no EMT while EMT correlated with the formation of tumoroids on the 3P scaffold. Scale bar= 20 µm.
Timeline of EMT

To determine the timeline of EMT induction, LLC-1 cells were cultured on 3P scaffolds and stained for vimentin and E-cadherin expression from day 1 to day 4. From day 1 to day 2 cells did not form tumoroids. E-cadherin expression was observed but there was an absence of vimentin expression indicating the absence of EMT. However, it was observed that cells begin to form tumoroids beginning at day 3 and there was a loss of E-cadherin expression and a gain in vimentin expression that signified the onset of EMT. Tumoroid formation and EMT induction continued into day 4. The timeline of EMT coincided with the self-assembly of the cells into tumoroids (Fig. 3.2).

EMT in other tumoroids

All of the cell lines that formed tumoroids on the 3P scaffolds expressed EMT markers. EMT correlated with tumoroid formation on the 3P scaffold. Cells cultured on monolayer for each of these cell lines showed E-cadherin expression and the absence of vimentin and no EMT. However, with the formation of tumoroids, EMT marker expression was observed. PC3 (human prostate cancer) expressed EMT markers at day 5, B16 (mouse melanoma) at day 5, MCF-7 at day 7, and MDA-MB-231 (human breast cancer) at day 4 cells (Fig. 3.3). LLC-1 cells cultured on 3P/chitosan composite scaffolds did not form tumoroids and there was an absence of EMT marker expression, however cells cultured on the 3P scaffold formed tumoroids and were positive for EMT (Fig. 3.4).
Figure 3.2. Timeline of EMT. LLC-1 cells (5x10^3) were cultured on 3P scaffolds from day 1 to day 4 and immunostained with anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue) for staining nuclei. (A) E-cadherin expression was observed in cells cultured in day 1 and day 2 but absent in tumoroids that started to form in day 3 and continued to day 4. (B) Cells cultured from day 1 to day 3 showed no vimentin expression but there was vimentin expression in tumoroids formed in day 3 and continued to day 4. EMT coincided with the onset of tumoroid formation at day 3. Scale bar =20μm.
Figure 3.3. EMT in other tumoroids. B16 melanoma, BG-1 ovarian cancer, MCF-7 breast cancer, MDA-MB 231 breast cancer and PC3 prostate cancer cells were cultured on monolayer and 3P scaffold, then were fixed and immunostained with anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue) for staining nuclei. Cells cultured on monolayer showed E-cadherin expression, no vimentin expression and absence of EMT. Cells on the 3P scaffold formed tumoroids and showed vimentin expression while no expression of E-cadherin was observed. B16 tumoroids expressed EMT markers at day 5, BG-1 at day 5, MCF-7 at day 7, MDA-MB-231 at day 4 and PC3 at day 5. Scale bar =20 µm.
**Figure 3.4. Absence of EMT in 3P/chitosan tumoroids.** LLC1 cells (5x10³) were cultured on 3P/chitosan composite scaffold or 3P scaffold for 3 days, then immunostained with anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue) for staining nuclei. Cells on the 3P/chitosan composite scaffold did not form tumoroids and showed expression of E-cadherin, the absence of vimentin expression and EMT. Cells on the 3P scaffold formed tumoroids and showed the absence of E-cadherin expression, the presence of vimentin and EMT induction. Scale bar = 20µm.
Chapter 4

The 3P scaffold can be used to assess chemotherapeutic efficacy and serve as a platform for the growth of tumor biopsies\(^3\)

4.1 Introduction

Three-dimensional (3D) culture systems such as the 3P scaffold, that mimic the tumor microenvironment may serve as optimal tools for drug screening. An eventual goal for such systems is to include the ability to incorporate individual patient tumor biopsies to test patient-specific responses to chemotherapeutic drugs and produce responses more predictive of clinical efficacy. The use of these 3D systems could improve drug testing in at least two ways: 1) speed decision-making for whether a particular therapeutic agent is worth pursuing thereby reducing time, cost and pain to the patient and 2) lead to fewer trial failures because of faster, more relevant results. Tumoroids represent physiologically relevant platforms for drug screening because they mimic the micrometastasis of tumors. With increasing size, they develop regions of hypoxia and central necrosis that have been shown to cause drug resistance [179,180]. They exhibit chemical gradients and mass transport limitations that are spatially correlated with

---

\(^3\) A portion of this chapter have been previously published by the author in an article entitled “A 3D Fibrous Scaffold inducing Tumoroids: A Platform for Anticancer Drug Development”, PlosOne, October 16 2013, Vol.8, Issue 10 and have been reproduced with permission from the publishers.
cellular physiology and are associated with alterations in sensitivity to a wide variety of anti tumor agents. In addition the sphere like symmetry may allow for simple theoretical analysis for the prediction of, for example, drug penetration and binding. The cell-cell interactions via cytoskeleton proteins such as tight junctions and the consequent signal transduction communication have been found to influence the response of cells to drugs [181]. Tumoroids are amenable to co-culture of different cell types and it has been shown that different cellular fractions in the tumoroid affect behavior and response to drugs similar to that observed in vivo [182]. Cocultured tumoroids may provide a model for deciphering the responses of different cell types to drug resistance in vivo. Our goal is to produce a 3D culture system that is validated against known effective anticancer agents to demonstrate the system's utility as a predictive tool and screening assay. We hypothesize that the 3P tumoroids can be used to assess chemotherapeutic efficacy, support the growth of biopsy tumoroids and can be utilized to culture primary patient samples for personalized drug therapy. Both PI3K and MAPK pathways have been implicated in EMT in cancer cells. The inhibitors, such as LY294002 that inhibits the PI3K pathway and U0126 that inhibits the MAPK pathway, are control modulators of EMT and have shown broad antiproliferative activity amongst tumor cell types including melanoma, liver and breast cancer cells [183-186] [187,188]. We show that 3P tumoroids treated with antitumor agents LY294002 and U0126 inhibitors displayed a dose dependent cytotoxic response. Thus, our results demonstrate that the 3P tumoroids can be used to assess chemotherapeutic efficacy in both prevention and treatment modalities using the inhibitors. Finally, we evaluated the possibility of generating 3P tumoroids from tumor biopsy specimens of mice and testing them for
anticancer drug sensitivity. Tumor-stroma interactions affect cancer growth, metastasis and the acquisition of drug resistance. 3P scaffolds supported the growth of both tumor and stromal cells. Our results show that 3P scaffolds can be used both to study the mechanism of tumorigenesis and to evaluate anticancer drugs in the arena of individualized and patient specific therapy.

**Antiproliferative drugs Ly29402 and U0126**

Both MAPK and PI3K/AKT signaling pathways are known regulators of tumor invasion and EMT. For example, growth factors such as HGF stimulates the EMT process by activating MAPK signaling resulting in the activation of the transcription factor, Snail-1, leading to the transcriptional repression of E-cadherin gene expression, the hallmark of EMT. Additionally it has been shown that cells expressing a constitutively active form of PKB/AKT, the most important downstream effector of PI3K signaling, induce expression of Snail-1, which in turn represses E-cadherin gene transcription and induces EMT. The PI3K pathway inhibitor LY294002 and MAPK pathway inhibitor U0126 have shown broad antiproliferative activity amongst many tumor cell types including MDA-MB468 and ZR-75 breast cancer, and 451LU and SKMEL 28 melanoma cell lines [189,190]. LY294002 inhibits the PI3K/AKT pathway by dephosphorylation of AKT thereby inducing G1 arrest followed by apoptosis. U0126 selectively inhibits MEK a dual specificity kinase in the MAPK cascade from phosphorylating ERK1/2 and prevents activation leading to apoptosis.
4.2 Methods

Inhibition of tumoroid formation and IC-50 determination

MCF-7 cells were cultured in 3P scaffolds at a cell density of $7 \times 10^3$ /scaffold for two days in complete medium. The culture medium was carefully removed and replaced with 100 µl of fresh media with LY294002 (at concentrations 0.0 µM, 0.1µM, 1.0µM and 10µM) or U0126 (at concentrations 0.0nM, 10nM, 100nM, and 1000nM). Cells were incubated for three days in a humidified atmosphere under 5% CO$_2$/95%air at 37°C then stained with calcein AM / EthD-1 stain. The viable cell number was counted using the ImageJ software. The cell viability was estimated by dividing the treated viable cells by untreated viable cell number. The IC-50, the concentration of drugs required to inhibit 50% cell growth was calculated from the dose response curve using GraphPad Prism Software (version 5.01).

Treatment of established tumoroids with inhibitors

For evaluating effects of inhibitors on established tumoroids, LLC-1 cells were seeded in the 3P scaffolds at a cell density of $5 \times 10^3$/scaffold and cultured for four days in complete DMEM media to allow for tumoroid formation. The culture media was replaced with fresh media containing different concentrations of the LY294002 inhibitor (0.0µM, 0.01µM, 0.1µM and 1.0µM) and U0126 inhibitor (0.0nM, 1.0nM, 10nM and 100nM). The media were replaced with fresh media or media containing the drugs every two days. Tumoroids were stained with calcein AM/EthD-1 at 24hrs, 48hrs and 96hrs post-treatment and evaluated for tumoroid size and numbers.
**Celltracker staining**

MCF-7 tumoroids cultured on the scaffolds for 7 days were washed with PBS twice. Celltracker Green (0.3125uM) in serum free media was added to scaffold and incubated at 37°C for 15 mins. The solution was removed and the scaffolds were incubated in serum-free media at 37°C for 30 minutes. The scaffolds were placed in a glass-bottomed dish containing a 10uM solution of doxorubicin and timed uptake of the drug was observed using an Olympus 1X81 inverted microscope with a microcell culture chamber attached. Images were taken using a 60X water lens. Celltracker green was excited at 488nm and collected with a 525/50-band pass emission filter. Doxorubicin was excited at 488 and emission collected with a 617/73 nm band pass filter. Serial images (20 slices, 6 tumoroids) were taken at half hour intervals up to four hours until nuclear uptake of the drug was observed in both monolayer and tumoroid cells.

**Culturing fine needle aspirates (FNAs) of implanted mouse tumors**

LLC-1 cells (5x10^5) were subcutaneously injected into the flanks of wild type C57BL/6 mice (National Cancer Institute). Tumor formation was monitored for two weeks after which tumor biopsies were collected as FNAs. Briefly, a 23 gauge needle was inserted into the tumor using a rotating motion and the tissue samples were flushed from the needle by attaching a syringe filled with tissue culture medium and expelling the contents into a sterile tube. This process was repeated twice. The three collected tumor samples were pooled and single cell suspensions were cultured on the scaffold.
**Immunofluorescence staining**

For dual immunostaining, biopsy tumoroids were incubated with the first primary antibody in 1% BSA in phosphate-buffered saline plus Tween 20 (PBST) overnight at 4°C, washed three times with PBS, and incubated with the corresponding secondary antibody in 1% BSA in PBST for one hour at room temperature in the dark. After washing three times with PBS, cells were blocked for a second time with 10% serum from the species the secondary antibody was raised for 30 minutes at room temperature. Cells were then incubated with the second primary antibody in 1% BSA in PBST overnight at 4°C, followed by the second secondary antibody in 1% BSA for 1 hr at room temperature. The cells were stained with the nuclear stain DAPI and imaged with a fluorescent microscope. Carcinoembryonic antigen (CEA) antibody (mouse monoclonal) (GeneMed) was used at 1:200, CD31 (mouse monoclonal) (Abcam) at 1:50, F4/80 (rat monoclonal) (Abcam), SMA (goat polyclonal) (Abcam) at 1:100 dilutions, Vimentin (rabbit polyclonal) (Cell Signaling) at 1:200 and E-cadherin (mouse monoclonal) (Santa Cruz) at 1:200 dilutions [132]. Secondary antibodies were goat anti-mouse conjugated to Alexa 594, (Invitrogen), rat anti-mouse conjugated to Alexa 488, goat anti-rat conjugated to Alexa 488, and donkey anti-goat conjugated to Alexa 488, goat anti-rabbit conjugated to Alexa 594 and goat anti-mouse conjugated to Alexa 488 (Invitrogen). Secondary antibodies were diluted 1:100 in 1% BSA.

**4.3 Approval declaration for animal studies.**

All animal studies were approved and conducted according to guidelines of the University of South Florida Institutional Animal Care and Use Committee.
4.4 Statistical analysis
Quantitative data were expressed as mean +/- standard deviation. Student’s t-test was used to analyze the statistical significance of the data between groups. Values with p<0.05 were considered statistically significant. All experiments were performed in triplicate.

4.5 Results
Prevention of tumoroid formation by inhibitors
To test the feasibility of the 3P scaffold to assess drug sensitivity, we examined the effects of known antitumor agents as control modulators of EMT. First, to evaluate if treatment can prevent tumoroid formation, MCF-7 cells were cultured on 3P scaffolds in the presence or absence of the LY294002 or U0126. Both inhibitors prevented tumoroid formation but cells that were untreated formed tumoroids (Fig.4.1). Cells that were treated with inhibitors showed the presence of E-cadherin expression and the absence of vimentin indicating the absence of EMT while cells that were untreated formed tumoroids and were positive for vimentin expression and an absence of E-cadherin and were positive for EMT (Fig.4.2).

IC-50 determination
Cells were treated with LY294002 and U0126 inhibitors at day 2 of culture before tumoroids were formed. The cells were incubated with or without the inhibitors for 3 days then cells the were stained with calcein AM/EthD-1. The viable cell number was
counted using the ImageJ software. The cell viability was estimated by dividing the treated viable cells by untreated viable cell number and the IC-50 was calculated from the dose response curve using GraphPad Prism Software. Scaffold-grown MCF-7 cells showed reduced sensitivity to inhibitors compared to cells on monolayer, as determined by the IC-50. The IC-50 of LY294002 for the monolayer was 0.1µM and for the 3P scaffold was 1.092 µM. Similarly, the IC50 of U0126 on the monolayer and 3P scaffold was 6.72 nM and 652 nM, respectively (Fig.4.3).

**Treatment with LY294002 inhibitor**

Our second approach was to determine if these inhibitors were effective in modulating established tumoroid growth. Formed LLC-1 tumoroids (day 3) were treated with various concentrations of Ly294002 for 24hrs, 48hrs and 96 hrs then assessed for changes in diameter and numbers. Measurements of tumoroid size and numbers revealed a dose-dependent cytotoxicity in treated tumoroids compared to untreated tumoroids. With increasing concentration of drugs, we observed more cell death accompanied with the dissolution of the tumoroids (Fig.4.4A). At 24 hours, tumoroids treated with LY294002 at concentration of 0.01µM, 0.1µM and 1.0µM demonstrated an average decrease of 17%, 22% and 37% in size respectively compared with untreated tumoroids. At 48 hours post treatment tumoroid size decreased on average 29%, 33% and 51% respectively and at 96 hours post treatment, those treated with 0.01 µM decreased 63% while those treated with1.0 µM and 0.1µM of inhibitor appeared dead. Tumoroid numbers decreased significantly from 14%, 26% and 37% respectively at 24hrs post treatment to 40%, 53% and 62% respectively at 48hrs. Tumoroids treated with 0.01µM inhibitor decreased by
93% while tumoroids treated with 1.0uM, 0.1uM of inhibitor dissipated at 96 hours after treatment. (Fig 4.4B).

Figure 4.1. Inhibition of tumoroid formation. MCF-7 breast cancer cells were cultured for 2 days on the 3P scaffold then treated with 1µM LY294002 and 100nM of U0126 for three days. The inhibitors prevented tumoroid formation on treated cells while untreated control cells grew tumoroids. Scale bar =50µm.

Figure 4.2. Inhibition of tumoroid formation abrogates EMT. MCF-7 cells were cultured on the 3P scaffold for two days then treated with 1µM LY294002 and 100nM U0126 for three days. Cells were immunostained with anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue) for staining nuclei. Cells that were treated with the inhibitors showed the presence of E-cadherin and the absence of vimentin and were negative for EMT. Untreated cells formed tumoroids that were positive for EMT markers. Scale bar = 20 µm.
Figure 4.3. Cytotoxicity response of MCF-7 cells to inhibitors. MCF-7 cells were cultured on monolayer and 3P scaffolds for two days then treated or not treated with LY294002 or U0126 inhibitors. Cells were incubated for three days then stained with calcein AM / EthD-1. The IC-50 was calculated from the dose response curve using GraphPad Prism software. Cells cultured on the 3P scaffold demonstrated more resistance to the LY294002 and U0126 than cells cultured on monolayer. The IC-50 for cells treated with LY294002 on the 3P scaffold was 1.092 µM and on monolayer 0.1 µM. The IC50 for cells treated with U0126 on the 3P scaffold was 652 nM and for cells cultured on monolayer 6.72 nM. Data is presented as mean +/- SD, n= 4 scaffolds/concentration of drugs. Experiments were performed in triplicates.

Treatment with U0126 inhibitor

LLC-1 tumoroids treated with U0126 followed a similar trend. LLC-1 tumoroids (day 3) were treated with different concentrations U0126 for 24hrs, 48hrs and 96 hrs then assessed for changes in tumoroid diameter and numbers. With increasing concentration of drugs, we observed more cell death accompanied with the dissolution of the tumoroids (Fig.4.5A). At 24 hours, tumoroids treated with U0126 at concentration of 1.0 nM, 10nM and 100nM demonstrated an average decrease of 18%, 30% and 35% in size
respectively compared to untreated tumoroids. At 48 hours post treatment tumoroid size decreased on average 35%, 45% and 51% respectively and at 96 hours post treatment, those treated with 1.0 nM and 10nM decreased 60% and 80% respectively while those treated with 100nM of inhibitor appeared dead. Tumoroid numbers decreased from 29%, 30% and 43% respectively at 24hrs post treatment to 45%, 45% and 51% respectively at 48hrs. Tumoroids treated with 1.0 nM and 10nM inhibitor decreased by 71% and 88% while spheroids treated with 100nM of inhibitor dissipated at 96 hours post treatment (Fig.4.5B).

**Diffusion of doxorubicin into 3P tumoroids**

It has been proposed that decreased sensitivity to anticancer drugs in tumoroid cultures may be attributed to factors related to micro-environmental mechanisms operating at the multicellular level as well as a function of the synthetic *in vitro* 3D conditions. Since these factors may limit drug penetration into the interior of the spheroid we utilized the intrinsic auto florescent ability of doxorubicin to evaluate if diffusion limitations were a factor in drug resistance. After incubating MCF-7 tumoroids on the 3P scaffold and on monolayer in the presence of doxorubicin, it was observed that the drug completely penetrated the tumoroid in 3 hours compared to monolayer that took two hours (Fig.4.6) suggesting that drug resistance in the 3P system cannot merely be explained by effects on drug transport.
Figure 4.4. LY294002 induced cell death in LLC-1 tumoroids. (A) LLC-1 tumoroids (day 4) were exposed to various concentrations of LY294002 for the indicated days and then stained with calcein AM/EthD-1. Significant cell death with the dissipation of tumoroids occurred at 96 hrs post-treatment. Scale bar =50 µm. (B) The average change in tumoroid size and numbers compared with untreated tumoroids show systematic decrease in tumoroid numbers and diameter and total cell death by 96 hrs post-treatment. The data is presented as mean ± SD (n= 4 scaffold /time point and 5 tumoroids/scaffold); * p<0.05.
Figure 4.5. **U0126 induced cell death in LLC-1 tumoroids.** (A) LLC-1 tumoroids (day 4) were exposed to various concentrations of U0126 for the indicated days and then stained with calcein AM/EthD-1. Cell death and dissipation of tumoroids occurred at 96 hrs post-treatment. Scale bar =50 µm. (B) The average change in tumoroid size and numbers compared with untreated tumoroids show gradual decrease in tumoroid numbers and diameter and total cell death by 96 hrs post-treatment. The data is presented as mean ± SD (n= 4 scaffold /time point and 5 tumoroids/scaffold); * p<0.05.
Figure 4.6. Diffusion of doxorubicin into MCF-7 tumoroids. MCF-7 tumoroids and monolayer cells were treated with 10µM doxorubicin and observed for timed uptake of the drug by confocal microscopy. Cells (green) were stained with CellTracker dye. Utilizing the intrinsic autofluorescence ability of doxorubicin the drug appears red in the nucleus using the red filter of the confocal microscope. Doxorubicin completely diffused into the nuclei of the monolayer cells in 2 hours and the tumoroid in 3 hours.

Treatment with inhibitors abrogates EMT

To determine if treatment with these inhibitors inhibited EMT in fully formed tumoroids, we administered the same concentrations of the drugs to tumoroids cultured for four day on the 3P scaffold. Results showed that after 48hrs, treatment with LY294002 and U0126, EMT was abrogated. This was demonstrated by the absence of vimentin and the presence of E-cadherin expression (Fig.4.7). Control tumoroids that were not treated by the inhibitors showed vimentin expression and the absence of E-cadherin and were positive for EMT. These findings implicate both PI3K and the MAPK signaling as significant contributors to tumoroid formation and EMT on 3P scaffolds.
Growth of biopsy tumoroids

To determine if the 3P scaffold can be utilized to culture tumor biopsies, single-cell suspensions of FNAs of implanted mouse tumors were cultured on 3P scaffolds. The average diameter increased from day 1 to day 5 was 152.29µm to 287.5 µm at approximately the same rate observed in the experiments with tumor cell lines. Immunostaining of biopsy tumoroids showed loss of expression of E-cadherin and gain of expression of vimentin and EMT induction (Fig. 4.8).

Presence of tumor stroma in biopsy tumoroids

Biopsy tumoroids were examined for the presence of stromal cells that are typical of the in vivo tumor microenvironment by immunostaining with antibodies to respective cell surface markers. Biopsy tumoroids were found positive for CEA and the stromal cell markers CD-31, F4/80 and SMA. LLC-1 tumoroids, used as control expressed only CEA (Fig.4.9).

Chemosensitivity of tumor biopsy grown on 3P scaffolds

To determine if the biopsy tumoroids cultured on 3P scaffolds can be utilized to assess chemotherapeutic efficacy, FNA tumoroids were cultured in the presence or absence of the inhibitors, LY294002 and U0126. Results showed that inhibitors were effective in modulating biopsy tumoroid growth as revealed by decrease in tumoroid size Fig.4.10). However, the IC50s of LY294002 and U0126 were >1uM indicating a greater resistance of biopsy tumoroids to the drugs than LLC-1 tumoroids. drugs than LLC-1 tumoroids.
Figure 4.7. EMT in tumoroids treated with inhibitors. LLC-1 tumoroids were treated with LY294002 (1 µM) and U0126 (100nM) for 48hrs then immunostained using anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue). (A) Untreated control tumoroids expressed vimentin while tumoroids treated with the inhibitors showed no expression of vimentin. (B) Untreated control tumoroids lacked E-cadherin expression while tumoroids treated with the drugs showed expression of E-cadherin. The inhibitors abrogated EMT in the treated tumoroids while untreated tumoroids expressed EMT markers. Scale bar=50µm.
Figure 4.8. Biopsy tumoroids on 3P scaffolds. (A) Biopsy tumoroids were cultured on the 3P scaffolds from day 1 to day 3. Cells were stained with calcein AM/EthD-1. Scale bar =200 µm. (B) The average size of biopsy tumoroids ranged from 152µm to 288 µm respectively. Data represents mean+/- SD, n= 12 tumoroids/ timepoint. (C) Tumoroids (day 3) were fixed and immunostained using anti-E-cadherin (green), anti-vimentin (red) and DAPI (blue). Tumoroids showed the absence of E-cadherin expression and the presence of vimentin expression and were positive for EMT. Scale bar =100µm.
Biopsy tumoroids (day 3) stained positive for tumor cell marker CEA, macrophage marker F4/80, endothelial progenitor cell marker CD-31 and cancer-associated fibroblast marker SMA, indicating the presence of stromal components in the tumoroids. LLC-1 cell line tumoroids, used as control expressed only CEA. CEA was stained red, CD31, F4/80 or SMA stained green, and DAPI (blue). Scale bar=100µm.

**Figure 4.9. Tumor stroma in tumor biopsies.**
Figure 4.10. Chemosensitivity of biopsy tumoroids. (A) Biopsy tumoroids were cultured on the 3P scaffold and treated with 1uM of LY294002 or U0126 for 3 days. Tumoroids displayed a dose dependent cytotoxicity in response to the drugs. The size of biopsy tumoroids decreased by 37.3% after treatment with LY294002 and 56% after treatment with U0126. This was compared with untreated control tumoroids. (B) Calcein AM/EthD-1 staining of biopsy tumoroids after treatment show live cells (green) and dead cells (red). Scale bar =200µm. Data represent mean+/- SD (n= 10 controls, 55 LY294002 and 50 U0126) * p<0.05.
In this study, we report on a novel 3P fibrous scaffold that induced the formation of a micrometastatic compact aggregate of tumor cells that we term a tumoroid. Our platform has several advantages over existing 3D technologies. (1) The 3P fibrous scaffold is produced from FDA-approved synthetic polymers. (2) It is conveniently and cheaply manufactured by electrospinning, which creates a mat of randomly distributed nano- to micro-scale fibers and can permit scale up production. (3) The scaffold mats can be cut into smaller pieces for placement in the wells of standard plastic cell-culture dishes. (4) Cancer cells seeded onto these scaffolds with growth medium containing the appropriate factors grow as tumoroids that show the EMT characteristic of \textit{in vivo} tumorigenesis. (5) This platform allows the coculture of tumor cells and stromal cells to identify changes in specific factors, gene expression and invasion potential. Tumoroids are able to respond to the same biochemical, nanotopographical and mechanical cues that drive tumor progression in the native ECM. (6) The 3P platform can be used to evaluate therapeutic strategies for simultaneously targeting tumor cells and the stromal cells that are components of the stem cell niche. (7) The 3P platform can also be

\[4\]

A portion of this chapter have been previously published by the author in an article entitled “A 3D Fibrous Scaffold inducing Tumoroids: A Platform for Anticancer Drug Development”, PlosOne, October 16 2013, Vol.8, Issue 10 and have been reproduced with permission from the publishers.
adapted for use in a point-of-care device to culture a patient’s own cells from a biopsy, test them *ex vivo* with different anticancer compounds and tailor a personalized treatment. Scaffold construction required systematic progression from the first PLGA scaffold that provided the blueprint for the appropriate topographical and physical features, to chemical modifications and final optimization to produce the 3P scaffold. To create a 3D surface that mimics the *in vivo* tumor microenvironment we utilized electrospinning to produce the 3P scaffold that induced all of the cancer cells used in this experiment, to self-assemble into tumoroids. Although the mechanisms are presently unknown there are two possibilities: (i) cell-cell interaction leading to tight aggregates that form the tumoroids, and (ii) outward proliferation of a fiber-attached cell to form a tumoroid. These possibilities are not mutually exclusive. Electrospun fibrous mats mimic ECM structures and the pore size, diameter and thickness of the fibers may regulate cell behavior [191]. Further, scaffold topography and architecture have been shown in previous studies to influence tumor cell behavior [45,84,85,192-194]. The 3P scaffold is composed of random fibers of a mixture of PLGA and PLA-PEG block-copolymer. While PLGA and PLA possess good mechanical properties, controlled degradability, and excellent biocompatibility [195-197], PEG alters the electrostatic binding properties of cells and promotes cell-cell interactions leading to assembly of tumoroids [9,198-200]. Although not fully elucidated, these characteristics may explain the spatial and temporal forces required to trigger tumoroid formation on the 3P scaffold. When we cultured LLC-1 lung cancer cells on the 3P scaffold they formed tumoroids that persisted, while cells cultured on the same polymer deposited as a film formed tumoroids that quickly disassociated. We also found that similar-sized scaffolds
of PLGA did not form tumoroids, underscoring the contribution of PEG-PLA block copolymer in the fiber, which by itself is not amenable to electrospinning. We changed the scaffold from a neutral charge to a positive charge by surface modification with cationic chitosan; this would increase the hydrophilicity and improve cellular interactions with scaffold. However cells proliferated on the scaffold but did not form tumoroids possibly because of strong preferential interactions of cells to the positive amine groups on the scaffold over cell-cell interactions required to form tumoroids. Moreover, tumor cells migrated from 3P tumoroids that had been transferred from the scaffold to tissue culture plates. These results showed that chemical and physical cues from the 3P scaffolds contributed to tumoroid formation. Tumoroid on 3P scaffolds underwent EMT with a loss of E-cadherin expression, a condition that imparts invasive and migratory capacity to cells and poor prognosis in several cancers [152,153]. Concomitant with down-regulation of E-cadherin was the expression of vimentin, a major cytoskeleton protein ubiquitously expressed in mesenchymal cells and tumor cells undergoing metastasis [202,203]. Tumor cells cultured as monolayer or on unmodified PLGA scaffolds maintained epithelial marker expression and did not undergo EMT. The majority of in vitro studies of EMT involve cultured cells that are induced either by forced expression of selected transcription factors or prolonged exposure to inducers such as growth factors and cytokines, whereas in our experiments the EMT occurred as a consequence of tumoroid formation. The EMT is considered an early event in the metastatic process and studies have suggested that dissemination may occur prior to tumor development [204,205]. In most experimental systems, a complete change in EMT marker expression requires ten days or longer [148]. Expression of EMT markers
by LLC-1 tumoroids on the 3P scaffolds occurred at day three of culture. The 3P scaffold may supply physicochemical cues that trigger the EMT by altering cell-cell and cell-matrix interactions, promoting architectural reorganization, or affecting the binding of proteins to cell surface receptors involved in signal transduction pathways [9,198-200]. It has been shown that pathways involving PI3K/AKT, MAPK and TGF-β communicate via growth factors and cytokines to repress E-cadherin and up-regulate mesenchymal genes during EMT [206]. TGF-β is the major inducer of EMT and acts through canonical and noncanonical pathways to affect PI3K/ AKT, MAPK signaling [164,207]. In support of this observation, we found that treatment of cancer cells with LY294002 (PI3K inhibitor) and U0126 (MAPK inhibitor) prevented tumoroids underscoring the importance of EMT in in vitro tumoroid formation. Treatment of tumoroids with the PI3K pathway inhibitor Ly294002 or the MAPK pathway inhibitor U0126, on 3P scaffolds vs. monolayer culture showed higher drug resistance of MCF-7 cells on the scaffold. The IC-50 values for 3P cultures were 10-fold higher for Ly294002 inhibitor and 100-fold higher for U0126 inhibitor than for monolayer cultures. These results are supported by other studies showing that tumor cells grown as 3D tumoroids develop multicellular resistance to most cytotoxic drugs compared to those grown in monolayer culture [16,215]. For example, the in vivo drug-resistant variant, EMT-6, of mouse mammary tumor cells lost resistance when cultured as a monolayer but regained it when regrown in vivo as a solid tumor or in vitro as tumoroids [216]. The reasons for the differences between 3D and monolayer cultures remain unknown. Pathophysiologi cal gradients to nutrients, oxygen and drugs and the concentric arrangement of heterogeneous cell populations within the 3P tumoroids may affect RNA
and protein expression. For example, it has been reported that quiescent cells near the necrotic core of a tumoroid up-regulate the cyclin-dependent kinase inhibitor p27Kip1, which arrests cells in the G0/G1 phase and induces cell cycle resistance [217]. The expression of p27Kip1 is fifteen times greater in tumoroid culture than in monolayer culture. In addition, inhibition of apoptosis via the Bcl-2 pathway [218], and the down-regulation of PMS2 and topoisomerase 11 DNA mismatch repair proteins [219,220] have been proposed as mechanisms that desensitize tumor cells in tumoroids to antitumor agents. We showed that this resistance was not a result of a mass transport defect. This is consistent with other drug penetration data that have shown that despite efficient drug penetration, tumoroids still exhibit higher drug resistance than monolayer cultures [221,222]. There is growing recognition that mechanical aspects, such as applied forces or the rigidity of the ECM, crucially influence cellular behavior and function in the induction of EMT [208]. These interactions are observed in vivo as well as in vitro on 3D artificial substrates. To cope with the constant mechanical stress, cells evolved specialized mechanosensing mechanisms. Several proteins undergo conformational changes in response to applied force, including mechanically gated ion channels [209], the cytoskeleton network [210], and ligand–receptor binding [211]. Integrins interact with both the ECM and focal adhesion proteins and function as ubiquitous mechanotransducers [212,213]. Integrin clustering leads to the phosphorylation of focal adhesion kinase (FAK) suggesting that FAK may mediate the transmission of mechanical stress into biochemical signals related to EMT. EMT preferentially occurs in response to TGF, a major inducer of EMT, at locations within tissues where mechanical stress is concentrated [213]. For example, regions of high
mechanical stress correlate with patterned expression of EMT markers during branching morphogenesis [214]. Increasing the rigidity of 3D substrates induced the expression of EMT transcription factor Snail. All of the cell lines cultured on the 3P scaffolds underwent EMT induction. We have not explored the effects of these forces on the induction of EMT on the 3P scaffold; however, these observations underscore the need to examine tumorigenesis as a consequence of changes at both the cellular and physical level of the ECM. Our novel 3P scaffold can be used to provide a 3D platform to study the metastatic cascade as well as other aspects of tumorigenesis that are exclusively observed in a 3D environment. Tumor-stroma interactions affect cancer growth, metastasis and the acquisition of drug resistance. To determine if the 3P scaffold was an appropriate in vitro model for anticancer drug screening we cultured tumor biopsy specimens on the scaffold. 3P scaffolds supported the growth of both tumor and stromal cells from the biopsy and this in vitro platform therefore mimic in vivo tumor growth. Moreover, tumoroids derived from the biopsies were more resistant to MEK and PI3K inhibitors than the tumoroids from cancer cell lines. Thus, while tumoroids from a cell line exhibit a distinct drug sensitivity profile, they do not accurately reflect the in vivo condition. Our biopsy results suggest that co-cultures of tumor cells with stromal cells will produce tumoroids that better reflect the in vivo context. These tumor/stroma effects on chemosensitivity underscore the importance of using a 3D platform such as the 3P scaffold for growth of actual patient tumor biopsies to test the efficacy of anticancer drugs.

In conclusion, our scaffold-based platform provides an elegant approach for developing customized anticancer treatments. Tumor biopsies can be cultured on a 3P scaffold,
differentiated into tumoroids and screened with an array of anticancer drugs to determine the most effective drug combinations for a particular cancer patient within a week. Rapid advances in genetics, genomics and related technologies are promising a new era of personalized cancer therapy based on molecular characterization of a patient's tumor and its microenvironment with the intent to improve outcomes and decrease toxicity [223]. However, these molecular predictors of tumor response are far from perfect. The heterogeneity of tumors, the lack of effective drugs against most genomic aberrations and the technical limitations of molecular tests hamper the current approaches for predicting the response to anticancer drugs. Our novel 3P scaffold may provide the solution for overcoming these hurdles to rapid drug screening and customized cancer therapy [224].
Chapter Six
Future Work

We have developed a novel 3D scaffold with potential for HTS and personalized medicine applications. The scaffold could be used to screen and identify novel small molecules that target metastatic diseases and their respective stromal cells as well as identify new uses for existing antitumor agents. The inhibitor studies indicate the significance of signal transduction pathways in tumoroid formation on the scaffold and further work is needed to identify the genetic pathways involved as well as those that contribute to drug resistance. The physicochemical mechanisms responsible for tumoroid formation on the scaffold need further elucidation. Future investigations are needed to understand how mechanical signals such as topography, are transduced into cancer cells and translated into signal transduction pathways that drives tumoroid formation and EMT induction on the 3P scaffold. These investigations may include the effects of mechanical forces such as tensile strength, strain and elasticity of the scaffold as it has been shown that these properties of the ECM affect cancer dissemination and impart resistance to chemotherapeutic drugs. Challenges exist to satisfying the criteria for HTS mainly automation of the electrospinning technique, scalability to satisfy multiple formatting requirements, low cost and wide applicability. Developing the automated system for scaffold production would increase production over the present system, would allow for adaptation to larger multiplate formats and would significantly
reduce time and the cost of production. Wide applicability requires that a larger library of cancer cell lines be tested to determine the potential for tumoroid formation including coculture with stromal cells.
References


Appendix A
Permission to use published work

Dear Yvonne,

Thank you for your enquiry.

We are an open access publisher and as such everything we publish is freely available online throughout the world, for you to read, download, copy, distribute, and use (with attribution) any way you wish.

No permission required.

For information on the Creative Commons Attribution License please follow this link http://www.plos.org/about/open-access/license/

Hope this is helpful.

Thank you,
Tamaira Witherspoon
PLOS 23 October 2013
Yvonne Girard was educated in Kingston Jamaica and received her degree in Medical Technology from the University of Technology in 1984. On immigrating to the US, she received a BS degree in Medical Technology from the University of South Florida where she was a recipient of the Multicultural Award. She worked as a clinical laboratory scientist (ASCP certified) in area hospitals before entering the PhD program in Medical Sciences at the University of South Florida in 2006. She received her MS degree in Medical Sciences in 2010. She is a recipient of the Sloan Foundation, Hope Foundation and IGERT awards. She has presented both oral and poster presentations at conferences including the the New England Science Symposium at Harvard University, ABRCMS, Nano-Bio and USF Health research conferences. Below is the list of her publications.


