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Effects of Cell Adhesion Peptides, pH, and Matrix Shape on Maintenance of Breast Cancer Stem Cells in an Engineered Hydrogel Matrix

Leily Daneshian
University of South Carolina - Columbia

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Breast Cancer Stem Cells in an Engineered Hydrogel Matrix

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Leily Daneshian

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Esmaiel Jabbari, Director of Thesis

Michael Gower, Reader

Michael Shtutman, Reader

Lacy Ford, Senior Vice Provost and Dean of Graduate Studies

Dedication:

To my family

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Abstract

Metastasis, resistance to chemo- and radiotherapy, and eventual relapse has been attributed to a tumor subpopulation known as cancer stem cells (CSCs). CSCs are regulated in their tumor microenvironment by various factors. Synthetic hydrogels can be used to investigate the effects of individual environmental factors on CSCs by providing inert 3D matrices. In this thesis, poly ethylene glycol diacrylate (PEGDA) hydrogel with 5kpa modulus has been used as a culture system to study the effect of; I) integrin and heparin binding peptides, 2) pH, and 3) the shape of the microenvironment on breast CSCs maintenance and tumorsphere formation in PEGDA. Human breast cancer cells were encapsulated in PEGDA hydrogels and the effect of the peptides, pH, and the shape of the environment on tumorsphere formation was investigated by fluorescent microscopy, qRT-PCR and DNA content assay.

All peptides including RGD, RYD, IKLLI, LIGRKK, VAPG, WQPPRARI, and SPPRRARV affected breast cancer cells by reducing their capability of sphere formation. Among peptides, RGD, RYD, and WQPPRARI were the most effective peptides in reducing sphere formation of breast CSCs.

Moreover, different shapes of micropatterned PEGDA including circle, square, and rectangle did not influence CSCs maintenance and behavior in forming tumorsphere.

Breast CSCs formed spherical tumors regardless of the shape of the micropatterned PEGDA and had the minimum surface area for a given volume. Furthermore, breast CSCs showed more resistance to acidic pH compared to non-stem breast cancer cells and normal breast epithelial cells.

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Chapter 1

Introduction

Breast cancer is the second leading cause of cancer death in women. About 1 in 8 (12%) women in the US will have invasive breast cancer during their life (American Cancer Society 2015). Regardless of advancements in diagnosis/treatment of metastatic breast cancer, the rate of death from this disease remains high. This is due to the fact that, the available therapies are limited by the existence of therapy-resistant cancer cells. Thus, metastatic breast cancer is an irrepressible disease by current treatment approaches. That means, further investigation needs to be performed on the breast cancer research area. Therefore, this research has been designed to achieve a better insight about these therapy-resistant cancer cells (cancer stem cells) behaviors and interactions in their microenvironment. Hopefully, the collected information will open up a new useful path toward the eradication of metastatic breast cancer.

1.1. Tumor Heterogeneity

Tumor heterogeneity refers to the existence of different cells within tumors or between tumors. Tumor cells are different in morphology, metabolism, proliferation, and ability of metastasis (Marusyk and Polyak 2010). There are two models that explain the tumor

heterogeneity: "Cancer stem cell" model and "clonal evolution" model (Shackleton et al. 2009)Cancer Stem Cell (CSC) model: CSCs are a subpopulation of cancer cells that form tumors. They self-renew and differentiate to other cancer cells, which are not able to form tumors. The idea that CSCs contribute a small population of cancer cells comes from this view that if almost all of the cancer cells would proliferate extensively and metastasize throughout the body, then all of them should be eradicated by available therapies. In reality, current cancer treatments are truly able to remove most of the cancer cells in the body, however the observation of cancer relapse illustrates that there should be a small population of cancer cells left in the body which initiate the disease later (Reya et al. 2001). CSCs are capable of self-renewing and differentiating. The heterogeneity that has been observed between differentiated cells refers to differences between cancer stem cells that they have originated from. The difference between cancer stem cells usually arise from epigenetic changes simultaneous with natural selection of advantageous genetic mutated cancer stem cells (Shackleton et al. 2009). This model proposes that CSCs behave the same as normal stem cells. They undergo epigenetic changes and differentiate to cells that are phenotypically different and have limited proliferation. These differentiated cancer cells make the majority of cells in a tumor (Shackleton et al. 2009).

Cancer stem cell model has been observed in multiple tumor types such as leukemias (Campbell et al. 2008), breast cancer(Jabbari et al. 2015), and prostate cancer(Alvarado et al. 2005). In these cancers, only a small population of cancers could form tumor

spheres in NOD/SCID mice, and also specific markers for these tumorigenic cells have been identified (Campbell et al. 2008, Jabbari et al. 2015, Alvarado et al. 2005).

1.2. CSCs and Their Microenvironment

Breast cancer stem cells exist in an environment (niche) which is responsible for the maintenance of specific stem cell properties like self-renewal and remaining in an undifferentiated state. The population in niche contains both stem cells and surrounding differentiated cells. Biochemical signals that breast cancer stem cells get from the interaction with ECM components and neighbor cells have critical roles in maintenance of them. Cancer stem cells niche is responsible for the control of the essential pathways that have critical roles in determination of stem cells destiny. Critical pathways such as STAT, Notch, and Wnt have been recognized in CSCs niche while some features of cancers like hypoxia and angiogenesis regulate these pathways. Investigating on processes within breast CSCs niche can provide a better understanding of these CSCs for prevention and treatment of metastasis breast cancer.

1.3. CSC microenvironment and peptides

The extracellular matrix (ECM) is a collection of extracellular molecules secreted by cells that provide structural and biochemical support to the surrounding cells. Cell adhesion, and cell-to-cell communication are common functions of the ECM (Abedin and King 2010).

Fibronectin, laminin and elastin are 3 components of ECM. These proteins which are long chains of amino acids, and peptides derived from these proteins which are short chains of amino acids are well known for mediating cell adhesion. It has been shown that the occurrence of breast cancer is concurrent with changes in these proteins expression, degradation and expression of their binding receptors.

VAPG (derived from elastin), IKLLI (derived from laminin), RYD (derived from streptavidin), and RGD (derived from fibronectin) are integrin binding peptides and WQPPRRARI and SPPRRARV (derived from fibronectin) are heparin binding peptides. Integrins and heparan sulfate proteoglycans are receptors that play critical roles in development of metastasis breast cancer as they activate focal adhesions mainly focal adhesion kinase (FAK). FAK is an intracellular non-receptor tyrosine kinase. It has been shown that FAK is highly overexpressed in breast cancers and it has key roles in promoting tumorigenesis and metastasis (Luo and Guan 2010).

Therefore, we chose these cell binding peptides from different domains of these proteins to investigate their effects on breast cancer stem cells maintenance in PEGDA.

The importance of using peptides over proteins is due to difficulties such as protein denaturation and degradation, and problems with protein absorption. Peptides are part of the ECM proteins which have similar stimuli of proteins while they are more stable and easier to conjugate on material surfaces (Ventre, Causa, and Netti 2012).

Some of the most recent usage of peptides in breast cancer research has been reviewed in the following 5 parageraphs.

Patched receptor binding peptides have shown to have a growth inhibitory effect in tumors with activated hedgehog signaling (Smith et al. 2014). Remarkable growth inhibition has been observed in breast cancer cell lines treated with patch-blocking peptides (Smith et al. 2014).

Cancer research has been illustrated that connexin 43 is effective in proliferation, differentiation, and migration of breast cancer cell. There are drugs available related to this but there is a lack of knowledge in specificity of these agents. In a study, α-connexin carboxyl-terminal (ACT1) peptide, which modulates connexin 43 has been tested in breast cancer. The peptide is able to regulate the connexin 43 activity in breast cancer to sustain connexin 43 -mediated gap junctional activity which cause the decrease of malignant progression. ACT1 peptide also is able to enhance the activity of lapatinib and tamoxifen (Grek et al. 2015).

L-peptide has showed to bind to a wide variety of cancers including breast cancers. Treatment of mice with breast cancer patient derived xenografts (PDX) with L-peptide-conjugated lipodox (LD-L) has been illustrated to result in greater suppression of tumor growth than lipodox (LD) alone (Lee et al. 2015).

Cell surface nucleolin is known to be overexpressed in cancer cells and also it is a marker for tumor angiogenic (Fonseca et al. 2015). To investigate whether nucleolin was a

common receptor among breast CSC and non-stem cancer cell (non-SCC), a group of researchers functionalized liposomes with the nucleolin-binding F3 peptide which targeted both nucleolin-overexpressing putative breast CSC and non-SCC. An *in vivo* assay showed that surface nucleolin overexpression could be related to the triple negative breast cancer cells which potentially connect the nucleolin expression to the stem-like properties in triple negative breast cancer cells (Fonseca et al. 2015) .

Proliferating cell nuclear antigen (PCNA) is one of the important regulators in the DNA replication and repair process. A peptide (caPeptide) as a mimic of PCNA has been synthesized and delivered into cells using a nine-arginine linking mechanism. R9-cc-caPeptide displayed cytotoxicity in MDA-MB-436, a triple-negative breast cancer cell line. R9-cc-caPeptide has also been resulted in blocking the association of PCNA with chromatin (Smith et al. 2015).

1.3.1. Importance of Fibronectin and its Peptides

Fibronectin (FN) is one of the important ECM glycoprotein that exists in fibrillar form in all tissues during life. Its formation is a cell-mediated process and is essential for life. FN fibrils form linear and branched meshworks in order to connect neighboring cells to each other. FN is a multidomain molecule that has different domains for interacting with other ECM proteins including other FN proteins, cell receptors, and glycosaminoglycans (GAGs). This arrangement of domains allows FN to bind to cells and molecules at the same time. FN has binding sites for collagen/gelatin, heparin, fibrinogen, heparin sulfate glycosaminoglycans, integrins, and other molecules (Singh, Carraher, and Schwarzbauer

2010). It plays a major role in cell adhesion, growth, migration, and differentiation (Pankov and Yamada 2002). Altered fibronectin expression, degradation, and organization have been associated with a number of diseases, including breasr cancer and fibrosis (Williams et al. 2008). Observing tumors and tumor-derived cell lines have been attributed to the decreased fibronectin expression, increased fibronectin degradation, and/or decreased expression of fibronectin-binding receptors such as α5ß1 integrins (Hynes 1990). So far, the effects of several peptides derived from different domains of fibronectin such as WQPPRARI (Hettick, Ruwona, and Siegel 2009, Van Den Heuvel, Jefferson, and Jacobs 2005, Yun, Kim, and Jang 2013, Woods et al. 1993, Hoesli et al. 2014, Mooradian et al. 1993, Ouchani et al. 2012, Wilke and Furcht 1990, Sagnella et al. 2005, Björklund and Koivunen 2005, Garagorri et al. 2008), SPPRRARV (Mooradian et al. 1993, Sagnella et al. 2005), LIGRKK (Hettick, Ruwona, and Siegel 2009, Tong 2000), RGD (Wierzba et al. 1995, Fischbach et al. 2009, Naghdi et al. 2014, Panda et al. 2010), and RYD (Murray et al. 2002, Knight 2001, Guo et al. 2005) on different cell lines behavior such as cell adhesion, proliferation and migration have been studied. In the following seven paragraphs a brief summary of these studies has been provided.

1.3.1.1. WQPPRARI Peptide

WQPPRARI is one of the well-known heparin binding peptides which is derived from the COOH terminal heparin binding domain of fibronectin. This peptide is famous for its cell adhesion features (Hettick, Ruwona, and Siegel 2009, Van Den Heuvel, Jefferson, and

Jacobs 2005, Yun, Kim, and Jang 2013, Woods et al. 1993). It has been shown that WQPPRARI is able to improve umbilical vein endothelial cell adhesion, expansion, and motility through focal adhesion formation and FAK activation (Hoesli et al. 2014); enhance cell adhesion, spreading, and migration of rabbit corneal epithelial cells directly (Mooradian et al. 1993); promote cell adhesion, spreading, and migration of normal and leukemic progenitors through direct interaction with α4ß1 (Ouchani et al. 2012); increase cell adhesion and spreading of human keratinocytes and saphenous vein endothelial cells (Wilke and Furcht 1990); and improve human pulmonary artery endothelial cell adhesion and spreading through local adhesion (Sagnella et al. 2005). The WQPPRARI peptide is able to stimulate expression of MMP-1 and MMP-9 in fibroblast plates on a fibronectin fragment, lacking the heparin binding domain. This stimulation is mediated by α5β1 and α4β1 integrins (Björklund and Koivunen 2005). It should be pointed out that, there is also a study on Keratocyte behavior in three-dimensional photopolymerizable poly (ethylene glycol) hydrogels which used the sequence WQPPRARI, tethered to hydrogels, and showed that it enhances adhesion, spreading, and migration of corneal epithelial cells to the hydrogels (Garagorri et al. 2008).

1.3.1.2. SPPRRARV Peptide

The other COOH-terminal heparin-binding domain of fibronectin is SPPRRARVT. This peptide can support cell attachment of fibroblasts (Sagnella et al. 2005); RCE cell

adhesion and spreading (but not RCE cell migration) (Mooradian et al. 1993); and also human pulmonary artery endothelial cell adhesion and growth (Sagnella et al. 2005).

1.3.1.3. LIGRKK Peptide

KNNQKSEPLIGRKKT is another heparin-binding peptide that derived from the COOH-terminal heparin binding domain of fibronectin which mediates cell adhesion for a variety of cell types and promotes neurite outgrowth. The basic structural features necessary for the activity have been identified in the COOH-terminal residues, LIGRKK (Hettick, Ruwona, and Siegel 2009). This biologically "active" sequence has been found in several other heparin/heparan sulfate-binding peptides such as LIGRKK derived from laminin, which helps fluoropolymer surfaces for the enhancement of nerve cells interactions (Tong 2000).

1.3.1.4. RGD Peptide

RGD, another well-known peptide, is a sequence in extracellular matrix proteins such as fibronectin, collagen, and laminin that mediates cell attachment by interacting with proteins of the integrin family of cell surface receptors (Wierzba et al. 1995). It has been shown that human breast cancer cells, MDA-MB-123, cultured in a hydrogel based culture system coupled to RGD are able to secrete more interleukin 8 (IL-8) compare to the cells cultured in a hydrogel based culture system without any conjugated peptide. Up regulation of IL-8 is critical in control of tumor vascularization. Therefore, 3D RGD coupled culture systems could regulate cancer cell angiogenic signaling, and controlled local and systemic blockade of IL-8 signaling (Fischbach et al. 2009).

Polyethylene glycol hydrogel (PEG) has been studied as a 3D culture system for neuron cells as well. It has been reported that neurite outgrowth was improved in systems with conjugating RGD to PEG polymer. Therefore, NSC survival, proliferation and differentiation are enhanced when the cells are cultured in 3D-PEG–RGD compared to 3D-PEG environments (Naghdi et al. 2014).

Moreover, proliferation and growth of mammalian cells (HeLa and L929) in a 3D environment with a dipeptide hydrogel chemically functionalized with a pentapeptide containing Arg-Gly-Asp (RGD) motif has been investigated. The functionalized gel exhibited enhanced cell growth promoting properties, and promoted 3D growth and proliferation of cells for almost 2 weeks (Panda et al. 2010).

RGD peptide also has been used to examine the effect of substrate stiffness on melanoma cell treatment responsiveness. Human cell lines derived from radial growth phase (WM35) and metastatic melanoma (A375), PEG hydrogels as a cell culture system and PLX4032 as pharmacological inhibitor were used. In this study, it was found that in A375 cells, matrix elasticity did not alter cell morphology or apoptosis with PLX4032 treatment. But in WM35 cells, matrix elasticity increased apoptosis and smaller focal adhesions on compliant substrates (Tokuda, Leight, and Anseth 2014).

In a study, polyethylene glycol (PEG) hydrogels, attached to RGD was used and its ability to support the growth of androgen-dependent LNCaP prostate cancer cells was investigated. It was found that, the mechanical properties regulate the growth of LNCaP cells in the PEG hydrogel. They showed that after 28 days of culture, LNCaP cells

formed tumor-like structures in 3D culture, with hypoxic and apoptotic cores (Sieh et al. 2012).

In another study, by using PEG, modified with RGD and another laminin derived peptide, murine models of lung adenocarcinoma investigated. The focus was on how matrix can influence epithelial morphogenesis of a metastatic cell line (344SQ). 344SQ encapsulated in bioactive peptide-modified, matrix metalloproteinase–degradable PEG hydrogels formed lumenized epithelial spheres. Changing matrix stiffness and peptide concentrations affected epithelial morphogenesis, apoptosis , proliferation, and expression of epithelial polarity markers (Gill et al. 2012).

Streptavidin is a biotin-binding tetrameric analogue of avidin, produced by the soil bacterium Streptomyces avidinii. Streptavidin, like fibronectin, contains an RGD-like sequence RYD, which promotes adhesion to the integrin receptor α5β1. This sequence, Arg- Tyr-Asp-Ser (RYDS), exhibits structural homology to Arg-Gly-Asp-Ser (RGDS). Binding of streptavidin to cell surfaces mediated through this RYDS domain, can be inhibited by using fibronectin as well as RGD- and RYD-containing peptides (Murray et al. 2002). Synthetic peptides containing such a sequence are able to mimic the integrin-mediated binding of the entire protein(s).

1.3.1.5. RYD Peptide

It was proposed that RYD in ARRSPSYY**RYD**GAGPYYAMDY functions as an analogue to RGD in fibrinogen. This peptide comprised the binding domain for the αIIbβ3 receptor. A linear peptide which is consistent with the sequence above was synthesized and was

found to be an inhibitor of both fibrinogen and PAC-1 binding to activated platelets. Exchanging RGD for RYD in the aforementioned peptide, increased its activity 10-fold (Knight 2001). Also the sequence RYD has been introduced into the dendroaspin scaffold in order to replace RGD. The RYD sequence produced a similar IC50 value to the RGD sequence, in inhibiting A375-SM cell (β-3 integrin) adhesion to collagen (Guo et al. 2005).

1.3.2. Importance of Laminin and its Peptides

Basal lamina is mostly made of Laminin. This protein plays a critical role in cell differentiation, migration, and adhesion, as well as cell phenotype and survival. Laminin is linked to type IV collagen via entactin (Smith and Ockleford 1994), fibronectin (Ockleford et al. 1993),and perlecan [28]. Moreover, this glycoprotein, Laminin, binds to cell membranes through integrin receptors and other plasma membrane molecules, such as the dystroglycan glycoprotein complex (Haralson, Hassell, and Streuli 1995). Through these interactions, laminin causes cell attachment, differentiation, shape, and movement (Haralson, Hassell, and Streuli 1995, Colognato and Yurchenco 2000).

As it has been shown in [30], laminin plays a critical role in regulating cancer cell migration and facilitating tumor cell invasion. Laminin establishes one of the essential components of basement membranes (BMs) as it is involved in cellular adhesion to BMs and ECM. Invading tumor cells are capable of attaching to the matrix through specific laminin receptors which is present on their cell membranes. Afterward, these invading

tumor cells release protease in the interstitial, consequently, promote BM disruption and cell diffusion (Albrechtsen et al. 1981).

1.3.2.1. IKLLI Peptide

IKLLI is a sequence shown to be active in laminin (Fischbach et al. 2009). Several peptides containing the IKLLI sequence in the α 1 chain of laminin-1 such as CSRNLSEIKLLISRARK, EIKLLIS, and SEIKLLIS were found to mediate heparin binding and cell adhesion of PC12 cells as well as promoting neurite outgrowth in these cells. Furthermore, the CSRNLSEIKLLISRARK and SEIKLLIS sequences also mediated proliferation in PC12 cells. As noted above, an IKLLI-containing peptide derived from the laminin α 1 chain may be an active site of laminin and its cell adhesion maintenance may be due to interaction with both integrin a3b1 and cell surface heparan sulphate proteoglycan (TASHIRO et al. 1999).

These Neurons expressed integrin b1, beside the fact that the treatment of cultures with an antibody against integrin b1 eliminated the protective effect of laminin. Moreover, neurons maintained on laminin displayed a continued activation of the Akt signaling pathway. The IKLLI-containing integrin-binding peptide is capabale of mimicking the neuroprotective effect of integrin engagement by laminin. Due to this fact, it can be well-understood that the IKLLI is an active sequence of laminin.

1.3.3. Importance of Elastin and its Peptides

Degradation is a requirement for cancer progression. This is due to the fact that, ECM degradation is vital to allow cell migration through its three-dimensional architecture and also to generate ECM fragments. ECM proteolysis causes the release of matrix fragments that exhibit proper biological activities. This degradation of ECM is coincident with the degradation of Elastin, a major component of ECM that confers elasticity to tissues (Panda et al. 2010). Protease-driven elastin degradation happens during physiopathological processes such as cancer progression, which generates bioactive elastin-derived peptides that are thought to contribute to tumor progression (Devy et al. 2010). In another words, Elastin peptides control proliferation, chemotaxis, and protease expression.

1.3.3.1. VAPG Peptide

VGVAPG is an elastin-derived peptide shown to block ceramide-induced apoptosis in human skin fibroblast cells. The elastin peptide treatment leads to activation of the pro-apoptotic protein Bad, and caspase-9 (Cantarelli et al. 2009). As mentioned in [36], elastin-derived peptides raise invasive capacities of lung cancer cells by post-transcriptional regulation of MMP-2 and uPA.

The VAPG peptide sequence is repeated several times in human elastin and most likely it is one of the breakdown products after the degradation of elastin. The VAPG elastin peptides could bind to three identical receptors, namely; *(i)* galectin-3, *(ii)* integrin αvβ3, and *(iii)* elastin-binding protein. It is also have been investigated that, VAPG is able to

increase the invasive potential of melanoma cells mostly by galectin-3 (Pocza, Falus, and Darvas 2009).

In a study, VAPG peptide sequence attached to a hydrogel material, and its effects on smooth muscle cell adhesion and spreading have been studied. The VAPG sequences was specific for adhesion of smooth muscle cells while fibroblasts, endothelial cells, and platelets cannot adhere to VAPG (Gobin and West 2003).

The effects of cell adhesion due to the VAPG peptide on vascular smooth muscle cells were also examined in (Gill et al. 2012). These cells more strongly adhered to the surfaces modified with adhesive ligands. In addition, cell migration was higher on surfaces with the adhesive ligand than on control surfaces. Moreover, cell proliferation was lower on adhesive surfaces. Likewise, in hydrogel which is functionalized with VAPG, cell proliferation was lower in comparison with control groups (Gill et al. 2012).

Matrix protein synthesis by cells cultured on materials that was modified by cell adhesion ligands, like the VAPG peptide, were examined in (Smith and Ockleford 1994). While initial adhesion of smooth muscle cells, endothelial cells, and fibroblasts increased on the higher density of peptides on surfaces, all cell types had less production of matrix on the more highly adhesive surfaces. This result may actually pose limitations for the use of bioactive materials, such as in tissue engineered scaffolds since matrix production is an important aspect of tissue formation (Mann et al. 1999).

In this Study, the role of these integrins binding peptides (RYD, RGD, IKLLI, and VAPG) and heparin binding peptides (WQPPRARI, SPPRRARV, LIGRKK, and IKLLI) on the maintenance and behavior of breast CSCs encapsulated in PEGDA has been investigated.

1.4. CSC microenvironment and pH

Tumor microenvironment is extremely acidic (pH ~6.8) compared to normal tissue (pH ~7.4) which affect tumorigenesis, angiogenesis and metastasis activity of cancer cells exclusively (Song, Griffin, and Park 2006). It has been shown that tumor microenvironment has lower pH and is more acidic in comparison with normal tissue, and this is due to anaerobic and aerobic pathways. In a tumor, vascularization is not homogeneous and adequate to feed enough nutrition, specifically oxygen to rapid dividing cancer cells. Lack of oxygen is the main reason for acid production. In hypoxia, cells undergo glucose uptake and glucose goes through glycolytic pathway instead of respiratory pathway which causes the production of lactic acid and reduction of pH in the microenvironment (Song, Griffin, and Park 2006, Tannock and Rotin 1989). Moreover, under hypoxia condition, ATP hydrolysis also causes the acidification of tumor microenvironment. On the other hand, it has been shown that in cancer cells, glucose undergo glycolysis even in the presence of sufficient oxygen in order to produce lactate.

Cancer cells require high amount of lactate because it helps them to escape from immune cells. In the presence of lactate produced from tumor cells, T cells do not secrete lactate and this interferes with T cells function. Lactate can inhibit the function

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of dendritic cell activated by antigen-specific autologous T-cell stimulation. Moreover, it also inhibits monocyte migration and cytokine release (Kato et al. 2013).

Moreover, tumor cells undergo glycolysis even in sufficient oxygen condition because of their advantage. The glycolysis pathway produces acid and the acidic extracellular environment help cells to become invasive and proliferative (Gatenby and Gawlinski 2003).

On the other hand, it has been shown that the pentose phosphate pathway is highly active in tumor cells. CO_2 is one of the products of this pathway while there is a large amount of carbonic anhydrase (CA) is also present in a tumor. CO_2 can be processed to H^+ and HCO_3^- by CA as a catalyzer (Gatenby et al. 2006). Therefore, CO_2 is another important reason for acidic PH of tumor microenvironment (Helmlinger et al. 2002).

It has been shown that Acidic pH can induce EMT in some types of cancer cells such as lung and melanoma cancer cell lines (Peppicelli et al. 2014, Suzuki et al. 2014). Moreover, acidic pH has also been associated to the expression of some genes that are contributed to metastasis of cancer cells such as metastasis of melanoma cells to the lungs (Rofstad et al. 2006). Acidic pH increases the expression of some genes that are involved with pro-metastatic factors. It has been shown that when melanoma cells incubated in acidic culture medium, had a higher metastatic rate accompanied by proteinase MMP-9 and NHE activation (Gatenby et al. 2006, Kato et al. 2013) and a higher angiogenesis by acid induced production of VEGF-A and IL-8 (Gatenby et al. 2006).

The glycolytic activity in tumor cells enhances tumor invasion. Protons (H+) produced by cancer cells diffuse (carried by a buffering agent) from tumor to the normal tissue nearby by using transporter proteins such as Na+/H+ exchanger (NHE) ,and cause the decrease of pH in normal tissue. It has been shown that the Na+/H+ exchanger type 1 (NHE1) was an important regulator of H+ efflux in breast cancer cells MDA-MB-231 (Stock et al. 2005).

Therefore the intracellular pH of cancer cells does not become acidic while it is slightly alkaline and in fact this alkaline pH is suitable for cell proliferation. The acidic pH causes normal cells to undergo cell p53-dependent apoptosis but some of the tumor cells survive which are resistant to acidic pH, which is probably due to mutation in p53 or other components of apoptosis pathway. Low pH causes the extracellular matrix to degrade by proteolytic enzymes that highly active in low pH and produces by fibroblasts and macrophages (Gatenby and Gawlinski 2003). Moreover, it enhances angiogenesis by using acid induced vascular endothelial growth factor and interleukin 8, and inhibits immune system to response to tumor antigen (Kato et al. 2013). Therefore tumor cells become more invasive as they disrupt the environment, and provide a better condition for them to proliferate. Cell-cell junctions of tumor cells become separated when cells move in to their surrounding tissue. Acidic pH helps tumor cells to destruct the adherence junction. This is by Src activation, that causes E-cadherin degradation through a the protein kinase activity (Gatenby et al. 2006).

To wrap up, pH is one of the important factor in tumor microenvironment and although the effect of pH has been studied on some cancer cell lines (Peppicelli et al. 2014, Suzuki et al. 2014, Rofstad et al. 2006) , the effect of pH on breast cancer stem cells has net been investigated yet.

Herein, we used PEGDA having modulus of 5 kpa to study the role of acidic pH (6.8) in the maintenance of breast CSCs. Having a good understanding of the role of this factor on regulation of CSCs can offer important information on the behavior of breast CSCs in their tumor microenvironment.

1.5. CSC Microenvironment and Shape

Tumor transformation and metastasis is involved with changes in mechanical properties of cells and cell's microenvironment including mechanics, shape, and topology of ECM. Cells are able to sense the rigidity (elastic resistance) of ECM and balance this force by exerting contractile stresses. The balance force is very important in regulating the structure, motility, proliferation, and differentiation of tumor cells. Cells exert this force by the use of adhesion receptors (e.g., integrins), intracellular focal adhesions, cytoskeletal networks, and molecular motors. Therefore, direct connection between the extracellular matrix (ECM) and the intracellular environment can control fundamental behaviors such as differentiation, morphology, motility changes, and alterations in cell cycle which can contribute to tumor transformation, invasion, and metastasis (Kumar and Weaver 2009, Tse, Weaver, and Di Carlo 2012, Kilian et al. 2010). It has been shown

that gene expression, self-renewal, and differentiation of malignant cells can be affected by geometrical confinement. Confinement of human cervical carcinoma cells changed the average tumorsphere size and cluster size affected MCF10A cells proliferation (Jabbari et al. 2015).

Previously we have encapsulated MDA-MB-231 cells in circle shape 50, 75, and 250 μm patterned PEGDA gels and incubated for 9 days in CSC medium. It was shown that as the patterns became smaller the expression of breast CSCs became higher. Therefore, using micropatterning and the confinement of breast CSCs can help to get a higher expression of CSCs markers (Jabbari et al. 2015) which can be used in further studies on breast CSCs. So far, breast cancer stem cells encapsulated in PEGDA or in micropatterned PEGDA form tumors in spherical shape. Herein, we hypothesize that if the shape of micropatterned changed, the shape of tumors may change as well. Therefore, we designed photomask with circle, square and rectangle micropatterns in order to shape the PEGDA and investigate the effect of them on maintenance of breast cancer stem cells in PEGDA.

1.6. Why Choosing PEGDA for Investigation on CSCs and Their Microenvironment?

CSCs are a small population of tumor cells that are drug resistant, capable of differentiation, metastasis, and self-renewal through specific pathways provided in the CSCs niche. On the other hand, cancer development is a hard process to follow as it may take many years in vivo. Thus, it is required to design in vitro systems in order to

investigate cancer tumor formation and progression on the molecular level. This molecular level investigation has been performed in two or three dimensional cell culture systems.

1.6.1. 2D Cell Culture Systems

Classical-two dimensional cell culture systems have provided the majority of modern cancer biology science. For instance, the most common substrates for supporting cell growth have been made from polystyrene or glass and have been made in shape of a flat two-dimensional (2-D) surface. However, the main problem for this 2-D system is the assumption that body physiology can be correctly reproduced using a cellular monolayer. Obviously, a eukaryotic cell cannot develop same properties on a two-dimensional glass or polystyrene substrate compared to the 3D extracellular matrix found in innate tissue. When cells are cultured in 2-D plates, they are attached to rigid and flat substrates which cause cells to be polarized and get sheet-like morphology (Alemany-Ribes and Semino 2014, Haycock 2011). These cells are exposed to excessive nutrition and oxygen. Moreover, in 2-D cultures cells have different surface receptors' orientation and clustering; therefore, they have different ECM secretion in composition, configuration and amount and as a consequence, they don't have normal signaling that comes from natural ECM (Alemany-Ribes and Semino 2014). Also, their cell growth rate, migration and apoptosis change in this classical culture systems (Chen et al. 2012). For the purpose of CSCs culturing, suspension (non-adherent plates) are commonly used which again have similar problems as adherent substrates.

Based on the above discussion, it can be concluded that, the culture of cells in 2-D systems is certainly too simple and neglects many important parameters for reproducing the cell and tissue environment, such as of mechanical cues, cell-matrix and cell-cell communication (Haycock 2011).

To be more specific in cancer, metastatic cells are not adherent and cannot form tight focal adhesions. Thus, 2-D cultures are not applicable for them. Furthermore, in multiple passages of cancer cells, those that have rapidly proliferation are the target of natural selection, whereas these rapidly proliferative cells are sensitive to therapies that target rapidly dividing cells while this is not the same condition for all cells in a tumor (Alemany-Ribes and Semino 2014).

Some parameters such as gradient of nutrients and growth factors as well as cell-cell and cell-matrix communication which are known to play crucial roles in cancer initiation, progression, and metastasis cannot be mimicked accurately in 2-D culture systems. As an example, cancer cells which are cultured in 2-D plates are less malignant in comparison with those under in vivo conditions (Kitai et al. 2005). Therefore, results from drugs that are designed to target cell-cell interaction, epithelial to mesenchymal transition (EMT), and CSC are not trustable(Chen et al. 2012).

1.6.2. Animal Models in Cancer Research

Animal models act as another alternative that are commonly used for the study purposes of molecular pathways and drug reaction in cancer research. In these cases, either animal tumors grown in syngeneic animals or human tumors grown in

immunocompromised animals are used for the research purposes. However, animal models may not satisfactorily reproduce the structures of human cancers in vivo (Yang et al. 2013).

Animal models constitute a wide range of models for cancer study:

1- Ectopic xenografts of tumor-derived cell lines or tissue, embedded into syngeneic or immunecompromised.

2- Orthotopic xenografts of tumor cell lines or tumor tissues are implanted within the proper organ or tissue.

3- Germ-line transgenic and conditional transgenic models (GEMMs)

4- Primary human tumorgrafts

5- Carcinogen-promoterinduced multi-stage tumor models

A review of pros and cons of these methods are available (Ruggeri, Camp, and Miknyoczki 2014).However, they are not explained here in details as describing them is out of scope of this thesis. As some important and most common examples of disadvantages related to these methods are lack of native tumor microenvironment, limited engraftment rates, labor intense, time consuming, and ethically problematic (Ruggeri, Camp, and Miknyoczki 2014, Sachs and Clevers 2014).

1.6.3. 3D Cell Culture Systems

To have a suitable cell culture system, it should provide a 3D matrix having tunable mechanical properties with a capacity of co-culturing cells in order to provide cell-cell interactions and the exchange of growth factors and other biological effectors.

Available 3D in vitro co-culture models satisfy these requirements (Kim 2005) which are also necessary in CSC research.

In many 3-D models, cell lines or cells from dissociated tissues are implanted and cultured in 3-D matrices in order to promote cell–cell interaction, adhesion, migration, and in vivo–like morphogenesis. There has been a big difference in all aspects of cell behavior; (i) cell shape, (ii) cell growth, (iii) gene expression, and (iv) the response to stimuli between 2-D and 3-D culture systems. Based on these differences, increasing the attraction of researches toward utilizing 3-D environments for the most recent biomaterial directed stem cell manipulation researches can be better understand (Yang et al. 2013).

In order to work in 3-D environments, in vitro or in vivo, biomaterial-based matrices have been used as an important tool. These 3-D environments provide ideal matrices for cell-cell and cell-matrix interaction. Moreover, their properties can be adjusted for specific features such as desired fluid transport, delivery of bioactive molecules, and induction of signal transduction. These properties are important since they direct cell adherence, nutrient/waste transport, cell proliferation, cell differentiation, and in cancer stem cells studies, tumor sphere formation. Most of these materials can be modified in order to adjust all of the mentioned critical matrices characteristics.

There are a variety of synthetic and natural materials that have been used for studying SC and CSCs behavior by manipulating biomaterial-based matrices properties. One of the most commonly type of these materials is Hydrogels. Hydrogels are a 3-D polymeric

network that can swell under biological conditions. With the ability of swelling, hydrogels have high water content, therefore they can offer environments that are closer to natural soft tissue in comparison with other polymeric materials. Additionally they are more biocompatible as they are highly permeable for oxygen, nutrients, and other water soluble metabolites. Therefore, hydrogels are ideal for cell encapsulation (Albrechtsen et al. 1981, TASHIRO et al. 1999, Devy et al. 2010, Cantarelli et al. 2009, Pocza, Falus, and Darvas 2009, Gobin and West 2003, Mann et al. 1999). Most hydrogels can be made by photopolymerization under mild conditions with consistent seeding of cells throughout the scaffold (Ifkovits and Burdick 2007, Chung and Park 2009, Slaughter et al. 2009).

Hydrogels are divided in to three main groups; (i) natural, (ii) synthetic and (iii) synthetic/natural hybrid hydrogels. There are many types of natural polymers such as;

• Proteins: gelatin, collagen, Matrigel™, fibrin, silk, and lysozyme (Glowacki and Mizuno 2008, Sakai et al. 2009, Wang et al. 2006, Mol et al. 2005, Yan et al. 2008, Kleinman and Martin 2005).

• Polysaccharides: hyaluronic acid (HA), agarose, dextran, and Chitosan (Leach et al. 2003, Denizli et al. 2004, Kuo and Ma 2001, Kim et al. 2008).

However, using natural biomaterials has some disadvantages. These disadvantages include limitation in the adjustment of physical and chemical properties, difficulty in modifying degradation rates, and the difficulty in sterilization and purification as well as

pathogen/viral issues when isolating from different sources. Furthermore, there are problems with isolating and studying cell response to the individual factors in the microenvironment. This problem is associated with the fact that naturally derived matrices have many interactions with cell surface receptors which interfere with cell responses to specific factors (Yang et al. 2013).

In order to overcome the disadvantages of natural hydrogels, synthetic hydrogels have been considered as a desirable alternative. Synthetic polymers have more reproducible physical and chemical characteristics, which is important for the production of tissue engineered scaffolds. One of these synthetic polymers is Poly ethylene glycol.

1.6.4. Using PEGDA with 5kpa Modulus for this Research

Poly ethylene glycol (PEG) polymer is investigated extensively because of its specifications, including solubility in water and organic solvents, low protein adhesion, nontoxicity, and nonimmunogenicity (Buxton et al. 2007, Beamish et al. 2010). Additionally, the end hydroxyl groups of PEG molecules can be grafted with various functional groups such as acrylate in order to create hydrogels (Zhu 2010).

Among the parameters in the microenvironment, stiffness (elastic modulus) plays a crucial role in regulating cell function in 2-D and 3-D culture systems (Rehfeldt et al. 2007). As it has been shown in (Sachs and Clevers 2014, Ifkovits and Burdick 2007, Chung and Park 2009), in 3-D culture systems, encapsulated stem cell differentiation, and the balance of cell proliferation, and apoptosis can be directed by the stiffness of the hydrogel. Cells need to respond properly to the environmental signals for survival.

As mechanical properties and composition of hard and soft tissues are different, cells can sense and respond to the matrix stiffness in their natural environment by making the proper ECM composition. Similarly, the proliferation, differentiation, migration, and apoptosis of cancerous cells in the tumor tissue are regulated by matrix stiffness (Discher, Janmey, and Wang 2005, Schrader et al. 2011, Verbridge, Chandler, and Fischbach 2010).

In this thesis, the inert poly ethylene glycol diacylate hydrogel (PEGDA), in a certain moduli based on previous studies, as a 3-D cell culture system has been used in order to investigate the role of cell binding peptides, microenvironment pH, and matrix shape on the maintenance of breast CSCs. It has been shown that only breast CSCs among breast cancer cells can form tumor sphere in PEGDA (Jabbari et al. 2015). Having a good understanding of the role of these environmental factors on regulation of CSCs can provide significant information on the behavior of breast CSCs in their tumor microenvironment.

Chapter 2

Results and Discussion

2.1. Results and Discussion for Peptide Experiment

We have shown that cancer cells can form tumorspheres in the PEGDA gel with respect to the gel modulus (Yang et al. 2013). MDA-MB-231 human breast carcinoma cells formed cancer tumorspheres in the gel with an optimum modulus of 5 kPa, and the sphere formation was correlated with the expression of CSC markers (Yang et al. 2013). In order to determine if synthetic cell binding peptides derived from ECMs' components, attached to PEGDA gel, could affect the sphere formation of MDA-MB-231 cells in the PEGDA gel, different peptides such as; (i) VAPG, (ii) IKLLI, (iii) WQPPRARI, (iv) SPPRRARV, (v) LIGRKK, (vi) RYD, and (vii) RGD have been conjugated to the gel with a 5 kPa modulus in concentrations of 1%, 2%, 4%, 6%, and 9% (mg/mg). Sphere formation in samples was compared with the sphere formation of MDA-MB-231 cells encapsulated in PEGDA polymer with a 5 kPa modulus and no peptide. To achieve this goal, different measurements such as; fluorescent images, cell number, sphere number, sphere size, and expression of CSC markers have been performed.

2.1.1. Comparison of Sphere Formation of Tumor Cells Encapsulated in PEGDA with Different Conjugated Peptides in Fluorescent Images

Figure 2.1 illustrates the effects of different peptides on the sphere formation of cells, encapsulated in PEGDA gel in fluorescent images. As the concentration of peptides increase, the ability of the cancer stem cells to form spheres decreases. That means, there are a larger number of tumor spheres in groups with no peptide compared to the 1%, 2%, 4% concentrations. There was no sphere formation for all peptides in 6% and 9%.

As can be seen in all of the subfigures of Figure 2.1, larger spheres exist in the control group. By the increase of the peptides concentratons to 1% and 2% the tumors sphere size decreases gradually. In Figure 2.1, there are only four different concentrations (0%, 1%, 2%, and 4%) that have been shown. Concentrations of 6% and 9% are not shown here as there was no tumorsphere formation in these concentrations for any of the peptides.

2.1.2. Comparison of Cell Density, Sphere Size, and Sphere Density of Tumorspheres for Different Peptides

The cell density for the cells encapsulated in PEGDA gel and incubated for 9 days in stem cell culture medium are shown in Figures 2.2 (a). The cell density of MDA-MB-231 cells encapsulated in PEGDA in the presence of all of the peptides decreased gradually as the concentration of peptides increased, suggesting that peptides crosslinked to the PEGDA
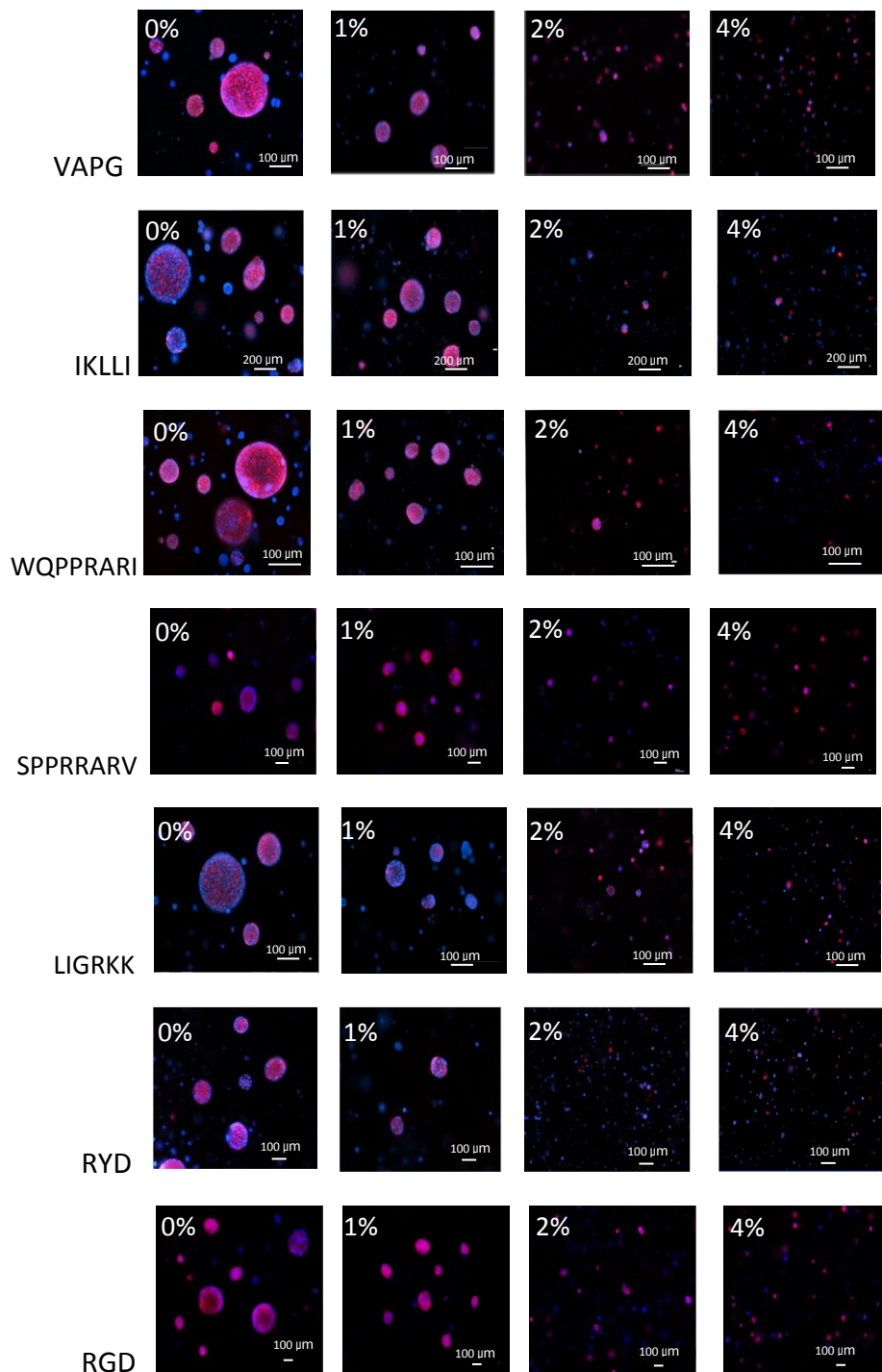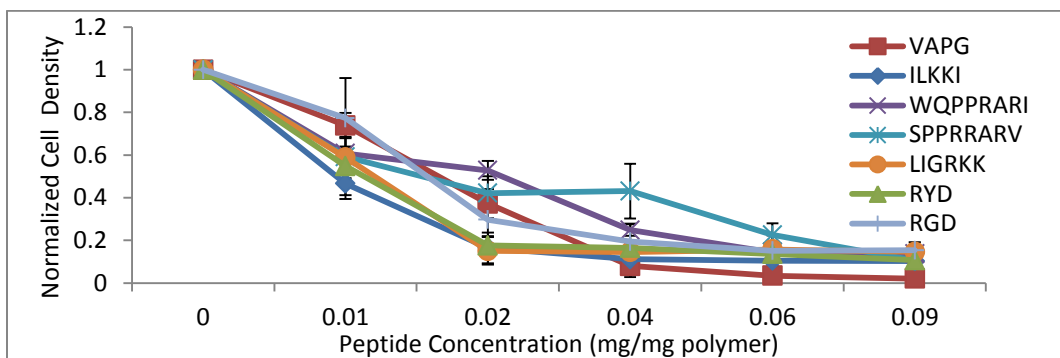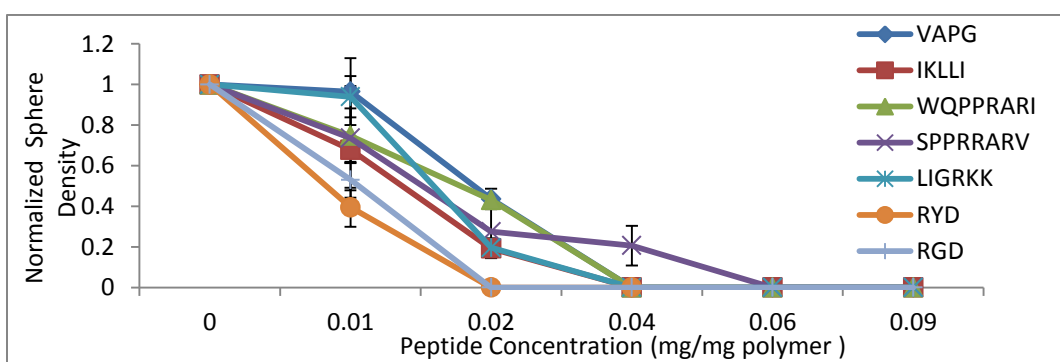
Figure 2.1 Comparison of tumorsphere formation in PEGDA gels conjugated with peptides. Illustrate of fluorescent images of the tumorsphere size and distribution for MDA_MB_123 tumor cells encapsulated in PEGDA gels with different concentrations of VAPG, IKLLI, WQPPRARI, SPPRRARV, LIGRKK, RYD, and RGD peptides.

are able to decrease the proliferation of tumor cells and the rate of this decrement increased with respect to the concentration increment.

The cell density was normalized with respect to the control groups without any peptides for all of the peptides. The cell density decreased significantly for all peptides with a concentration of 2% or more (mg/mg). Figure 2.3 (a) Displays the comparison of cell density for the peptides used in a 0.02 concentration. The cell density of the samples with a 2% peptide concentration decreased to 0.738469 ± 0.059071, 0.372907 ± 0.068205, 0.16477 ± 0.071641, 0.52825 ± 0.044298, 0.150917 ± 0.065204, $0.362006962\pm0.044315007$, and $0.296782544\pm0.117010065$ per cell density of the control groups, for VAPG, IKLLI, WQPPRARI, SPPRRARV, LIGRKK, RYD, and RGD, respectively. Cell density decreased significantly for all of the peptides in this concentration with respect to the control group. Moreover, there was a significant difference in cell density between peptides and their scrambled ones, whereas cell density did not change considerably with respect to the control group for scrambled peptides, meaning that the effect of reducing cell number is specific for the peptides. In comparison with 2%, a 1% peptide concentration showed less success for reducing the cell number density with respect to the control groups, shown in figure 2.3. (b). There was no significant difference between cell number of cells encapsulated with peptides in a 1% concentration compared to the control group with no peptide except for RYD.
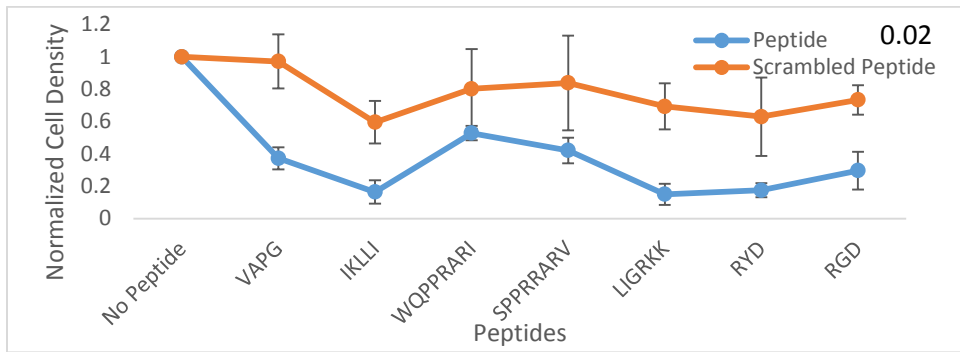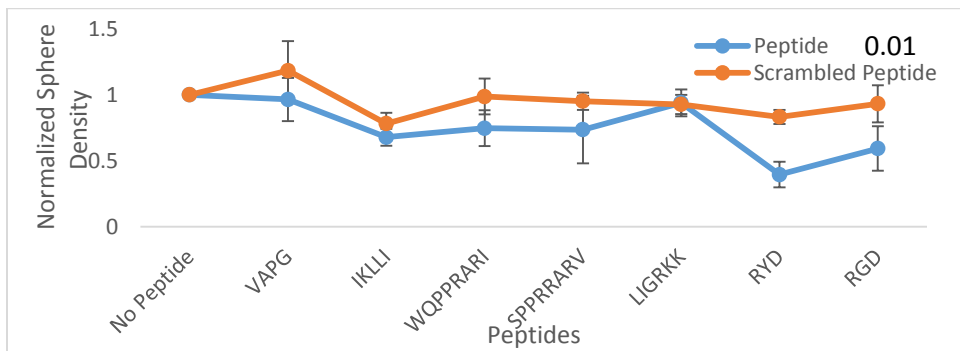
(a)

(b)
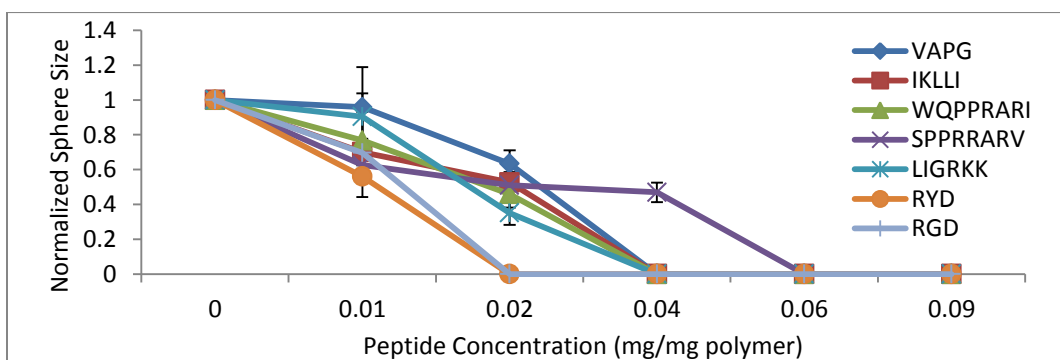
(c)

Figure 2. 2 Comparison of cell density, tumoresphere density and and sphere size of the cells in PEGDA gels conjugated with peptides. Illustrate the effect of the peptides on normalized cell density (a), tumorsphere density (b) and sphere size distribution (c) for MDA-MB-123 tumor cells encapsulated in PEGDA gel and incubated in CSC medium for 9 days.

(a)

(b)
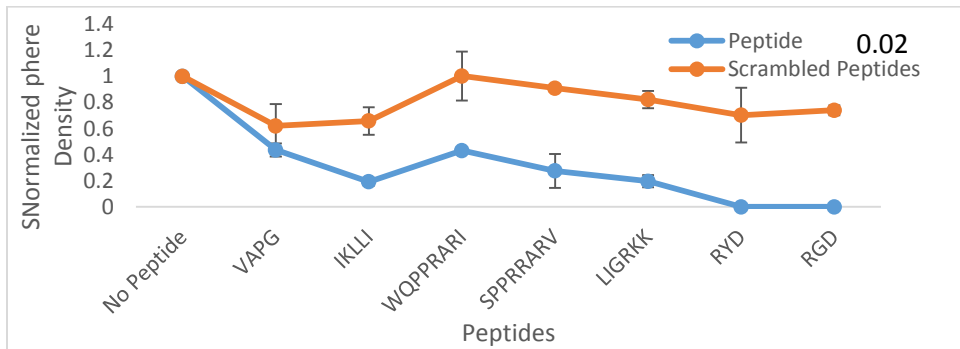
(c)

Figure 2.3 Comparison of cell density in 0.01 and 0.02 concentrations, and tumoresphere density in 0.02 of the cells in PEGDA gels conjugated with peptides. Representative the effect of the peptides in 0.02 concentration (a) and 0.01 concentration (b) on normalized cell density, and tumorsphere density in 0.02 concentration (c) for MDA-MB-123 tumor cells encapsulated in PEGDA gel and incubated in CSC medium for 9 days.

The variation in the cancer tumorspheres density has similar trends with respect to cell density. As the concentration of the conjugated peptides increased, the cell density decreased, meanwhile the cancer tumor spheres density reduced as well. In Figure 2.2 (b), the sphere density was normalized with respect to the control groups for all the peptides. The highest densities of sphere were within samples with no peptide concentration. In a 2% concentration, shown in figure 2.3 (c), all of the peptides reduced the ability of cancer stem cell in order to form tumorspheres considerably with respect to the control group. The cell number density decreased to 0.43628 ± 0.050591, 0.19286 ± 0.031074, 0.431222 ± 0.016621, 0.275115 ± 0.129692, 0.196425 ± 0.046058, per cell density of the control groups, for VAPG, IKLLI, WQPPRARI, SPPRRARV, LIGRKK, respectively. There were no spheres for RYD and RGD in this concentration. On the other hand, the scrambled peptides were not able to have the same effect on the cancer cells; not only the tumorspheres densities did not have a major difference with respect to the control, but also they had a significant variance with the density in relative peptides samples. In addition, in a 1% concentration did not show any significant effects on the decrease of tumor density with respect to the control groups and scrambled peptides except RYD peptide, shown in Figure 2.3 (b). In all of the 4% concentrations of the conjugate peptides except one, SPPRRARV, as well as higher concentrated ones the cancer stem cells were unable to form tumorspheres. This was also true for the scrambled peptides as there were no sphere formation in high concentrations, proposing that the prevention of the cancer stem cells from forming tumor spheres in 4% and above concentrations is not specific to the peptides. Also, cell

number was significantly reduced in 4% and higher concentrations for both peptides and scrambled ones which shows that the reduction is through nonspecific interactions.

Human breast cancer stem cells formed larger tumorspheres in groups with no peptide after 9 days of culturing in the gel. This has been shown in Figure 2.2 (c). The average sphere size of groups with different peptides and concentrations were normalized with respect to the control groups. The average sphere size of the cells encapsulated in PEGDA with conjugated peptides having 2% concentration decreased to 0.633892042±0.076894548, 0.526900821±0.030774561, 0.459564566±0.076799477, and 0.509764726±0.075889146 per average sphere size in control groups for VAPG, IKLLI, WQPPRARI, SPPRARV, and LIGRKK, respectively. In addition, there was no sphere formation for tumor cells encapsulated in PEGDA with conjugated RYD and RGD having a 2% concentration. The average sphere size did not change significantly for scrambled peptides in a 2% concentration showing that peptides are able to reduce the size of spheres. Moreover, in comparison with 2%, the average size of sphere also did not change fundamentally for both peptides and scrambled ones in a 1 % concentration with respect to the control group with no peptide except for RYD.

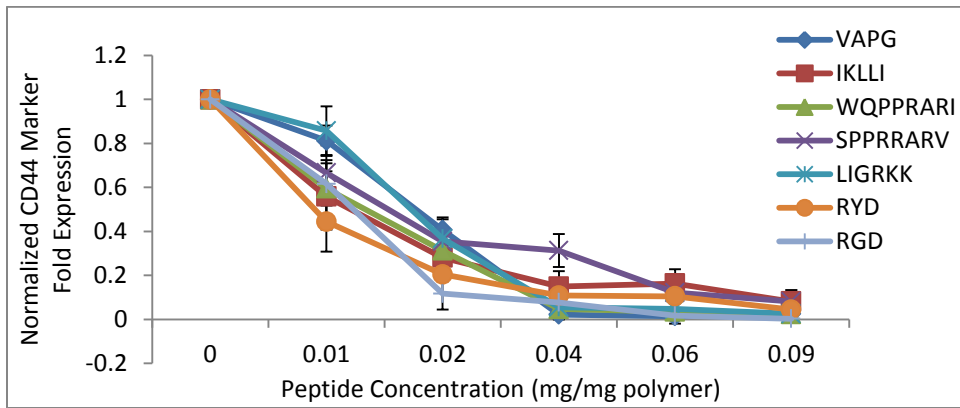### 2.1.3. Comparison of CD44, ABCG2, and TGF β Expressions of Tumor Cells Encapsulated in PEGDA with Different Conjugated Peptides

Figures 2.4. (a)- (c) shows the expressions of breast CSC markers CD44, ABCG2, and TGF β for the encapsulated cells, normalized with respect to the control group. CD44 is a cell surface molecule that has multiple structures and functions. This molecule is involved

35

in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, and reducing of proteases at the cell membrane, as well as in signaling for cell survival. All these biological properties are critical to the physiological activities of cancer cells (Eibl et al. 1995).

ABCCG2 (ATP-binding cassette sub-family G member 2) is a membrane-associated protein encoded by ABCG 2 gene. ABCG2 protein is part of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport several molecules across extra- and intra-cellular membranes. Therefore, ABCG2 referred to as the Breast Cancer Resistance Protein. This protein functions as a xenobiotic transporter which probably play a role in multi-drug resistance to chemotherapeutic agents (Allikmets et al. 1996).

TGF-β (Transforming growth factor beta) is a secreted protein that mostly controls cellular proliferation and differentiation (Schoenhoff et al. 2009). Normally it is acting through its signaling pathway, stops the cell cycle at the G1 stage to prevent proliferation, cause differentiation, or stimulate apoptosis. In many cancer cells, parts of the TGF-β signaling pathway are mutated, therefore, TGF-β no longer controls the cell proliferation. As a consequence, the cancer cells and surrounding stromal cells (fibroblasts) proliferate. Both cells produce higher levels of TGF-β. This TGF-β acts on the

(a)
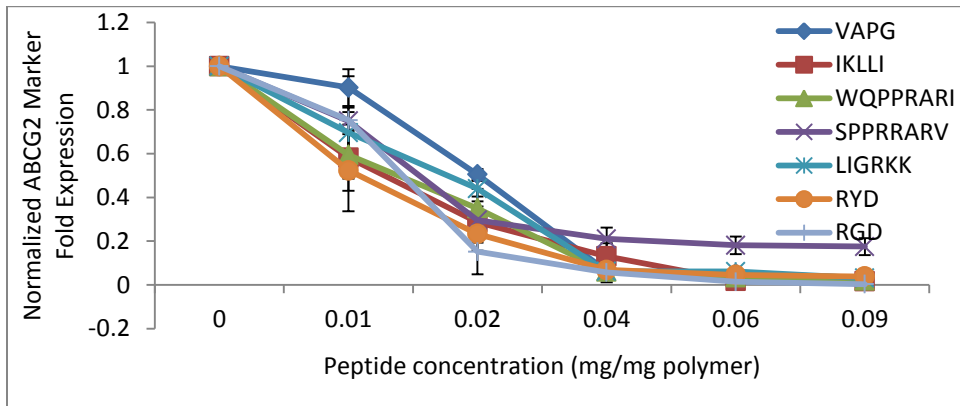
(b)
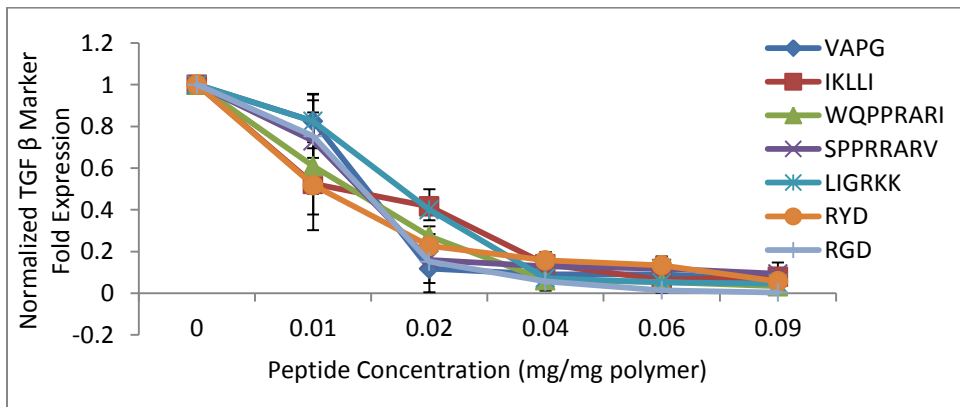
(c)

Figure 2.4 Comparison of CD44, ABCG2, and TGF β expressions of the tumor cells in PEGDA gels conjugated with peptides. Illustrate the effect of peptides on the normalized CD44 (a), ABCG2 (b), and TGF β (c) marker fold expressions of MDA-MB-123 cells encapsulated in PEGDA gel for 9 days in CSC medium.
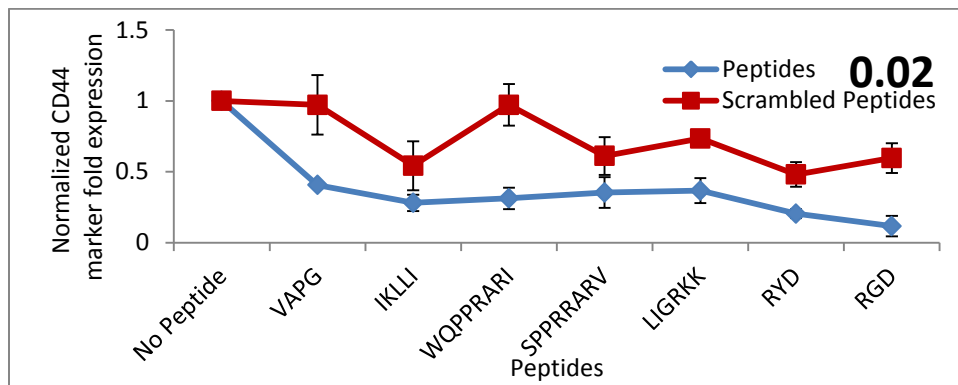
(a)
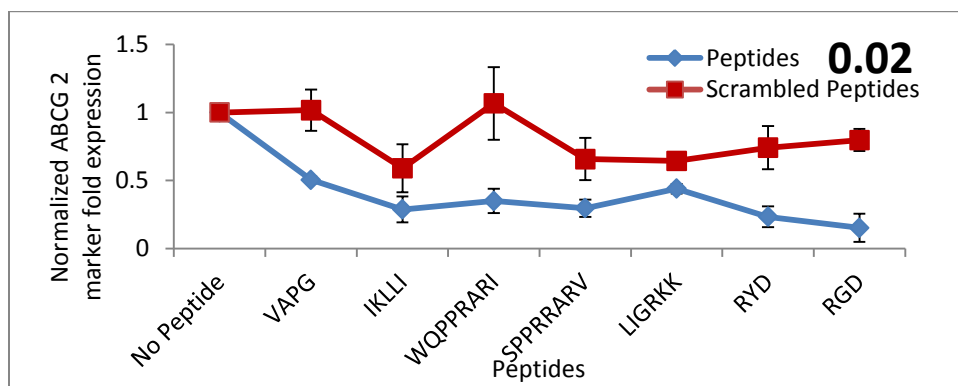
(b)
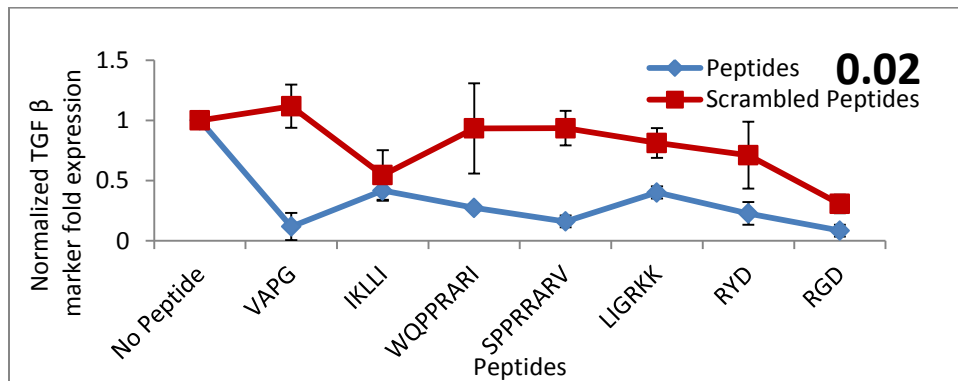
(c)

Figure 2.5 Comparison of normalized CD44, ABCG2, and TGF β expressions of the tumor cells encapsulated in PEGDA gels with 2% concentration of different conjugated peptides. Representative of the comparison of the effect of peptides in 2% concentration on the normalized CD44 (a), ABCG2 (b), and TGF β (c) marker fold expressions of MDA-MB-123 cells encapsulated in PEGDA gel for 9 days in CSC medium.

surrounding stromal cells, immune cells, endothelial and smooth-muscle cells. It causes immunosuppression and angiogenesis, which makes the cancer more invasive. TGF-β also changes effector T-cells, which under normal condition attack cancer with an inflammatory (immune) reaction, into regulatory (suppressor) T-cells, which turn off the inflammatory reaction (Epstein et al. 2000).

After 9 days of incubation, CD44, ABCG2, and TGF β expression levels in the cells encapsulated in PEGDA with no peptide was significantly higher than the level of expressions in the cells encapsulated in PEGDA conjugated with peptides.

Figures 2.5. (a)- (c) shows the comparison of normalized expressions of breast CSC markers CD44, ABCG2, and TGF β for the tumor cells, encapsulated in PEGDA with a 2% concentration of different peptides for 9 days in CSC medium. As shown in Figure 2. 5. (a), (b), and (c) for all of the peptides with 2% concentration, there is a significant decrease in CD44, ABCG2, and TGF β expression levels of cancer cells with respect to the control group. On the other hand, in groups with 2% concentration of respective scrambled peptides, there is no major difference in the levels of CD44 and ABCG2 expression of cells with respect to the control group while there is a significant variance between CD44 and ABCG2 expression of cells encapsulated with each peptide and its scrambles one. In a 1 % concertation, WQPPRARI and RYD were more effective on reducing the markers expression among the peptides. In a 2% concentration WQPPRARI, RYD and RGD were more effective on reducing the markers expression among the peptides. In 4% and higher concentrations both peptides and scrambled ones decreased

the markers expression significantly, suggesting that the reduction is not specific to the peptides.

2.1.4. Comparison of the Effect of Conjugated Peptides on Sphere Formation of Tumor Cells Encapsulated in PEGDA for 9 Days in CSC Medium

Results for the measurements of cell and sphere density together with marker expressions illustrated that in a 0.01 concentration of conjugated peptides, RYD and WQPPRARI were the most effective peptides in reducing tumor sphere formation. In a 0.02 concentration, WQPPRARI, RGD, and RYD were the most effective peptides in reducing the sphere formation among peptide. Plus, 4% and higher concentrations are too high amounts of peptides which change the PEGDA matrix and cause nonspecific interactions with breast cancer stem cells.

2.1.5. How Cell Adhesion Peptides Affect the Sphere Formation of Tumor Cells Encapsulated in an Inert PEGDA System?

When MDA-MB-231 cancer cells are cultured in the inert PEGDA hydrogel matrix, there is a population of these cells that have high expression of CD44 cell surface glycoprotein. This population of cells interacts with each other to grow and divide, and eventually form a tumorsphere. These cells which have breast cancer stem cells properties are non-adherent and through the cell-cell interaction instead of the cell-matrix interaction survive in the matrix; however, conjugating cell binding peptides to the matrix will cause a competition for the cells to interact with each other or with the matrix. By binding of the cells to the peptide ligands, cells adhere to the matrix and do not form

tumorspheres. There should be a preference of the cell adhesion peptides for the cells which cause some of these cell binding peptides to be more effective in reducing the tumorsphere formation of breast cancer stem cells. The reason probably refers to the cell surface binding receptor and intracellular pathways which will be investigated in future studies.

2.2. Result and Discussion for pH Experiment

In this study, we investigated the effect of acidic pH 6.8, equivalent to the pH of tumor microenvironment (Pellegrini et al. 2014), on the maintenance of breast CSCs encapsulated in PEGDA and incubated for 9 days in CSC medium. Fluorescent Imaging, qRT-PCR, and DNA Content measurements have been done in order to determine the differences between breast CSC maintenance and behavior in acidic pH 6.8 and normal pH 7.4.

2.2.1. Comparison of Sphere Formation of MDA-MB-231 Tumor Cells Encapsulated in PEGDA and Incubated in CSC Medium with Different pH in Fluorescent Images

Figure 2.6. Illustrates the effects of pH of the CSC medium on the sphere formation of cancer cells encapsulated in PEGDA gel for 9 days. In CSC medium with pH 7.4 which was used as a control, there were not a significant difference in number of tumor spheres and their diameters compared to that in pH 6.8. Therefore, tumor cell's capability of forming spheres in pH 6.8 was the same as that in pH 7.4.
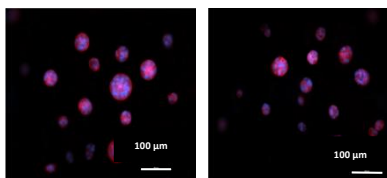
Figure 2.6 Comparison of tumorsphere formation in PEGDA gels, encapsulated in CSC medium with PH=7.4 and 6.8. Illustrate of fluorescent images of the tumorsphere size and distribution for MDA_MB_123 tumor cells encapsulated in PEGDA gels and incubated in CSC medium with pH 7.4 and pH 6.8. The scale is 100 µm.

2.2.2. Comparison of Cell Number, Sphere Size, and Sphere Number of Cancer cells in CSC Medium with Different pH

The cell density for the MDA-MB 231 cells encapsulated in PEGDA gel and incubated for 9 days in stem cell culture medium are shown in Figures 2.7.(b). The cell density of MDA-MB-231 cells encapsulated in PEGDA end incubated in pH 7.4, and pH 6.8 was 8.1079±0.82 and 6.4191±0.53, respectively.

MCF10a cell line is a normal breast epithelial cell line which was used as control group for the experiment. This cell line is not cancerous and does not form tumor spheres when encapsulated in 3D PEGDA. The cell number of MCF10a cells in PEGDA with pH 6.8 and 7.2 was 0.8511±0.2183 and 2.9803±0.6523 respectively, shown in Figure 2.2. b). this results suggests that the decrease of cell number due to the reduction of pH is more effective on normal cells than cancer cells.

The average sphere size of MDA-MB-231 cells encapsulated in PEGDA end incubated in pH 7.4 was 50.9189±8.45 and was 43.3028±8.05 in pH 6.8. The average sphere number of MDA-MB-231 cells encapsulated in PEGDA end incubated in pH 7.4 was 4854.27±1321.16 and was 4045.23±934.20 in pH 6.8. MDA-MB-231 and MCF10a cell

lines also cultured in 2D flasks for 9 days in CSC medium with pH 6.8 and 7.4. Cell number was reduced for both of these cell lines by the decrease of pH, while the reduction was higher for non-cancerous MCF10a cells. The results together show that cancer cells are more resistant to acidic pH.
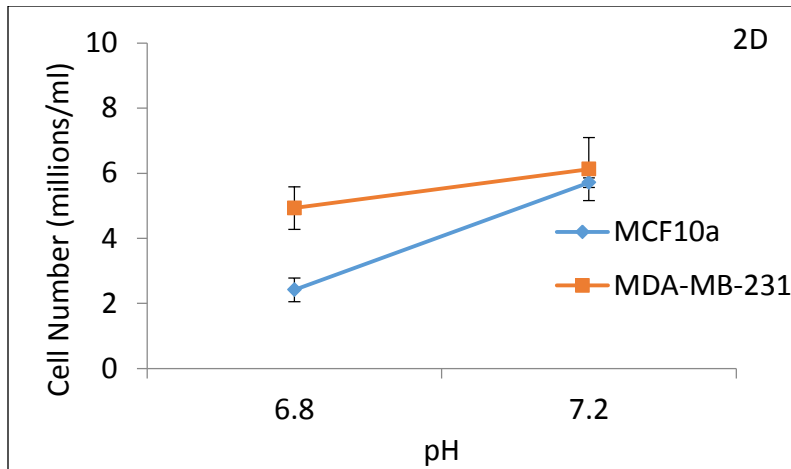
(a)
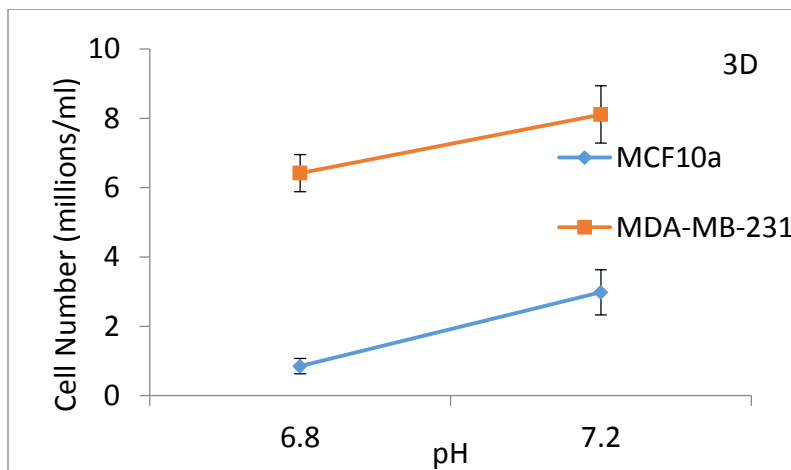
(b)

Figure 2.7 Comparison MDA-MB-231 and MCF10a cell numbers cultured in CSC with pH 7.4 and pH 6.8. Illustrate the effect of PH on (a) cell density and (b) tumorsphere number for MDA-MB-123 tumor cells encapsulated in PEGDA gel and incubated in CSC medium with two different pH for 9 days.

2.2.3. Comparison of CD44, and ABCG2 expressions of MDA-MB-231 and MCF10a Cells Incubated in CSC Medium with pH 7.4 and pH 6.8

Figures 2.8.(a)- (d) shows the expressions of breast CSC markers CD44, and ABCG2 for the cultured cells in CSC medium with different pH. After 9 days of incubation CD44, and ABCG2 expression levels for the MDA-MB-231 cells encapsulated in PEGDA, in CSC medium with pH 7.4 was not significantly higher than the level of expressions for the cells encapsulated in PEGDA, in CSC medium with pH 6.8. However, when MDA-MB-231 cells cultured on a 2D substrate, they had a higher expression of CD44 and ABCG2 in pH 6.8 compared to that in pH 7.4. While, the expression of these markers decreased for MCF10a cells in both 2D and 3D cultures when pH was 6.8.

2.2.4. How Did Acidic PH Affect the Maintenance of CSCs?

Fluorescent imaging showed that, MDA-MB-231 cells formed tumor spheres with almost same sizes in pH 7.4 and pH 6.8. Also, the density of tumor spheres which was counted in fluorescent images, was not significantly different form that in normal pH. Consistently, the cell density, measured by DNA Content measurement, as well as breast CSC markers; including CD44, and ABCG2, measured by the use of qRT-PCR, were also the same in acidic pH and normal PH. The populations of these cells that are encapsulated in PEGDA and formed tumorspheres are mostly cancer stem cells. Therefore, the data shows that CSCs are resistant to acidic pH. On the other hand, when MDA_MD-231 cells were cultured in a 2D culture system the cell number decreased while the marker expressions increased in pH 6.8. The majority of the cells that are cultured in 2D are cancer cells and a few percentages of them are cancer stem cells. The

reduction in cell number shows the sensitivity of cancer cells to pH 6.8 and the increase in expressions shows the resistance of breast cancer stem cells to pH reduction. On the other hand, normal breast epithelial cells had higher level of reduction in both cell number and markers expression in 2D and 3D cultures compared to cancer cells. The results show that CSCs are more resistant to acidic pH compared to cancer cells and normal cells.

2.3. Results and Discussion for Matrix Shape Experiment

In this study, we investigated the effect of micropatterned PEGDA with different shapes, on the maintenance of breast CSCs encapsulated in PEGDA and incubated for 9 days in CSC medium. Fluorescent Imaging, qRT-PCR, and DNA Content measurements have been used in order to determine the differences between breast CSC maintenance and behavior in different microshaped PEGDA.

2.3.1. Comparison of Sphere Formation of Tumor Cells Encapsulated in Micropatterned PEGDA Gels in Fluorescent Images

Figure 2.9. Illustrates the effect of micropatterns including circle with 50 μm diameter, square (50 μm*50 μm), rectangular (50 μm*150 μm), and rectangular (50 μm*250 μm), on the sphere formation of cells, encapsulated in PEGDA gel. Figure 2.9. (a) is the schematic pictures while Figure 2.9. (b) is the fluorescent images. Each arrow shows an individual 3D micropattern (orange), corresponded to the micropattern in fluorescent images, made of PEGDA located on the surface of a substrate (gray) which is made of PEGDA with higher stiffness.
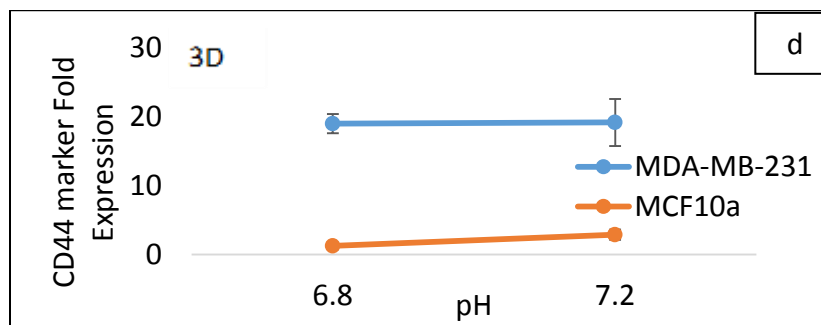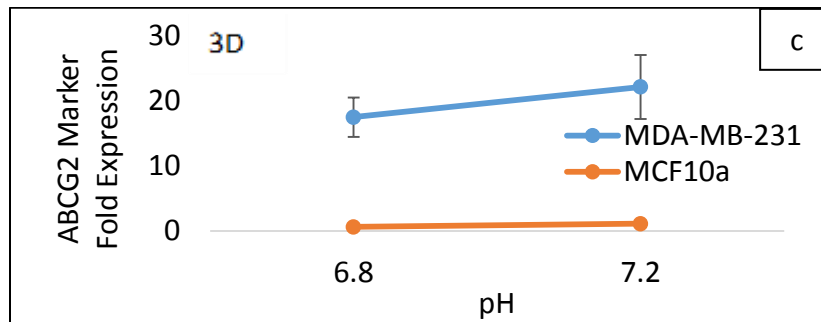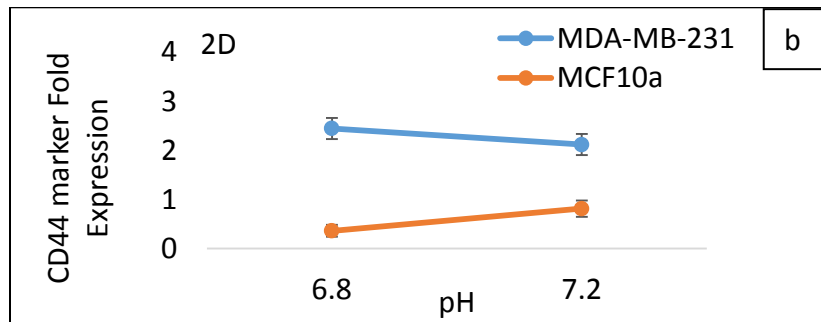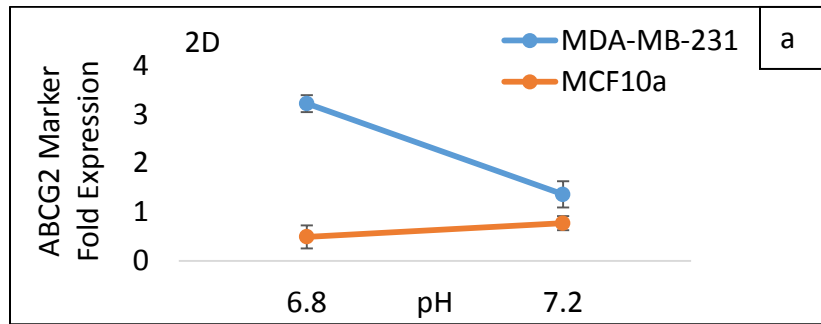
Figure 2.8 Comparison of CD44, and ABCG2 expressions of the cells in CSC medium with pH 7.4 and pH 6.8. Illustrate the effect of pH on the CD44 (b,d), and ABCG2 (a,c), marker fold expressions of MDA-MB-123 and MCF10a cells cultured for 9 days in CSC medium with pH 7.4 and pH 6.8.

By florescent imaging and then shape analysis it was found out that cancer stem cells

still try to make the tumors in spherical shape regardless of the shape of the PEGDA,

which means that they try to have a minimum surface area for a given volume.
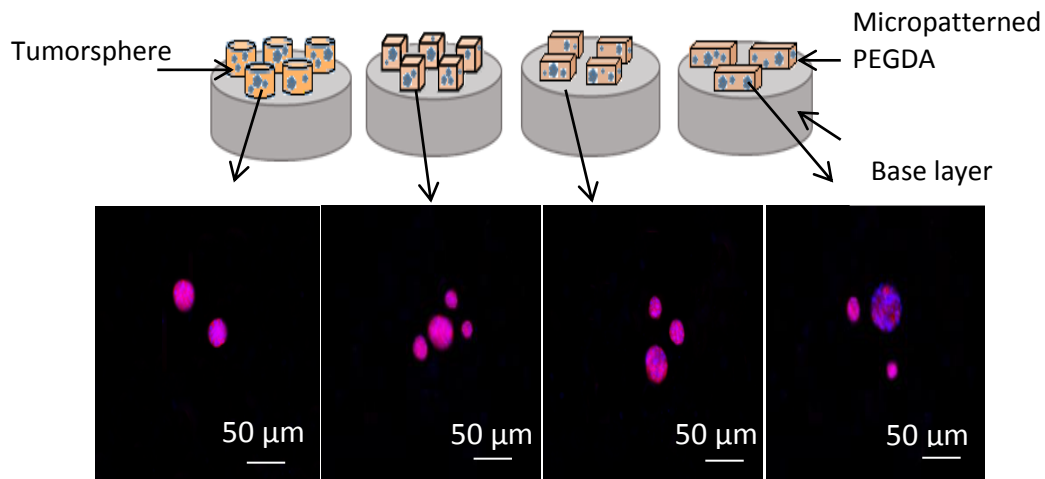
Figure 2.9 Comparison of tumor formation in micropatterned PEGDA gels. Illustrate of schematic pictures (a) and fluorescent images (b) of the tumor size and distribution for MDA_MB_123 tumor cells encapsulated in PEGDA gels with different micropatterns. Each arrow shows an individual 3D micropattern (orange) made of PEGDA located on the surface of a substrate (grey) which is made of PEGDA as well. The scale is 50 μm.

2.3.2. Comparison of Sphere Density, Sphere Diameter, and Cell Density of Tumorspheres within PEGDA Gels Shaped with Different Micropatterns

The sphere density/ cell density for the cells encapsulated in PEGDA gels which were

shaped in circles with 50 μm diameter, squares (50 μm*50 μm), rectangulars (50

μm*150 μm), and rectangulars (50 μm*250 μm), incubated for 9 days in stem cell

culture medium are shown in Figures 2.10. (a).
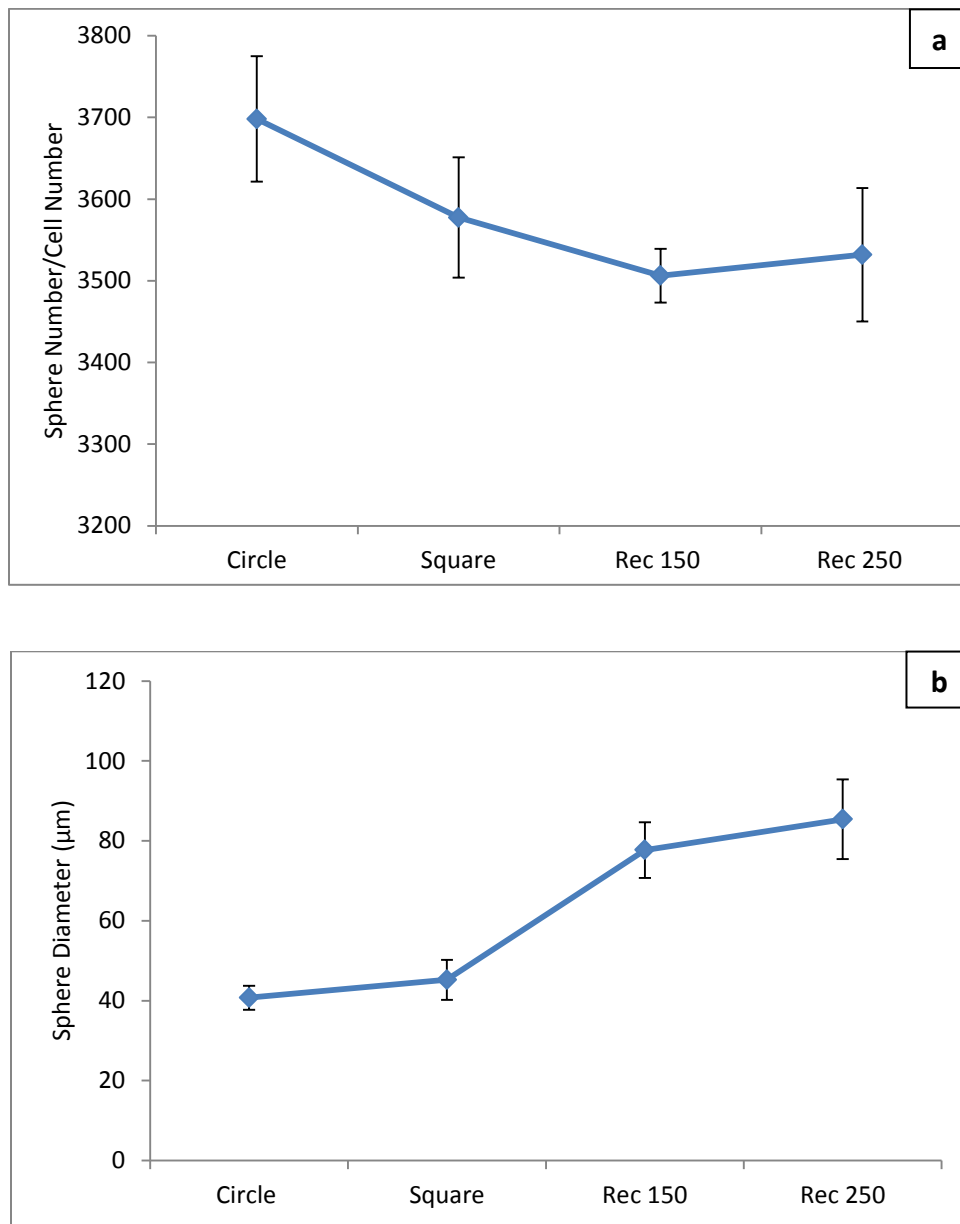
Figure 2.10 Comparison of tumoresphere density/ and sphere size of the cells encapsulated in pmicroatterned PEGDA gels. Illustrates the effect of the micropatterns on tumorsphere density/ cell density (a), and sphere size distribution (b) for MDA-MB-123 tumor cells encapsulated in PEGDA gel and incubated in CSC medium for 9 days.

The cell density of encapsulated MDA-MB-231 in patterned PEGDA was 1.6±0.15, 1.87±0.16, 2.60±0.18, 2.86±0.20 in circles, squares, rectangulars 75, rectangulars 150, and rectangulars 250, respectively. Having different micropatterns did not have a significant effect on the cell number of tumor cells encapsulated in PEGDA. The tumorsphere density of MDA-MB-231 cells was 6256.33±397.86, 6700.53±459.09, 8866.67±711.63, 10121.15±942 in PEGDA gels patterned in circles, squares, rectangulars 75, rectangulars 150, and rectangulars 250, respectively. Results do not show any considerable difference between groups.

2.3.3. Comparison of CD44, ABCG2, and TGF β Expressions of Tumor Cells Encapsulated in Micropatterned PEGDA Gels

Figures 2.11. (a)- (c) shows the expressions of breast CSC markers CD44, ABCG2, and TGF β for the encapsulated cells in micropatterned PEGDA for 9 days. CD44 is a cell surface protein that has multiple functions in breast cancer development (Eibl et al. 1995). ABCG2 referred to as the Breast Cancer Resistance Protein (Allikmets et al. 1996). This protein is critical in multi-drug resistance(Allikmets et al. 1996). TGF-β is a secreted protein that mostly controls cellular proliferation and differentiation (Schoenhoff et al. 2009). It is highly expressed by cancer cells and surrounding cells in cancer microenvironment (Epstein et al. 2000).

After 9 days of incubation in CSC medium, CD44, ABCG2, and TGF β expression levels in the cells encapsulated in patterned PEGDA were measured and no significant difference between micropatterns have been observed. The results suggest that the shape of

PEGDA gel did not have any effects on marker expression of breast cancer stem cells. To wrap up, breast cancer stem cells form spherical tumors regardless of the shape of the microenvironment suggesting that they try to have the minimum surface area for a given volume.
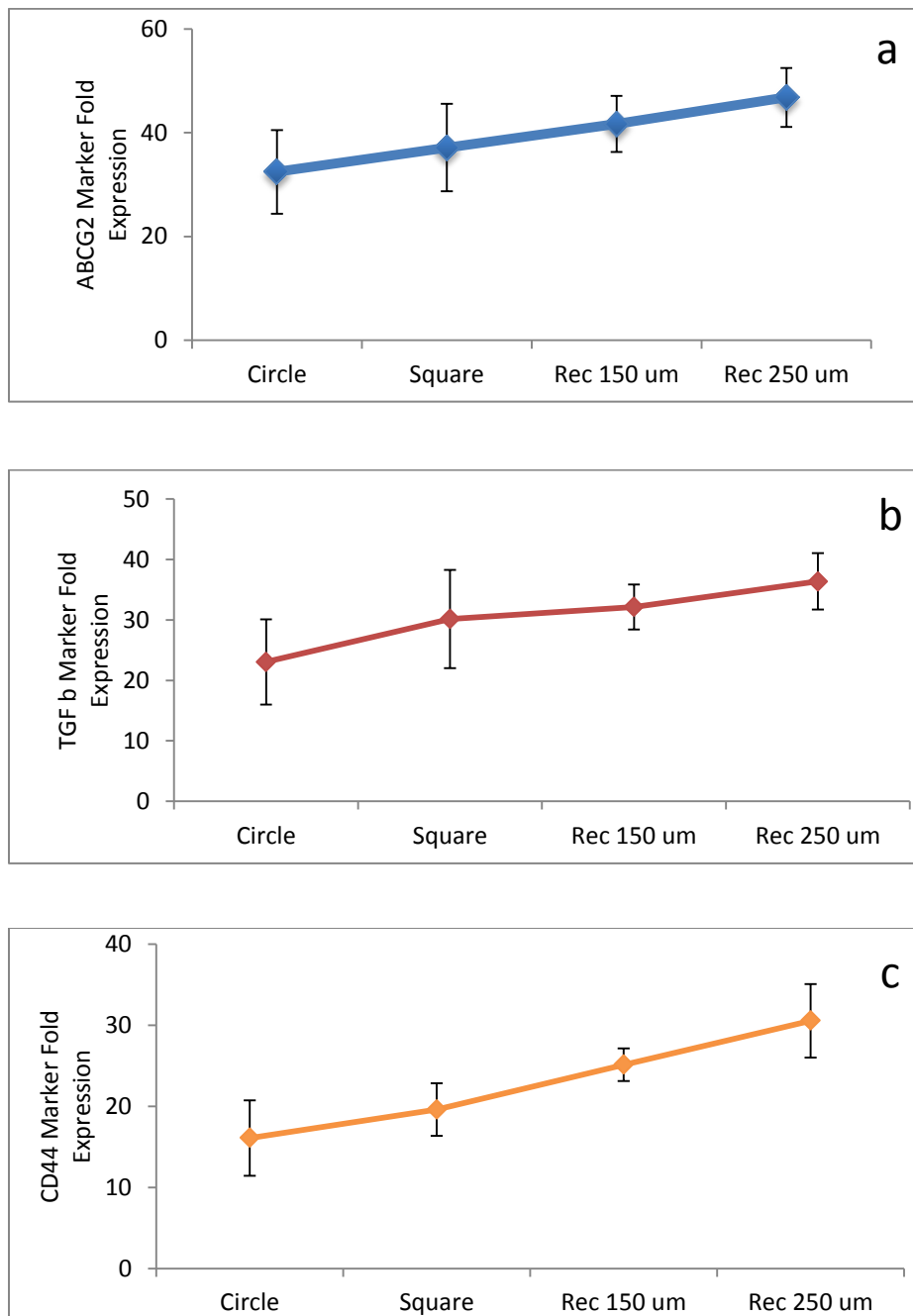
Figure 2.11 Comparison of CD44, ABCG2, and TGF β expressions of the tumor cells in micropatterned PEGDA gels. Illustrates the effect of the micropatterns on ABCG2 (a), TGF β (b), and CD44 (c), marker fold expressions of MDA-MB-123 cells encapsulated in PEGDA gel for 9 days in CSC medium.

Chapter 3:

Methods

3.1. PEGDA Synthesis

PEGDA was made by acrylation of hydroxyl end-groups of PEG with acrylate groups. In that regard, acryloyl chloride was reacted with the hydroxyl groups of PEG at the end of macromeres. Trimethylamine (TEA) was the reaction catalyst. At the beginning, PEG was dried using azeotropic distillation from toluene in order to remove residual moisture. The polymer was then dissolved in dried DCM and then cooled by the use of ice bath. For the reaction, 5.6 mL of acryloyl chloride and 9.7 mL TEA dissolved in dichloromethane (DCM), then were added to the reaction while constantly stirring. The reaction continued for 12 hours under nitrogen flow.

After the reaction was finished, the solvent was removed through rotary evaporation and then the rest was dissolved in anhydrous ethyl acetate. This was performed to precipitate the by-product of the reaction which is trimethylamine hydrochloride salt. Afterwards, vacuum distillation was used to remove ethyl acetate. Next the polymer was dissolved in DCM and precipitated in cold ethyl ether two times. Next, the polymer was dissolved in dimethylsulfoxide

(DMSO) and dialyzed against distilled deionized (DI) water in order to eliminate the by-products. The PEGDA powder was freeze-dried and stored at -20°C.

3.2. Peptide Synthesis and Characterization

3.2.1. Preparing the Chromatographic Column and Amine Resin

In order to synthesize the peptide chains, 160 mg of amine resin (at room temperature) was added into a 3 mL chromatographic column (CC). The bottom of the CC was capped and 3 mL of dimethylformamide (DMF) was added to the column and the top of the CC was capped. The CC was then vortexed and the resin was allowed to swell for 10 minutes. The CC was drained by uncapping and inserting the bottom tip into a vacuum, trapping the resin in the filter. The bottom cap was replaced and the first amino acid was prepared for addition.

3.2.2. Addition of an amino acid

The reaction for adding an amino acid must be performed in an oxygen-free environment, in this case inside a nitrogen-gas filled pyramid (BRAND). For each reaction, the following was placed inside the pyramid: the CC with dry resin, 160 µL diisopropylcarbodiimide (DIC), 1 mmol of target amino acid and 2 mmol of hydroxybenzotriazole (HoBT) dissolved in 6 mL of DMF, and 14 mg of dimethylaminopyridine (DMAP) dissolved in 1 mL of DMF. Oxygen was vacuumed from the pyramid and nitrogen was added until pyramid was fully inflated. The reaction was performed as followed: First, the DMAP solution was added to the amino acid and HoBT

solution. This solution was then added to the CC and 160 µL of DIC was pipetted into the CC. The CC was capped tightly and shaken. The pyramid was opened and both ends of the CC were wrapped with ParafilmTM and taped closed. The CC was then placed in a shaker for a minimum of six hours for, unless adding the initial amino acid, then the CC was placed in a shaker for at least 13 hours. After the waiting period, a Keizer test was performed to determine if the amino acid was added correctly (color should stay yellow).

3.2.3. Removal of the FMOC Group

In order to add an amino acid to the resin or an existing chain, the FMOC protecting group must be cleaved of the last added amino acid. The resin was washed twice by adding 6 mL of DMF, vortexing, and draining. Afterwards, 6 mL of 20% piperidine was added, vortexed, and placed in a shaker for 12 minutes. Following the waiting period, the piperidine was drained and the resin was washed twice again. Piperidine addition was then repeated. After draining the piperidine, the resin was washed twice and a Keizer test performed in order to determine if FMOC group was removed. If the Keizer test turned blue, this means that there are unprotected amino acids and the reaction can proceed. If the Keizer test turns yellow, then the FMOC group was not removed and piperidine addition must be repeated.

3.2.4. Keiser Test

The Keiser test was used to determine the presence of unprotected amino acids. A small amount of resin/DMF was extracted from the CC and added to a miniature CC. The DMF

was drained and 1 mL of dichloromethane was added to the mini CC and then drained. The following reagents were added to the mini CC: 7 drops of Ninhydrin, 2 drops of Phenol, and 2 drops of KCN. The solution was mixed with a pipette and transferred to a 1.5 mL Eppendorf tube. Leaving the cover open, the tube was covered with aluminum foil and placed in a heating block, preheated to 110 °C for ten minutes. If the solution turns blue, there are unprotected amino acids and the amino acid addition reaction can proceed. If the Keizer test produces a yellow solution, then the amino acid is successfully added.

3.2.5. Addition of additional amino acids

In order to add additional amino acids, the same three procedures were taken as described above, beginning with Addition of an amino acid.

Following the addition of the last amino acid, depending on the desired application, either the FMOC group was cleaved or also terminated with acrylic acid or capped with acetic anhydride.

3.2.6. Cleaving the Resin from the Peptide

Following the completion of the addition of the last amino acid, the resin must be cleaved from the peptide chain. First, 45 mL of ethyl ether is cooled in a 50 mL tube. The cleaving solution was prepared and composed of 95% trifluoroacetic acid (TFA), 2.5% tiisopropylsilane (TIPS), 2.5% deionized water. The resin is then washed twice with DCM, draining with a vacuum and placing the CC in a 15 mL tube. The CC tube was gently

tilted for 1 minute and then 3 mL of the cleaving solution was added. After the bubbling had ceased, the top was capped and placed on its sign for 1 minute. Every 20 minutes, the CC was gently tilted; then the resin was allowed to resettle to the bottom. After 6 hours, the cooled ether was placed under the CC. The CC was quickly drained into the ether containing 50 mL tube. 3 mL of cleaving solution was added to the CC and repeat steps above. After cleaving the resin twice, the 50 mL tube to cooled to – 20 C and held overnight at this temperature in the freezer to further precipitate the peptide. The 50 mL tube was centrifuged for 10 minutes at 4000 RPM. The supernatant was decanted and freeze dried until a free-flowing powder was formed.

The acrylamide-terminated (Ac) peptides were further purified by preparative HPLC on a 250 x 10 mm, 10 μm Xterra Prep RP18 column (Waters, Milford, MA) with a flow rate of 2 mL/min using a gradient 5- 95% MeCN in 0.1% aqueous TFA at detection wavelength of 214 nm. The HPLC fraction was lyophilized and the product was characterized with a Finnigan 4500 Electro Spray Ionization (ESI) spectrometer (Thermo Electron, Waltham, MA). The peptides and their scrambled ones are written in Table 1:

Table 3.1 Peptides and their scrambled sequence.

Peptide	LIGRKK	IKLLI	VAPG	SPPRRARV	RGD	RYD	WQPPRARI
Scrambled peptide	KGIKRL	ILKLI	PGVA	APRPVSRR	RDG	RDY	RPQIPWAR

3.3. Hydrogel Preparation and Modulus Measurement

To prepare hydrogels, PEGDA polymer crosslinked within an aqueous solution, using ultraviolet initiated radical polymerization with 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl) ketone photoinitiator (Irgacure 3959; CIBA, Tarrytown, NY). Ten mg of initiator was dissolved in 1 mL PBS at 50°C. A 13% PEGDA hydrogel precursor solution was prepared by mixing 130 mg PEGDA macromer with 870 μL of the initiator solution. The hydrogel precursor solution was filtered with 0.45 μm filter and degassed, then transferred to a Teflon mold, covered with a piece of glass and sealed using clips. The solution and irradiated with Omnicure Series 1500 UV light for 15 seconds. Following crosslinking, the elastic modulus of the hydrogel was determined. Disc shape gel samples were cut by an 8 mm cork-borer and were put in PBS for 24 hour at 37°C to swell. The samples were then loaded onto the Pentier plate of a rheometer (TA Instruments, New Castle, DE) and uniaxial compressive force at a displacement rate of 7.5 μm/s was used. The slope of the linear fit to the stress-strain curve for 5-10% strain was taken as the elastic modulus of the hydrogel.

3.4. Cell Culture and Encapsulation in the Hydrogel

MDA-MB-123 human breast cancer cells were cultured in RPMI-1640 medium with 10% FBS ,100 U/ml penicillin, and 100 mg/ml streptomycin under 5% CO_2 at 37°C. Cells were harvested by trypsin and suspended in 1640 after reaching 70% confluency. Cell density was determined using a hemocytometer and Trypan blue assay. The cell density was adjusted to 1.0×10^6 cells/mL. Then peptides were added to the hydrogel solution and

the mixture was sterilized using a 4.5 µm filter. Afterwards, 1 million /ml cells were

added to the hydrogel precursor solution (13 wt%) and mixed softly. The cell-suspended

hydrogel precursor solution was added to a Teflon mold, covered with glass, and

crosslinked using UV (Omnicure Series 1500 Standard Filter) for 1 minutes. Following

crosslinking, the gel was cut and incubated in CSC medium consists of DMEM-F12

supplemented with 5 mg/ml insulin, 40 ng/ml bFGF, 20 ng/ml EGF, 5% horse serum, 100

U/ml penicillin, and 100 mg/ml streptomycin on ultra-low attachment tissue culture

plates for 9 days under 5% CO_2 at 37°C .

3.5. PH Adjustment at 6.8

The following steps were used to incubate encapsulated cancer cells in CSC medium

with PH 6.8. First the PH of CSC media was reduced from 7.4 to 6.8 by using 1 molar HCl.

Then, the media was filtered with 0.1 µm filter and kept in an incubator for 2 days to

ensure the PH remains at 6.8. For the experiment, samples were kept in CSC media with

PH 6.8 in ultra-low

attachment tissue culture plates for 9 days and the media was changed every 24 hours.

3.6. Micropatterning

To encapsulate MDA-MB-231 cancer cells in a micropatterned gel. First, 50 µl of the

PEGDA precursor solution (25 wt%) were added to a glass slide. The edges of the glass

slide were covered with an adhesive tape to control gel thickness. Then the solution was

covered with a glass cover slip and kept under UV light for 8 min. After the gel was

formed, the glass slide edges were covered with another layer of adhesive tape in order

to make a mold and 40 µl of the hydrogel precursor solution with cells was added on top of the previous gel layer. Next, the UV mask was transferred at the top and then the cell-hydrogel solution were places under UV light for 5 mins. The UV masks (in different shapes including circle, square, and rectangles) were designed with AutoCAD software (AutoCAD 2010, Autodesk, San Rafael, CA) and printed on a transparent sheet. After the gel was formed, it was washed with PBS to remove any remain material. Then the two-layer micropatterned gel was transferred to an ultra-low attachment tissue culture plates and incubated in CSC medium for 9 days under 5% CO_2 at 37°C.

3.7. Fluorescent Imaging

First samples (gel pieces) were removed from CSC medium and washed with PBS two times. Then samples were fixed with 4% formaldehyde for 24 hours. Next, samples were washed with PBS three times and then cells were permeabilized with PBS having 0.1% Triton X-100 for 5 minutes. Samples again washed with PBS three times and then incubated with Alexa 488 phalloidin (1:200 dilution) for 25 mins to stain cell s' nucleus. Afterwards, samples were washed with PBS six times; each time for 10 mins. Next, cells were incubated with DAPI (1:5000 dilution) to stain actin filaments of the cells 'cytoskeleton. Then samples were washed with PBS six times, each time for 10 mins. Prepared samples were imaged with a Nikon Eclipse Ti-e inverted fluorescent microscope.

3.8. Determination of Tumorsphere Number and Size

Fluorescent imaged were derived in to small sections and sphere size and density was measured by using Image J software.

3.9. DNA Extraction from PEGDA Hydrogel and Cell Number Measurement

In order to extract the DNA from cells encapsulated in PEGDA hydrogel, the hydrogel needs to be degraded or destroyed. In that regard, first hydrogel samples were submerged within liquid nitrogen. The gel samples were then pulverized using a mortar and pestle until resembling a fine powder. The samples must be well pulverized and liquid nitrogen was intermittently poured on the samples to keep them cold. Following pulverization, the samples were added to 0.1 mL of lysis buffer (0.2% triton+10 mM Tris) consists of inside a 2 mL glass homogenizer and were homogenized with lysis buffer until well incorporated. The samples were then transferred to a 1.5 mL centrifuge tube and centrifuged for 5 minutes at 12000 RPM in order to remove gel debris. Up to 100 µl of supernatant was transferred to a sterile centrifuge tube. To measure the cell number, the double stranded DNA (dsDNA) content using a Quant-it PicoGreen assay was performed. Regarding that, 100 µl of working solution was added to 100 µL of the cell lysate and incubated for 4 min at room temreture. The fluorescence of the solution was measured with a plate reader (Synergy HT, Bio-Tek, Winooski, VT) at emission and excitation wavelength of 485 and 528 nm, respectively. Flourescent intensities were linked to cell numbers using a calibration curve made with cells of known concentration ranging from zero to 10 million cells/ml.

3.10. RNA Extraction from PEGDA Hydrogel and qRT-PCR

In order to extract the RNA from cells encapsulated in PEGDA, the hydrogel needs to be destroyed. This was done by first submerging hydrogel samples within liquid nitrogen. The gel samples were then pulverized using a mortar and pestle until resembling a fine powder. The samples must be well pulverized and liquid nitrogen was intermittently poured on the samples to keep them cold. Following pulverization, the samples were added to 1 mL of TRIzol® inside a 2 mL glass homogenizer. The sample was homogenized with TRIzol® until well incorporated. Sample was then transferred to a 1.5 mL centrifuge tube and centrifuged for 6 minutes at 9000 RPM in order to remove gel debris. Up to 1 mL of supernatant was transferred to a sterile centrifuge tube. Up to 200 μL of chloroform was added to the sample (1:5 chloroform:sample) and shaken for 30 seconds. The sample was put on ice for 3 minutes and then centrifuged for 10 minutes at 13200 rpm. Following centrifugation, the supernatant was transferred to a new, sterile centrifuge tube. An equal amount of ethanol (95-100%) was added to the supernatant and pipetted to mix. The sample RNA solution was then transferred to a pink QIAGEN RNeasy spin column, centrifuged for 30 seconds at 9000 rpm, and flow through discarded. Next, 700 μL of RW1 Buffer (QIAGEN) was added to the column, the column was then centrifuged, and flow through discarded. The sample was then washed twice by adding 500 μL of RPE Buffer (QIAGEN, diluted according to the bottle), centrifuging, discarding the flow through and then repeating a second time. After washing the sample twice, the collection tube was then replaced with a sterile 1.5 mL centrifuge tube. In order to dissolve the RNA, 30-50 μL of RNAse free water was added

to the sample and allowed to site for 1 minute. The sample was then centrifuged for 1 minute at the highest rpm. Flow through, containing the RNA, was then stored at -80 °C for further use in qRT-PCR.

Following TRIzol RNA extraction as described above, by using Nano drop, RNA concentration of samples was equalized. Purified RNA with equal amount for all samples was reverse transcribed to cDNA by using SuperScript II Reverse Transcriptase (Invitrogen) with random primers. The cDNA was amplified using quantitative real time polymerase chain reaction (qRT-PCR) with the appropriate gene specific primers. The differential expression of CSC markers: CD44, ABCG2, and TGFβ genes with SYBR green RealMasterMix (Eppendorf, Hamburg, Germany) using Bio-Rad iCycler PCR system (Bio-Rad, Hercules, CA) was measred. GAPDH gene was used as an internal control. The gene specific primer sequences that were used are as following. Human GAPDH: forward 5'-GAGTCAACGGATTTG GTCGT-3', reverse 5'-TTGATTTTGGAGGGATCTCG-3',

human CD44: forward 5'-GGCTTTCAATAGCACCTTGC-3', reverse 5'-ACACCCCTGTGTTGTTTGCT-3', human ABCG2: forward 5'-CACCTTATTGGCCTCAGGAA-3', reverse 5' CCTGCTTGGAAGGCTCTATG-3', human TGF-β: forward 5'-CCGGAGGTGATTTCCATCTA-3', reverse 5'-CTCCATTGCTGAGACGTCAA-3'.

3.11. Statistical Analysis

Data were reported as means ± standard deviation. A two-tailed Student's t-test was used to determine significant differences between groups. A value of $p<0.05$ was considered statistically significant.

Conclusion

We have used a 3D PEGDA culture model and demonstrated that cell binding peptides such as RGD, RYD, IKLLI, LIGRKK, VAPG, WQPPRARI, and SPPRRARV cross-linked to the hydrogel can reduce the ability of breast CSCs to form tumorspheres in PEGDA. Among peptides RGD, RYD and WQPPRARI were the most active peptides in reducing the sphere formation of breast cancer stem cells. It has also been shown that, breast cancer stem cells are more resistance to acidic pH compared to breast cancer cells and normal breast epithelial cells. Also, breast cancer stem cells formed spherical tumors in micropatterned PEGDA, regardless of the shape of the micropatterns and tried to have minimum surface area for a given volume

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