ABSTRACT

Title of Dissertation: ON CHIP ISOLATION AND ENRICHMENT OF TUMOR INITIATING CELLS

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We report for the first time a microdevice that enables the selective enrichment and culture of breast cancer stem cells using the principles of mammosphere culture. For nearly a decade, researchers have identified breast cancer stem cells within heterogeneous populations of cells by utilizing low-attachment serum-free culture conditions, which lead to the formation of spheroidal colonies (mammospheres) that are enriched for cancer stem cells. While this assay has proven to be useful for identifying cancer stem cells from a bulk population, ultimately its utility is limited by difficulties in combining the mammosphere technique with other useful cellular and molecular analyses. However, integrating the mammosphere technique into a microsystem can enable it to be combined directly with a number of

functions, including cell sorting and analysis, as well as popular molecular assays. In this work, we demonstrate mammosphere culture within a polydimethylsiloxane (PDMS) microsystem. We first prove that hydrophobic PDMS surfaces are as effective as commercial low-attachment plates at selectively promoting the formation of mammospheres. We then demonstrate the culture of mammospheres as large as 0.25 mm within a PDMS microsystem. Finally, we verify that reagents can be delivered to the cell culture wells exclusively by diffusion-based transport, which is necessary because the cells are unattached. This microsystem component can be integrated with other microfluidic functions, such as cell separation, sorting, and recovery, as well as molecular assays, to enable new discoveries in the biology of cancer stem cells that are not possible today.

ON CHIP ISOLATION AND ENRICHMENT OF

TUMOR INITIATING CELLS

by

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DEDICATION

To My Parents.

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Chapter 1

Introduction

Despite all the scientific advances in cancer research, cancer continues to be a lethal disease due to the disease relapse in the primary site or its metastasis to a distant organ. According to a data collected in 2007 approximately 1.4 million men and women in only US are diagnosed with cancer where more than one third of this population loses the battle to the disease [1]. The same record estimates that about 1 in 3 people born today will be diagnosed with cancer in their lifetime. According to the same survey, the 5 year survival rate for these patients is 65.3 % compared to those without cancer.

Cancer, which is a genetic disease, is characterized by uncontrolled cell division and growth with a high potential for metastasis in most diagnosed cases. In other words, cellular aberrations caused by genetic or epigenetic alterations, accumulate to form tumors in solid organs such as lung, brain or breast, or cause malignancies in tissues such as blood or lymph [2,3]. One of the difficulties in curing cancer comes from the genetic heterogeneity in the tumor cell population where different genetic

profiles can be identified even from tissue samples taken from the same specimen. The tumor heterogeneity was also characterized by the striking variability in features such as cell size, morphology, antigen expression, membrane composition as well as behaviors such as proliferation rate, cell-cell interaction, metastatic potential and sensitivity to treatments such as chemo- and radiotherapy [4, 5].

In studying genetic pathways that lead to the establishment and maintenance of tumors, two separate models, "clonal evolution" and "cancer stem cell" hypothesis were separately proposed to support the heterogeneity of tumor cell population. These two models, which shed light on different cell events that might be responsible for driving the growth and prospect of a tumor, can have different therapeutic implications. In clonal evolution model, which was first proposed by Nowell in 1976, it was hypothesized that genetic mutations that occur throughout the lifetime of a tumor can lead any cancer cell to become invasive and therapeutic-resistant [6–12]. On the other hand is the cancer stem cell model, which was initially proposed by Virchow over a century ago [13]. This model suggests that only a small subpopulation of cells within the tumor express stem cell like properties and can drive tumor initiation, progression and recurrence. While each model explains different cellular events differently, they both agree on the assumption that the existence of a cell population with stem cell characteristics is the main reason for the inefficiency of current treatments. Although there is a strong body of evidence in support of each model, our focus in this work will be solely on the cancer stem cell model and its implications in breast cancer research.

1.1 Cancer Stem Cell Hypothesis

A common characteristic to all tumors and malignancies is the aberrant cell division, which results in an uncontrolled tumor growth. Rudolf Virchow was the first to propose in 1855 that tumors are similar to other tissues in that they arise from the activation of a small subpopulation of stem cells that reside in the tissue [13]. Stem cells, which have a high capacity for self-renewal, give rise to multiple tissue lineages upon asymmetrical cell division in appropriate conditions. In this process, where normal stem cells behave according to a well conserved and predictable rule, the size of the stem cell compartment of the particular tissue remains intact. In other words, in each cycle of division, one stem cell and one progenitor or a more differentiated cell is generated. The cancer stem cell hypothesis, on the other hand suggests that tumors arise from an uncontrolled cell division that is caused by several mutations. These mutations occur either within the stem cell pool, or in the more mature and differentiated cell populations that have gained self-renewal capacity. In this unstable process, the frequency of stem cells is no longer predictable.

The first solid evidence in support of the cancer stem cell hypothesis came in 1994, more than one century after it was first proposed. In that observation, Lapidot and his research group isolated cancer stem cells (CSCs) from an acute myeloid leukemia (AML) using cell surface markers [14]. In a later experiment, it was shown that these cells can initiate a tumor when injected at very low numbers into immunocompromised mice. Subsequent analysis confirmed that these cell subpopulations express characteristics common to stem cells [15]. In this observation

the possible role of mature, differentiated blood cells in initiation and progress of the disease was completely excluded.

Cancer cells with stem like properties have since been identified in breast [16], colon [17], brain [18], pancreas [19,20], and prostate [21,22] tumors, using cell surface markers. It should be noted that while the tumorigenicity of the other subset of cancer cells, i.e. the non-stem ones, are not completely ruled out, they are less likely to initiate a tumor as was shown in a report by Clarke, *et al.* in 2003 [16]. In this observation, which was reported on breast tumors, it was shown that while as low as few hundred cells with stem like properties are enough to form a tumor upon injection into immunocompromised mice, it probably takes tens of thousands or millions of cells in the other category to regenerate a tumor.

It is noteworthy to mention that the cancer stem cell hypothesis is mainly defined based on the characteristic of that fraction of cancer cells that are capable of initiating a new tumor and recapitulating the heterogeneous properties of the original tissue. While controversies still exist among cancer biologists in defining the source of cancer stem cells, it was decided in 2006 by the American Association of Cancer Research (AACR), that "cancer stem cell" is the most scientifically accurate label to refer to a malignant cell that fulfills the classical stem cell criteria [23]. Based on this definition, the term "cancer stem cell" refers to a tumor cell that has a high capacity for self-renewal and can give rise to all cell lineages within the tumor. It should be noted that this definition does not infer that cancer stem cells are necessarily originated from stem cells in the tissue, as they can as well be the offspring of progenitor and/or differentiated cells that have gained self-renewal

capability as a result of mutations. Some groups thus refer to this cell subpopulation as "cancer initiating cells" to avoid confusions around the origin of these tumorigenic cells.

1.2 Pathways that Give Rise to Cancer Stem Cells

While most researchers agree on the stem cell-like properties of tumorigenic cancer cells, the pathways that lead to the formation of mutant stem cells is still a subject of debate. Understanding these pathways has different therapeutic implications and can be used as a guide to target the most dangerous tumor cell subpopulation. While different malignancies might arise from different subsets of cells, there are in general three hypotheses about the cellular precursors of cancer cells.

The first hypothesis suggests that cancer cells are driven by stem cells in the tissue wherein the tumor is formed. This theory is widely supported by the evidence that tumors are comprised of a heterogeneous population of stem as well as differentiated cells similar to those in the tissue where the malignancy is raised. Additionally, the self-renewal ability of stem cells gives them a lifespan that is long enough (as compared to more mature, differentiated cells) to acquire multiple mutations necessary for the formation and metastasis of the tumor [24]. Some of these mutations include alterations in the signaling pathways such as *Notch*, *Wnt* and *Hedgehog* pathways [25–27]. It should be noted however that most observations on the stem cell origin of CSCs comes from studies on leukemia-inducing cells [28].

More investigations on the characteristics of tumor initiating cells in solid tumors are therefore necessary before making conclusive statements on the origin of CSCs.

In the second hypothesis, progenitor cells are held responsible for the initiation and growth of the tumors, instead of stem cells. Progenitor or precursor cells are the intermediate cells that originate from a stem cell and can ultimately differentiate to a more mature tissue cell. These cells, which exist in higher numbers compared to stem cells, have partial self-renewal capacity[1], which makes them interesting subjects in studying CSCs [29, 30].

The third hypothesis on the other hand suggests the possibility that cancer stem cells arise from differentiated cells. This hypothesis, which is more in support of the cloning evolution model, is based on the assumption that a subpopulation of mature, differentiated cells undergoes oncogenic mutations and de-differentiates into a stem-like cell population. This model though does not discuss the possibility that all tumor cells could have tumorigenic potential. However, it suggests that based on the laws of probability, from a large pool of differentiated cells, some cells can actually acquire the genetic mutations that are necessary for de-differentiation. In support of this hypothesis, researchers have recently shown that by reprogramming human adult somatic cells, these cells can display pluripotent properties, a characteristic common to stem cells [31, 32].

While each model is useful in describing different tumorigenic events, the one thing all three have in common is that tumors are initiated and derived by a subpopulation of cells that expresses stem cell properties and possess a high potential

[1]Progenitor cells have less self-renewal capacity as compared to stem cells.

for self-renewal. Whether this characteristic is an inherent property of the tumor initiating cells or is acquired through several mutations is an open question to be answered.

1.3 Cancer Stem Cells in Relapse and Metastasis

Metastasis which is referred to the recurrence of the disease in an organ distant from which the tumor is first diagnosed, is believed to be the result of cancer stem cells dissemination through the patients' blood or lymph nodes. In this process, loss of E-cadherin, which is an adherent molecule at cell-cell junction will cause some cancer stem cells to break loose from the surrounding epithelial tissue and acquire mesenchymal or migratory characteristic through accumulation of genetic mutations [33]. It is recognized that in this transition from epithelial to mesenchymal behavior (referred to as EMT[2]), an important factor is the deregulation of the *Wnt* signaling pathway [36], a signaling behavior that in normal conditions plays a pivotal role in stem cell formation, tissue development and maintance of cell homeostasis [37–44]. Aberration in *Wnt* signaling activity can therefore cause some cancer stem cells to detach from the tumor tissue and get into the bloodstream where they remain dormant until further mutations or an environmental stimulus reactivates their tumorigenic machinery. It is often believed that tissue microenvironments or niches similar to the cells' original environment can attract these disseminated cancer

[2]Epithelial to mesenchymal transition (EMT), is a crucial component in early developmental processes, and is also activated in adult tissues in occasions of wound healing and tissue regenerating. Activation of EMT in tumors, which occurs as a result of several mutations caused by extracellular or microenvironmental stimuli has been additionally suggested as a trigger in generation of cancer stem cells from a differentiated cell population [34, 35].

stem cells and stimulate them into forming a new tumor. Based on observations, these cells can then recapitulate the stem as well as differentiated phenotypes of their primary tumors in the corresponding metastases [45, 46].

The question here would be whether all cancer stem cells are capable of metastasis or there are two subsets of CSCs where one leads to the relapse and the other to metastatic events. Some theories suggest that a tumor is comprised of a heterogeneous population of cancer stem cells, stationary and migratory, which are defined based on their expression patterns [47]. While both classes of CSCs reside in the epithelial tissue of their microenvironment or niche, stationary cancer stem cells are embedded in deeper sites. This subset therefore cannot disseminate and is rather active in the progression of the primary tumor. The existence of stationary CSCs also justifies partly, the relapse that occurs after the cancer is thought to be treated. The migratory or metastatic cancer stem cells on the other hand refer to that fraction of CSCs that predominantly reside at the interface of the tumor and its host organ [48, 49]. It should be noted that while a single mutation can cause normal stem cells (also residing in the niche [50]) to become stationary cancer stem cells, migratory stem cells are the result of additional mutations [51] and/or unusual microenvironmental triggers [41, 52–54]. In colon cancer for instance, a transformation from benign adenoma to malignant carcinoma happens when CSCs try to cross the thin muscle layer that separates mucosa from submucosa [47]. This transmigration might in turn stimulate some aberrations in the environmental signaling pathways that would consequently lead to a malignant transformation of cells. In a different experiment, it was shown that the frequency of cancer stem cells at the tumor-host

interface with high EMT expression levels and/or high degrees of aberrations in the *Wnt* signaling pathways are related to malignant progression and a poor prognosis in patients with rectal cancer [48,49]. This phenotype was also detectable in tumors of breast [55–58], and pancreas [59], the intestinal type of gastric cancer [60] and squamous cell carcinomas [61].

The migratory cancer stem cells in circulation remain quiescent until they find a microenvironment that reflects that of the parent tumor. It should be noted however that other environmental factors such as oxygen gradients and other chemoattractants that are secreted at the niche play an important role in trafficking cells toward specific organs [62–65]. In the metastatic organ, the genetic instability of CSCs then plays an active role in the survival and maintenance of cancer stem cells in the new environment. In other words, the tumor stem cells' plasticity makes CSCs and their progenies more adaptable to the growth and signaling molecules of the new environment [66].

Another interesting characteristic of metastatic tumors that is explained in the of stationary versus migratory cancer stem cell model is the morphology and differentiation heterogeneity of metastatic lesions that recapitulates that of the primary tumor [47, 55–61]. As mentioned earlier, while some cancer stem cells at the tumor-host interface manage to leave their microenvironment and metastasize to a distant organ, some others are left behind to further participate in the progression of the primary tumor. Since these two subsets originate from the same adult tissue stem cells, they inherit identical genetic programs. In other words, according to this model, the cancer stem cells that are involved in the development of both primary

tumor and their metastases, originate from the same tumor and consequently share same genetic profiles. This in turn explains the heterogeneity of cell populations in the metastases that mirrors that of the primary tumor. It should be noted however that metastatic cells are capable of evolving into a more mutant stem cell as a result of communication with the new environment.

1.4 Implications in Therapy

The cancer stem cell hypothesis was also proposed to explain the inefficiency of current treatments in curing the disease. This is due to the fact that these treatments are mainly focused on targeting differentiating cells (especially in the primary tumor) and have therefore failed to address metastatic events.

Since metastasis accounts for about 90% of lethality in cancer patients [67], studying the cellular and molecular mechanism that underlies cancer metastasis can lead to significant implications in therapy. In a previous section, we briefly described a hypothetical model in which tissue stem cells acquire tumorigenic characteristics as a result of mutations believed to be caused by microenvironments or niches. These mutant stem cells can then orchestrate major cancer events such as relapse and metastasis. If the model proves to be valid, targeting cancer stem cells in general and metastatic CSCs in particular instead of differentiated cells (which are the target of current treatments) can lead to significant improvement in cancer therapies.

In designing new treatments that would include both stationary and migratory stem cells, one has to take into account the distinctive characteristics of CSCs that

has made them resistant to current treatments. One inherent property of cancer stem cells for instance is their high capability in pumping chemotherapeutic drugs out of the cell through a family of ABC drug transporters. New drugs have therefore been designed to target these transporters, inactivate their pumping machinery and make CSCs more susceptible to chemotherapeutic agents [68]. Cancer stem cells on the other hand respond to radiotherapy by stimulating their DNA repair activity, making themselves more resistant to the treatment [69]. In this scenario, a strategic approach might be to combine radiation with drugs that impair the DNA repair mechanism. Cancer stem cells (as mentioned earlier in this document) also have a high capacity for self-renewal. This property, which accounts for the growth of the primary tumor and its metastasis, can have new implications in treatments. New therapies have looked into targeting signaling pathways such as Wnt/β catenin that are critical in the process of stem cell self-renewal [70]. Another approach, which has proved to be more successful, attempts to indirectly eliminate the self-renewal ability of CSCs (especially at the site of the primary tumor) by forcing stem cells to differentiate [66].

While targeting migratory cancer stem cells can be very challenging, a prospective approach can aim at signaling pathways such as Wnt and genes that are associated with EMT. Another useful strategy might be using drugs that render migratory stem cells dormant by targeting their proliferation activity (both self-renewal and differentiation) at the site of the secondary tumor [66]. Therapies based on this idea are however, far from materialization as dormancy models need first to be validated and characterized.

While recent studies focus on new treatments that target cancer stem cells instead of more mature and differentiated cells, there is a major concern that these new therapies might not be able to differentiate between a normal stem cell and a mutant cancerous one. This concern arises from the fact that many molecular mechanisms that are involved in the machinery of normal stem cells, also play important roles in the survival of cancer stem cells. Another challenge in applying treatments that mainly target cancer stem cells is measuring the efficacy of the treatment as CSCs constitute only a very small portion of the tumor. In other words, unlike in current treatments that target differentiated cells that populate the bulk of the tumor, the efficacy of new treatments targeted at minor CSC population, cannot be measured by the degree of tumor shrinkage. In this regard, new treatments should look instead for the level of reoccurrence as a measure of effectiveness.

While several characteristics of cancer stem cells and their relationship with relapse and metastasis still needs to be validated before any new treatment can be taken into effect, some researchers have focused on isolating these rare cells in in-vitro assays and using them as a model for further analysis. This dissertation, which is in concert with current in vitro techniques, will focus on the development of a microsystem for the isolation and enrichment of tumor initiating cells. In the rest of this document I will first discuss CSCs in the context of Breast Cancer (Chapter 2) and will then focus on several techniques in isolating CSCs from patient samples or propagated cell lines (Chapter 3). The main emphasis in Chapter 3 (and following chapters) is on mammosphere assay, which is a 3-dimensional in vitro model of breast cancer. In Chapter 4, I will first discuss the important factors that are involved in

cell-substrate interactions and their significance in driving spheroid formation as in mammospheres. I will then move on to describe different approaches I took in developing a homemade mammosphere assay and compare the results from each assay. In Chapter 5 I will discuss how the homemade assay was translated into a microsystem, the difficulties of a long term culture of mammospheres and the strategies that I developed to overcome those challenges. I will finally conclude by demonstrating a COMSOL simulation on the fluidics part of the micro-system.

Chapter 2

Breast Cancer Stem Cells: Biological and Medical Insights

Breast cancer is the most prevalent malignancy among women in western countries and the second leading cause of death in women diagnosed with cancer. Every year, breast cancer accounts for more than 40,000 deaths in the United States alone as reported by the National Breast Cancer Foundation [71]. While early detection of the disease can lead to a better prognosis, the survival rate among those diagnosed with advanced, metastatic breast cancer is still significantly low. This is mainly due to a lack of systematic observations pertaining to cellular and molecular mechanism of events such as relapse and metastasis that are exclusively involved in the progression of tumors. In this regard, as discussed in the previous chapter, the cancer stem cell hypothesis was proposed to bring new insights into molecular pathways that lead to the recurrence of the disease. This hypothesis brings attention to a minority subpopulation of tumor cells that have the potential to initiate and develop a tumor, even after the disease is believed to be cured. This model argues that a small group of tumor cells that have retained or acquired stem cell phenotypes as a result of genetic or epigenetic mutations is invulnerable to stressful therapeutic

conditions (such as chemo- and radiation-therapy) and can regenerate the primary tumor or metastasis to a foreign organ. While the first solid evidence in support of the cancer stem cell hypothesis came in 1994 where CSCs were isolated from acute myeloid leukemia [14], breast cancer stem cells were the first to be identified in a solid tumor [16]. In this chapter, breast cancer stem cells, their characteristics and their implications in therapy will be discussed along with resources available for their isolation. Specific markers of breast cancer stem cells will be identified in the following chapters where current isolation techniques based on the detection of surface markers as well as label free detection methods will be discussed.

2.1 Signaling Pathways Involved in the Regulation of Breast Cancer Stem Cell Function

Several findings have demonstrated the involvement of signaling pathways of epithelial origin in stem cell activities such as self-renewal, proliferation, differentiation and survival. These pathways include *Wnt*, *Notch*, *Nanog*, *Oct-4*, *hedgehog* and BMI-1 whose alterations can lead to a mutant stem cell or cancer stem cell. In breast cancer, signaling pathways of integrin [72], insulin-like growth factor-1 [73], ER and progesterone receptors (PgR) [74], epidermal growth factor (EGF)-like/EGF receptor (EGFR) and *HER2/Neu* [75], BRCA-1 [76], leukemia inhibitory factor [77], SDF1/CXCR4 [78], and interleukin-6 [79] are additionally involved in the functional activities of cancer stem cells of the mammary gland. Another mechanism, important in cancer metastasis, is the epithelial-mesenchymal transition (EMT) [34]. It

has been shown that let-7, a distinct family of miRNA is involved in EMT through the regulation of stemness related pathways and silencing of multiple genes [80]. Finally, it has been demonstrated that molecular pathways such as telomerase and antiapoptotic proteins (survivin and Bcl-2) and pro-angiogenic factors that are involved in the maintenance and survival of cells are activated and/or overexpressed in breast cancer stem cells [81]. In what follows we will discuss in more detail some of these pathways that have been used in clinical and experimental models.

2.1.1 Wnt Signaling

Wnt signaling pathway, which is a key component in embryonic development has been found in certain human tumors and malignancies including leukemia and colorectal cancer, which occurs as a result of genetic mutations in components such as tumor suppressor APC or in Axin and β-catenin [82]. While deregulation of this signaling pathway was initially detected in the stem/progenitor portion of breast tumors in mice models, its relevance to human breast cancer has remained unclear till recently. In a study conducted by Kumar, *et al.*, it was demonstrated that an increased level of MTA1 protein (metastasis associated protein 1) and MTA1s (a shorter variation of MTA1) can cause oncogenic alterations in certain types of human breast cancers [83]. These proteins indirectly activate the *Wnt* signaling pathway by reducing the level of Six3, a protein that is known to inhibit *Wnt1* signaling activity in normal circumstances. Another line of evidence indicates that MTA1s is also involved in deregulation of *Wnt* pathways by directly affecting ERK mediated

GSK3β pathways, which are *Wnt* related. These observations suggest that MTA1 and its variations can be used as potential targets in therapeutic applications in breast tumors.

2.1.2 Oct-4 Signaling

Oct-4, which is a transcriptional factor that has an essential role in self-renewal of embryonic stem cells, is also involved in regulation of pluripotent stem cells in tumors [84]. In is shown that in breast cancer, Oct-4 is significantly higher in the tumor lesion than its surrounding tissues. Using $CD44^+/CD24^-$ and non- $CD44^+/CD24^-$ breast cancer cells, it has been demonstrated that *Oct-4* expression is much higher in the $CD44^+/CD24^-$ subtype that is enriched in cancer stem cells [85]. An additional line of evidence indicates that the expression level of *Oct-4* is highly related to the prognostic rate in post-operational cases. These observations therefore suggest that targeting this molecular pathway can lead to better therapeutic outcomes.

2.1.3 Nanog Signaling

Similar to Oct-4, Nanog is highly involved in the maintenance of pluripotent characteristic of embryonic stem cells and plays a pivotal role in the self-renewal of stem cells. Several studies have shown that this transcriptional factor might also be active in breast tumors and can therefore be used as a marker to identify and target undifferentiated cancer stem cells [86]. In fact, in a recent report by Grudzien, *et al.*, Nanog was demonstrated to be preferentially overexpressed in tumorspheres formed

from several breast cancer cell lines and primary specimen as compared to the cells from the bulk of the tumor [87]. The authors further demonstrate that the activity of Nanog is elevated in ALDH positive cells but not in ALDH negative ones. The upregulation of Nanog in tumorspheres and $ALDH^+$ cells which are both enriched in cancer stem cells suggests that this signaling pathway can be used as a therapeutic target in breast tumors.

2.1.4 Hedgehog Signaling

The *Hedgehog* signaling pathway is an essential component of embryonic stem cell differentiation. Activation of *Hedgehog* cascade in adult tissues has been implicated in the development of several cancers, including brain, lung, prostate, skin, and breast tumors. Additionally, this signaling pathway has been demonstrated to play a crucial role in angiogenesis and metastasis [88]. It also controls the tumor progression by upregulating angiogenetic factors [89] and anti-apoptotic genes by downregulating apoptotic genes [90]. In breast cancer, sonic *Hedgehog*, which is one of the three components of the *Hedgehog* family, is involved in initiation and development of inflammatory breast cancer, which is an aggressive form of a primary breast tumor [91]. The *Hedgehog* signaling pathway can also trigger molecular activity of proteins such as BMI-1 to suppress genes that are involved in the senescence and death of human mammary epithelial cells, rendering them invulnerable to death inducing stimuli. BMI-1 is also overexpressed in mammosphere cultures of breast cancers that are enriched in cancer stem cells [92]. The BMI-1-based genetic profile

has proven to be a powerful therapy-independent predictor of recurrence, distant metastasis and death in 11 epithelial and non-epithelial cancers [93]. These observations have therefore led several pharmaceutical companies to actively develop drugs that will selectively target hedgehog signaling pathway and its downstream molecules.

2.1.5 Notch Signaling

Notch and most of its ligands are transmembrane proteins that are involved in cell-cell communication and gene regulation activity in the process of cell differentiation and proliferation. In mammary gland, they play an important role in determining cells' fate during the developmental stages [94]. Its deregulation has been associated with many cancers including murine and human breast tumors [95–100]. For instance, *Notch 1* and *Notch 4* are involved in breast carcinoginesis in mice and show an increased growth potential in anchorage independent assays [97, 98]. Overexpression of *Notch 1* in patients with breast cancer has been associated with poor prognosis, while *Notch 4* has the opposite effect [97–100]. Since inhibiting *Notch* signaling has anti-proliferative effect, it has been a subject of study in developing anti-cancer drugs [101].

2.1.6 HER-2 Signaling

HER-2 or *Neu*, which is a member of EGFR (epidermal growth factor receptor) family, is a regulator of stem/progenitor cell population in normal and cancerous

mammary epithelial cells. Out of every 4 patient with breast cancer, about 1 case has indications of *HER-2* overexpression [102]. Amplification of this protein has been correlated with high tumorigenicity and a poor prognosis outcome [99]. In addition, using human breast cancer cell lines, overexpressed *HER-2* cell populations demonstrate a higher growth rate in non-adherent mammosphere cultures, and an increased ALDEFLUOR activity, and have a better success rate in regenerating tumors upon xenograft transplantation into non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice models [103].

2.2 Resources for the Characterization of Cancer Stem Cells

2.2.1 Breast Cancer Cell Lines

Cell lines have been extensively used for decades in cancer research as they have been proven to retain the molecular signatures of the parental tumor. Whether they can reconstitute the hierarchical organization of the tumor they were isolated from, however is still a subject of debate. In breast cancer, cancer stem cells have been identified in both murine and human cancer cell lines, using recognized stem cell-surface markers [104,105]. Both subpopulations were able to form spherical colonies in anchorage-independent cultures, displayed resistance to chemotherapeutic agents, and were more tumorigenic than the parental line.

Using cell lines has many advantages over patient samples as they are more accessible, require less manual steps in sample preparation, and have a higher yield

and reproducibility. More importantly, they inflict less pain on patients as using cell lines minimizes the need for blood or biopsy samples for basic investigations. While use of cell lines can accelerate studies that focus on the characterization of stem cells' regulatory pathways and markers that can facilitate better therapeutic targets, it does not offer an ultimate and perfect solution. This is mainly due to the fact that major key factors that are consistently involved in tumor development and progression such as environmental and inflammatory stimuli are absent in experimental observations that include cell lines instead of real samples. In other words the temporal and spatial plasticity, which is part of the dynamic nature of cancer cells, cannot be investigated using cell lines, which in turn might lead to inaccurate results.

2.2.2 Xenograft Models

While in vitro models have proved useful in studying the biology of cancer stem cells and their implications in therapeutic strategies, they fail to portray a complete picture of tumorigenic events. Identified cancer stem cells in vitro, are therefore subsequently validated in vivo using immunodeficient animals. While these models do not represent the exact tumor environment in human, the xenograft model of patients' sample seems the closest that one can get to a dynamic system for studying carcinogenesis in human patients. In some experimental studies of xenografts, it has been demonstrated that animal models do not naturally support the growth of a tumor (or a healthy gland) upon transplantation of human tissue fragments or dis-

sociated epithelial cells. This was specifically the case in xenograft transplantation of human normal or cancerous mammary cells where the mice model had to be first humanized by introducing normal fibroblasts into the clear fat pad of NOD/SCID mice, before any subsequent experiment [106]. Additionally, it has been demonstrated that in transplantation experiments, the site of injection plays an eminent role in the experimental outcome of xenografted models [107]. While animal models have in some cases offered a reasonable substitute to human physiological environments, it should be noted that in order to get the most accurate and reliable results, the choice of xenograft models should be as close as possible to the native human environment.

2.3 Breast Cancer Stem Cells in Response to Systematic Treatments

The cancer stem cell hypothesis, which was partly proposed to justify the inefficiency of current treatments, was recently validated in breast cancer stem cells. In this clinical observation conducted by Li and colleagues, it was demonstrated that the postchemo residues of tumor cells were enriched in $CD44^{+}/CD24^{-/low}$ stem cells and had higher mammosphere formation efficiency [108]. Another line of evidence in support of cancer stem cell hypothesis came from observations on *HER-2* positive breast cancer patients in which an inhibitory drug was tested against this signaling pathway, which is involved in self-renewal [75]. As was indicated in this clinical trial, the residual tumor cells did not show any increase in tumor cells with putative

stem cell features, suggesting that inhibition of specific signaling pathways involved in stem cell regulation can have significant therapeutic implications. On the same line of evidence, it was shown that therapeutic approaches targeting *Notch* signaling pathway can reduce self-renewal capacity of breast cancer stem cells and limit the mammosphere formation efficiency in non-adherent cultures derived from breast tumor samples or cell lines [103]. In another set of experiment, it was found that simultaneous targeting of several signaling pathways such as EGFR and *hedgehog* that are involved in self-renewal activity, can improve cytotoxicity of drugs in metastatic tumors [109, 110]. Other reports have shown similar results in breast tumors by inhibiting EGFR and *HER-2* signaling cascades [75, 100, 103]. As was mentioned previously, another treatment possibility in eradicating tumors is deactivating the self-renewal capacity of tumor stem cells by generating a forced differentiation. This approach, which was shown to be very effective in treating breast tumors in mice models [34], can have promising implications in curing human breast tumors.

While studying therapies that target breast cancer stem cells have been implicated in xenograft mice models, it should be noted that they are not completely defect free. For instance, serial transplantation experiments are highly time consuming where they require several months of observation. Additionally, large scale drug screening in several types of tumors such as breast have proven to be difficult as they have a low success rate of transplantation into immunodeficient mice. The significance of microenvironment that plays an eminent role in tumor initiation and progression is also sacrificed in mice models. On the other hand are the in vitro assays, which offer a rapid and quantitative approach but are highly protocol depen-

dent and lack a systematic outcome. One logical approach to overcome limitations associated which each method, will therefore be to combine in vitro and in vivo assays in validating new therapeutic strategies.

Chapter 3

Identification and Isolation of Breast Cancer Stem Cells

While several techniques have been proposed and utilized in identification of cancer stem cells, translating cancer stem cell research into clinical applications is strongly dependent on the thoroughness and accuracy of these characterization techniques and the reliability of markers used to study CSCs. In what follows, four widely used techniques will be discussed where our emphasis will be more on functional assays for the isolation and characterization of breast cancer stem cells.

3.1 Cell Surface Marker Assays

All cells, including normal and cancer stem cells, display a unique pattern of proteins on their surface membranes that can be used as an identifying signature. While different tissues and species express different cell surface markers, the markers described below have been extensively used in studying human breast cancer stem cells. Flow cytometry techniques are used in these studies to purify cells for CSCs based on their unique surface markers. To examine the tumor initiating capability

of cells sorted out for CSCs, these cells are later transplanted into a mammary fat pad of mice models. It should be noted however that, for successful cell transplantations, the microenvironment in xenografted models should closely resemble that of the cells' origin [111]. In other words, for these cells to survive and colonize in their new environment, the experimental mice models should be first humanized. In studying breast cancer stem cells, therefore normal fibroblasts are first introduced into the clear fat pad of non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice [106]. Later, epithelial cancer cells sorted out for certain cell-surface markers (as described below), are orthotopically injected into the clear humanized fat pad of mice models for further studies. It's noteworthy to mention that among a large variety of tumors transplantable into immunodeficient mice, breast cancers are the most difficult to establish [112], which might explain the lack of consistency in the results reported by different groups.

- **$CD44^+/CD24^{-/low}/lin^-$**

In a pioneering study by Al-Hajj and colleagues, it was demonstrated that breast cancer cells with a $CD44^+/CD24^{-/low}/lin^-$ phenotype[1] obtained from pleural effusions, express a high tumorigenic potential [16]. In that seminal work, it was shown that as few as 200 cells with a combined expression of cell surface markers $CD44^+/CD24^{-/low}/lin^-$, are adequate to initiate a tumor upon xenograft transplantation into the clear fat pad of NOD/SCID mice models whereas 20,000 cells

[1]To isolate $CD44^+/CD24^{-/low}/lin^-$ cell types, lin^+ cells (CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) had to be removed from the mixed cell population.

that did not display this phenotype had no tumorigenic potential. Several other groups have since reported the presence of $CD44^+/CD24^{-/low}/lin^-$ cells in the enriched breast cancer stem cells obtained from the primary breast tumor [113, 114], its metastases [78], and in the bone marrow specimens of patients with breast cancer [115]. Additionally, it has been demonstrated by several studies that chemotherapy and radiation therapy enrich for breast cancer stem cells by increasing the level of $CD44^+/CD24^{-/low}/lin^-$ as was measured in cases with administered neoadjuvant therapies [104, 116].

While $CD44^+/CD24^{-/low}/lin^-$ is a suitable precursor for identification of cancer stem cells, it should be noted that not all cancer cells with this phenotype are capable of forming a tumor. The work by Dr. Max Wicha's group has shown that only a subset of these cells that express Aldehyde dehydrogenase enzymatic activity (as will be discussed in subsequent sections) has the potential to regenerate a heterogeneous tumor in xenografted models [81, 107]. In that study, it was demonstrated that as few as 20 cells with a combined expression of $CD44^+/CD24^{-/low}/lin^-$ and ALDEFLUOR activity (which is an enzymatic activity identified in some tissue stem/progenitor cells as well as CSCs from some tumor samples) are adequate to initiate a tumor, while none of the cells in the other subset that lacks ALDEFLUOR activity could grow a tumor in mice models. One can therefore conclude that the expression of $CD44^+/CD24^{-/low}/lin^-$ does not by itself define a breast cancer stem cell and should rather be combined with other biomarkers or enzymatic activities.

- **ITGA6/α6-integrin**

In a study conducted by Cariati, *et al.*, it was shown that using the MCF7 luminal breast cell line, a subpopulation of cells with an overexpression of α6-integrin is highly capable of forming tumor-spheres in anchorage-independent assays (a characteristic of stem cells) [117]. This subpopulation shows more resistance to pro-apoptotic agents and has a higher tumorigenic potential as compared to the rest of the cell line. In addition to α6-integrin, it was shown that other cell-surface markers such as ITGA6 play an important role in tumorigenicity of cancer cell lines. This was exclusively demonstrated by inhibition of α6-integrin/ITGA6 in the MCF7 cell line, which caused the tumor-sphere derived cells to lose their colony forming ability in anchorage-independent assays, and a weakened tumorigenicity upon transplantation.

It should be noted that, unlike stem cell populations of mouse mammary glands, in human mammary glands the markers are scarce and the assays are more difficult to standardize. Furthermore, the cell-surface markers used to isolate stem cell populations in mice models are rarely valid in humans. This in turn has made the field of breast cancer research more challenging and its clinical implications less successful, due to a lack of human experimental models. Other techniques in addition to cell-surface markers expression, however, have been proposed and widely used to isolate human breast cancer stem cells. These techniques, which are described in more detail in the following sections, exploit enzymatic activity and functional characteristics specific to stem cells.

3.2 ALDEFLUOR Assay

ALDEFLUOR assay is based on enzymatic activity of aldehyde dehydrogenase 1 (ALDH1). While this enzymatic marker has proven to be useful in identifying several types of murine and human hematopoietic, neural and mammary stem/progenitor cells [107, 118–121] as well as cancer stem cells in patients with breast, myeloma, and leukemia cancer [107,121,122], it is not a universal marker for stemness [123]. In breast cancer, ALDEFLUOR positive cells sorted by flow cytometry or immunohistochemistry (that measures the enzymatic activity at cytoplasmic subcellular fraction), are highly detected in basal and *HER2* positive subtypes and not in other mammary cells [124]. It should be noted that while ALDEFLUOR positive cells in general have a higher capacity to engraft in vivo upon transplantation in NOD/SCID mice, they display a heterogeneous tumorigenic characteristic. In breast cancer cell lines for instance, ALDEFLUOR positive cells within the $CD44^+/CD24^-$ and $CD44^+/CD133^+$ populations express the highest tumorigenic and metastatic potential [125]. Based on these observations, one can therefore infer that identification of highly tumorigenic cancer stem cells is strongly dependent on the isolation techniques. This in turn implies that a more accurate approach in isolation of CSCs with the highest tumorigenicity is to utilize multiple CSC markers for identification. Combining these isolation techniques with functional assays such as xenograft transplantation in immunodeficient mice or anchorage independent assays can lead to a more reliable result.

3.3 Side Population Technique

The Side population (SP) technique has been used for many years to isolate normal stem cells as well as cancer stem cells from different tissues and species [126–129]. This technique is based on the ability of cells with stem like properties to exclude vital dyes such as Hoechst 33342 or Rhodamin 123 due to high expression of transmembrane transporters, such as ABCG2 (ATP-binding cassette protein)/ BCRP1 (breast cancer resistance protein 1). In normal breast for instance, cells that remain negative for these dyes do not express luminal, myoepithelial or estrogen receptor markers; an indication of a stem/progenitor phenotype [130]. Using the side population technique as a potential approach in isolating stem cells was further validated in work done by Dontu, *et al.* using uncultured mammary cells in parallel to mammospheres (which are enriched in stem cells). In that study, it was shown that while the side population fraction of uncultured cells constitute only 1% of the cells, the SP fraction in mammospheres is as high as 27% [131]. On the other hand, SP cells isolated from the MCF7 breast cancer cell line was shown to represent 2% of the entire population. This fraction was further demonstrated by xenograft transplantations into NOD/SCID mice to be highly tumorigenic and capable of reconstituting the initial heterogeneity of the cell line [132]. In another report, it was shown that side population cancer cells are additionally highly drug resistant and might be enriched in CSCs [133]. It should be mentioned that while the side population technique might be a useful approach in isolating cancer stem cells, the functional studies of cells stained with Hoechst is highly limited by the

toxicity of this dye. For this reason and the controversies around proper identification of the side population, this technique is no longer the preferred approach for stem cell studies.

3.4 Mammosphere Assay

Another characteristic specific to normal as well as cancer stem/progenitor cells is their capability to form 3-dimensional spherical structures in selective culture conditions. In these conditions, single cell suspensions obtained from a human or animal model or alternatively from propagated cell lines are cultured onto a low-attachment surface in a serum free medium that is supplemented with growth factors. In this technique, which takes advantage of the anchorage independent characteristic of stem cells, cells with stem like properties are isolated and enriched in the form of spheroidal suspensions. In other words, unlike differentiated cells that require a solid substrate for survival, stem/progenitor cells are independent of their substratum and can proliferate to form undifferentiated cell clusters that are enriched in stem cells [131].

The first demonstration of the spheroidal culture technique was in studying the self-renewal capacity of neural stem cells [134, 135]. In those studies that were implemented about two decades ago, the suspension culture of neuronal cells resulted in the selective formation of floating spherical colonies, referred to as neurospheres. These neurospheres consisted of 4% - 20% stem cells which demonstrate multipotent characteristics. Later, Singh, *et al.* and Hemmati, *et al.*, utilized sim-

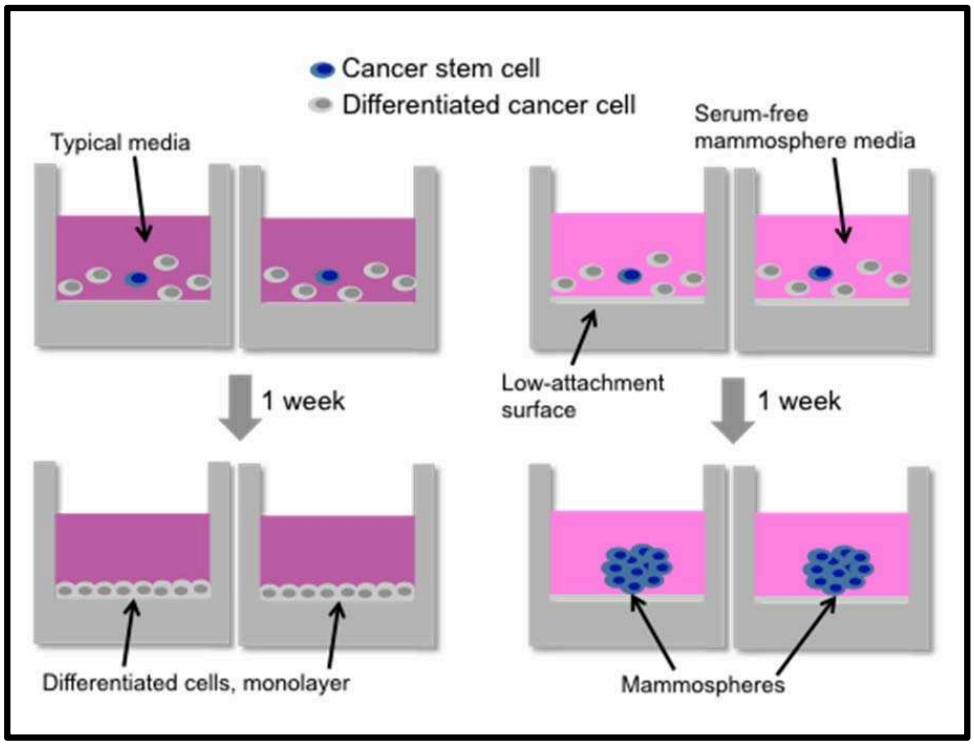

Figure 3.1: In conventional cell culture (left), cells are cultured in serum-rich media within wells or flasks that have a bottom surface that is optimized for cell attachment. Cells attach and form a monolayer. In mammosphere culture (right), cells are cultured in serum-free mammosphere media on low-attachment surfaces. Under these conditions, differentiated cells do not survive, but tumor-initiating cancer stem cells survive and proliferate to form floating colonies enriched in stem cells.

ilar culture conditions to form neurospheres from samples taken from solid brain tumors, demonstrating that the neurosphere technique may also be applicable to cancer stem cells [136, 137]. This technology was later adopted for human breast epithelial cells by Dontu, *et al.* [131] where the suspension culture of human mammary epithelial cells (hMECs) taken from reduction mammoplasties resulted in the formation of spherical colonies called mammospheres. The subsequent monolayer culture of these mammospheres under differentiating conditions interestingly can lead to the formation of mixed colonies of luminal-like and myoepithelial-like cells,

an indication of multipotency, which is a stem cell characteristic [81,131]. Soon after this revelation that stem cells from breast tissue could be enriched through the simple mammosphere culture technique (illustrated in Fig. 3.1), it was demonstrated that mammospheres could also be formed from breast cancer cells. Ponti, *et al.*, were able to culture mammospheres from breast tumor lesions and from the MCF7 breast cancer cell line [81][2]. Cells recovered from mammospheres had protein expression patterns that are recognized as being common to stem cells, and were able to initiate new tumors when as few as 1000 cells from MCF7 mammospheres were injected into the mammary fat pad of mice (one million MCF7 cells were required to form tumors when mammosphere enrichment was not used). Similar results were also achieved in later work by Grimshaw, *et al.*, in which mammosphere colonies containing a relatively high fraction of tumor-initiating cells were generated from pleural effusions from breast cancer patients [138]. These reports demonstrate that the mammosphere technique is capable of enriching the highly tumorigenic CSCs – probably the most dangerous cells within a tumor – from the bulk population of tumor cells using a simple in-vitro culture technique[3]. It should be noted however, that while mammosphere technique is used to enrich for stem cells, the spheroidal colonies that form consist of a heterogeneous population of stem vs. non-stem cells. In one report for instance it was demonstrated that while the vast majority of cells in the colonies are enriched for stem cell markers, only 10%-20% of these cells have

[2]Other cell lines reported to form mammospheres in low-attachment culture include SK-BR-3, MDA-MB-231 [138], MA-11 [139], BT474, T47D, ZR75-1 [140].

[3]The tumorsphere culture technique (as in mammosphere assay) that is used to enrich for CSCs have been since reported for a number of different tissues, including melanoma [141] and prostate [142], as well as a number of cell lines, including glioblastoma, mammary carcinoma, and melanoma [139].

self-renewal capacity [81].

Today the mammosphere technique is on a path to becoming a relatively common tool in the study of CSCs due to its simplicity and higher efficiency as compared to conventional isolation techniques that exploit fluorescent activated cell sorting (FACS), which isolates CSCs on the basis of their cell surface markers. In fact unlike FACS, which requires tedious sample preparation steps and highly trained personnel for sorting CSCs from a large number of cells[4], isolation of cancer stem cells via mammosphere culture can be implemented in a few simple steps using low cell concentrations. This is highly beneficial as in many cases, the total number of CSCs available in a biopsy sample or in patient blood (as circulating tumor cells) may be extremely low. For these reasons, mammosphere culture is gaining popularity in research labs for enriching potential breast cancer stem cells using low-attachment plates and serum free media that are commercially available.

The mammosphere culture technique may have great promise to enable those who study cancer to unlock some of the mysteries associated with the cancer stem cell hypothesis, such as the role of CSCs in the growth of primary tumors and more importantly the cellular and molecular pathways that are involved in the recurrence of the disease. Meanwhile, isolating and enriching CSCs from cultured cell lines can aid researchers to determine whether particular cells have been transformed to be highly tumorigenic (i.e., capable of forming mammospheres), which in turn enables one to study the triggers involved in tumor growth, relapse, and metastasis [34]. In other words, the cells within the mammospheres can be analyzed for biomarkers

[4]A large fraction of the sample may be lost using the FACS technique.

that indicate metastatic potential. Furthermore, drug screens can be performed on the tumorspheres (enriched in CSCs) to determine directly their resistance and susceptibility[5] as mammospheres are a more accurate 3D tumor representation for cancer drug investigations [145]. In what follows, a brief overview will be given on certain biomarkers expressed on CSC containing mammospheres as well as their drug screens.

Mammosphere Assay in the Discovery of Biomarkers and Therapeutic Identification

Currently, the mammosphere technique is being used by many research laboratories to establish pathways toward diagnosis and treatment. For example, the assay was initially used to discover and validate biomarkers associated with CSCs, which can then be used to provide a more thorough diagnosis of a patient's cancer in the clinic. In one of the pioneering reports on mammospheres, Ponti, *et al.*, cultured mammospheres from patient breast cancer tumors and reported that the cells within the mammospheres had reduced expression of epithelial markers, and that these markers returned upon differentiation of the cells in culture [81]. In addition, this work verified that CSCs were $CD44^+/CD24^-$, as had been reported earlier [16]; this biomarker profile is now commonly associated with breast CSCs. Later, in another important report on CSC biomarkers, it was shown that cells that were stained positively for aldehyde dehydrogenase (ALDH) activity formed mam-

[5]Cancer stem cells have been shown to be resistant to a number of chemotherapeutic agents, and thus it is critical to evaluate cancer drugs on mammosphere cultures [68, 104, 113, 143, 144]

mospheres, while those with low ALDH activity did not form mammospheres [107]. As a result, ALDH activity is now a marker for CSCs. In another biomarker validation, Cicalese, *et al.*, reported that cells from ErbB2$^+$ breast tumors were far more likely to form mammospheres [146]. Similarly, the technique of spheroid formation in low-attachment culture was used to discover that CD133 is a biomarker for CSCs in non-small-cell lung cancer [147].

More recently, the mammosphere assay has been used in research laboratories to identify important signaling pathways involved in maintaining the CSC phenotype; these pathways could serve as identifiers for CSCs and potentially as drug targets. Liu, *et al.* discovered that cells enriched for mammosphere-forming capabilities showed high expression of *hedgehog* signaling elements, and that activation of the *hedgehog* signaling pathway increases mammosphere formation [148]. Later, the same group demonstrated that recombinant IL-8 increased mammosphere formation in breast cancer cell lines, thus verifying that IL-8 is involved in maintaining the CSC phenotype [149]. Similarly, Fillmore, *et al.*, reported that estrogen stimulation increases the capability of MCF7 breast cancer cells to form mammospheres [150]. Further experiments confirmed that estrogen signaling increases the number of CSCs through the FGF/FGFR/Tbx3 signaling pathway. In another example of research that may lead to drug targets, it was reported that Musashi1 (Msi1), a regulator of the well-known stem-cell-related *Notch* and *Wnt* pathways, is correlated with mammosphere-formation in breast cancer cell lines [151]. Upon identifying it as an indicator of CSCs, the authors then demonstrated that knockdown of Msi1 decreased mammosphere formation. In addition, Chiou, *et al.*, applied the mammosphere cul-

ture techniques to lung cancer cell lines and demonstrated that ectopic expression of *Oct4* and *Nanog* increased spheroid formation, as well as drug resistance and the tendency to undergo an epithelial-mesenchymal transition, which is associated with metastasis [152].

Finally, the mammosphere assay is also useful in the identification and validation of drugs that combat CSCs in patient tumors. In one important study, Gupta, *et al.*, utilized the mammosphere technique to demonstrate that many popular cancer drugs, including doxorubicin, paclitaxel, and others, are not effective against CSCs [144]. The authors screened a number of chemical compounds, and ultimately identified salinomycin as a potential drug for CSCs, as it greatly reduced the mammosphere-forming capability of breast cancer cells as compared to paclitaxel. Bandyopadhyay, *et al.*, confirmed that doxorubicin is ineffective against CSCs, but proved using the mammosphere technique that doxorubicin in concert with TGFβ type 1 receptor kinase inhibitor was effective against CSCs [153]. In other work on identifying drug candidates, Li, *et al.*, discovered that sulforophane inhibits mammosphere formation in breast cancer cell lines [154]. Another compound that has potential as an anti-CSC drug is tranilast, which was shown to reduce mammosphere formation in MDA-MB-231 breast cancer cells [155]. Botchkina, *et al.*, used the mammosphere technique to demonstrate that SB-T-1214, a new derivative of taxol, may be an effective treatment against CSCs [156]. Together, this collection of recent results on signaling pathways that represent targets for drugs, as well as the assessment of drug candidates, suggests that the mammosphere technique may be an effective tool in the clinic as well.

Chapter 4

Fabrication of Mammosphere Culture Promoting Substrates*

As was mentioned in earlier chapters, progress in cancer stem cell research has been overwhelmingly slow, despite all the investment that has been put forth into the field. This is mainly due to the lack of systematic techniques for the identification of cancer stem cells and their progenies, which has in turn led to inconsistent results and theories. On the other hand, functional assays such as anchorage-independent cell cultures offer a more reliable technique in studying cancer stem cells, as tumorspheres that are enriched in CSCs also closely mimic a 3-dimensional tumor phenotype.

Today mammosphere culture assays use Low-attachment multi-well culture plates and serum-free media that are commercially available. Figure 4.1 shows micrographs of MCF7 cells (a commonly studied breast cancer cell line) cultured in a typical commercial attachment flask (Fig. 4.1 (a)) and in a commercial low-attachment plate (Fig. 4.1 (b)). The spherical colony in Fig. 4.1 (b), which resulted

*Some of the material in this Chapter is included in "A Tunable 2- and 3-Dimensional Cell Culture Microenvironment for the Isolation, Enrichment, and Study of Breast Cancer Stem Cells" submitted to the journal of Biomaterials.

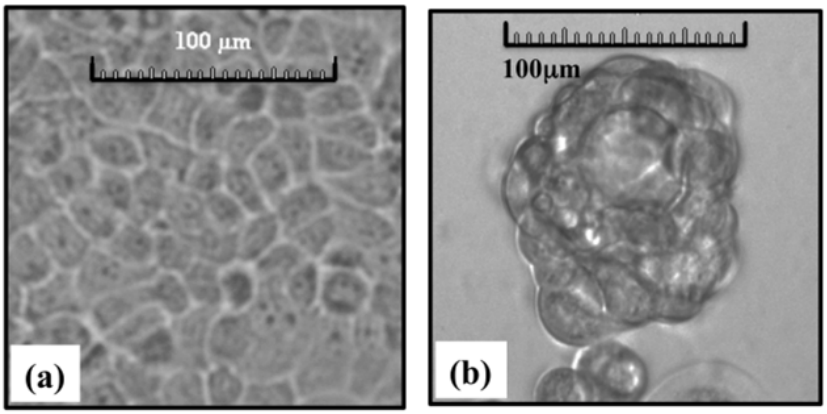

Figure 4.1: MCF7 cells cultured in (a) tissue culture treated flasks with typical cell culture media and (b) low-attachment plates with mammosphere media (after seven days in culture).

after seven days in culture, is a mammosphere.

While the multi-well format is somewhat useful for studies of tumorigenicity and cancer stem cells, a number of manual steps are required, and it is essentially impossible to perform subsequent assays on the cultured mammospheres, such as immunostaining and drug screens because reagents cannot be exchanged within the well that contains the suspended mammosphere colonies. It is clear that it would be highly beneficial to integrate the mammosphere culture assay into a microsystem with other microfluidic functions, including tumor cell enrichment and purification, in-situ immunofluorescence, drug screens with concentration gradients, and reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, microsystem integration reduces the consumption of expensive reagents, the opportunity for sample contamination, and the number of difficult sample manipulation steps.

To implement a mammosphere culture micro-system, a low attachment, transparent and biocompatible surface must be identified, which can additionally be in-

corporated with microfabrication techniques. This in turn implies that surfaces that normally enhance cell attachment through hydrophilic-hydrophilic interactions or in some cases through protein-protein encounters are the least desirable substrates for non-adherent cultures as in mammospheres. On low-attachment surfaces where the majority of cells with a more differentiated phenotype go through anoikis[1] as a result of delay in attachment, cells with stem like properties survive this stressful condition by changing their growth machinery as they proliferate into suspended tumorspheres. These spheroids (or mammospheres in the case of breast cancer cells), which are composed of non-polarized undifferentiated cells, have the ability to form large numbers of cyst shape structures over several weeks of culture. These colonies continue to grow for as long as they are supplied with culture media.

While techniques that are used in cell culture systems for the purpose of cell attachment are well documented in literature, a non-adherent cell culture device for studying tumorigenic events have not been sufficiently addressed. Non-adherent surfaces, which are mainly used in tissue engineering and in cell patterning, exploit antifouling materials to minimize protein and cell attachment onto certain regions and environments. Among several materials that inhibit cell-surface interactions, some polymers have been more widely used due to certain characteristics displayed by these materials. In developing a mammosphere culture microdevice, we therefore investigated two of such materials that are commonly used in cell culture studies. Surface characterization of the mammosphere promoting material was then carried

[1]Anoikis is a programmed cell death that occurs when cells are deprived from environmental signals that support their attachment.

out to investigate the key factors involved in cell-surface interactions that determine the cells' fate. After the successful culture of mammospheres on the polymeric substrate, subsequent growth capacity of mammospheres or the cells isolated from these tumorspheres were investigated. The role of media in cancer stem cell proliferation and differentiation on the culture substrate and the growth rate of tumorspheres over the time course of the culture then led to interesting revelations that are presented in more details in this chapter.

4.1 Poly(ethylene glycol) as a Protein/Cell Repellent Substrate

Poly (ethylene glycol) or PEG and its derivatives are the most commonly used biomaterials in the fabrication of protein and cell repellent surfaces [157, 158]. In fact, the steric hindrance effect of PEG plays an eminent role in inhibiting protein and/or cell attachment onto surfaces that are coated with this material. PEG and its hydrogels were therefore the first candidates to be tested for a mammosphere culture system as will be discussed in the following subsections.

4.1.1 Surface Modification of Glass Substrates with Poly(ethylene glycol) Monolayers

EXPERIMENAL

Surface modification with MM(PEG)$_{12}$

In order to modify glass substrates with poly(ethylene glycol), the surface of

glass should be first functionalized with a functional group that has a high affinity for PEG. One such reagent is (3-Mercaptopropyl)trimethoxysilane (3MTS), that is used to silanize the glass surface. In this reaction silane will covalently bond to the glass, exposing the thiol functional group of 3MTS for further surface modifications with PEG.

To prepare the glass surfaces, $1'' \times 1''$ size glass slides were first washed in a 1:4 solution of H_2O_2:HCl (piranha acid) for 30 minutes, rinsed in DI water, blow dried with air and put in the oven for 45 minutes. Clean glass slides were then functionalized using 1% (v/v) 3MTS in 90%/10% Ethanol/acetate buffer for 1 and a half hour at room temperature using a shaker. This was then followed by an Ethanol rinse and a 2 hours bake in a 65°C oven. Silanized glass slides were then PEGylated using 1% (v/v) $MM(PEG)_{12}$ for 1 and a half hour followed by a water rinse. These substrates were then stored in 6 well trays for cell culture experiments.

Surface modification with mPEG-silane (methoxy PEG-silane)

To eliminate the silanization step, in this experiment we functionalized glass surfaces with methoxy PEG-silane; a PEG derivative that is conjugated with a silane group. In this surface treatment, the silane terminal of mPEG-silane reacts with glass, leaving PEG exposed on the surface. For the surface treatment, $1'' \times 1''$ size glass slides were first washed in piranha acid (as described earlier). mPEG-silane was made in two stock solutions, one as a 0.5% (v/v) mPEG-silane in 90%/10% v/v Ethanol/acetate buffer and the other as 1% (v/v) mPEG-silane in adhydrous toluene. Piranha-cleaned glass slides were then treated with either mPEG-silane

solution for 1.5 h at room temperature followed by a rinse. Functionalized glass slides were then baked in a 65°C oven for 40 minutes and put in 6 well trays for cell culture experiments.

Commercial PEG-silane Treated Glass Slides

Commercial PEG-silane treated coverslips were used as a comparison template to investigate the efficiency of PEG modified glass surfaces in preventing cell attachment onto each substrate. For cell culture experiments, the cover slips were placed into 6 well trays that were later loaded with cell containing media.

Cell Culture

MCF7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 4 mM L-glutamine, 4.5 g/L glucose and reduced sodium pyruvate supplemented with 5 μl/mL Penicillin/Streptomycin, 1% Non-essential amino-acids, and 10% FBS (this media is referred to as regular media throughout this dissertation). Cells were subcultured in T25 flasks every 3-5 days by trypsinization with 0.25%/0.02% trypsin/EDTA in a 37°C incubator with 5% CO_2.

To prepare cells for culture on surface-modified substrates, MCF7 cells removed from flasks were spun down and resuspended in fresh media. To obtain a single cell suspension, the cell mixture was passed repeatedly through a 0.45 mm syringe needle. Cells were diluted to approximately 0.5×10^4 cells/mL and loaded into the wells of the trays. The trays were placed in a 37°C incubator with 5% CO_2.

RESULTS

Cell attachment is a phenomenon that occurs as a result of cell-substrate interactions such as hydrophilic and/or protein interactions. While commercial tissue-culture treated surfaces that are most commonly used in cell culture systems take advantage of the hydrophilicity of the plasmonic treated polystyrene or plastic materials to enhance cell attachment, other cell culture substrates that are developed in research labs are modified with attachment promoter proteins[2]. In the latter scenario, the substrate is either pretreated with proteins such as fibronectin and/or vitronectin or will be indirectly functionalized through the culture media that is rich in serum proteins. Cell attachment will then occur as a result of interaction between cells' membrane proteins and the protein coated material. In order to inhibit cell attachment therefore one approach would be to utilize surfaces that have a minimum affinity for proteins. Poly(ethylene glycol), which have been used in several contexts as an antifouling material, was therefore used in these experiments to fabricate a substrate that is inert to protein and cell attachment. Self-assembled monolayers (SAM) of PEG compounds such as $MM(PEG)_{12}$, methoxy PEG-silane as well as commercial PEG-silane treated glass slides were used in these experiments to study the efficacy of PEG surface treatment in repelling proteins. Clean glass slides were used as a control in these experiments. Cell attachment was then used as a detective mechanism to measure possible protein absorptions onto PEG-grafted surfaces.

The results of these experiments demonstrate that a self-assembled monolayer

[2]In cell culture conditions where none of these attachment promoting factors exist, cell attachments are attributed to other factors such as electrostatic or hydrogen interactions between cells and their substratum.

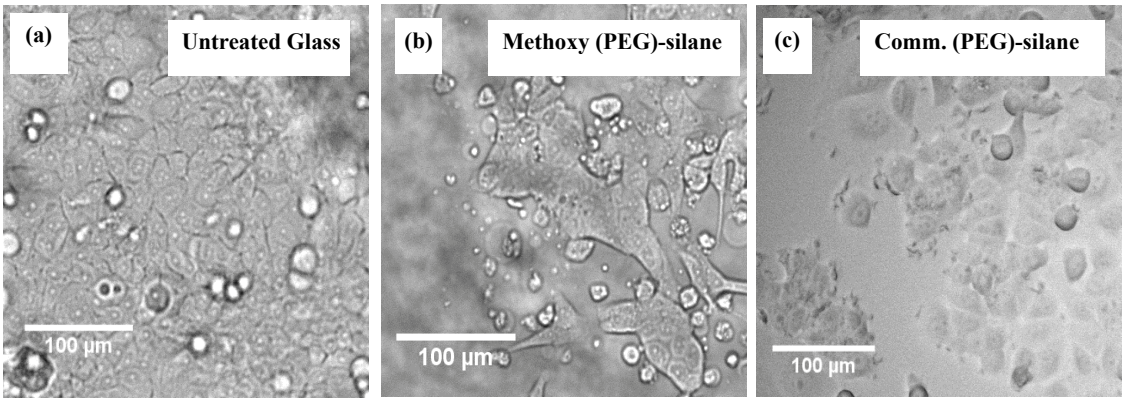

Figure 4.2: Self-assembled monolayer PEG on glass slides. Images are from day 7.

of PEG is not sufficient for inhibiting cell attachment as MCF7 cells (an epithelial breast cancer cell line) formed a monolayer after one week of culture. Possible explanations could be inhomogeneous PEG binding or degradation of the thin PEG layer that occurs overtime. Modification of the surface treatment protocol, which included variations in PEG concentration, and/or prolonging treatment and baking time, still resulted in cell attachment, as can be seen in Fig 4.2. Similar results were obtained using commercial PEG-silane treated cover slips.

4.1.2 Poly(ethylene glycol) Hydrogels

Polymer hydrogels have been extensively used in tissue engineering and for cell encapsulation. In these experiments, the hydrogel serves as the extracellular matrix for the cells of study. In should be noted however that while some polymers such as poly(ethylene glycol) are intrinsically non-adherent to cells and tissues, when in direct contact with a tissue, adhere strongly by interdigitation with the microscopic texture of their environment [159]. In this dissertation where the focus is on cell

culturing onto surfaces (rather than cell encapsulation), the cell repellency of PEG is exploited as will be discussed later in this chapter.

EXPERIMENTAL

Chemically Crosslinked Poly(ethylene glycol)-diacrylate (PEGDA)

PEGDA disks were fabricated by making a solution of ammonium persulfate[3] (15 mM) and tetramethylethylenediamine (15 mM) in a 30% v/v PEGDA (M_n=575) in DI water. The crosslinked PEGDA was then rinsed twice with phosphate buffered saline (PBS) or water to remove uncrosslinked radicals. PEGDA substrates were then immediately used for cell/mammosphere culture.

Photo-Crosslinked Poly(ethylene glycol)-diacrylate (PEGDA)

In this method, a UV source is used to initiate the crosslinking process in PEGDA, instead of chemical compounds. The PEGDA precursor is prepared by adding 1% (v/v) 2-Hydroxy-2-methyl-propiophenone (a UV light photoinitiator) to the PEGDA solution. 96 well trays are then filled with 50 μL of the precursor and illuminated by 220 nm UV light for 6 seconds. After photopolymerization, the hydrogels are rinsed twice with PBS or DI water to remove the uncrosslinked agents.

Cell Culture

MCF7 cells were grown on T25 tissue culture treated flasks as described in

[3]Ammonium persulfate (APS) is used as an initiator and tetramethylethylenediamine (TEMED) as an accelerator in the crosslinking of PEGDA chains.

the previous section. For mammosphere culture on PEGDA hydrogels, MCF7 cells removed from flasks were spun down and resuspended in MammoCult^{TM} Basal Medium (Human) supplemented with 10% MammoCult^{TM} Proliferation Media (this media is referred to as mammosphere media throughout this document). As a control, MCF7 cells in regular media were also cultured onto PEGDA surfaces. To obtain a single cell suspension, the cell mixture was passed repeatedly through a 0.45 mm syringe needle. Appropriate cell dilutions (250 cells/mL for 96 well PEGDA coated wells and 0.5×10^4 cells/mL for 6 well hydrogel coated trays) were then made and loaded into the wells of the trays. The trays were placed in a 37°C incubator with 5% CO_2.

RESULTS AND DISCUSSION

As can be seen in Fig. 4.3, MCF7 culture on PEGDA hydrogel substrates using either media (regular and mammosphere) did not result in any cell attachment as was expected. However, these substrates did not induce mammosphere formation either, which indicates that PEGDA is not an effective surface for mammosphere growth. While the number of MCF7 cells on PEGDA substrates remained constant over the two week culture period, where they are observed mostly as single cell entities, in some cases they cluster in one spot to form aggregates. Although cells on PEGDA substrates did not show any growth activity (consistent with previous reports [157]), the fact that they keep their original size, and round morphology (as demonstrated in Fig. 4.3) and are in some cases stained partially live with a Live/Dead stain, brings further attention to the concept of tumor cell dormancy.

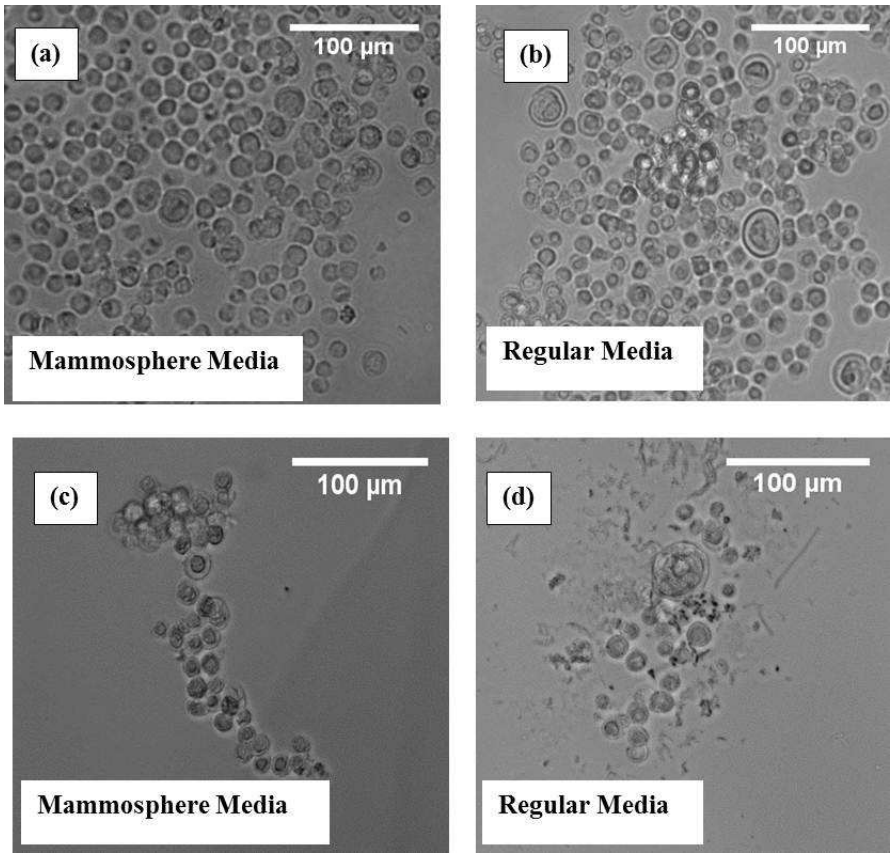

Figure 4.3: MCF7 cell culture on PEGDA hydrogels: 10^3 cells/mL cells cultured onto chemically crosslinked PEGDA hydrogel in (a) mammosphere media and (b) regular media. 250 cells/mL cultured onto photo-crosslinked PEGDA hydrogel in (c) mammosphere media and (d) regular media. Images are taken on day 7.).

Further investigation on this matter is however out of the scope of this dissertation.

4.2 Polydimethylsiloxane (PDMS) as a Mammosphere Culture Substrate

Polydimethylsiloxane or PDMS, which is a silicone-based organic polymer, is widely used in biomedical devices and in fabrication of microsystems owing to its unique characteristics [160–162]. These properties include biocompatibility (an essential element of biological assays that require non-toxic environments [160, 163],

optical transparency with wavelengths down to 256 nm for optical and fluorescence microscopy [164], elasticity and durability. The low cost and mechanically flexible characteristic of PDMS has further made this polymer a suitable material for prototyping microsystems using soft-or photolithography based microfabrication techniques [165–167].

The unique characteristics of PDMS as mentioned above, has made this polymer to become one of the most widely used materials for cell culture devices. However, normally several surface modifications are implemented on PDMS based cell culture systems, some which require labor intensive processes and expensive reagents for surface treatments. While these modification techniques are well established, no studies have been implemented to date on certain surface characteristics of PDMS that alone can be exploited for a modification free cell culture device that can be equally used for both cell attachment and non-adherent suspension cultures. In this study therefore, we first characterized surface properties of PDMS that are commonly involved in cell-material interactions. Certain characteristics of this polymer were then exploited to fabricate a mammosphere culture as well as cell attachment based devices, as will be discussed in more details. A comparison between these polymeric surfaces, clean glass as well as commercially available tissue culture treated (TC-treated) and commercially low-attachment (hydrogel treated) mammosphere plates was then performed.

EXPERIMENTAL

Formation of Substrates for Cell Culture

PDMS substrates for cell culture were created by filling the wells of 96-well plates with the elastomer. PDMS was prepared by mixing a 1:10 ratio of curing agent with the prepolymer and was poured into the wells, degassed in a vacuum desiccator, and cured in an oven (65°C) for one hour. For cell culture on glass substrates, glass cover slips were diced to fit into the wells of 96-well plates and were cleaned in piranha acid. A thin layer of PDMS (1:10 ratio) was poured into wells of a 96-well plate, and then the diced and cleaned cover slips were placed on top of the thin layers of PDMS. The plates were placed in an oven (65°C) for one hour to cure the PDMS, which fixes the cover slip fragments into the wells, with the glass serving as the surface. In parallel to glass and PDMS coated wells, cell culture was implemented on commercial cell-culture-treated as well as hydrogel-coated (non-adherent) 96-well trays in order to compare the efficiency of our engineered substrates with that of the well-established commercial plates.

Cell Line Selection

MCF7 and MDA-MB-231 epithelial breast cancer cell lines as well as MCF10A, which is a non-tumorigenic breast cell line, were selected for our culture experiments. Mammosphere formation has been reported for MCF7 and MDA-MB-231 cell lines [138,168–172]. While neither MCF7 nor MDA-MB-231 are commonly considered as a cancer or normal stem cell, prior reports of mammopshere formation indicate that a subset of cells from each sub-culture have stem-like properties [173]. Further reinforcing this notion, Chaffer, *et al.*, verified that a subset of human mammary epithelial cells (HMEC) has stem-like properties, and interestingly, that

a sub-population of the cells is capable of de-differentiating into stem-like cells [174]. This demonstrates that within each sub-culture, cell lines may continually consist of a heterogeneous population that includes some stem-like cells. As a result, it is not surprising that mammosphere formation can occur from cultured MCF7 and MDA-MB-231 cells.

Cell Culture

MCF7 and MDA-MB-231 cells were grown in regular media as described previously. For MCF10A cell culture, DMEM/F12 media was supplemented with 5% Horse Serum, 0.02% Epidermal Growth Factor[4], 0.05% Hydrocortisone, 0.01% Cholera Toxin, 0.1% Insulin and 0.1 μL/mL Penicillin/Streptomycin. Cells were subcultured in T25 flasks every 5-7 days by trypsinization with 0.25%/0.02% trypsin/EDTA in a 37°C incubator with 5% CO_2.

To prepare cells for mammosphere formation, cells removed from flasks were spun down and rinsed with PBS before any mammosphere culture to ensure the complete removal of serum from the cells. The cell pellets were then resuspended in mammosphere media. For regular cell culture, another set of cells was simultaneously prepared using regular serum-rich media. To obtain a single cell suspension, the cells were passed repeatedly through a 0.45 mm syringe needle. Cells were diluted to approximately 250 cells/mL and 200 μL of the cell suspension was loaded into the wells of the plates. At least 5 wells were used for each experimental condi-

[4]A stock of 0.01% w/v EGF was prepared by dissolving the growth factor into 10 mM Acetic Acid.

tion. The plates were placed in a cell culture incubator at 37°C with 5% CO_2.

Surface Roughness Measurement

Surface textures of PDMS and glass were measured using a profilometer (Tencor TP-20 Profilometer, AlphaStep 200 Inc.), which provides a profile for each surface. The data acquired from these measurements was then analyzed to determine the surface roughness of each material.

Contact Angle Measurements

The hydrophobicity/hydrophilicity of each surface was determined using the static sessile drop technique, which utilizes a goniometer (Tantec A/S, Denmark) to measure the contact angle of a water droplet that is pipetted onto the surface. For each substrate the contact angle was averaged from measurements of at least three droplets.

Protein Adsorption Determination

The protein adsorption onto PDMS and glass surfaces was measured by passing a serum-rich and serum-free (mammosphere) media through a microchannel with either a glass or PDMS base. Typical soft lithography procedures were used to fabricate 600 μm$\times$30 μm microchannels with PDMS. Briefly, Hexamethyldisilazane (HMDS) was spin coated onto a silicon wafer for 40 seconds. Shipley 1813 photoresist was then spin-coated onto the wafer for 40 seconds at 4000 rpm. The wafer was then baked for 1 min on a hot plate, rehydrated at room temperature for 1 min, UV

exposed at 365 nm through the mask for 9 seconds using an MJB-3 mask aligner and developed in a CD-30 developer. A deep reactive ion etcher (DRIE) was then used to etch the negative of the microsystem features to a depth of 30 μm.

To facilitate PDMS removal after replica molding, the master mold was coated with trichloro(1H,1H,2H,2H,-perfluorooctyl)-silane. PDMS was mixed in a 1:10 ratio of curing agent and prepolymer, poured onto the master, degassed in a vacuum desiccator and cured in an oven (65°C) for two hours. The PDMS was then washed in ethanol, plasma oxidized using a corona discharge and brought into contact with the appropriate substrate (glass or PMDS), to which it is permanently bonded after 30-minutes at 65°C.

In each experiment serum-rich or serum-free media was run through microchannels with a PDMS or glass bottom surface for 30 minutes at a speed of 10 μL/min. The microchannels were then rinsed with water for 10 minutes in order to remove unattached proteins. Fluorescein isothiocyanate (FITC), which is an amine-reactive dye was then passed through the channel to fluorescently label any adsorbed protein. The FITC was prepared at 0.1% (w/v) in 90/10 (v/v) de-ionized (DI) water/DMSO. Channels were rinsed with DI water to remove unconjugated dye molecules from the surfaces. Channels were observed with a fluorescence microscope using a 1 second integration time. For comparison, FITC was also passed through channels with glass and PDMS bottom surfaces that were not treated with either media. Images were also taken before FITC exposure using the same filter setting to establish a baseline for each device.

Imaging

An Olympus IX-51 inverted microscope was used to record all images in this work. Monolayers of cells were imaged using phase contrast imaging. To image FITC staining of protein layers, the fluorescence imaging capabilities were utilized with a filter set designed for green fluorescent protein (GFP) imaging.

RESULTS AND DISCUSSION

PDMS Substrates Selectively Drive Mammosphere Formation

MCF7 and MDA-MB-231 cells, which have been shown to form mammospheres [138, 168–172], were loaded in mammosphere media onto four surfaces: PDMS, commercial low-attachment plates (hydrogel-treated), piranha-cleaned glass, and commercial TC-treated plates. Figure 4.4 presents representative images of all four experimental conditions after seven days in culture. As shown in the micrographs in Figs. 4.4 (a-b) and 4.4 (e-f), mammospheres formed from MCF7 and MDA-MB-231 cells on the PDMS and the commercial low-attachment plates. MCF7 cells form tightly packed mammospheres while the MDA-MD-231 cells form loosely structured mammospheres; this is consistent with previous reports [138]. Mammospheres continued to thrive in culture on PDMS up to 14 days and beyond, as shown in Fig. 4.5.

The MCF7 mammosphere on PDMS in Fig. 4.4 (a) is approximately 200 μm in diameter and, assuming a spherical geometry of the mammosphere, contains approximately 500 cells (using a cell diameter of 23 μm and a packing factor of 0.74). Meanwhile, the MCF7 mammosphere on the low-attachment surface in Fig. 4.4

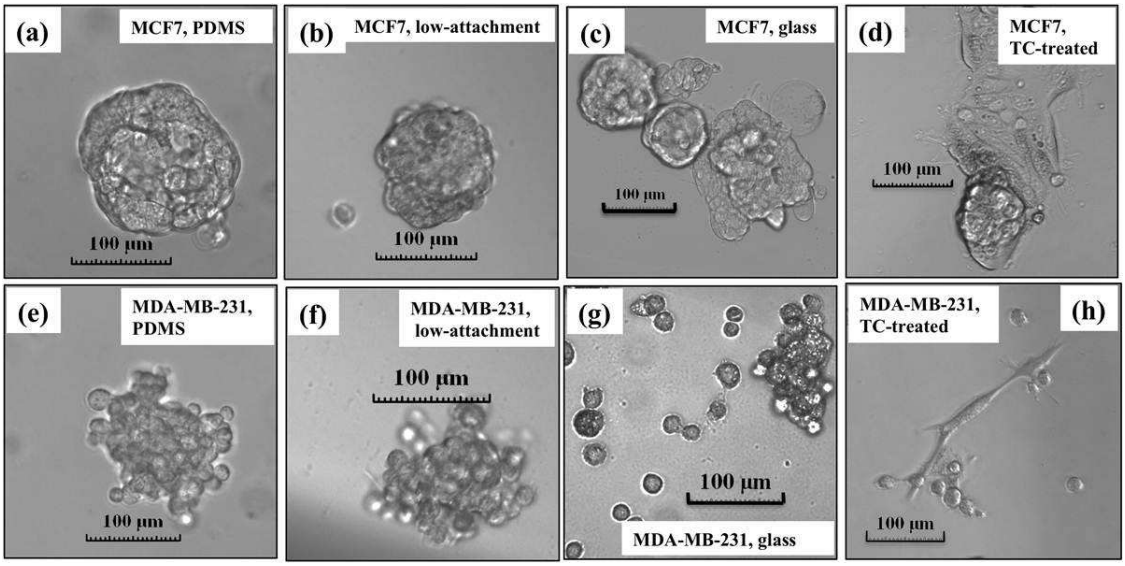

Figure 4.4: MCF7 cells (a-d) and MDA-MB-231 cells (e-h) cultured in mammosphere media on four different surfaces: PDMS (a, e), commercial low-attachment plates (b, f), glass (c, g), and commercial TC-treated plates (d, h). The colonies in a, b, e, and f are mammospheres, which remain in suspension. On glass and commercial TC-treated plates both mammospheres and attached monolayers could be found (c, d, g, h) however, on these surfaces mammospheres are attached to the underlying monolayer. (Images were taken after seven days in culture. TC-treated = tissue-culture-treated. All scalebars = 100 μm.).

(b) has approximately 100 cells, indicating that the exponential growth on PDMS is about two cycles ahead. However, commercial low-attachment plates contained an average of 6.6 mammospheres (N=9 wells), while PDMS wells contained one or two mammospheres (N=9 wells). The reason for these two differences is likely because the PDMS wells have a gradual bowl-shaped surface, causing cells to collect in the center shortly after loading. As a result, each mammosphere on PDMS likely resulted from a few cells brought together in close proximity by the shape of the surface. In contrast, in low attachment plates, cells drift to the outer edge of the well, and thus the mammospheres likely evolved from single cells. The concave geometry of PDMS coated substrates provides an additional advantage over commercial

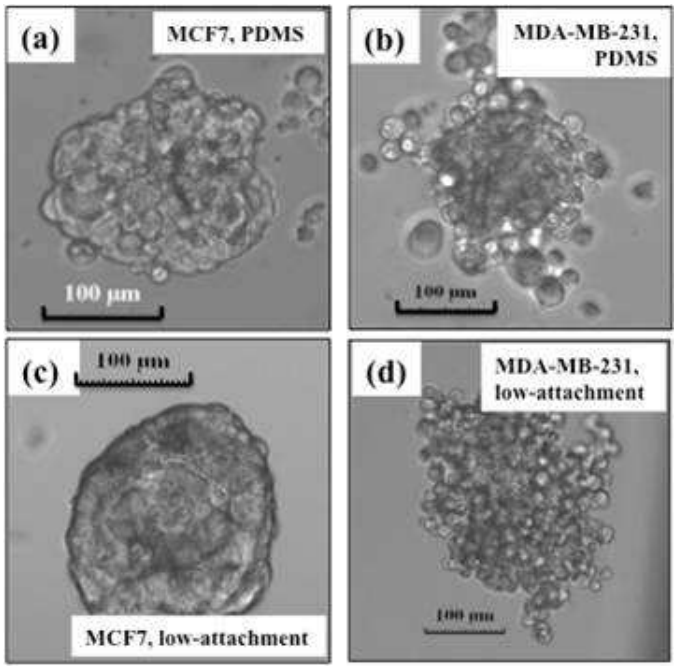

Figure 4.5: MCF7 cells (a,c) and MDA-MB-231 cells (b,d) cultured in mammosphere media on PDMS (a-b) and commercial low-attachment plates (c-d) after 14 days in culture.

plates as it makes it less challenging to monitor the growth of cell colonies over a period of time as they are found only at one single spot. In other words, on PDMS wells mammosphere(s) are restricted and trapped at the bottom of the bowl shaped surface and cannot roll around with the same ease that mammospheres do on flat commercial plates. This also assures us that the huge colonies that form over a few weeks period are the result of just a few cells, and not an aggregation of several smaller colonies. It is noteworthy to mention that unlike other reports in which mammospheres are cultured using at least 1000-10,000 cells, in all experiments implemented in this research, on average fewer than 20 cells were used and yet they successfully formed tumorspheres[5]. This is a significant result as it confirms the

[5]Even though we aimed for 50 cells/well (200 μL of ρ_{cell}=250 cell/mL per each well), this number is an over estimation of the cells that are actually distributed into each well as was shown

hypothesis that only a few numbers of cells would suffice to initiate a tumor in-vivo. These colonies then continue to grow uncontrollably even in non-ideal environmental conditions[6]. The fact that in some experiments we formed mammospheres from single cells also indicates that these cells can survive well in isolation[7]. The second important conclusion that can be drawn from these experiments is that in contrast to the common belief, a larger percentage of cells that have gone through several differentiation cycles are in fact capable of rewinding into a more stem/progenitor state. This in turn can further confirm the new hypotheses that suggest that tumor cells have a dynamic behavior and can switch back and forth between a stem and a non-stem state upon external triggers. More detailed investigation is however required to verify the accuracy of this hypothesis.

For the control experiments, we studied mammosphere formation on glass and tissue culture (TC) treated plates. While clean glass and TC-treated plates represent optimal surfaces for attachment, both of these surfaces produced a heterogeneous distribution of cells in which both mammospheres and attached monolayers could be found (Fig. 4.4 (c-d) and (g-h). In general, MCF7 cells continued to survive and grow in this heterogeneous distribution, while the MDA-MB-231 cells mostly attached to the surface, and then contracted and died.

An interesting observation in this picture of a heterogeneous cell distribution

by cell counting.

[6]In all these experiments even though media was not replenished over the life span of the experiments (more than two weeks), mammospheres continued to grow until the wells were completely dried out. This in turn indicates that once mammospheres are formed, they continue to grow uncontrollably even when they are low in nutrition.

[7]Single cell culture of mammospheres were implemented using a mammosphere culture microdevice that will be discussed in the following chapter.

is that unlike mammospheres that form on PDMS and commercial low-attachment surfaces, tumor-spheres that form on the surface of glass and TC treated substrates (which are optimized for cell attachment), are not in suspension. In other words, they grow as 3-dimensional structures on top of attached monolayers and are therefore fixed in place. Although it is not completely clear which pathway the cells decide to take first when they proliferate, it seems that they initially form attachment and then choose a few spots to grow colonies. A logical explanation might be the existence of some unattached cells in the culture well that eventually interact with the cell monolayer and start colonizing. In determining the order of proliferation paths that cells take in this mixed condition, one might also consider the possibility that cells form colonies before any attachment occurs. In this scenario, the colonies then interact with the surface (that is optimized for cell attachment) and, as a result of making an attachment with the substrate, proliferate as monolayers. Regardless of the pathway that cells take in forming this heterogeneous mixture, the surprising fact is that monolayers form despite the lack of serum protein that is necessary for all cell attachment based assays. This observation might suggest that surfaces play a more dominant role in the growth and differentiation of tumor cells than a media component does. Whether this holds true for non-cancerous cells would be an interesting subject to investigate.

Role of Media in Mammosphere Formation

As was shown in previous section, PDMS substrates drive mammosphere formation when combined with a serum free medium (or mammosphere media). In

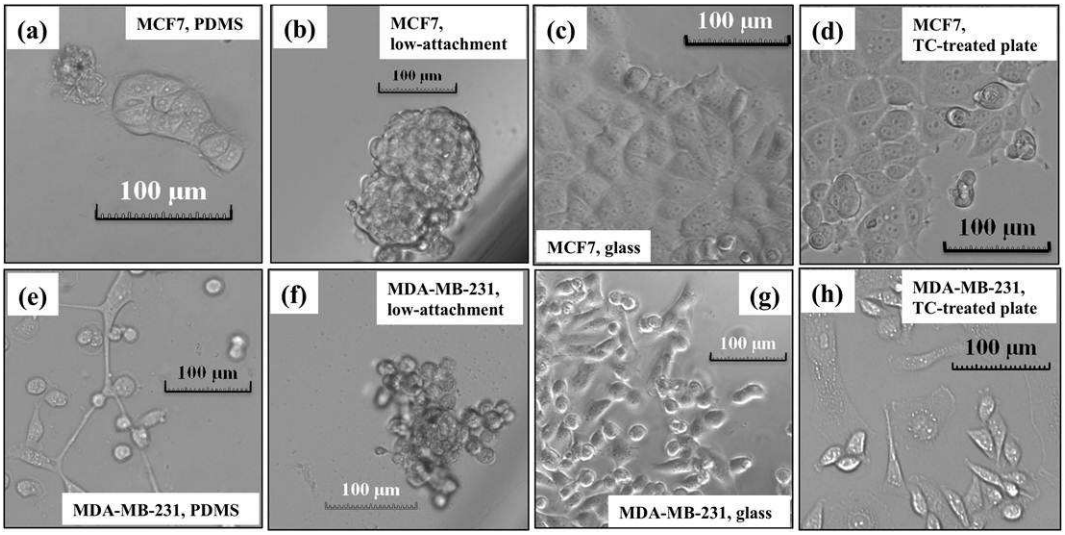

Figure 4.6: MCF7 cells (a-d) and MDA-MB-231 cells (e-h) cultured in regular media on PDMS (a, e), commercial low-attachment plates (b, f), glass (c, g), and commercial tissue-culture-treated plates (d, h). Images were taken after seven days in culture. TC-treated = tissue-culture-treated. All scalebars = 100 μm.

fact the ability of this polymer to induce spheroid formation is comparable with that of the hydrogel treated, low attachment commercial plates. We also showed that glass and TC-treated commercial plates do not selectively drive mammosphere culture while inhibiting non-mammosphere forming cells. To investigate the role of media in mammosphere formation, we also loaded MCF7 and MDA-MB-231 cells onto these same four surfaces in regular media. Figure 4.6 presents representative micrographs after seven days in culture. As expected, when MCF7 and MDA-MB-231 cells are loaded onto clean glass and TC-treated plates, they form attached monolayers and multiply quickly. A comparison between cell attachment onto these substrates using serum free and serum rich media (Figs. 4.4 (c, d, g, h) and 4.6 (c, d, g, h)) indicates that while cells can still attach without serum, the proliferation rate is much slower. In addition a close look at the morphology of cells attached

in these two media conditions clearly indicates that cells without serum are more stressed and are therefore not as healthy.

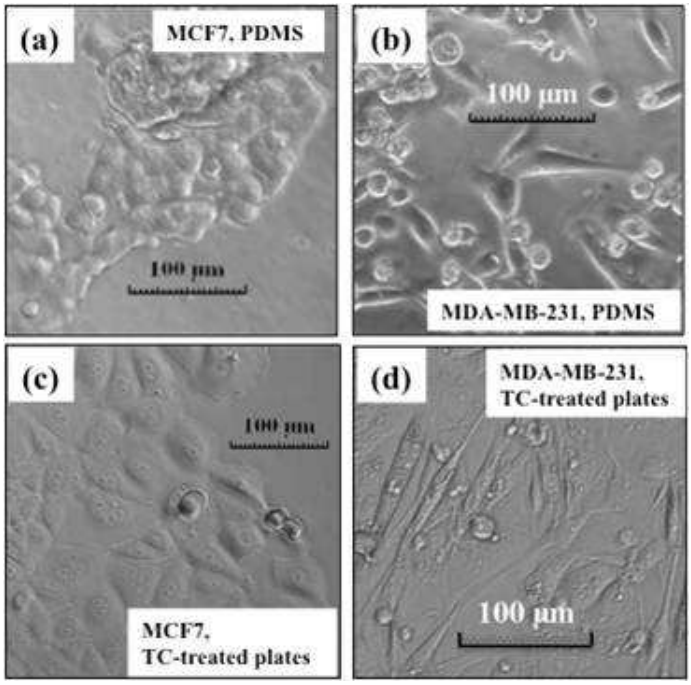

Figure 4.7: MCF7 cells (a,c) and MDA-MB-231 cells (b,d) cultured in regular media on PDMS (a-b) and commercial tissue-culture-treated plates (c-d) after 14 days in culture.

As for PDMS, when regular media is used, cells can attach to PDMS and form monolayers as can be seen in Figs. 4.6 (a, e). Attachment and growth was initially slow (compare Figs. 4.6 (a, e) with Figs. 4.4 (c, d, g, and h)), but cells continued to grow over extended periods of time. Figure 4.7 compares monolayers of cells on PDMS and TC-treated plates after 14 days in culture. In contrast to PDMS, Figs. 4.6 (b, f) show that mammospheres formed in the commercial low-attachment plates, even though regular media was used. In general, however, fewer mammospheres were found in each well as compared to when mammosphere media is used. Additionally, after three days in culture, many cells were observed to form

	Mammosphere Media	Regular Media
Glass	Attachment + Small Colonies	Monolayer Attachment
TC-Treated Plates	Attachment + Small Colonies	Monolayer Attachment
PDMS	Mammosphere Formation	Delayed Attachment
Low-Attachment Plates	Mammosphere Formation	Mammosphere Formation

Table 4.1: Tumor cell's response to different environmental conditions imposed by substrate and culture medium.

attachment to the commercial low attachment plate, but these cells died before the seven-day time point.

Together, these results (which are summarized in Table 4.1) show that PDMS provides a resistance to the attachment of epithelial cells, and that this resistance is sufficient to drive mammosphere growth of stem-like cancer cells in mammosphere media. While Fig. 4.6 shows that cell attachment can occur on PDMS (though more slowly than glass), Fig. 4.6 confirms that the delay in attachment onto PDMS is sufficient to drive mammosphere growth in mammosphere media. Importantly, this result demonstrates for the first time that PDMS, a common microfabrication material, can be utilized to form the substrate for mammosphere assays. In addition, the combination of the results in Fig. 4.4 and Fig. 4.6 indicate the potential of utilizing PDMS as a multi-functional cancer cell culture substrate.

MCF7 Cell Culture on PDMS of Varying Stiffness

As discussed in previous sections, PDMS substrates selectively drive mammo-

sphere formation in the absence of serum proteins and promote cell attachments when signaled by a medium that is rich is serum proteins. These observations were made on a PDMS substrate that had a 1:10 curing agent to prepolymer ratio, which is the commonly used protocol for the fabrication of PDMS substrates. We then studied how variations in the elasticity of PDMS substrates affect mammosphere formation and cell attachment onto this material in the absence and presence of serum. For this experiment, PDMS was prepared by mixing 1:15, 1:10, 1:7.5, 1:5 and 1:3 ratios of curing agent to prepolymer and poured into the wells of 96-well plates, degassed in a vacuum desiccator, and cured in an oven (65°C) for one hour. For simplicity we will designate these substrates as $PDMS_{1/15}$, $PDMS_{1/10}$, $PDMS_{1/7.5}$, $PDMS_{1/5}$, and $PDMS_{1/3}$. For cell culture onto these substrates, MCF7 cells were then prepared as discussed previously. A 200 μL single cell suspension that was prepared at a density of 250 cells/mL in either mammosphere or regular media was then loaded into the wells of the PDMS-coated 96 well trays. The plates were placed in a cell culture incubator at 37°C with 5% CO2. Images of mammospheres or cell monolayers that formed on these substrates were then taken at one week point, and 17 days after the culture.

While PDMS substrates pertaining to different rigidities displayed almost a similar trend in the interaction with cells (with the exception of $PDMS_{1/5}$, and $PDMS_{1/3}$), a few differences were noticeable between different PDMS templates as is discussed below.

- ***Cell Culture in Mammosphere Media on Varying PDMS Stiffness***

Several reports have indicated that cells behave differently on substrates with a different elasticity [175,176]. While these observations were mainly in the context of cell attachments, substrate rigidity can also have minor effects on mammosphere formation on PDMS, as will be discussed here. As can be seen in Figs. 4.8 (a-e), after one week of culture in mammosphere media, an average of 1-3 colonies form on each PDMS substrate. However, it is noticeable that colonies formed on surfaces with the highest curing/base ratios, i.e. $PDMS_{1/5}$, and $PDMS_{1/3}$ are much smaller in size than those formed on the three other surfaces. As a matter of fact these cells, which were more in the form of cell aggregates (Figs. 4.8 (d, e)), did not look healthy and displayed unusual dark spots on their surface membranes. This cell behavior, which was more pronounced on $PDMS_{1/3}$, might be a sign of toxification

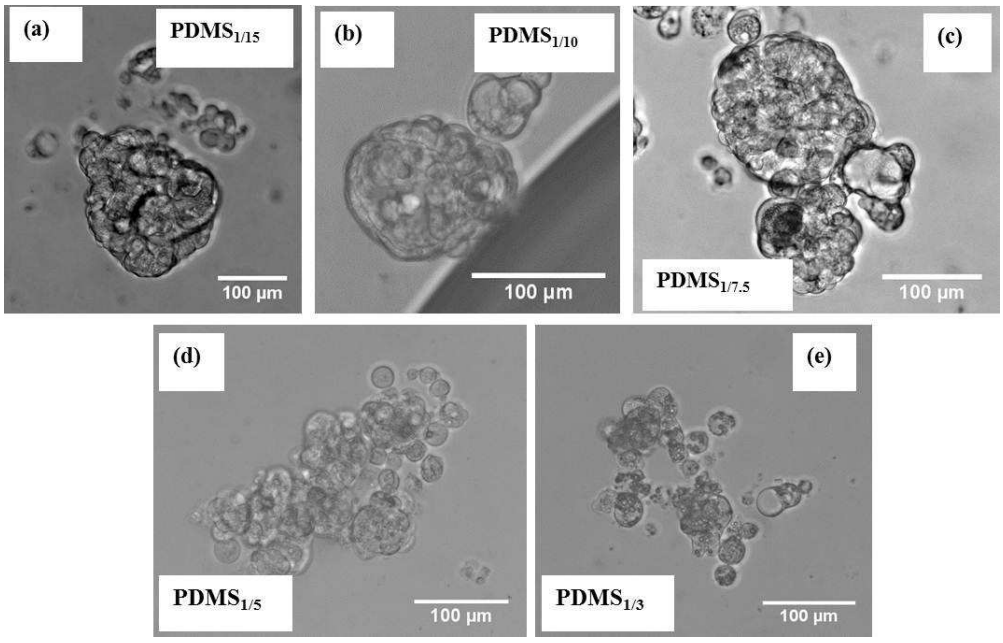

Figure 4.8: MCF7 mammosphere culture on PDMS substrates with different rigidities. The images were taken after 7 days.

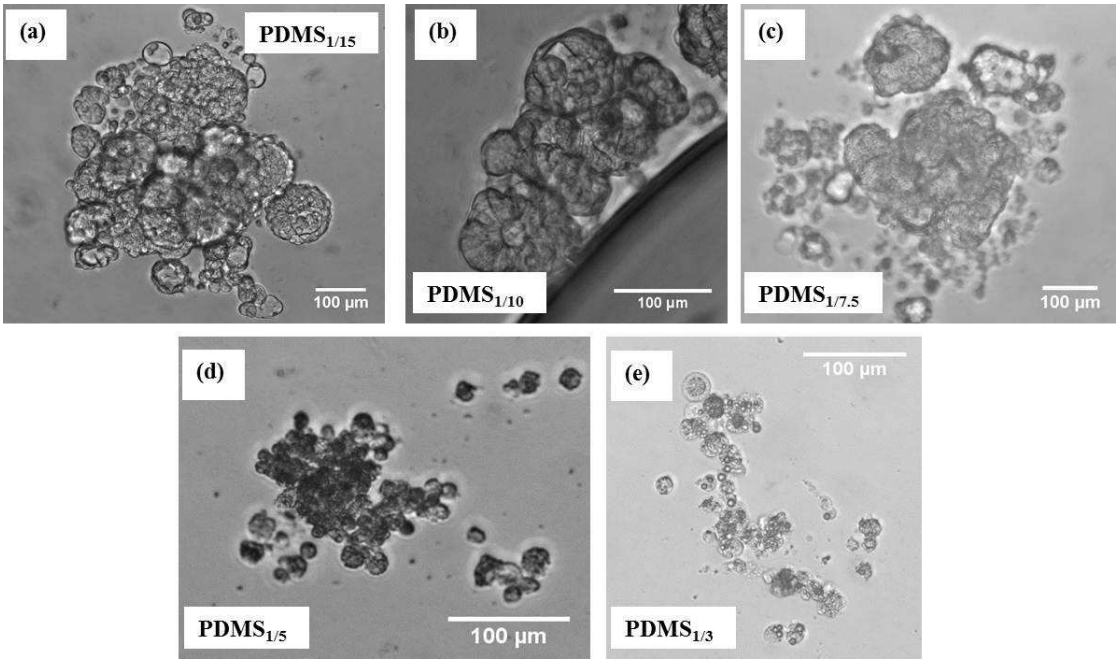

Figure 4.9: MCF7 mammosphere culture on PDMS substrates with different rigidities. The images were taken after 17 days.

due to the higher ratio of curing agent in PDMS, which is composed of toxic materials. In some instances the un-crosslinked low molecular weight polymer leaches into the culture medium where the monomer can then affect cellular activities by interacting with the hydrophobic part of the cells' membrane. The effect of high curing agent/base ratio PDMS substrates on cell culture was further confirmed by the fact that cell growth was arrested soon after this time point (week 1), as can be seen from images of day 17, where cells are all dead (Figs. 4.9 (d, e)). On the other hand, on other PDMS surfaces, i.e. on $PDMS_{1/15}$, $PDMS_{1/10}$, $PDMS_{1/7.5}$, mammospheres continued to grow and multiply. Mammosphere formation might specifically be enhanced by lowering the ratio of the curing agent to prepolymer as in few cases the 1-2 colonies observed on $PDMS_{1/15}$ on day 7 (Fig. 4.8 (a)), continued to grow into multiple colonies after 10 additional days (Fig. 4.9 (a)). Mammospheres on

other two PDMS substrates only doubled after 17 days of culture (Figs. 4.9 (b, c)).

- ***Cell Culture in Regular Media on Varying PDMS Stiffnesses***

Using a serum rich medium, differential cell attachment was observed on PDMS substrates that were prepared by mixing different ratios of curing agent with the prepolymer. That is while cells attach on $PDMS_{1/15}$, $PDMS_{1/10}$, $PDMS_{1/7.5}$ substrates (Figs. 4.10 (a-c)), on $PDMS_{1/3}$, $PDMS_{1/5}$ cells remain as inactive single entities over the one week culture (Figs. 4.10 (d, e)), similar to what we had previously observed on PEGDA hydrogels (Fig. 4.3). However, unlike PEGDA hydrogels on which cells retain their symmetrical and round morphology over an extended pe-

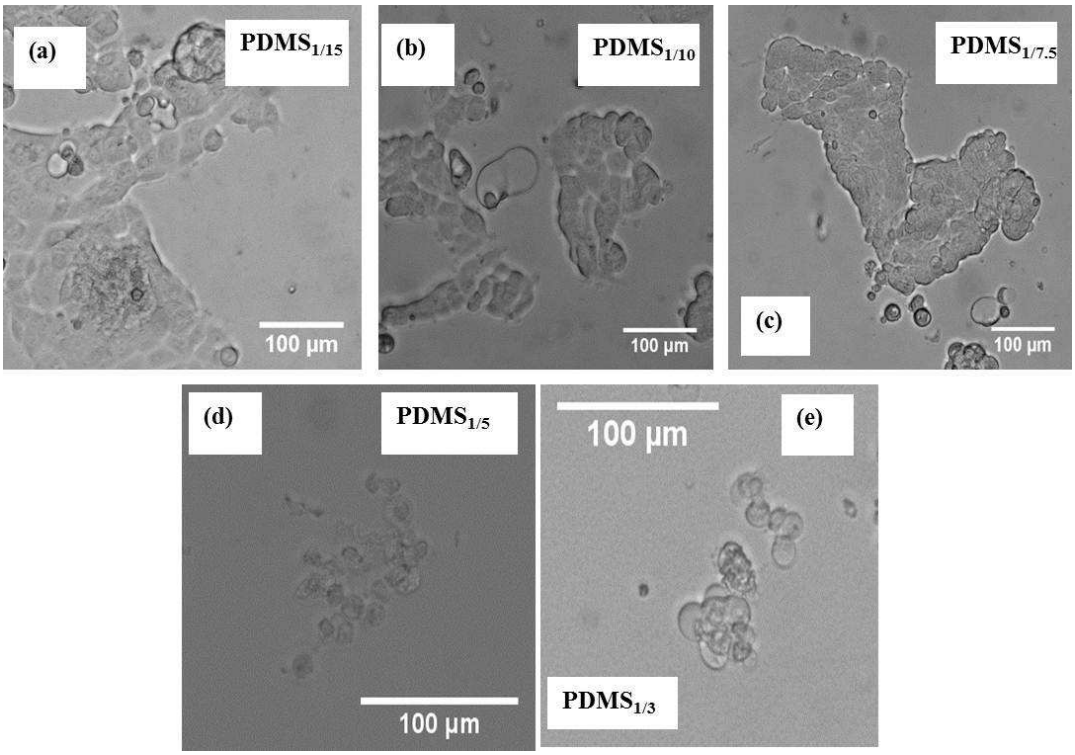

Figure 4.10: MCF7 culture in regular media on PDMS substrates with different rigidities. The images were taken after 7 days.

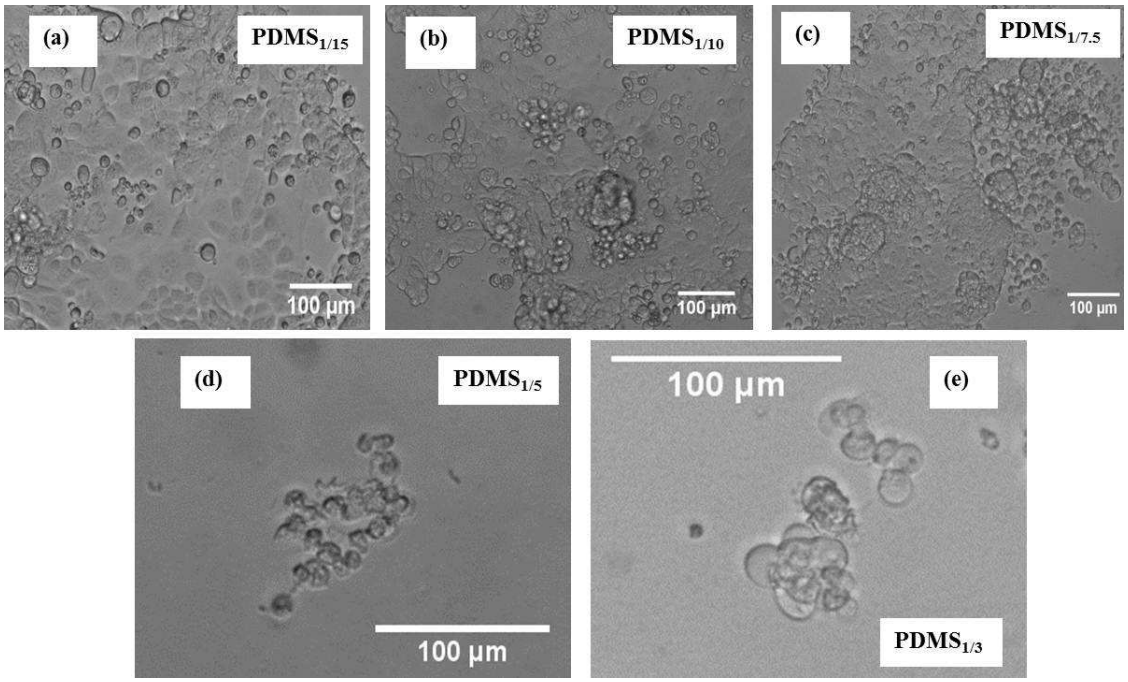

Figure 4.11: MCF7 cell culture in regular media on PDMS substrates with different rigidities. The images were taken after 17 days.

riod of time (which can be an indication of cell viability), on $PDMS_{1/3}$, $PDMS_{1/5}$ cells eventually undergo apoptosis. This can be seen by the dramatic decrease in the cells' size and uneven cell surface membrane after 17 days of culture on $PDMS_{1/3}$ and $PDMS_{1/5}$ (Figs. 4.11 (d, e)). On the other hand, cells adhered on all three $PDMS_{1/15}$, $PDMS_{1/10}$, $PDMS_{1/7.5}$ substrates where no significant difference in attachment was observed. This can be seen in Figs. 4.10 (a-c), where cells display a slow attachment on $PDMS_{1/10}$, $PDMS_{1/7.5}$ and $PDMS_{1/15}$ over the first week of culture but eventually form a monolayer after two weeks (Figs. 4.11 (a-c)). One can also notice some free and unattached cells on top of these monolayers, which are specifically higher in numbers on $PDMS_{1/7.5}$ and $PDMS_{1/10}$. From the size of these cells, one could infer that these floating cells that were not observed over the first

week of culture are in fact alive and capable of growing. However, it is not clear how their function might be different from the original cells that were seeded onto the PDMS wells.

All in all, these results indicate that using a bare (or unmodified) PDMS substrate, PDMS surface rigidity does not in general play a significant role in cellular activities such as attachment or colony formation (except in the case of $PDMS_{1/3}$ and $PDMS_{1/5}$, that might be a toxicity effect). Therefore from here after, all mammosphere culture experiments are implemented on $PDMS_{1/10}$ (referred to as PDMS from here on) as the 1:10 mixture of curing agent/prepolymer has proven to be more fabrication friendly in the design of microsystems. In what follows a few functional characteristics of mammospheres formed on PDMS or cells isolated from these spheroids will be discussed.

Time Dependent Growth and Cell Shedding of Mammospheres on PDMS

To measure the time dependence growth of colonies on PDMS substrates, PDMS coated wells were filled with a dilution of MCF7 cells in mammosphere media (250 cells/mL). The mammospheres growth rate over a two-week culture period was then determined by measuring the diameter of the colonies (assuming a spherical structure) and calculating the average number of cells that the mammospheres are composed of. As can be seen in Fig. 4.12, these colonies continue to grow consistently for approximately 10 days. Their size then suddenly drops after this time point at which they have reached an average diameter of 160-170 μm (in some cases

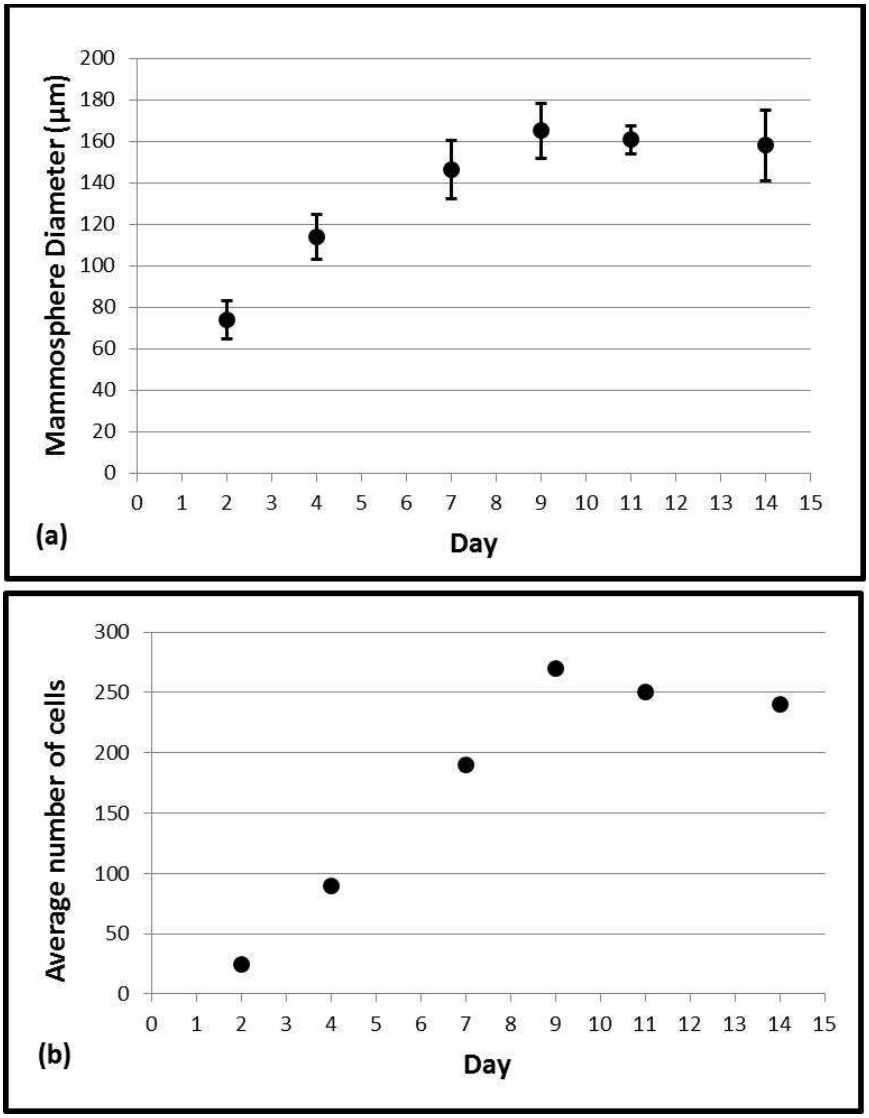

Figure 4.12: MCF7 mammosphere growth rate on PDMS substrate over a two weeks culture period. These data were obtained by measuring the average diameter of colonies (n=4) (a) and the average number of cells that constitute these colonies (b).

however a re-growth was observed after a couple of days). This might suggest that cells that are located deep inside the colony undergo necrosis when the colonies get to a certain size. In other words, in large dense spheroids, cells that are at the center of the colony might not receive enough nutrition and will therefore experience cell death. This size reduction of the colonies might alternatively be explained by the

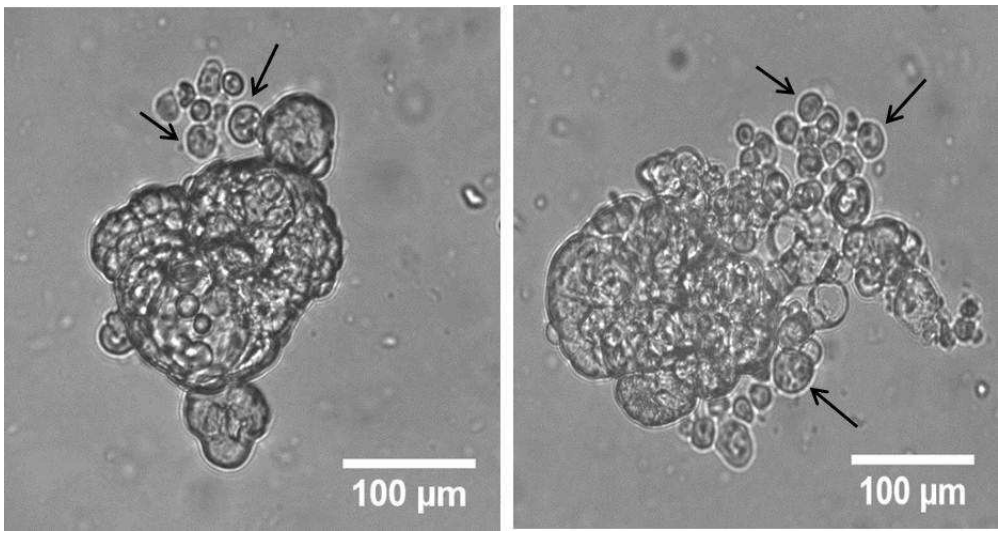

Figure 4.13: Mammospheres can shed viable cells into their surrounding as shown by the arrows in the figures. These cells appear to be larger in diameter than a typical MCF7 cell that colonies originated from.

fact that mammospheres can shed cells into their surrounding environment when they get to a certain point in their growth, as is noticeable in Fig. 4.13. These cells that might be similar to migratory stem cells in vivo, have the ability to form new tumorspheres over time as demonstrated by their multiplication potential in several experiments (also compare Figs. 4.8 (a) and 4.9 (a)).

Propagation of Cells Isolated from Mammospheres that Form on PDMS Substrates

To ensure that PDMS surfaces do not interfere or alter cells' regular activities, we then propagated cells that were isolated from mammospheres formed on PDMS substrates. In these experiments, cell attachment and colony formation capability of these isolated cells were separately examined. For cell attachment experiment, cell isolated from mammospheres formed on PDMS were dissociated and resuspended

in regular media. These cells were then cultured onto commercial tissue culture treated 96 well trays. Images were taken after one and two weeks of culture. As can be seen in Fig. 4.14, these cells make complete monolayer attachments and continue to proliferate consistently. This cell attachment is in fact comparable with that of the pre-mammosphere cultures.

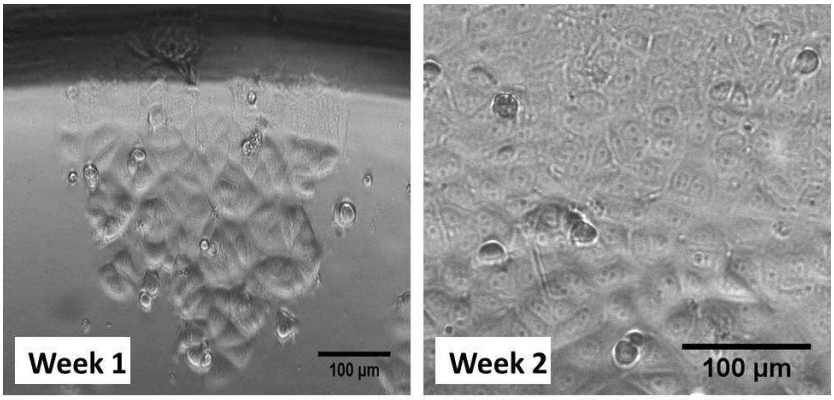

Figure 4.14: Propagation of cells isolated from mammospheres that had formed on PDMS substrates. These isolated cells that are cultured onto commercial tissue culture treated plates in serum rich media, form monolayer attachments after two weeks in culture.

To examine the spheroid formation capability of cells isolated from primary mammospheres formed on PDMS, the isolated cells were resuspended in fresh mammosphere media and cultured onto commercial low attachment plates. Images of mammospehres were then taken after one and two weeks of culture. As can be seen in Fig. 4.15, these cells successfully form spheroidal colonies, which are in fact larger in size than primary mammasopheres formed on both PDMS and low-attachment plates (Figs. 4.4 (a,b) and 4.5 (a,c)).

The result of these experiments together indicate that PDMS can successfully be utilized as an alternative to commercial hydrogel-treated low attachment plates.

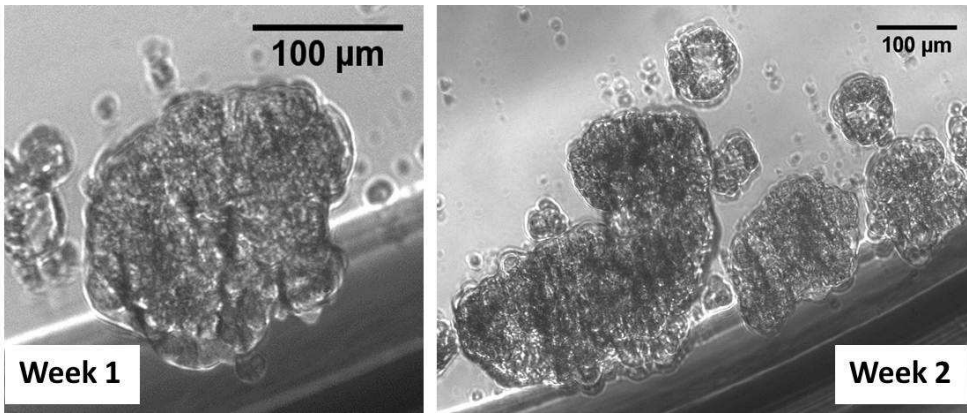

Figure 4.15: Propagation of cells isolated from mammospheres that had formed on PDMS substrates. The isolated cells formed secondary mammospheres when cultured onto commercial low attachment plates in serum free media.

In fact, PDMS has a few advantages over commercial plates, as it not only offers a low cost material for the isolation and enrichment of cancer stem cells, it also offers a practical solution for miniaturizing the assay into a microsystem as will be discussed in more detail in the following chapter.

Non-Transformed MCF10A Mammosphere Culture on PDMS

In previous sections, we demonstrated that PDMS is capable of driving mammosphere formation of epithelial breast cancer cell lines when a serum free culture medium is used. We also showed that this characteristic of PDMS is fairly comparable with that of the low-attachment commercial plates. To investigate the interaction of PDMS with non-cancerous cells, we then cultured MCF10A cells onto the polymer substrates as well as commercial low-attachment plates using mammosphere media. The MCF10A cell line, which is an immortalized non-transformed epithelial cell, is derived from human fibrocystic mammary tissue. MCF10A cells, which

are considered non-tumorigenic[8], have a near diploid karyotype and are normally used as normal breast epithelial cells in experiments. It has been shown in several reports that these cells do not have the ability to grow in anchorage-independent assays [177], however, they form 3-dimensional acini shaped structures upon culture onto a mixture of collagen and laminin [178, 179].

Consistent with previous reports [177], our experiment with MCF10A cells on commercial low-attachment plates did not result in colony formation as cells died soon after they were cultured in serum free medium onto these substrates. Interestingly though, MCF10A cells formed fairly round 3-dimensional structures upon culture onto PDMS substrates. Unlike mammosphere culture of MCF7 and MDA-MB-231 cells on PDMS, which resulted in 1-2 colonies per well, MCF10A cells formed quite a few number of colonies, which were spread across the PDMS surface as can be seen in Fig. 4.16. In rare cases though, one could find one or two cell attachments in addition to the colonies. These colonies as depicted in Fig. 4.16, continued to grow up until four weeks, where they then start to form dark shaped structures.

These results indicate that the PDMS surface is not only a suitable substrate for isolation and enrichment of cancer stem cells from breast cell lines, it also performs well in enrichment of normal stem-like cells. Consequently as demonstrated by this experiment, this polymeric substrate can be a better candidate for anchorage independent assays as compared to commercial plates. Whether PDMS substrates

[8]MCF10A cells do not have the ability to form tumors upon transplantation into mammary fat pad of mice models and are therefore considered non-tumorigenic.

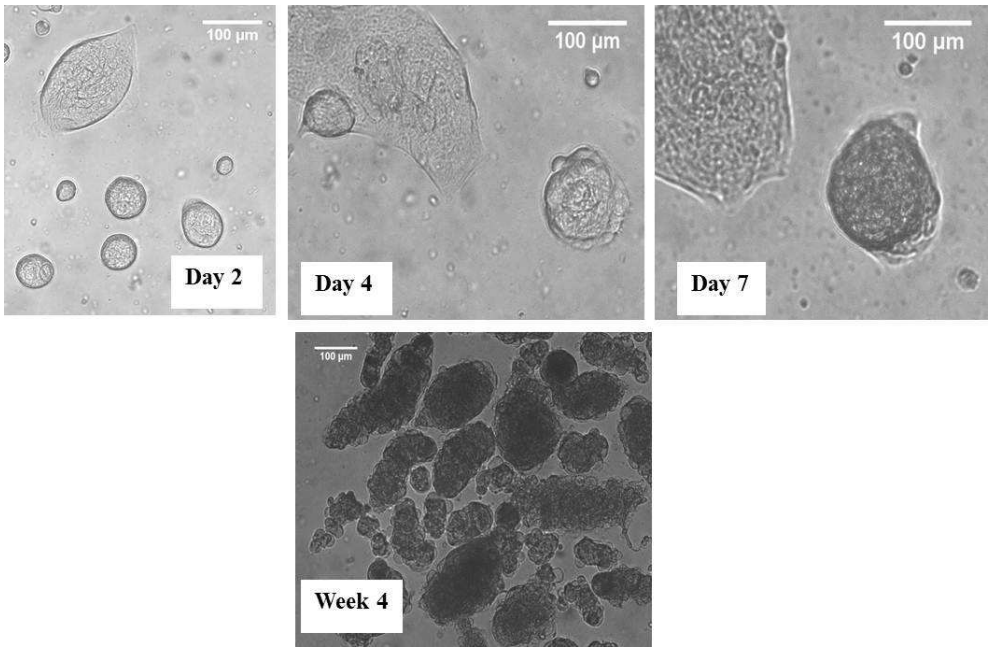

Figure 4.16: MCF10A mammosphere culture onto a PDMS substrate.

are as effective in isolation and enrichment of normal and cancer stem cells from other tissues and organs as they were in breast cell lines, is yet to be investigated.

4.3 Factors Involved in Cell-Material Interactions

As was demonstrated in previous sections, PDMS substrates offer a useful, straightforward and cost effective substrate for the isolation and enrichment of stem-like cells, especially in the study of tumorigenicity. In this section, we will continue to probe the characteristics of this polymer in driving mammosphere formation in the form of suspension culture, where the emphasis would be on characteristics important in cell-surface interactions. Studies have shown that there are a number of factors that are involved in the interaction between cells and their substrate. In

general, the transfer of signals from the substratum into the cells depends on the physiochemical nature of the material [180]. Properties such as surface chemistry or surface functional groups [181], roughness [182], hydrophobicity/hydrophilicity (or level of surface free energy) [181, 183–186], rigidity [187], surface charge [184, 188] and finally specific interaction with the cell surface can all affect cell activities. However, since cell-material interaction is a very complicated phenomenon, it is not clear which property plays the dominant role. In other words, the dominant factor might differ significantly from one system to another. In what follows, some characteristics important in cell-surface interactions will be discussed in order to unravel the driving force in mammosphere formation on PDMS substrates that is induced in the absence of serum.

- ***Surface Charge***

In any cell culture system, electric charges of the surfaces onto which cells are exposed have an eminent role in determining the cells' fate. This is due to the fact that a large portion of cell and serum protein surfaces are negatively charged and are therefore electrostatically more attracted to positively charged materials[9]. Within the same context, polymers with a negative surface charge density such as PDMS, can serve as a protein/cell repellent substrate and inhibit or delay cell attachments. The delay in cell attachment can then causes cancer stem cells to form mammospheres in the absence of serum. In the presence of serum, however some protein

[9]All cells have a considerable amount of both positive and negative charges however, the total ζ-potential of a cells surface is negative.

attachments occur possibly due to *van der Waals* interactions. This interaction, which leads to an inhomogeneous protein coating on PDMS substrates, will in turn mediate cellular attachments as can be seen in Figs. 4.6 (a,c) and 4.7 (a,b). On the other hand as was demonstrated in Figs. 4.4 (c,g) and 4.6 (c,g), cell attach to the surface of glass regardless of the media content. While clean glass slides are electrically neutral, they become deprotonated upon exposure to aqueous solutions where the hydroxyl (-OH) group on the glass surface can then enhance cellular attachments through hydrogen bonding with the polar groups on the cells' surface. In the presence of serum proteins, cell attachment onto glass is further enhanced due to proteins in the culture medium that will be presented onto the glass surface.

- **Protein Adsorption–Mammosphere Formation is Inversely Related to Protein Adsorption**

To further confirm the hypothesis that mammosphere formation on PDMS is driven by a resistance to cell attachment, we also investigated protein adsorption onto our PDMS substrates relative to glass. It has been shown previously that cell attachment to surfaces is correlated with protein adsorption on surfaces [184, 186]. That is, the same mechanism that works in favor of protein adsorption onto a surface can mediate cell attachment. It should be noted however, that protein adsorption is a very complex phenomenon and is driven by factors such as the acidic or basic nature of the protein and the characteristic of the surfaces onto which proteins interact. Additionally, the type of energies exchanged between proteins and their substrates,

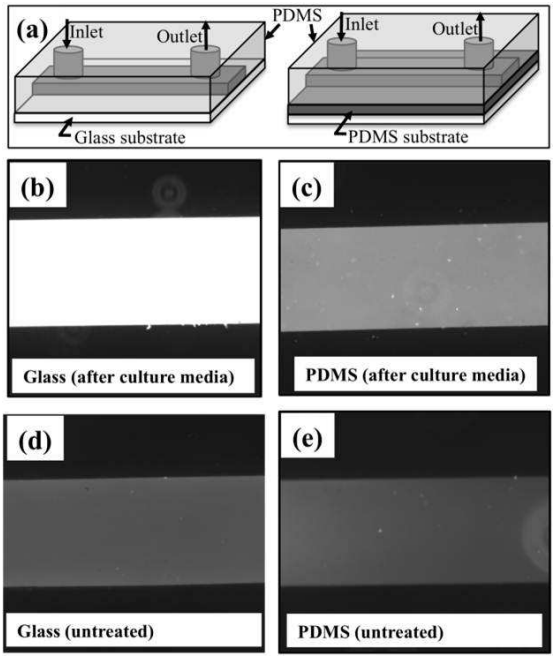

Figure 4.17: Protein adsorption to glass and PDMS. (a) Microfluidic channels with either a glass or PDMS bottom are used. Fluorescence images are aquired through the bottom surface. (b-c) Fluorescence image of FITC-labeled protein on (b) glass and (c) PDMS. (d-e) For reference, fluorescence images from (d) glass-bottom and (e) PDMS-bottom were collected after FITC-staining of untreated surfaces.

in the form of *van der Waals*, hydrophobic or electrostatic, play an important role in the protein-surface interactions.

To determine the protein adsorption to the substrates, we passed regular cell culture media, which contains protein-rich serum through a microchannel with either a glass or PDMS bottom (Fig. 4.17 (a)). After rinsing, we passed FITC through the channels; the FITC fluorescently labels any adsorbed proteins on the channel surface. To account for possible residues that FITC might leave behind, we also did a negative control on glass and PDMS substrates that were not exposed to media. The fluorescence micrographs in Fig. 4.17 show that serum protein adsorption is much lower on PDMS as compared to glass. A comparison between Figs. 4.17 (c)

and 4.17 (e) indicates that the fluorescent intensity on PDMS substrates is in fact mainly due to residues that FITC leaves behind on any surface, regardless of its coatings. Nonetheless, the fact that serum protein does not adsorb well to PDMS suggests that cells will have difficulty forming attachments, which is consistent with the formation of mammospheres (Fig. 4.4). Meanwhile, the high protein adsorption onto glass suggests that cells can quickly form attachments onto a glass substrate, as shown previously in Figs. 4.4 and 4.6.

- **Surface Wettability – Hydrophobicity Can Drive Mammosphere Formation**

Surface wettability (hydrophobicity/hydrophilicity), which is a measure of surface free energies has a great impact on how cells and proteins interact with the substrate. The hydrophobicity of a surface, which is determined by contact angle measurements, mainly depends on the functional groups that form the outermost layer of the material. For instance while hydroxyl groups on the surface of glass make it more hydrophilic, the highly hydrophobic property of PDMS can be related to the methyl groups that present the surface of the polymer. In addition to the effect that functional groups impose on the degree of surface wettability, surface roughness can also significantly affect hydrophilicity or hydrophobicity of a substrate. In other words, rough surface textures can serve as physical barriers and prevent water droplets from spreading/flattening on the surface of the material. This was in fact confirmed by our experiment in which a moderately rough PDMS surface with a

feature size of $\sim$ 80 nm displayed a higher contact angle than a smooth glass surface (R $\approx$ 4 nm). In the context of protein/cell attachment, it is recognized that a moderately hydrophilic surface with a contact angle of 40°-50° has a significantly higher level of attachment than highly hydrophobic or hydrophilic surfaces [183–186]. Since cells in suspension culture in general require a protein and cell repellent substrate to grow on, a suitable surface for mammosphere culture can therefore be one with a highly hydrophobic (or highly hydrophilic) property. Consistent with this theory, as has been discussed in previous sections we successfully formed mammospheres on PDMS substrates, which display a highly hydrophobic characteristic. In fact this hydrophobicity, which delays cell attachment, is the dominant factor (besides surface charge densities) that forces cells with stem like properties to survive and proliferate in serum free media by growing into unattached spheroidal colonies.

To quantify the wettability of our substrates, we measured the contact angle of the substrate materials used in Figs. 4.4 and 4.6. The hydrophobicity/hydrophilicity of each surface was determined using the static sessile drop technique to measure the contact angle of a water droplet that is pipetted onto the surfaces (Fig. 4.18). The contact angles are shown in Table 4.2. As can be seen from this table, while TC-treated plates exhibit an average contact angle of 46° (even before media exposure), clean glass slides with a contact angle of 22°, acquire the right hydrophobicity for cell attachment (contact angle of 42°) after they are exposed to a serum rich medium. In contrast, PDMS exhibited an average contact angle of 99°, which is significantly more hydrophobic than the two optimized cell attachment surfaces. As a result, the delayed cell attachment is logical, given that cells typically show the

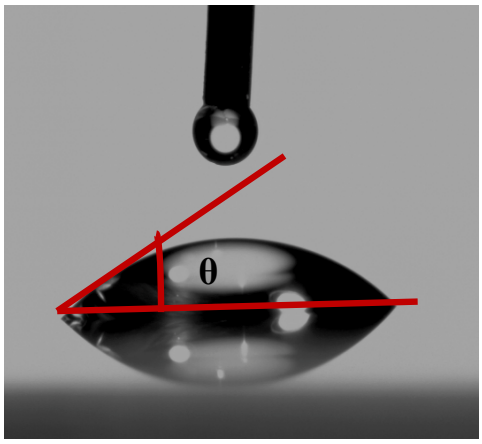

Figure 4.18: The hydrophobicity/hydrophilicity of a surface can be determined using the static sessile drop technique that is used to measure the contact angle of a water droplet pipetted onto the surface.

highest attachment to moderately hydrophilic surfaces and do not attach well to hydrophobic surfaces as in PDMS or hydrophilic substrates as in commercial low attachment plates [183–186].

Substrate	Contact Angle
Low-Attachment Plates	29°
TC-Treated Plates	45°
PDMS Plate	99°
PDMS conditioned with regular media	76°
PDMS conditioned with mammosphere media	93°
Glass (piranha washed)	22°
Glass conditioned with regular media	42°

Table 4.2: Contact angle of the surfaces used for cell culture.

Fig. 4.17 (c) indicates that minor protein adsorption occured on PDMS in the presence of serum proteins. In fact, upon exposing PDMS to serum-rich regular media for two days, the contact angle decreased from 99° to 76°, suggesting a chemical or conformational change at the solid-liquid interface, which might be due to a partial or complete protein adsorption onto PDMS. However, since the orientation of PDMS polymer chains are random, proteins will attach and spread randomly on the surface of the polymer. This was in fact proved by large variations in the contact angle values across the surface of PDMS. It should be noted that the change in contact angle of PDMS was significantly lower (less that 10 degrees decrease) when mammosphere media, which does not contain serum was used.

Regarding cell attachment onto PDMS substrates where cells were cultured in regular media, evidently, the modest protein adsorption from the serum in the media can be enough to mediate some degree of cell attachment via protein-protein interactions, as cells were able to attach slowly to PDMS in the presence of serum (Fig. 4.6 (a, e)). However, in mammosphere media, there is no serum present, and thus minimal quantities of proteins to modify the surface of PDMS. With little protein on the surface, the cell must attach through adsorption of the membrane proteins to the surface. According to Fig. 4.6, cells are able to attach to the moderately hydrophilic glass[10] and TC-treated surfaces, but not to the hydrophobic PDMS surface as discussed previously. Just as is the case for the commercial low attachment plates, this lack of attachment to PDMS drives cancer stem cells as well

[10]While acid washed glass slides used for these experiments had a contact angle of 22°, they become more hydrophobic after exposure to serum rich media as indicated in Fig. 4.17 and Table 5.2.

as non-tumorigenic MCF10A cells to form mammospheres.

CONCLUSION

In this chapter, we showed that PDMS can serve as a substrate for mammosphere formation from breast cancer cell lines. We conclude that the hydrophobic property of PDMS in addition to its negative surface charge density drives the mammosphere growth because it provides sufficient resistance to cell attachment. This result has tremendous implications for the study of cancer stem cells, as it implies that PDMS can be used to construct mammosphere culture microsystems as will be discussed in next chapter. This development will also enable mammosphere assays to be integrated into a microsystem with other microfluidic functions, including tumor cell selection and purification, in-situ immunofluorescence, RT-PCR, and drug screens with concentration gradients. These integrated microsystems have the potential to dramatically simplify current techniques for studying cancer stem cells and may lead to experiments that are not possible today, such as the enrichment and drug screening of cancer stem cells from a population of metastatic circulating tumor cells.

Chapter 5

Development of a Mammosphere Culture Microassay*

For nearly a decade, researchers have identified breast cancer stem cells within heterogeneous populations of cells by utilizing low-attachment serum-free culture conditions, which as was discussed in previous chapters, lead to the formation of spheroidal colonies (mammospheres) that are enriched for cancer stem cells. While this assay has proven to be useful for identifying cancer stem cells from a bulk population, ultimately its utility is limited by difficulties in combining the mammosphere technique with other useful cellular and molecular analyses. However, integrating the mammosphere technique into a microsystem can enable it to be combined directly with a number of functions, including cell sorting and analysis, as well as popular molecular assays that would enable new discoveries in the biology of cancer stem cells that are not possible today.

In this chapter, we will discuss the first ever mammosphere culture microsystem that was developed in our lab using concepts discussed in the previous chapter.

*Some of the material in this Chapter is included in "Breast cancer stem cell enrichment and isolation by mammosphere culture and its potential diagnostic applications" submitted to the journal of Expert Reviews in Molecular Diagnostics and in " Enrichment of tumor-initiating breast cancer stem cells within a mammosphere-culture microdevice", which is in preparation.

In chapter 4, we demonstrated that properties such as hydrophobicity can drive polymer surfaces such as polydimethylsiloxane (PDMS) to selectively promote mammosphere formation (a functional property that is comparable with commercial low attachment plates). However, in order to have a long term mammosphere culture in a microsystem there are few critical problems that have to be taken into account. These problems, including media evaporation and air bubble formation, which impose serious limitations on long term cell culture in any biological microassay, are more pronounced in mammosphere culture microsystems as these cell entities are in suspension and therefore more difficult to handle.

In this chapter, we will first discuss how biology and medicine can benefit from microsystems. Then we will return to the subject of mammosphere culture and its implementation into a microdevice. Issues such as evaporation and bubble formation, which are critical problems in long term cell culture, are discussed along with strategies that we exploited to overcome these problems. After the successful culture of mammospheres in the final design, the device was tested for applications such as zero velocity delivery of media and reagents to the spheroidal colonies that are in suspension. In order to incorporate a convection free transport system into the culture device, two different approaches were employed as will be discussed in this chapter. The final design was then utilized to isolate and enrich breast cancer stem cells in the form of suspension colonies, which were then stained through the diffusion system with a Live/Dead stain. The device was further used to transform stem cell colonies into a fully differentiated monolayer cell attachment on the same substrate. Finally the results of these experiments were analyzed using a finite ele-

ment analysis technique.

5.1 Microsystems for Biomedical Applications

Microsystems that were originally designed for electronic devices are usually referred to as miniaturized platforms and are made up of features that have micron sizes in at least one dimension. While the birth of this technology goes back to 1960, where miniaturized systems were developed in the form of microprocessors, the implications of this technology have been expanded to other fields such as chemistry, biology and biomedicine. A lot of efforts have been made in developing laboratory on a chip (lab on a chip) devices that would enable the integration of major diagnostic operations (performed in a hospital) on a single microfluidic device.

The first lab on a chip devices were developed on silicon or glass, using a heavy microelectronics infrastructure. In 2000 the technology shifted toward using polymers such as polydimethylsiloxane (PDMS) to mass produce biomedical microdevices using soft- or photo-lithography techniques. Since the first integration of PDMS into microdevices, this polymer has been widely used in microfabrication due to its unique characteristics such as optical transparency, biocompatibility, deformability (which also enables easy molding), thermal stability and most importantly its affordability, which is an important factor in production of biomedical devices.

In addition to biomedical applications of micrsystems designed for implants and/or diagnosis purposes, microdevices offer a unique platform in studying cellular and molecular biology of diseases, such as cancer [189–196]. The micron size struc-

tures, which can be made to approximately the size of a single cell have made it possible to study biological events at the cellular level. Microfabrication technology has also made it feasible to design microsystems with the capability of spatial and temporal bioscreening. Other applications include systems with concentration gradients of drugs and reagents [197–200]. These lab on a chip devices are especially useful in studying cells' response to different concentrations of drugs, reagents, and other stimuli such as pH, oxygen, etc. that are essentially impossible to implement using conventional assays.

In addition to single purpose microdevices, microsystems can provide increased automation and throughput by seamlessly integrating multiple functions, thus eliminating tedious manual steps. This is particularly advantageous in the case of samples that are difficult to manipulate, such as a small number of floating cells. Today the microsystems research community is actively developing a number of tools for diagnostics, as has been documented in a number of recent review articles [201–203]. All of these and many other microfluidic techniques simplify and automate assays in hopes of minimizing the cost and labor required for clinical diagnostics today. Clinical applicability of these techniques can further be improved by integrating multiple functions such as sample preparations into one single device.

5.2 A Mammosphere Culture Microsystem for the Isolation and Enrichment of Tumor Initiating Cells

Implementing the mammosphere assay in an integrated microsystem may lead to one of the first clinical applications of microfluidic cell culture specifically in the field of cancer research as it can be used as a reliable technique for enriching breast cancer stem cells from small sample volumes and for further drug screening. It should be noted however, that while cell studies in microfluidics and microsystems, including monolayer cell culture as well as spheroid culture have been reported in several contexts [194, 204–208], the few suspension culture microsystems that have been developed to this date are mainly focused on cell aggregates in the study of embryoid bodies [207, 208]. While these microsystems have enabled the formation of spheroidal colonies from embryonic stem cell aggregates, they require complex fabrication or surface modification of culture substrates in order to induce suspension cultures. In addition, in some of these devices, subsequent cell retrieval and analysis is almost impossible due to the structure of the device. While these enabling microsystems were developed to enrich embryonic stem cells, no report has been found on utilizing microdevices for the isolation of rare cancer stem cells from a population of differentiated cells and the following enrichment that is achieved through spheroidal culture. In this work, a tumorsphere culture microsystem was developed for the first time that would allow a label free isolation of breast cancer stem cells in an easy to fabricate and user friendly device. This platform, which enables 3-dimensional cell culture onto a microsystem (which recapitulates the in

vivo tumor morphology) can further be used for immunostaining and drug screening studies, some which are not possible using commercially available macro-assays. As will be discussed in this chapter, this device can additionally be used to stimulate cancer stem cell colonies to differentiate on the same device. In other words, using this microdevice we were able to demonstrate for the first time a polymeric system that can be used to visualize the transition from mammospheres to monolayer culture owing to certain characteristics of PDMS. This characteristic can be used in future studies to shed some light onto the mechanism of mesenchymal to epithelial transition (MET), one of the processes that are hypothesized to be responsible for tumor metastasis. Additionally, integrating the CSC enrichment assay with other on-chip functions, such as cell enrichment, immunofluorescence, drug screening, and molecular analysis, can lead to new methods for studying the biology of cancer stem cells.

Fabrication of an Anchorage Independent Cell Culture Microdevice

While cell culture has been performed in microfluidics in a number of reports [204–207], the requirements of a mammosphere culture microsystem are more severe. In cell culture microdevices with a convectional transport system, the cells are attached to the surface and reagents, such as fresh media, drugs, cytokines, and staining agents, can be loaded over the top of the cells. However, in a mammosphere culture microdevice, cells should be inhibited from attachment. Since the spheroids are unattached, it is practically impossible to apply convection flow to the microfluidic culture system in order to renew the media or deliver reagents.

Driving reagents to the cells with convective transport would force the unattached cells out of the microsystem. Therefore, it is necessary to deliver reagents to the cells via diffusion-based transport. To incorporate a diffusion compartment into the device we exploited different strategies such as vertical integration of diffusion microchannels or integration of microporous membrane into the culture device. In each approach, the diffusion component that is placed between the cell culture chamber and the reservoir on top, is used to mediate fluidics exchange between the two layers in a near-zero velocity manner. The details of each approach are discussed in following subsections.

Evaporation through the gas permeable PDMS based device poses another problem for the long term culture as the low volume liquids in the cell culture wells are quickly consumed or dried out. To resolve this problem, we incorporated a media reservoir on top of the cell culture chamber. This reservoir will shield the culture chamber from evaporation of media during the extended time required for mammosphere culture. In addition it provides cells in the bottom layer of the device with fresh media through diffusion based components that are integrated into the microsystem.

In all these designs PDMS was used as the base of the cell culture micro-wells as well as the sidewalls of the device as PDMS is (a) an excellent gas permeable material and would therefore enable the exchange of O_2 and CO_2 between cells and their environment, (b) hydrophobic, which delays cell attachment and successfully drives the formation of mammospheres (as discussed in previous chapter) and (c) it is conveniently used for soft-lithography based fabrication and assembly of microde-

vices.

5.2.1 Mammosphere Culture Microdevice with Integrated Diffusion Channels

The first design included a microfluidic system consisting of a cell culture layer with triangular wells, a microchannel for cell loading, and a media reservoir that was aligned on top (Figs. 5.1 and 5.2). In this design, the reservoir is connected to the wells through vertically designed diffusion channels where media and reagents are delivered to the non-adhered cells in a near zero velocity manner. The fabrication procedures and consequent mammosphere culture on this device is discussed below.

EXPERIMENTAL

Fabrication of the Mammosphere Culture Microsystem

The step by step assembly of the layered microfluidic device is presented in Fig. 5.1. Soft lithography was used to microfabricate the features in the PDMS mammosphere culture system. First, the 1 mm wide microchannels and $2 \times 2.2 \times 2.2$ mm^3 triangular cell culture wells were designed in AutoCad. In this design the dimensions of the wells are chosen such that they can contain spherical colonies of 200 μm in diameter (and larger) and are patterned such that an array of 3×4 wells can fit into $1'' \times 1''$ templates. The wells and the microchannels that pass the top of the wells in the final structure are approximately 5 mm apart (Fig. 5.1). This distance that is large enough, will prevent possible bridging and dislocation of

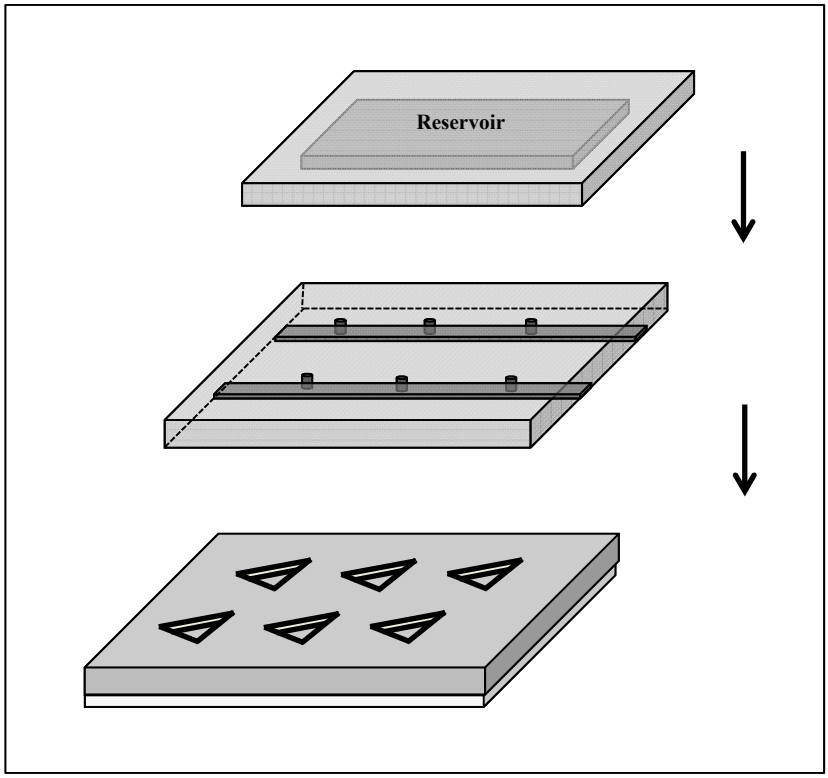

Figure 5.1: The PDMS micro-wells, microchannels and the reservoir are brought into contact and permanently bonded to each other. Before bonding the reservoir to the rest of the device, vertical diffusion channels are punched into the microchannels in order to connect the microwells to the reservoir in the final structure.

the contents in the adjacent wells; a phenomenon that normally occurs in closely designed micropatterns. Transparency masks of the designs were printed to fabricate the molds for soft lithography.

Two standard 4 in. silicon wafers (one for the micro-well layer and one for micro-channel layer; see Fig. 5.1) were used to fabricate the master molds. Each wafer was first washed in a 1:3 dilution of HF in DI water, followed by a water rinse. The clean wafers were then dehydrated at 120°C for 20 min. Then Hexamethyldisilazane (HMDS) was spin coated on the wafer for 30 s. To etch the designed features into the wafer we used AZ-4620 photoresist, which is suitable for relatively

deep etching. The photoresist was first preheated to room temperature and then spin coated onto the wafer for 1 min at 1500 rpm. The wafer was then baked for 3 min on the hot plate, rehydrated at room temperature for 20 min, UV exposed through the mask for 17 s using an EVG 620 mask aligner and developed in a 1:3 dilution of AZ 400K developer for 4 min. A Deep Reactive Ion Etcher (DRIE) was then used to etch the negative of the microsystem features to a depth of 200 μm. This thickness, along with other dimensions, was chosen to allow for large colony formation in micro-wells as mentioned earlier.

To facilitate PDMS removal after replica molding, the master mold was coated with trichloro(1H,1H,2H,2H,-perfluorooctyl)-silane in vapor phase for 45 minutes using a vacuum desiccator. PDMS was then mixed in a 1:10 ratio of curing agent and prepolymer, poured onto the master, degassed in a vacuum desiccator and cured in an oven (65°C) for two hours.

To fabricate the reservoir, we used multiple layers of electrical tape to assemble a $10 \times 10 \times 2$ mm structure on a petri-dish surface. This structure served as the mold for the reservoir. The PDMS mixture was then poured onto the mold and cured in the oven.

The PDMS replica of the micro-wells and microchannels was washed in ethanol, plasma oxidized using a corona discharge and then brought into contact such that the wells and channels were facing each other (Fig. 5.1). Before binding the reservoir to the rest of the device, using a 0.35 mm hole puncher, holes were punched into the microchannels directly above the cell culture wells. These columns serve as the diffusion channels that later connect the micro-wells to the reservoir that is

positioned on top of other two layers (Fig. 5.1).

Cell Culture

LA7 cells (rat mammary carcinoma cells) were grown in DMEM with 4 mM L-glutamine, 4.5 g/L glucose and reduced bicarbonate (1.5-2.0 g/L) supplemented with 5 μL/mL Penicillin/Streptomycin, 50 ng/mL hydrocortisone, and 10% FBS. Cells were subcultured every 5-7 days by trypsinization with 0.25%/0.02% trypsin/EDTA in a 37°C incubator with 5% CO_2. To prepare cells for mammosphere formation, cells removed from flasks were spun down and resuspended in DMEM media supplemented with B27, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor and 4 μg/mL heparin. To get a single cell suspension, the cell mixture was passaged repeatedly through a 0.45 mm syringe needle.

After full assembly of the device, the micro-chip was sterilized by a one hour UV exposure in the cell culture hood. Using a syringe pump, single LA7 cells in mammosphere media were then loaded into the microdevice (through tubings that connected the device to the pump) at a speed of 1 mL/hr. The cells in the microsystem were then incubated in a humidified atmosphere with 5% CO_2 at 37°C for 48 hours before the first observation.

RESULTS

Mammosphere culture in microfluidic chips

The fluidic mammosphere culture microdevice, as shown in Figs. 5.1 and 5.2, consists of three layers: a cell culture layer with an array of 3×4 micro-wells, a layer

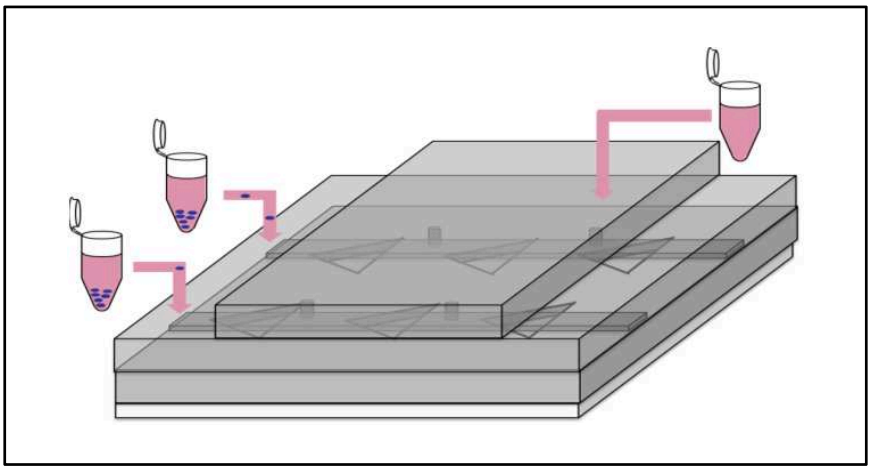

Figure 5.2: A three layer mammosphere culture microdevice. The micro-wells at the bottom of the device are loaded with cells through the microchannels, which pass the top of the wells. The reservoir shields the fluidics in the device from fast evaporation in addition to delivering media/reagents to the non-adhered cells through vertical diffusion channels.

with cell delivery micro-channels, and a reservoir layer that is designed to provide cells with media/reagents while preventing evaporation in the cell culture layer. The triangular geometry of microwells is selected because the initial random movements of the cells tend to carry them into the corners of the triangular wells where they become stationary, and thus it is easier to track individual colonies over weeks of culturing. This cell/colony trapping is not possible using a circular design.

Another consideration in designing cell culture microsystems is keeping cells that are exposed to significantly low amounts of media viable for a reasonably long period of time. Evaporation, which is unavoidable in any cell culture microsystem (made of vapor permeable polymers such as PDMS) imposes a serious stress on cells as it affects the osmolarity. In other words, osmalarity, which controls the balance between exterior and interior fluid pressure on cells' membrane can easily

be affected in cell culture microdevices. To keep the humidity of the cell culture microenvironment intact during the culture period, fresh media should be periodically delivered to cell containing microwells. While in attachment based cell assays this is accomplished by periodic or constant perfusion of media that is delivered from the top of the cells, using the same techniques in suspension cultures is not practical as it will force the unattached cells and colonies out of the microdevice. In order to refresh media in mammosphere culture microsystem in addition to keeping the level of media in the cell culture chamber intact, we therefore implemented diffusion channels into the system that vertically connects cell culture microwells to the nutrition rich chamber that is on top. These vertical channels were mainly intended to support the diffusion of fresh supplements into the cell contained microwells. These microchannels were designed such that cells in microwells would not sense any turbulence when media is replaced on the top chamber (reservoir).

While by using this microfluidic device we successfully cultured small colonies of approximately 150 cells after 3 days of culture (Fig. 5.3), we could not grow them beyond this time point as air bubbles that had filled the diffusion channels made it practically impossible to deliver fresh media to the drying microwells. In addition,

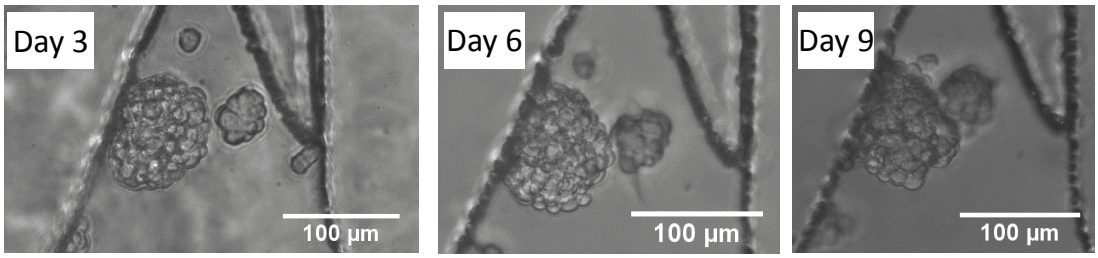

Figure 5.3: Time course growth of LA7 mammospheres within the triangular-shaped cell culture wells in the microsystem with integrated diffusion microchannels. Using this design, cells cease to grow after 3 days of culture.

nucleation of bubbles in cell delivery microchannels (second layer in Figs. 5.1 and 5.2), which are directly in contact with microwells, can rupture the cells' membrane and lyse them over time. To overcome these limitations, other approaches were taken, as will be discussed in next subsection.

5.2.2 Mammosphere Culture Microdevice with Diffusion Based Membranes

Microfluidics, which are widely used in cell culture microsystems for the delivery of media and reagents such as stains and drugs can also impose difficulties in the functional aspect of the device (as was mentioned previously) due to air bubble formation in micron size channels. These air bubbles that impede the flow path are often introduced to the system during the loading step where fluidics from wide tubings enter significantly smaller channels. As we demonstrated in the previous subsection, bubble formation in a microfluidic mammosphere culture microdevice can be even more problematic than attachment based culture devices. To avoid this problem, we exploited simple engineering techniques that would free us from microchannels as well as tubings in the cell culture microdevice. It should be noted that while these strategies are employed here for a suspension culture microdevice, they can as well be incorporated into any system including cell attachment based microassays.

In this design the mammosphere culture microsystem is composed of a cell culture chamber and a fluidic reservoir (no microchannels are used); culture wells

are connected to the reservoir on top by a microporous membrane. Reagents are exchanged within the reservoir where they diffuse through the membrane and into the cell culture microwells. In addition, as was mentioned earlier the reservoir also aids in preventing evaporation of media from the low-volume cell culture region.

A schematic representation of the microsystem is presented in Fig. 5.4. As in the previous design, a PDMS substrate with triangular microwells designed into it serves as the bottom surface of the cell culture device. The cell culture chamber is capped by a transparent and hydrophilic polycarbonate membrane with pores that are approximately 2 μm in diameter. The reservoir is attached onto the top of the membrane; reagents are loaded through the reservoir and are fluidically connected to the cell culture volume through the membrane.

Using this device, we were able to form mammospheres from MCF7 breast cancer cells where colonies as large as 250 μm in diameter formed over the course of 10 days in culture. To demonstrate that new media and reagents can be delivered to the mammospheres in the microsystem, colonies were stained through the diffusion membrane using a Live/Dead stain. A COMSOL simulation was then used to determine the concentration of dyes that had diffused to the bottom of the cell culture microwells. A detailed discussion on these simulations are given at the end of the chapter. In addition to staining colonies with a Live/Dead stain, we demonstrate for the first time that exchanging mammosphere media with regular media after the colonies are formed will result in a biological response where mammospheres start differentiating as they form monolayer cell attachments. This collection of results demonstrates that cancer stem cells can be isolated, enriched, and studied within a

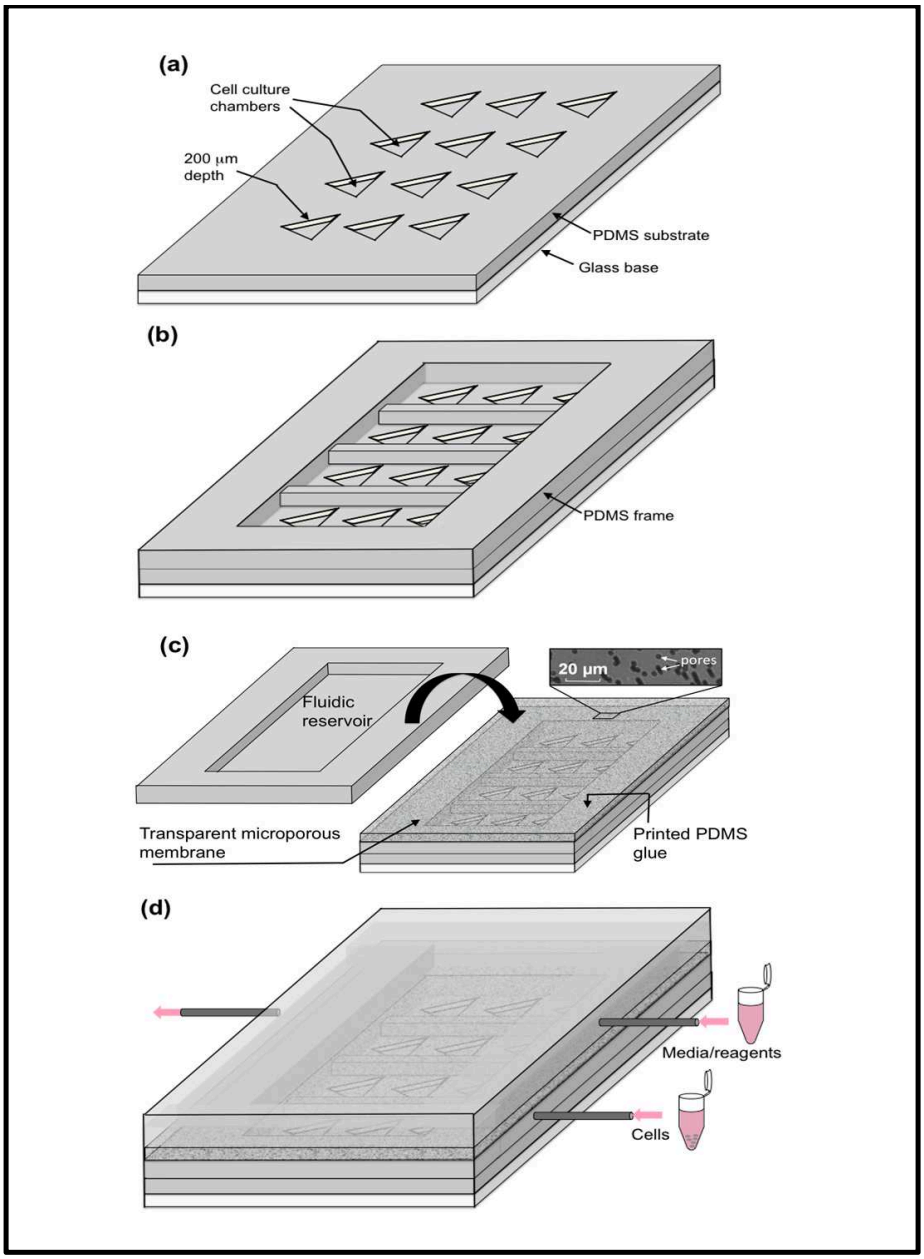

Figure 5.4: Diffusion based mammosphere culture mircodevice. (a) PDMS with triangular-shaped wells serves at the bottom surface of the mammosphere culture microsystem. (b) PDMS sidewalls are added to isolate rows of culture wells, if necessary. (c) A transparent microporous membrane is fixed above the culture wells by depositing PDMS around the edge of the device and curing the membrane in place. A PDMS reservoir is placed on top of the membrane. (d) To start the assay, cells are loaded into the culture wells with a syringe. Fresh media and other reagents are loaded through the reservoir. The microporous membrane enables diffusion-based exchange of molecules between the reservoir and the culture wells.

PDMS microdevice.

EXPERIMENTAL

Fabrication of mammosphere culture microsystem

The device assembly is illustrated in Fig. 5.4. Soft lithography techniques were used as discussed before to fabricate triangular cell culture microwells in PDMS with a perimeter of $2 \times 2.2 \times 2.2$ mm. 2 mm thick walls were then permanently bonded to the PDMS layer to separate the microwells. This layer would later form the base of the microsystem.

To contain the cells and culture media, a frame was cut from a PDMS slab 2 mm in thickness and attached on top of the PDMS substrate with triangular microwells (Fig. 5.4 (b)). In this step, a PDMS replica of the micro-wells and the PDMS frame were washed in ethanol, plasma oxidized using a corona discharge and brought into contact. The device was baked at 65°C for 30 min to allow the frame to bond to the cell culture layer. The reservoir was then aligned on top of the first layer with the opening of the two chambers facing each other. In this design, the porous membrane separates the two chambers as depicted in Fig. 5.4 (c). For the membrane we tested three different materials, a cellulose paper filter, a polyester membrane (0.4 μm pore size) and a hydrophilic polycarbonate membrane (2 μm pore size). To attach the membrane to both chambers, PDMS pre-polymer and curing agent were mixed at a 1:10 ratio and applied around the perimeter of the device, thus acting as glue. The device was baked at 65°C for 30 min to permanently attach the device.

Cell Culture

MCF7 cells were grown in DMEM with 4 mM L-glutamine, 4.5 g/L glucose and reduced sodium pyruvate supplemented with 5 μL/mL Penicillin/Streptomycin, 1% Non-essential amino-acids, and 10% FBS in tissue-culture-treated T25 flasks. Cells were subcultured every 5-7 days by trypsinization with 0.25%/0.02% trypsin/EDTA in a 37°C incubator with 5% CO_2. To prepare cells for mammosphere formation, cells removed from flasks were spun down and resuspended in MammoCult^{TM} Basal Medium (Human) supplemented with 10% MammoCult^{TM} Proliferation Media. To obtain a single cell suspension, the cells were passed repeatedly through a 0.45 mm syringe needle.

Mammosphere Culture in the Microsystem

Before loading the cells, the microsystem was sterilized by UV exposure in a cell culture hood for approximately one hour. A cell dilution of 10^3 cells/mL was then manually loaded into the bottom layer of the device by injection (Fig. 5.4 (d)). This was implemented by inserting the syringe needle into the PDMS frame of the cell culture chamber; after removing the syringe, the PDMS reforms, thus closing the hole formed by the syringe tip and preventing media from leaking. In addition, this tubing free technique (which is applied for the first time), eliminates the risk of evaporation and bubble formation, which occurs in long term culture microsystems due to wide openings at the inlet/outlet ports and long tubings. The same loading technique was used to load mammosphere media into the reservoir. After loading

cells into the culture chamber and media into the reservoir, the microchips are then placed in a cell culture incubator (37°C, 5% CO_2) for 48 hours before observing the first mammosphere formation.

Cell Staining in the Microsystem

A Live/Dead Viability/Cytotoxicity Kit was used to stain the cells in the micro-wells. Fresh media was supplemented with 0.1% Calcein AM (Live stain) and 0.1% Ethidium homodimer-1 (Dead stain). The Calcein AM probes the viability of the cell by measuring its intracellular esterase activity, while the Ethidium homodimer-1 determines the plasma membrane integrity. Once Calcein AM diffuses into the cell, an enzymatic reaction within live cells converts the non-fluorescent Calcein AM to Calcein, which is fluorescent. On the other hand, the cells with a damaged membrane allow the "Dead" stain to penetrate into the cell and bind to nucleic acids, resulting in a red fluorescent light when excited at 495 nm. Using a syringe pump, the stain containing media was loaded into the reservoir at a speed of 1 mL/hr for 1 hour. The microdevice was then placed in the cell culture incubator for another hour before observing the cells (in order to generate the fluorescent Calcein from Calcein AM, the cells must be warmed to 37°C). Images of the fluorescent cells were obtained with an Olympus IX-51 microscope.

Exchanging Mammosphere Media with Serum Rich Media in Mammosphere Culture Microdevice

After 10 days of culture, mammosphere media (including the growth factors)

in the reservoir was replaced with regular media, which is rich in serum proteins. This media exchanged was implemented using a syringe pump, at a speed of 1 mL/hr for 1 hour. Images of cells were obtained after 48 hrs.

RESULTS

Mammosphere Culture in Microchips

Figure 5.5 presents the formation of mammospheres over the course of 10 days at two different locations in the triangular-shaped culture wells of the microsystem. The micrographs in Figs. 5.5 (a-d) are imaged in one single location, while those in Figs. 5.5 (e-h) are recorded at another location. Mammospheres were also imaged at several other locations throughout the microsystems. Three days after cells were loaded into the microsystem, mammospheres on the order of 50-75 μm could be observed throughout the microwells (Fig. 5.5 (a,e)). Over the course of ten days, the mammospheres continually increased in size up to approximately 250 μm in diameter.

Diffusion Based Reagent Delivery

In conventional microfluidic cell culture assays, media renewal and reagent delivery is a straightforward process since cells are attached to the culture surface and thus can withstand convective delivery of solutions. However, as discussed earlier, the same delivery techniques are not applicable to suspension cultures as in a low-attachment cell culture microsystem, convective flow would flush the cells out of the chamber. As a solution, we fluidically connected the cell culture chamber

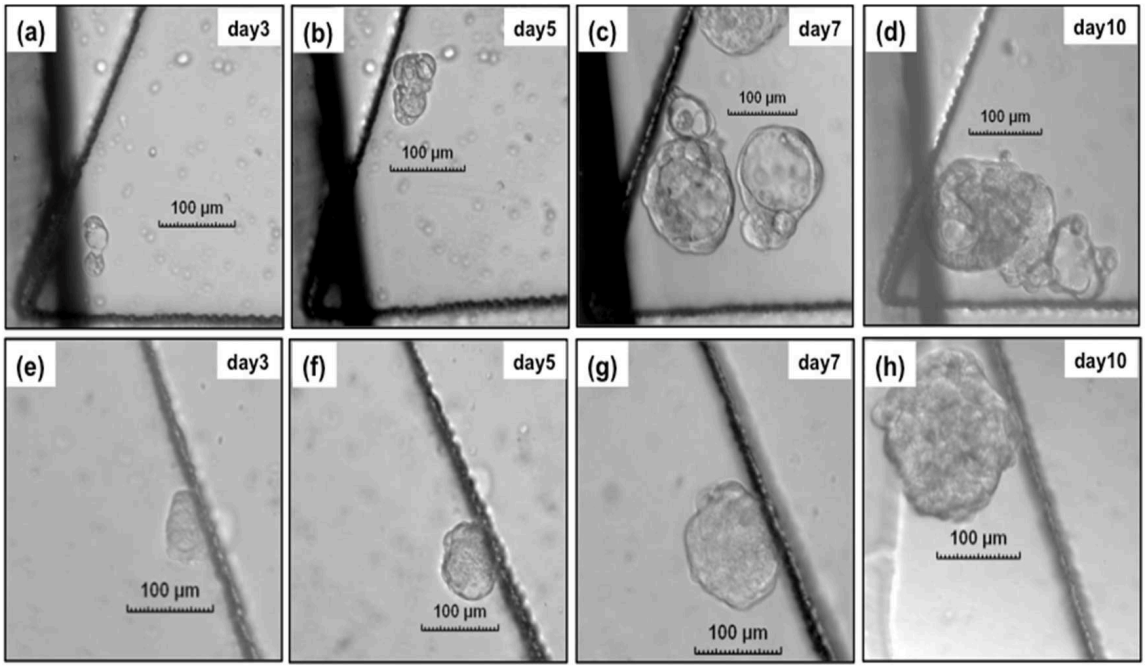

Figure 5.5: Time course growth of MCF7 mammospheres within the triangular-shaped cell culture wells in the microsystem with a membrane based diffusion system. (a-d) shows the emergence of a mammosphere in one location of the microsystem while (e-h) shows the emergenece of a mammosphere in another location.

with the reservoir using a microporous membrane (Fig. 5.4 (c)). For this purpose, three membranes were tested; cellulose filter paper, polyester membrane with a 0.4 μm pore size and a hydrophilic polycarbonate membrane with a 2 μm pore size. The pore sizes were chosen such that the risk of cell migration through the membrane is completely eliminated. In other words, the membrane ensures that all cells loaded into the cell culture chamber at the bottom layer are trapped, while also enabling diffusion-based mass transport of reagents from the reservoir.

While the polyester membrane proved to be ineffective in transporting fluidics due to its hydrophobic surface characteristic and its ultra-small pore size, we were able to exchange media between two chambers using either filter paper

or a hydrophilic polycarbonate membrane. However, since paper is an optically opaque material, it does not offer the best solution for a cell culture microsystem that requires periodic screening using a microscope. We therefore used transparent hydrophilic polycarbonate membranes as a transport system in our mammosphere culture microdevice. It should be noted that loading media or reagents through the reservoir, does not cause any convective flow through the membrane. However, molecules can be exchanged between the reservoir and the culture chambers due to diffusion. This enables media renewal and reagent delivery to the cells.

To verify that the porous membrane is functional in delivering media and reagents to the cell culture chamber, we loaded culture media that contained (a) small molecules such as stains (we used a Live/Dead fluorescent staining reagents) and (b) large protein molecules such as serum proteins in FBS. In the staining experiment, finding fluorescently labeled cells in the cell culture chamber indicates that the reagents are successfully delivered to the cells, while in the other experiment where mammosphere media is replaced with regular media, a biological response is expected.

In the Live/Dead staining experiment, after preparing the stains in cell culture media, the reagent was loaded into the reservoir using a syringe pump at room temperature, as described above. Following injection of the Live/Dead stains through the fluidic reservoir, the microsystem was placed in the incubator for one hour to warm the cells to 37°C, which is necessary to promote the cells' esterase activity. During the reagent loading and subsequent incubation, the staining reagents diffuse from the reservoir through the membrane and into the cell culture chamber.

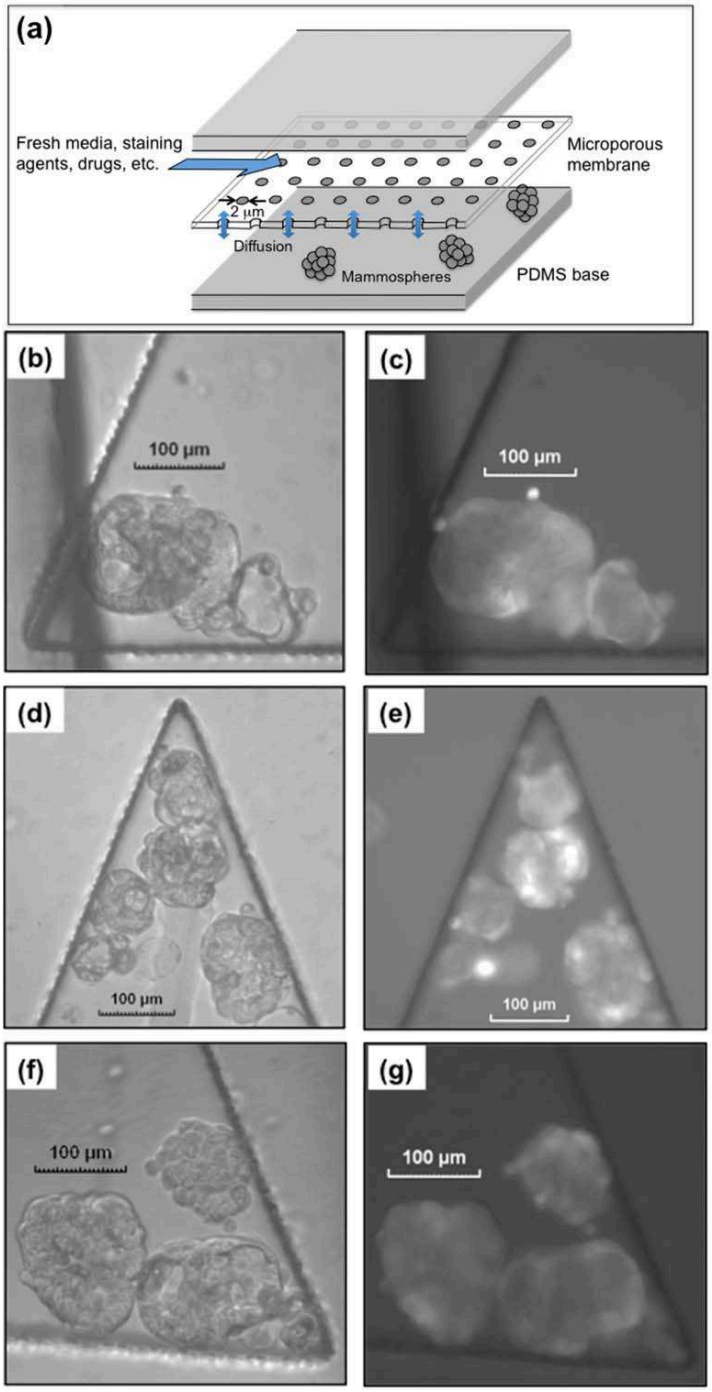

Figure 5.6: (a) Reagents are delivered to the cells via diffusion through the microporous membrane. (b-g) Fluorescent staining of the mammospheres in the microsystem. Bright-field images (b, d, f) and fluorescent images (c, e, g) were recorded for MCF7 mammospheres in three different locations of the microsystem. Cells in the mammospheres are stained with Calcein.

Images recorded at three different locations of the mammosphere culture microsystem are presented in Fig. 5.6. Figs. 5.6 (b, d, and f) are bright-field images, while Fig. 5.6 (c, e, and g) are fluorescent images (blue excitation, green emission filters to visualize the "Live" stain) at exactly the same locations. Fluorescent images for the "Dead" stain (green excitation, red emission filters) were also recorded at the same location, but no dead cells were identified in any of these locations. From these images, it is clear that the design of the mammosphere microsystem enables reagent delivery to the mammospheres without introducing convection into the cell culture chamber.

In the second experiment where mammosphere media in the reservoir is replaced with regular media (which is rich in serum proteins), spheroid colonies respond to the media exchange by initiating cell-surface attachments. In this experiment, serum proteins that adsorb onto PDMS, make it more available for attachment. Meanwhile, the reduction in growth factors such as EGF (epidermal growth factor) and FGF (fibroblast growth factor) that occurs as the mammosphere media is replaced with regular media, will result in the loss of stem cell characteristic of cultured mammospheres. This in turn will encourage cells in tumorspheres to differentiate as they make monolayer attachments. The transition in the state of the cells that occurred approximately 24-48 hrs after media exchange is depicted in Fig. 5.7. This experiment further demonstrates the capability of our mammosphere culture microdevice in manipulating cells and colonies in ways that are impossible in macrosystems. For instance, to emulate the same experiment in a macroscale, one has to manually collect single colonies from a macro-well and plate them onto

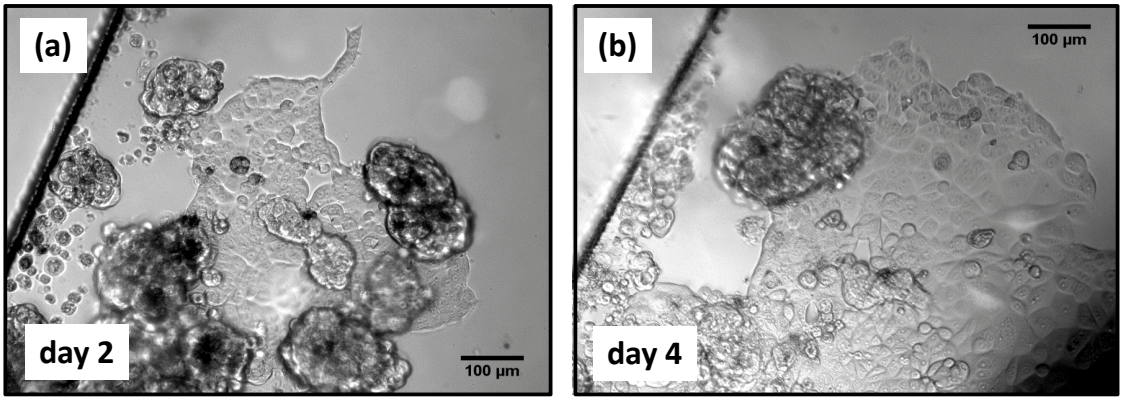

Figure 5.7: Transition of tumorspheres to monolayer cell attachment in microwells a) 2 days, b) 4 days after diffusion of serum into the microwells.

a culture treated plate that is filled with a serum rich medium. This labor intensive and tedious step however, is eliminated using a mammosphere culture microsystem as described here.

The experiments described above clearly demonstrate that the mammosphere culture microdevice with an integrated diffusion membrane not only enables long term spheroid culture (which are enriched in stem cells), it also facilitates the exchange of media and reagents, including small and large molecules and drugs, from the reservoir into the cell culture chamber while monitoring individual mammospheres. This in turn enables automated assays that are not possible in today's commercially available 96-well low-attachment plates.

COMSOL Simulations

To determine the concentration of dye molecules that have diffused to the bottom of the cell culture chamber through the porous membrane, we used COMSOL Multiphysics, which is a finite element analysis technique used in various physics,

engineering and design applications. The technique is mainly utilized to simulate the performance of a mathematical or engineering system that is defined by certain initial and/or boundary conditions. The simulations performed in this work were aimed to determine the concentration of Calcein AM (Live stain) that diffused to the cell culture chamber. In these simulations, different factors that impact the diffusion process were taken into account, such as the geometry of the microdevice, porosity of the miroporsous membrane, and the diffusion coefficient of the dye (which strongly depends on the molecular weight of the dye molecule as well as temperature). In these simulations that were performed using Convection and Diffusion Module, we initially used the geometry that is presented by the actual mammosphere culture microdevice as described in previous subsection. We then studied the effect of porosity and the height of the cell culture chamber on the diffusion process and travel time of dye molecules.

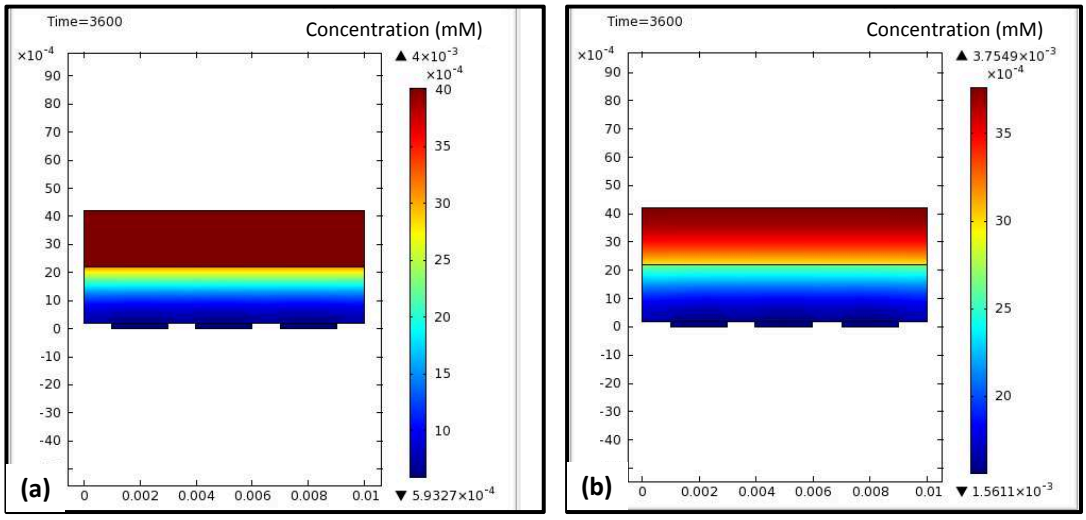

Figure 5.8: COMSOL simulation of diffusion of Calcein AM (Live Stain) through the mircoporous membrane into the cell culture chamber after a) 1 hr. constant flow of 4μM stain in the reservoir at $T = 22°C$ followed by b) 1 hr. diffusion in a static condition at $T = 37°C$.

For the first simulations where the actual mammosphere culture device geometry is considered, the distance between the 10 μm thick porous membrane and the bottom of cell culture microwells is approximately 2.2 mm. In the first phase, where constant flow is used to replace the serum-free medium in the reservoir with a 4 μM dye containing medium, the concentration of Calcein diffused to the bottom of the wells reaches a value of 0.59 μM after 1 hour fluid exchange at 22°C (Fig. 5.8 (a)). In the second phase, the flow is stopped and the dye is let to diffuse to the bottom chamber at 37°C (this temperature is necessary to promote the cells' esterase activity). After 1 hour incubation at 37°C, the concentration of Calcein at the bottom of the microwells reaches a value of 1.56 μM as calculated by COMSOL (Fig. 5.8 (b)). This value, which is within the range of concentrations at which Calcein functions on live cells (1-4 μM), is in agreement with our live cell imaging. In these simulations, the diffusion coefficient of Calcein at different temperatures, was calculated using the Polson equation

$$D = \frac{9.40 \times 10^{-15}}{\mu_B \sqrt[3]{M_A}} T \tag{5.1}$$

which is a semi-empirical equation used for biomolecules with molecular weights of approximately 1000 Da and larger [209]. In this equation, μ_B is the viscosity of the solvent ($\mu_B \approx 1$ Pa.s), M_A is the molecular weight of the solute ($M_A = 994.87$ Da for Calcein AM), and T is the absolute temperature of the solution in K. Using this equation, the diffusion coefficient of Calcein was calculated to be 2.77×10^{-10} m^2/s, and 2.9×10^{-10} m^2/s at $T = 22°C$ and $37°C$, respectively.

It should be noted that the height of the cell culture chamber has a significant

impact on the travel time of biomolecules and reagents in reaching cells/colonies that reside at the bottom of the device, as is shown in Fig. 5.9. In other words, for the mammsophere culture microsystem to be more effective in subsequent molecular analysis and/or drug screen, a device optimization in terms of dimensions is desired. The importance of size becomes especially more noticeable in using reagents that have a short lifetime. This in turn implies that one should design shorter intermedi-

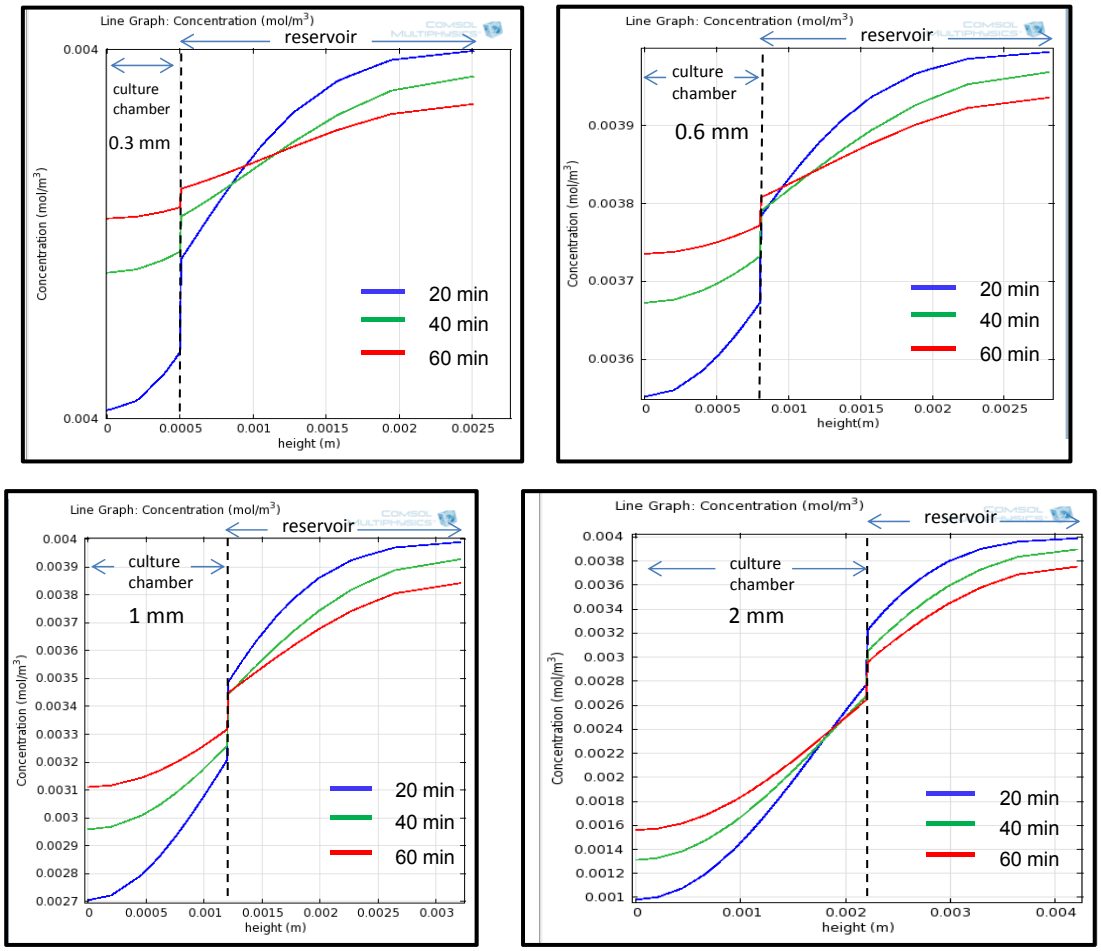

Figure 5.9: Height dependence diffusion. The concentration of Calcein at different distances form the porous membrane is strongly dependent on the thickness of the cell culture chamber. The above simulations were carried out in static conditions at $T = 37°C$.

ate steps (such as rinsing and/or fluidic exchange) via facilitating thinner chambers. A COMSOL simulation on the effect of thickness indicates that just a few hundred micron shorter in height can drastically reduce the time required for a reagent (such as Calcein) to propagate through the whole chamber and reach the cells/colonies (Fig. 5.9). These results can independently be confirmed using Fick's Law:

$$n(x,t) = n(0)\,erfc\frac{x}{2\sqrt{Dt}} \tag{5.2}$$

where $n(x,t)$ and $n(0)$ are respectively the concentration of the diffusing reagent in the sink and in the reservoir, *erfc* is the complementary error function, x is the distance from the source (reservoir), and t is the time at which concentration at distance x reaches a certain value (n), due to the diffusion coefficient, D. Eq. 5.2, can then be expanded to obtain

$$n(x,t) \simeq n(0)\Big[1 - \frac{2x}{2\sqrt{Dt\pi}}\Big] \tag{5.3}$$

which is valid solely for arguments that are small enough to converge the equation. For other values Eq. 5.4 is used instead:

$$n(x,t) \simeq n(0)\Big[\frac{e^{-y^2}}{\sqrt{\pi}y}\Big] \tag{5.4}$$

where $y = \frac{x}{2\sqrt{Dt}}$.

To determine the appropriate equation to be used for different thicknesses, we compared the solution of these equations with those obtained from COMSOL that was run for certain initial and final concentrations. For this purpose, we assumed an initial ($n(0)$) and final (n) concentrations of 4 μM, and 1 μM, respectively. These simulations/calculations were then carried out for different cell culture chamber

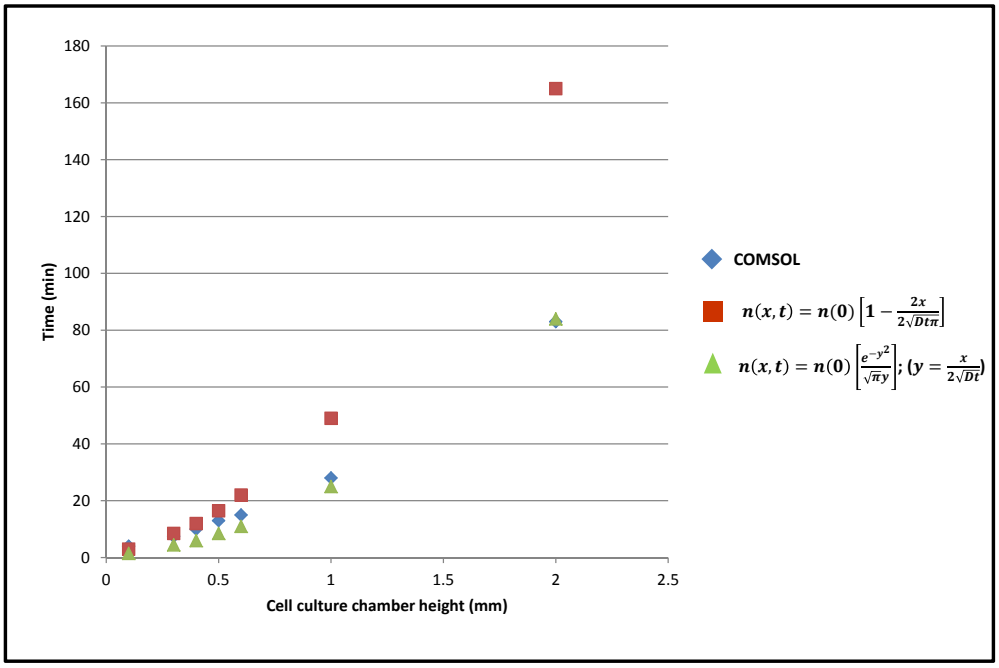

Figure 5.10: Height dependence propagation time. In this figure, different approaches were employed to calculate the time required for a 4 μM dye (Live Stain) in the reservoir to reach a concentration of 1 μM at the bottom of microwells through the diffusion membrane. In calculating these data, the height of the microwells (200 μm) were taken into account.

thicknesses ($0.1mm \leq x \leq 2mm$) at 22°C. In these calculations, Eqs. 5.3 and 5.4 were used simultaneously to measure the propagation time that would best match the results of the simulations. As can be seen from these results, depicted in Fig. 5.10 and Table 5.1, for chamber sizes of 0.6 mm and higher, Eq. 5.4 provides the best estimate for the propagation time of a dye molecule, while for thicknesses below 0.4 mm, Eq. 5.3 can be used for time evaluation. A 0.5 mm thickness is the threshold height for the culture chamber as for this thickness both equations are equally applicable.

Finally to investigate the effect of the membrane's pore size on diffusion, we performed a series of simulations by varying the membrane's porosity ϵ ($0.02 \leq$

Cell Culture Chamber Height (mm)	Propagation Time (min) (COMSOL)	Propagation Time (min) $n(x,t) = n(0)\left[1 - \frac{2x}{2\sqrt{Dt\pi}}\right]$	Propagation Time (min) $n(x,t) = n(0)\left[\frac{e^{-y^2}}{\sqrt{\pi}y}\right]$ $(y = \frac{x}{2\sqrt{Dt}})$
0.1	4	3	1.5
0.3	7.5	8.5	4.5
0.4	10	12	6
0.5	13	16.5	8.5
0.6	15	22	11
1	28	49	25
2	83	165	84

Table 5.1: Height Dependence propagation time. In calculating these data points it was assumed that the initial concentration of Calcein in the reservoir is 4 μM and the final concentration diffused through the porous membrane to the bottom of microwells is 1 μM. These measurements were made at 22°C using a constant flow condition. In calculating these data, the height of the microwells (200 μm) were taken into account.

$\epsilon \leq 1$, where $\epsilon = 1$ for a free flow) and using the actual sizes of the device. As can be seen from these simulations (Figs. 5.11 and 5.12), while for small values of ϵ, the variation in porosity can have a significant impact on the travel time of reagents through the membrane, for $\epsilon \geq 0.2$ the membrane is almost non-existent to the system. In other words, the transport mechanism resembles that of a free flow. This can be explained by the fact that at small porosities where the size of the pores are close to that of the transport molecules, the membrane can impose a resistance to the flow and is therefore detrimental to the flow rate. On the other hand there is a big tradeoff in using membranes with high porosities where cells will use the pores as a transport gate to migrate between two chambers, making it impossible to trace

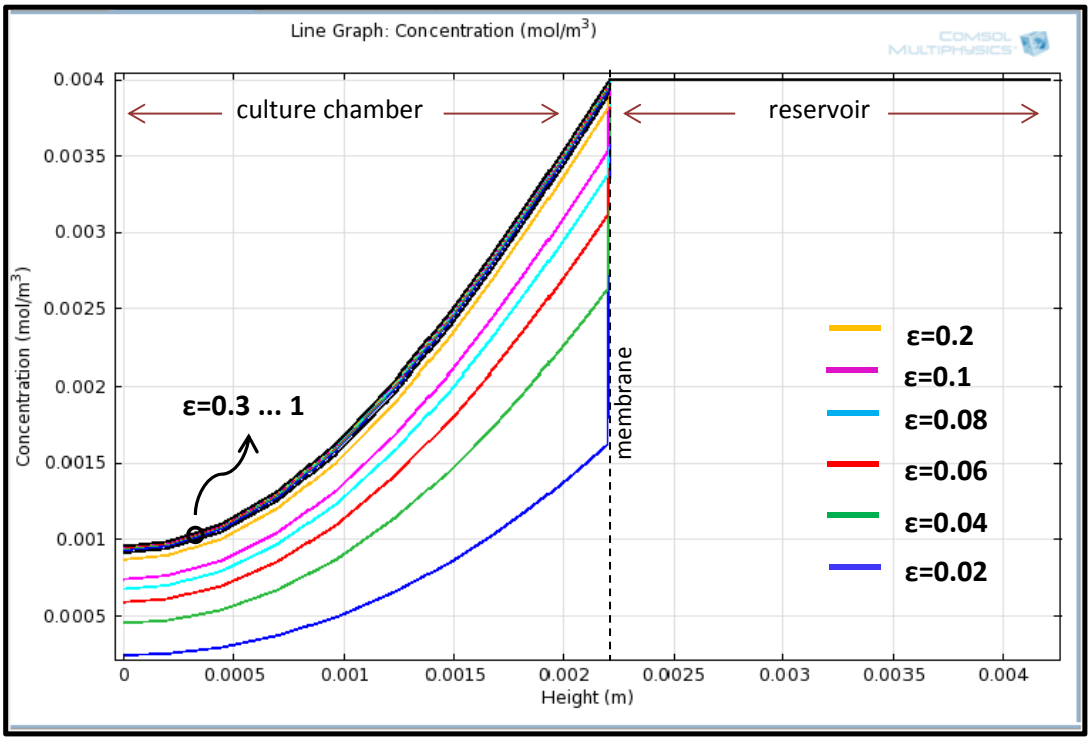

Figure 5.11: Porosity and Diffusion. While diffusion at high porosities resembles that of a free flow, at small pore sizes, porosity comes into effect.

cells and colonies over an extended period of time. Additionally, the application of a large pore size membrane can have a negative effect on the transport of fluidics between the two chambers as using membranes with a large pore sizes can result in convection in the bottom chamber as media is replaced in the reservoir. For these reasons, it is important to use membranes with a proper porosity to engineer a device that not only facilitates a simple transport system but it can also prevent cells from translocating between the two chambers.

All in all, these results as reported in this chapter indicate that our mammosphere culture microdevice with its convection-free mass transfer system offers an enabling platform for studying 3-dimentional cancer stem cell colonies at cellular

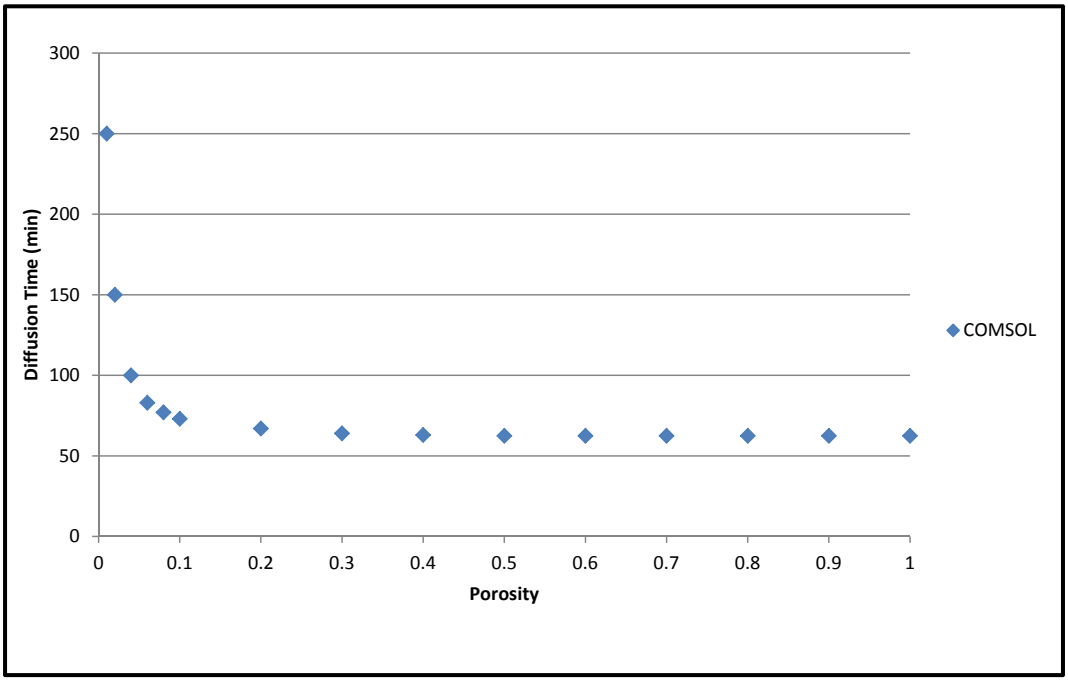

Figure 5.12: Porosity and Diffusion. COMSOL simulations were used to determine the dependency of diffusion on porosity and membranes pore sizes. In this simulation the height of the cell culture chamber is 2 mm and the initial and final concentrations are respectively 4 μM and 1 μM. All simulations were carried out at $T = 22°$C in a constant flow condition.

and molecular levels, in ways that are not possible using conventional macro-assays. This simple and cost effective microdevice can later be integrated with other microfluidic functions, some which has already been developed in other contexts.

CONCLUSION and DISCUSSION

The mammosphere assay has proven to be a useful technique for the identification of breast cancer stem cells by enrichment of these rare cells in suspension cultures. The current assay, which utilizes low-attachment 96 well trays to culture mammospheres, though simple, is not well suited to be coupled with other functions, such as cell enrichment and concentration techniques, as well as molecular assays,

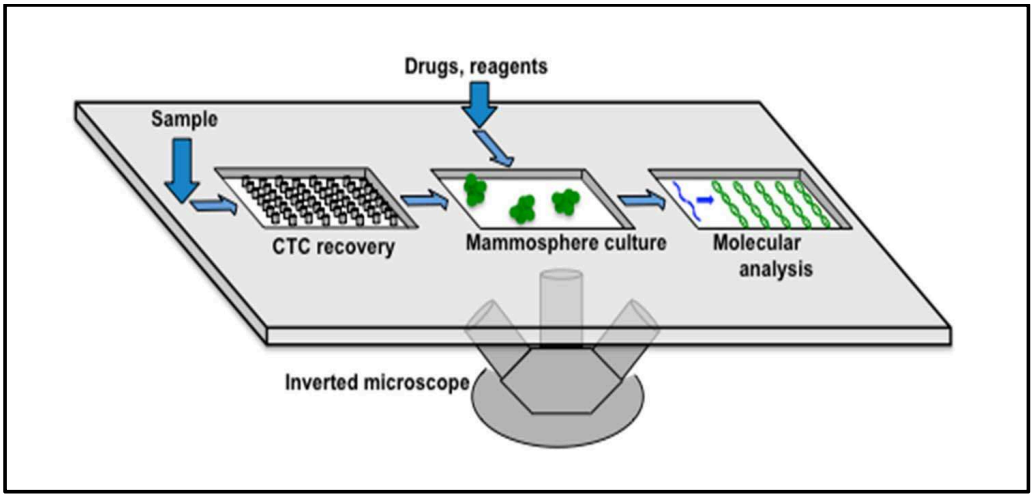

Figure 5.13: Conceptual integrated microsystem for the study of the tumor-initiating capabilities of captured circulating tumor cells.

such as RT-PCR. Microsystem integration offers the promise of creating a single automated system to investigate the tumor-initiating capabilities and associated CSC characteristics of metastatic cells. For example, one can envision integrating a microfluidic circulating tumor cell (CTC) recovery device, which has been vigorously investigated in recent years [210–215], with the microfluidic mammosphere assay, such that recovered CTCs can be interrogated for their tumor initiating properties. Any cells enriched for CSC properties through the microfluidic assay can then be investigated further with microfluidic drug screens [216] and microfluidic RT-PCR [217, 218]. This system integration concept is illustrated in Fig. 5.13.

In this study we have reported for the first time a microsystem that enriches breast cancer stem cells via the mammosphere assay technique. The surface of the cell culture region is formed from a PDMS substrate, which promotes mammosphere formation when a heterogeneous population of cells is cultured in serum-free media. We recorded the time-course growth of mammospheres over a period of ten

days within the microsystem. Mammospheres as large as 250 μm were observed, which is consistent in size with mammospheres cultured in commercially available macro-scale plates. We demonstrated that reagents, such as fresh media, drugs, or staining agents, can be delivered to the suspended cellular colonies by diffusion-based transport through an integrated porous membrane. As a result, this platform enables the study of potential tumor-initiating breast cancer stem cells. At this point, the mammosphere culture microdevice is poised for integration with other microfluidic functions, as illustrated in Fig. 5.13, which will enable new studies of cancer metastasis that are not possible today.

Chapter 6

Summary

In this dissertation we have shown that a biocompatible and optically transparent polymeric material such as polydimethylsiloxane (PDMS) can drive the growth of 3-dimensional spheroidal colonies in serum-free media as effectively as commercial hydrogel-treated low-attachment culture plates. The hydrophobic surface of PDMS is resistant to cell attachment, and thus upon culture onto this surface in the absence of serum proteins, cells with stem cell like properties such as cancer stem cells (CSCs) expand into suspended colonies while fully differentiated cells do not survive. In this work where the focus was on breast cancer stem cells, we show that PDMS substrates not only support the isolation and enrichment of CSCs in the form of tumorspheres, they in fact offer several advantages over the commercial low-attachment plates that are designed for the same purpose. We have shown that unlike suspension cultures on commercial low attachment plates that results in the formation of multiple colonies (scattered all over the substrate), mammosphere culture onto PDMS coated surfaces results in the formation of a single colony that can easily be screened over the time course of the culture. The single mammosphere formation on PDMS substrates, which is due to the bowl shape structure of PDMS

coated surfaces that allows cells to be collected at the bottom of curved surface before they initiate proliferation. This in turn has led to important observations that are reported in this dissertation for the first time. The growth of a single colony over a time period for instance was shown to result in cell shedding and the birth of new tumorspheres after few weeks of culture. This observation might provide some insights into the phenomenon of tumor cell dissemination in vivo that is attributed to cancer metastasis.

In addition to utilizing PDMS as an effective material for inducing mammosphere formation to isolate and enrich CSCs, in this work we demonstrated that PDMS enables the formation of 2-dimensional attached monolayers when cultured in serum-rich media, while hydrogel-treated low-attachment plates do not. Based on the collection of results included here, we predict that the proteins in serum modify the PDMS surface, thus allowing cells to attach. All in all the results of the experiments reported in this dissertation indicate that unlike commercial plates that are optimized for either cell attachment or suspension culture, PDMS can be utilized for either culture simply by using a serum free or serum rich media. This surface tunability of PDMS was then utilized to develop a microsystem that has a dual functionality in cell culture. Using this device we demonstrated for the first time the enrichment of CSCs by mammosphere culture and the subsequent transition to attached monolayers without the need to transfer the cells from one substrate to another.

This new cell culture assay based on switchable surface attachment properties creates new opportunities for the investigation of the biology of cancer stem cells.

Currently, if breast cancer stem cells are to be studied following enrichment by mammosphere culture, the spheroid colonies must be manually removed from the culture plate and transferred to a new culture plate while exchanging the media. This can be a difficult task, and can result in the loss of the rare cells in the sample. The technique demonstrated in this work enables enriched CSCs to be transitioned from mammosphere culture into attachment-based culture without transferring the cells. For example, using this technique, one can enrich CSCs from a heterogeneous sample and then monitor the transition of the CSCs into fully differentiated cells.

Importantly, we expect that our transitional culture technique will lead to new methodologies for investigating the link between cancer stem cells and metastasis. It has recently been observed that the induction of an epithelial-mesenchymal transition (EMT) leads to the acquisition of stem cell properties, including the ability to form mammospheres in low-attachment culture [34, 35]. This observation is an important link between CSCs and metastasis, as the EMT has long been hypothesized to lead to metastatic cells. Conversely, much less is known about the mechanisms and the role of the mesenchymal-epithelial transition (MET), which apparently must exist if mesenchymal CSCs eventually lead to solid metastatic tumors. The PDMS fluidic platform demonstrated in this work may lead to simple experiments that demonstrate the MET and that can shed light on the associated mechanisms and physiochemical cues.

The relevance of this potential investigation is further enhanced by the fact that the device is constructed from PDMS, one of the most commonly used materials for microsystem fabrication. As a result, the multi-functional cell culture device can

easily be integrated with microfluidic cell capture devices, which have recently been reported for the recovery of rare circulating tumor cells (CTCs) from whole blood samples [210–215]. One can envision a device that captures CTCs from the blood samples of metastatic cancer patients, enriches cancer stem cells from this population using mammosphere culture, and then enables the identification of the mechanisms involved in the MET. Thus, we expect that the tunable $(2 + 1)$-dimensional cell culture device that was developed in our lab as reported in this document will have significant applications in the investigation of cancer, cancer metastasis, and cancer stem cells.

The implementation of the mammosphere technique into an integrated microsystem can further be utilized for cellular and molecular analysis as well as drug screens of tumorspheres that are enriched in CSCs. The significance of this device is particularly recognized by the fact that the mammosphere culture microsystem (unlike conventional suspension culture macro-devices) can easily tolerate the delivery and exchange of drugs and reagents where the risk of losing suspension colonies is completely eliminated. This additional device capability is in fact due to the diffusion based transport system that was integrated into the device.

Finally, since the 3-dimensional cellular structures as in mammospheres are a better in vitro representative of the tumor constructs, the integration of analytical and diagnostic functions that follow the mammosphere assay might in fact have a significant impact on our view of cancer. This might particularly be important in exploiting more effective treatment regimen that can eventually result in a complete eradication of this deadly disease.

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