SUBSTRATE STIFFNESS ADJUSTABLE PDMS DEVICE/ARRAY FOR UNDERSTANDING ITS EFFECT ON CELL GROWTH, DIFFERENTIATION AND MIGRATION

by

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ABSTRACT

SUBSTRATE STIFFNESS ADJUSTABLE PDMS DEVICE/ARRAY
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AND MIGRATION

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The mechanical properties of the cellular environment play an important role in the functioning of the cell. Cells attach to the extracellular matrix or other surrounding cells, thus sensing their mechanical properties and tune their own internal mechanical properties accordingly. A number of *in vitro* studies have been performed to study various cellular mechanical phenomena, but most of them employ a very stiff substrate such as glass or plastic that does not encompass the physiologically relevant range. Also, for tissues of the nervous system, the relationship between the substrate stiffness and the cellular behavior is less understood.

In this study, we report the fabrication of a novel device having a cell growing substrate with adjustable elastic moduli ranging from 20kPa to 2.2MPa and systematically study the effects of substrate elasticity on cell behavior. The substrate can be further tuned to obtain a wider range of moduli for the intended applications. Also, the substrate has a thickness of 50

μm which is suitable for monitoring the cells at high magnifications. To study the effects of the varying stiffness on cell behavior, mouse neural stem cells, rat embryo derived cortical neurons, and human glioblastoma multiforme cells were cultured on these devices and monitored. We observed a significant difference in the outgrowth, differentiation, and migration of these cells based on the stiffness of the substrates, indicating that cells respond differently to substrate stiffness. For NSCs, it was seen that there was an increase in the proliferation and differentiation of these cells into astrocytes with increase in the substrate stiffness. Cortical neurons displayed increased outgrowth with stiffer substrates whereas for GBM too, migration of the cell from their clusters was seen to increase with the underlying substrate stiffness. In order to further improve the efficiency of the device and study two or more factors that could affect cell behavior simultaneously, a PDMS array has been fabricated having substrates of different stiffness, assembled on a 48X65 mm glass slide and coated with different ECM proteins. Human gliobastoma multiforme cells were cultured on the array to examine the effects of both the stiffness and the underlying ECM protein at the same time and it was observed that substrate stiffness strongly controlled cell migration for majority of the coated proteins. In conclusion, the device/array can be easily tuned for making substrates of varying stiffness over the entire physiologically relevant range on a single glass slide to mimic the *in vivo* substrate of the cells and to test cellular behavior on the differing substrates.

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CHAPTER 1

INTRODUCTION

1.1 Background

In living organisms, most cells are embedded in tissues that are viscoelastic in nature i.e. have the properties of being an elastic solid as well as a viscous liquid and are composed of other cells and extracellular matrices [1]. The mechanical properties vary across the different tissues and the developmental stages and are being increasingly recognized as an important and relevant cellular stimulus [2]. The entire evolutionary spectrum, comprising of cells and organisms from the simplest to the most complex systems posses the special ability of being mechanosensitive [3,4]. This ability of cells allows them to relay mechanical signals from their environment of from within the cell to various biochemical signals which in turn initiate and regulate a wide repertoire of the required physiological responses [5]. The cells can thus sense and regulate various external or cellular tensions and forces leading to cellular adaptation to the physical environment and ultimately tissue homeostasis [6]. A wide range of cellular activities such as protein synthesis, secretion, migration, proliferation, viability and apoptosis are dependent on the property of cells to relay the mechanical stimuli [5]. Cellular defects in processing the mechanical cues are often referred to as a cause of diseases such as muscle dystrophies [7,8], artheriosclerosis [9,10], immune system and central nervous system disorders [11-13] and also cancer progression [14]. The most common attachment site for mammalian cells that make up tissues is the surrounding extracellular matrix (ECM) or another similar cell [15]. Thus, cells within the tissues sense the mechanical properties of both the ECM and other cells and generate the required response. These mechanical forces may be fluid shear stress, pressure, elongation stresses and stiffness. The material property of the cell along with those of

the matrix or substrate to which the cell is attached plays an important role distributing these mechanical forces on the surface and within the interior of the cell [16].

1.2 Mechanics of cellular interactions

Most tissue cells require anchorage dependence for survival i.e. they need to attach to a solid for survival. Even if cells are suspended in a liquid having soluble proteins to promote cell adhesion, they are simply not viable [17].Thus cells adhere to the ECM or other cells through specific anchorage points, termed focal adhesions that form the loci of interaction between the cytoskeleton and adhesion proteins. Focal adhesions are highly dynamic and mechanosensitive in nature and are capable of changing their size, shape and number in response to the microenvironment of the cell [18-20]. These regions localize and concentrate the heterodimeric receptors for the extracellular matrix known as integrins which act as the primary cellular mechanosensors for adhesion dependent mechanical forces. Integrins thus link the extracellular physical environment to the cytoskeleton of the cell [21,22]. Cell-cell mechanosensing may be mediated by other adhesion proteins such as cadherins. Focal adhesions thus bind the cell to the substrate allowing the cell to pull on the substrate via its actin-myosin cytoskeleton and sense the resistance and generate the necessary response to that resistance through activated myosins or other motors leading to cytoskeleton organization. Thus, the transcellular focal adhesion molecules such as integrins, cadherins, talin and other proteins along with the myosin-based contractility of the cells primarily govern the transmission of cellular forces with the environment for anchorage and motility. Figure 1.1 gives a brief example of how force is transmitted between the ECM and the nucleus. The extracellular matrix consists of proteins such as laminin, collagen etc. which link to the cytoskeleton of the cell through focal adhesion complexes present at the cell surface. These signals are then transmitted through the cytoskeleton comprising of actin and intermediate filaments and myotubules which is coupled to the nucleus through nesprins and other proteins. Nesprins thus carry the signal into the luminal space through inner nuclear membrane proteins thus binding to

the nuclear DNA and transmitting the force generated in the ECM to the nucleus of the cell [5,23].

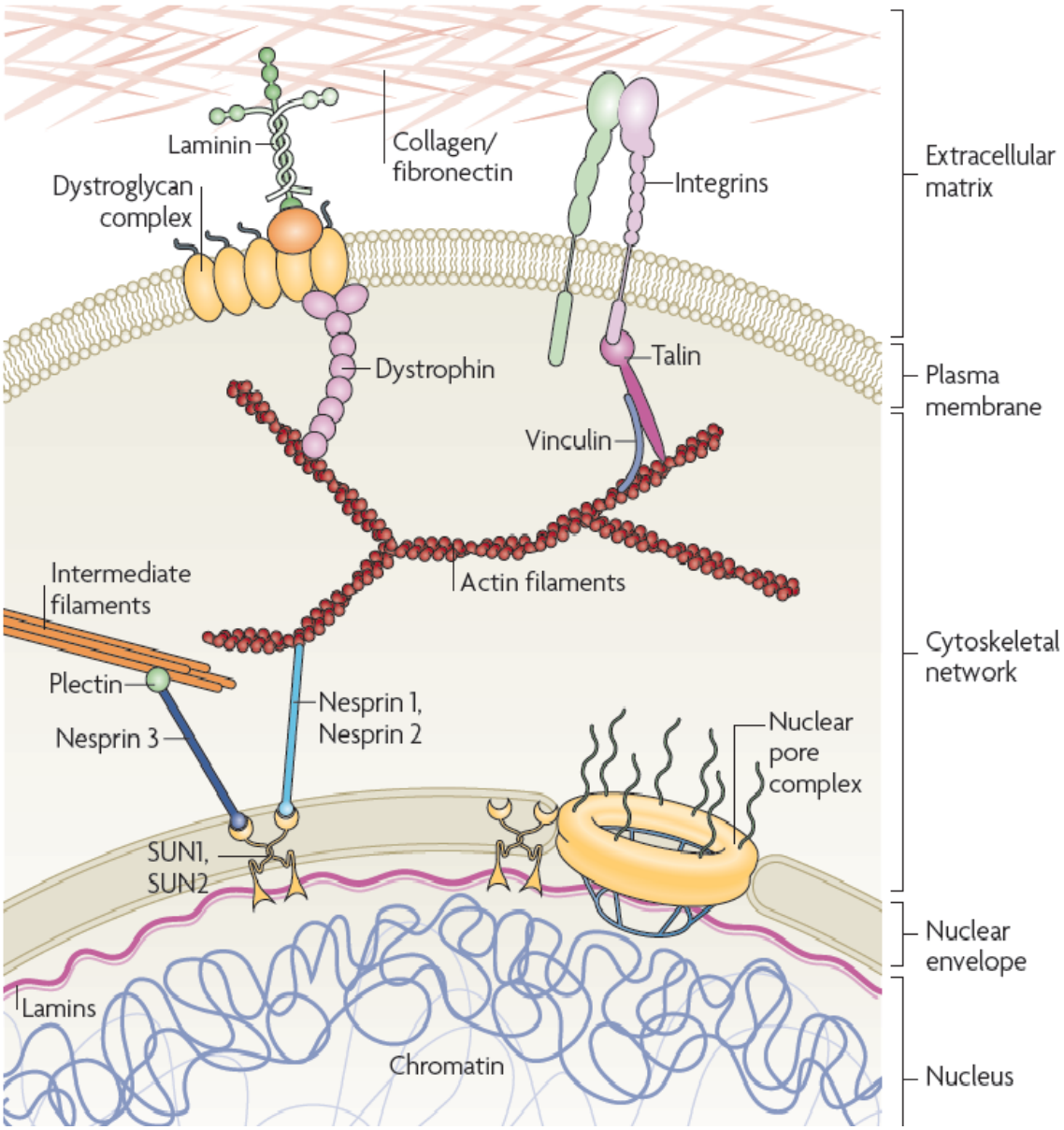


Figure 1.1 Force transmissions between the ECM and the nucleus [5]

Mammalian cells possess the ability to not only sense the applied mechanical forces, but also sense the mechanical properties of their environment, such as the elasticity of the substrate on which they grow [24]. Studies have shown that cells possess the inherent

capabilities to tune their internal stiffness with the environmental stiffness [25]. Anchorage dependent cells have been shown to be responsive to the mechanical properties of their matrix even though the molecular pathways behind these phenomena are yet to be characterized [26-28].

1.3 Regulation of cell behavior by matrix stiffness

The most common attachment site for a mammalian cell is the extracellular matrix or another cell. Cells adhere to solid substrates and the elastic modulus of the cell's microenvironment can span over many orders of magnitude ranging from 100 Pa for very soft tissues such as fat or brain to >10,000 Pa for muscle, cartilage and bone [29]. The intrinsic resistance of the cell to an applied force can be measured by its Young's elastic modulus *E.* This value gives the resistance offered by the cell to any deformation and is reported in units of Pascal. It can be calculated by plotting the graph of the stress applied versus the cellular deformation (strain). The elastic modulus can also be determined by controlled poking of indenters and atomic force microscopes [30]. At the cellular level, the elastic modulus *E* varies widely for different cell types and is largely dependent and determined by their actin-myosin based cytoskeletons [31]. Cells can only sense the mechanical forces applied by the ECM or any neighboring cells as they can only mechanosense over short distances [1].

The chemical makeup and organization of the extracellular matrix are primarily responsible for its stiffness. The cytoskeleton organization of various cells such as muscle cells depends strongly upon the matrix stiffness. The substrate stiffness also regulates the strength with which cells adhere to the substrate and their degree of spreading [32,33]. When the cell attaches to the matrix via focal adhesions, it develops a certain amount of tension within its membrane and in the underlying actin mesh which in turn depends upon the inherent material properties of the matrix [34]. Generally, studies have shown that stiffer matrices resist more cellular force than a softer matrix thus causing the cell to be more rigid and extended about its periphery [35]. Also cells on stiffer substrates have been seen to have stiffer and more stable

focal adhesions even though the cellular force-response relationships are not linear for all cell types [32]. Matrix connections to stiffer substrates allow the actin cytoskeleton to be involved in the cellular response thus strengthening the cell matrix interactions. Other cellular characteristics in response to a relatively stiff substrate include assembly of actin fibers [35], exhibiting a more spread phenotype [36], up regulating the expression of integrins [37] and activating signaling pathways that are characteristic of contractility that leads to an increase in the stress applied to cellular substrates leading to the formation of focal adhesions [38]. As shown in Figure 1.2, studies have also shown that in general, cells on soft matrices are rounded and minimally adhesive whereas those on stiff substrates are proliferative and have an increased number of integrin/ECM bonds. Also, cells have been seen to migrate from soft to stiff regions of the matrix and seem to be the most motile at intermediate stiffness [39].

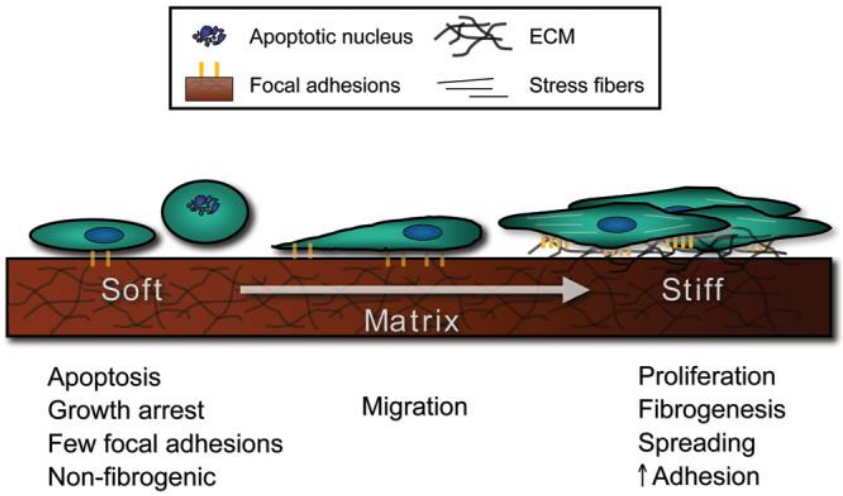


Figure 1.2 Effects of matrix mechanics on cell behavior [1]

1.4 Literature review

Substrate stiffness has been shown to have a variety of different effects on different cell types while performing *in vitro* studies. As seen above, substrate stiffness has been shown to regulate a number of cellular activities such as cell adhesion, proliferation, locomotion, morphology and differentiation. A review of the recent literature has been listed in Table 1.1.

Table 1.1 Comparisons of cellular responses to different stiffness

Cell type	Cellular Response	Reference
Aortic smooth muscle cell	Minimal spreading on substrates <5kPa, spreading saturates around 15kPa	19
Bovine vascular smooth muscle cell	Increased cell proliferation on soft substrates (48kPa)	41
Rat 3T3 fibroblasts	Reduced spreading and increased rates of motility on softer substrates (15kPa)	40
Human dermal fibroblasts	Increased adhesion with increased substrate stiffness (23kPa)	26
Alveolar macrophage	Increased cell stiffness and area on stiffer substrates (>10kPa)	44
Hepatocyte	Increased aggregation and differentiation markers on >150kPa	42
Astrocyte	Increased spread area and process extension (>500Pa)	27
Chondrocyte	Increased growth and proliferation on stiffer substrates (>10kPa)	43
Myoblast	Striated myotubes formation at 12kPa	26

From the above studies, it can be inferred that cells seem to attach and proliferate preferentially on stiffer substrates and often tend to migrate from soft to hard substrates exhibiting a broader and flatter morphology. However there are some studies indicate that this is not always true as shown in Figure 1.3. Neutrophils have been shown to have no preference for

any stiffness and spread equally well on both stiff and soft substrates [37]. Myocytes have been shown to be able to spread and form myotubes on a wide range of substrate stiffness but require substrates of intermediate elastic modulus (12kPa) for optimal striation [26,37]. Also, it has been shown that neurons preferentially branch on softer substrates whereas glial cells prefer stiffer substrates for survival [45]. Another study on neurons has shown that they are insensitive to the substrate stiffness and behave similarly on substrates of varying stiffness [46]. Mammary epithelial cells have been shown to undergo morphogenesis on substrates with stiffness as low as 200Pa. Thus different cell types have been seen to behave differently with varying stiffness and no general statement can be made about a cell's response to stiff or soft substrates. It is thus empirical to culture cells on substrates having stiffness of their native environment during *in vitro* studies.

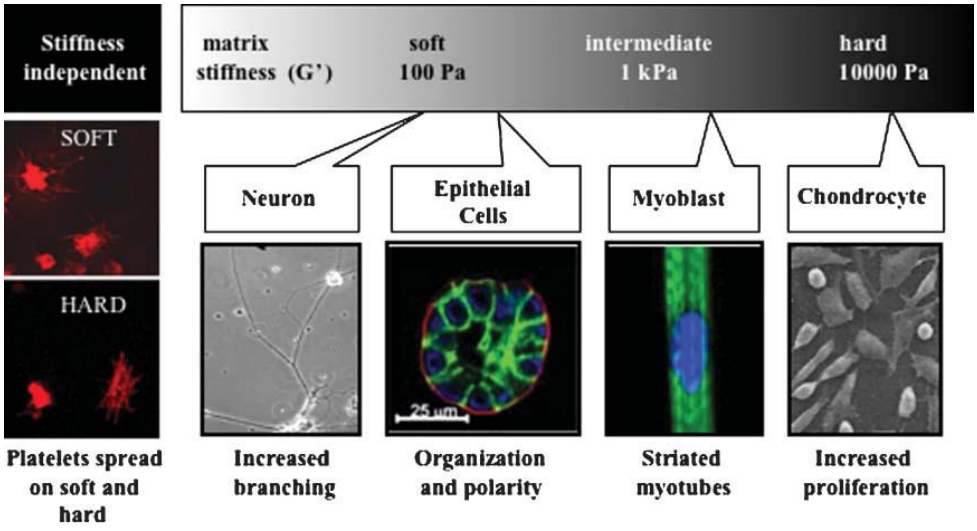


Figure 1.3 Effect of substrate stiffness on cell morphology [29]

1.5 Cellular substrates used for *in vitro* studies

1.5.1 Need for different substrates

Though countless substrata have been used to investigate cell–substrate interactions, surprisingly few have been used to systematically study the effects of substrate elasticity on cell behavior. While performing *in vitro* studies, it is important to maintain the stiffness of the

microenvironment similar to that of the body. Most cells in the tissues of multicellular organisms have an elastic modulus in the range of a several hundred pascal (brain) to about a few megapascals (tendon, cartilage) [26,27]. However, nearly all *in vitro* studies are performed on glass or tissue culture plastic surfaces whose elastic modulus is in the gigapascal range [37]. Other studies generally focus either on very soft or very stiff substrates using materials such as hydrogels [47]. These studies indicate that the current techniques for performing *in vitro* studies provide the cells with a highly nonphysiological mechanical environment. From the recent literature and studies that have been performed it can thus be inferred that a number of the cellular behaviors ranging from the cytoskeleton organization to the cell differentiation patterns that we attribute to the cells in culture may not be representing the true *in vivo* conditions and be artifactual [1]. The recent interest in cell mechanics and the effects of substrate elasticity on cell structure and function and also the ability to synthesize materials that approximate the mechanical nature of biological tissue have lead to the increased development of many different materials for use as the cell culture substrate.

1.5.2 Various materials used for cell-substrate mechanical interactions

Considering the importance of substrate elasticity on cellular response, it is critical to test a wide variety of substrata that span physiologically relevant ranges of elasticity. Recently, a number of methods to study the cells *in vitro* under more physiological conditions have been developed.

1.5.2.1 Natural hydrogels

Natural hydrogels have been shown to be a promising candidate for cell substrates. Protein based ECM gels such as fibrin, collagen or a mixture of a number of ECM proteins such as collagen, laminin and other proteins that form Matrigel, are commonly being used to create two or three-dimensional cell culture substrates that provide culture conditions of physiological stiffness [27]. Stiff collagen substrates made out of thin monomeric collagen and relatively soft substrates made of fibrillar collagen have been employed for cell cultures to study the effects of

relative stiffness [48]. Studies have shown that derivatives of fibrin and collagen are the most efficient at supporting neurite outgrowth in culture [49]. The elastic moduli of crosslinked polysaccharides such as alginate and agarose gels can also be adjusted to make it permissive for cell culture [36]. Hydrogels of alginate and agarose have been used as cell substrates and have shown to be the most efficient at supporting neurite outgrowth in culture [50,51].

1.5.2.2 Synthetic materials

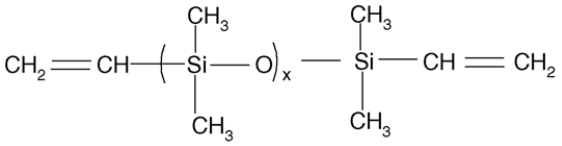
A number of synthetic materials have also been employed for used in substrate rigidity dependent studies. Synthetic hydrogels such as polyethylene glycol, polyacrylate derivatives, poly(2-hydroxyethyl methylacrylate) and polyelectrolyte multilayers are being designed for use in different cellular systems involving substrate rigidity [52-54]. However, polyacrylamide gels (PA) and poly(dimethylsiloxane) (PDMS) remain to be the most attractive choice for researchers for use in studies of the cellular microenvironment such as substrate stiffness studies. PA based hydrogels have been a popular choice for the study of cell-substrate mechanical interactions due to the multiple mechanical, chemical and optical advantages offered by them and are one of the easiest substrates to employ [34,55]. These materials produce a porous, bioinert substrate and have been utilized for stiffness research for many years. The first study using PA gels of different stiffness was performed using NIH3T3 fibroblasts and rat kidney epithelial cells where changes were seen in cell motility and cytoskeleton adhesion [40]. However, PA is chemically inert which makes covalent attachment of proteins sometimes difficult. A major disadvantage of PA gels is that they are typically characterized by elastic moduli (young's modulus) in the range of 10 kPa - 100kPa which is much lower than the elastic modulus of a variety of human tissues including tendons, cartilage and the blood vessel wall and hence cannot be used for the study of these cell types [41,56,57]. Hence, in this study, we have used PDMS as the polymer for fabricating the cell culture substrates to overcome these limitations of PA gels and to make substrates having stiffness similar to that of the native cellular environment.

1.6 Poly (dimethylsiloxane)

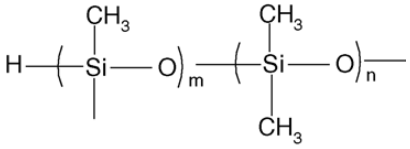
Microfluidic devices offer a unique tool for designing and performing *in vitro* experiments that allow for control of the cellular microenvironments. Polydimethylsiloxane (PDMS) is a material widely used for making microfluidic devices. It is a silicon based organic polymer that has been widely used in microfluidics, medical implant, biomedical devices and a number of other biological assays and hence has evolved to become one of the most favorable polymers in cell culture studies [58-60].

1.6.1Theoretical background

PDMS belongs to the family of polymeric organosilicone compounds commonly known as silicones [61]. Its chemical formula is $CH_3[Si(CH_3)_2O]_nSi(CH_3)_3$ where n is the number of repeating monomer $[SiO(CH_3)_2]$ units. The most widely used form of PDMS (Sylgard 184) derives from a two part polymer that consists of a base and a curing agent. The base consists of dimethylsiloxane oligomers with vinyl-terminated end groups, cis-dichlorobis(diethyl-sulphide)-platinum catalyst and silica filler (dimethylvinylated and trimethylated silica). The curing agent consists of dimethyl methylhydrogen siloxane that acts as the cross-linking agent and tetramethyl tetravinyl cyclotetrasiloxane that acts as the inhibitor [62]. The vinyl group from the siloxane oligomers (base) and the silicon hydride group from the siloxane crosslinkers (curing agent) undergo a hydrosilylation reaction to undergo crosslinking and form a Si-C bond [63,64]. This reaction relies on the ability of the hydrosilane bond of the cross linker (Si-H) to add across a C-C double bond that belongs to the prepolymer in the presence of the platinum catalyst [65]. The normal ratio of the base and the curing agent as per the manufacturer's protocol is 10:1. While curing, the number of the cross-linked and un-crosslinked groups generated largely depends upon the temperature and the time allowed for the curing process to take place. Any change in the base and curing ratio alteration largely affects the stiffness of the polymer [62]. The cross-linking reaction for the PDMS network formation is shown in the Figure 1.4.

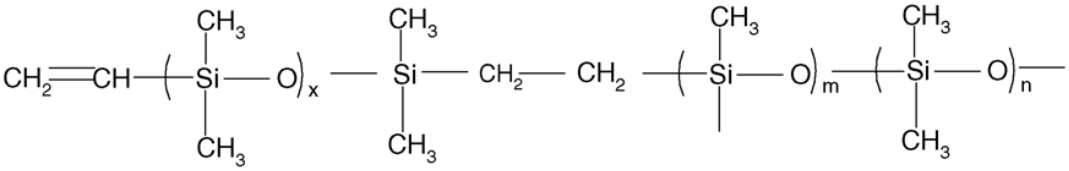


Siloxane oligomers



Siloxane crosslinkers

Pt catalyst



Crosslinked PDMS

Figure 1.4 Schematics of the cross-linking reaction for PDMS formation [65]

1.6.2 Rationale for using PDMS in this study

PDMS is a highly biocompatible and visco elastic polymer that can be easily fabricated and manufactured [58,66]. These polymers have low electrical conductivity, are permeable to gases but not water and have very high oxidative and thermal stability [59,62,67]. PDMS is optically transparent down to 230 nm, thus allowing for both optical and fluorescent microscopy of the contained cells and fluids [68]. It does not cause any irritation to the skin while handling and animal experiments have shown that it produces only mild inflammatory reactions when implanted subcutaneously [69]. PDMS also offers other advantages in *in vitro* studies – it allows respiration of cells enclosed within it as it is permeable to gas, is nontoxic and autoclavable and

does not swell or dissolve in culture medium [70]. All these properties along with its ease of being fabricated into various microstructures have made PDMS an attractive candidate in cell biology including those of contact guidance, chemotaxis and mechanotaxis, cell sorting, DNA sequencing, polymerase chain reactions and immunoassays [71-74]. PDMS has been used for creating substrates with a stiffness range of 20 kPa to 4 MPa in studies where a higher physiological stiffness is targeted. However, PDMS is very hydrophobic in nature and thus requires some chemical or electrical treatments before it can be used for cell culture and other biological applications.

1.6.3 Surface modification techniques employed

PDMS is a highly hydrophobic material and thus transferring and spreading of aqueous solutions on it and thus maintaining a long term culture of cells is very difficult. The hydrophobicity of the PDMS can be attributed to the repeating dimethylsiloxane – $OSi(CH_3)_2$- units [62]. The contact angle of water on these PDMS surfaces has shown to be about 100˚ - 120˚ [75]. Thus the surface of PDMS needs to be modified in order to achieve the desired surface energy or functionality. To ameliorate these negative effects of PDMS, various techniques have been employed. These include methods for eliminating the low molecular weight species such as treatments with oxygen plasma, ultraviolet (UV)/ ozone, UV-irradiation and electric discharge (corona treatments) or boiling the polymer which focuses on surface chemistry. Other techniques employ extracting the uncured monomers using various organic solvents [69,76]. Amongst the different techniques employed for surface modification of PDMS, plasma treatment is the most controlled means of achieving desired interfaces. The technique of plasma treatment can bring about surface modification of polymers by – functional group implantation, simultaneous grafting and post-treatment grafting[77]. In this study, we use air plasma for modifying the PDMS surface using the implantation method. In this process, the hydrogen atoms from the methyl groups ($Si\text{-}CH_3$) present in the polymer chains are first extracted to generate radicals within the polymer chains located at the surface. Finally, these

methyl groups are replaced with silanol (Si-OH) groups thus making the PDMS surface hydrophilic and reducing the water contact angle by 15˚ or less [62,78]. The plasma treatment creates functional groups on the surface of the PDMS along with a negative charge that is restricted to a depth of a few hundred nanometers [79]. However, the surface of the polymer has to be kept in continuous contact with water. If it is exposed to air for a long time, surface rearrangement occurs and new hydrophobic groups migrate to the surface owing to the high flexibility and mobility of the dimethylsiloxane chains in the PDMS caused as a result of its low glass transition temperature of -127˚C and lower the surface free energy [59,80]. Thus, the PDMS surfaces can be treated with air plasma and kept in contact with liquids constantly to retain its hydrophilicity so that it can be used for biological applications.

1.7 Overview of the project

In this study, we have developed a device having stiffness adjustable Polydimethylsiloxane (PDMS) based substrates and compared it with substrates generally used for *in vitro* studies (glass) to study and understand the effects of the substrate stiffness on cell outgrowth, migration, and differentiation as the substrate stiffness changes. The substrate stiffness is modulated by altering the material composition i.e. ratio of the PDMS base and curing agent. For the fabrication of varying stiffness PDMS substrates, different base:crosslinker ratios of the PDMS (10:1, 20:1, 30:1, 40:1 and 50:1) are used here thus yielding devices with the elastic modulus ranging from 2.2 MPa to 20 kPa as measured using a universal mechanical tester to mimic the *in vivo* cellular mechanical environment. The thickness of the substrate was also controlled so that visualization of the cells under high magnification was possible. To facilitate cell culture on the PDMS surfaces, surface modification was performed using plasma and Poly-D-Lysine Hydrobromide and the surfaces were coated with an ECM protein – protein to increase cell adhesion. The protein adsorption on the surface modified PDMS substrates was compared with the amount of protein adsorbed on glass so as to create the same conditions for cells seeded on both types of substrates. Then, three different cell types of the central nervous

system were used for cell culture since the dependence of substrate stiffness on these cells is poorly documented. E-18 rat embryo derived cortical neurons, mouse derived neural stem cells, and human glioblastoma multiforme cells, were seeded on the PDMS devices with five different stiffness substrates and each of the cell's response on the different stiffness substrates were monitored, imaged and quantified. Furthermore, to study the effect of substrate stiffness along with that of the underlying ECM protein, human GBM cells were seeded on a PDMS array having substrates of different stiffness and assembled on a single cover glass. The array was coated with five different ECM proteins – Fibronectin, vitronectin, collagen type I, laminin and Bovine serum albumin thus making it possible to study the effect of substrate stiffness along with the underlying ECM protein on cellular behavior simultaneously.

CHAPTER 2

METHODS

2.1 Fabrication of PDMS device of varying stiffness

For fabricating the varying stiffness device, firstly a PDMS block was prepared using a SYLGARD 184 silicone elastomer kit (Dow Corning). As per the manufacturer's protocol, the prepolymer base and curing agent were mixed thoroughly in a ratio of 10:1 in a plastic cup. This mixture was then placed inside a vacuum dessicator and then degassed (20 inches Hg vacuum) for about 45 minutes to completely remove any air bubbles that arise during mixing of the base and curing agent. The PDMS was then poured onto a silicon wafer (Addison Engineering) and heated at 150°C for about 5 minutes in order for the PDMS to cure. Once the PDMS cured completely, the silicon wafer was transferred onto another hot plate at 70°C for cutting the PDMS from the wafer. The cured PDMS sheet was peeled off and later cut into 10 x 10 mm square blocks using a razor blade. An 8 mm punch was made in each of the 10x 10mm PDMS square blocks using a biopsy punch (Miltex). Each punched PDMS block was treated with plasma and then placed above a 22x22 mm cover glass that had been cleaned previously using scotch tape.

Once the block assembly was complete, PDMS solutions having five different ratios of base to curing agent (by weight): 10:1, 20:1, 30:1, 40:1 and 50:1 were mixed and degassed in a similar way as explained above. 7µl of each of the different PDMS solutions was poured inside the punch of previously prepared assembly block. The assembly was then placed on a custom built slow spin coating apparatus for about 5 minutes so as to ensure the even spreading of the prepolymer inside the punch. After this, the devices having different base to curing agent ratios at the base of the block were kept in an oven at 80^{o}C for about 48 hours to ensure the freshly placed prepolymer cured completely. After complete curing of the PDMS, the devices were

removed from the oven and were used as the substrate for cell culture and other studies. The overall fabrication procedure of the varying stiffness PDMS devices is summarized in the Figure 2.1.

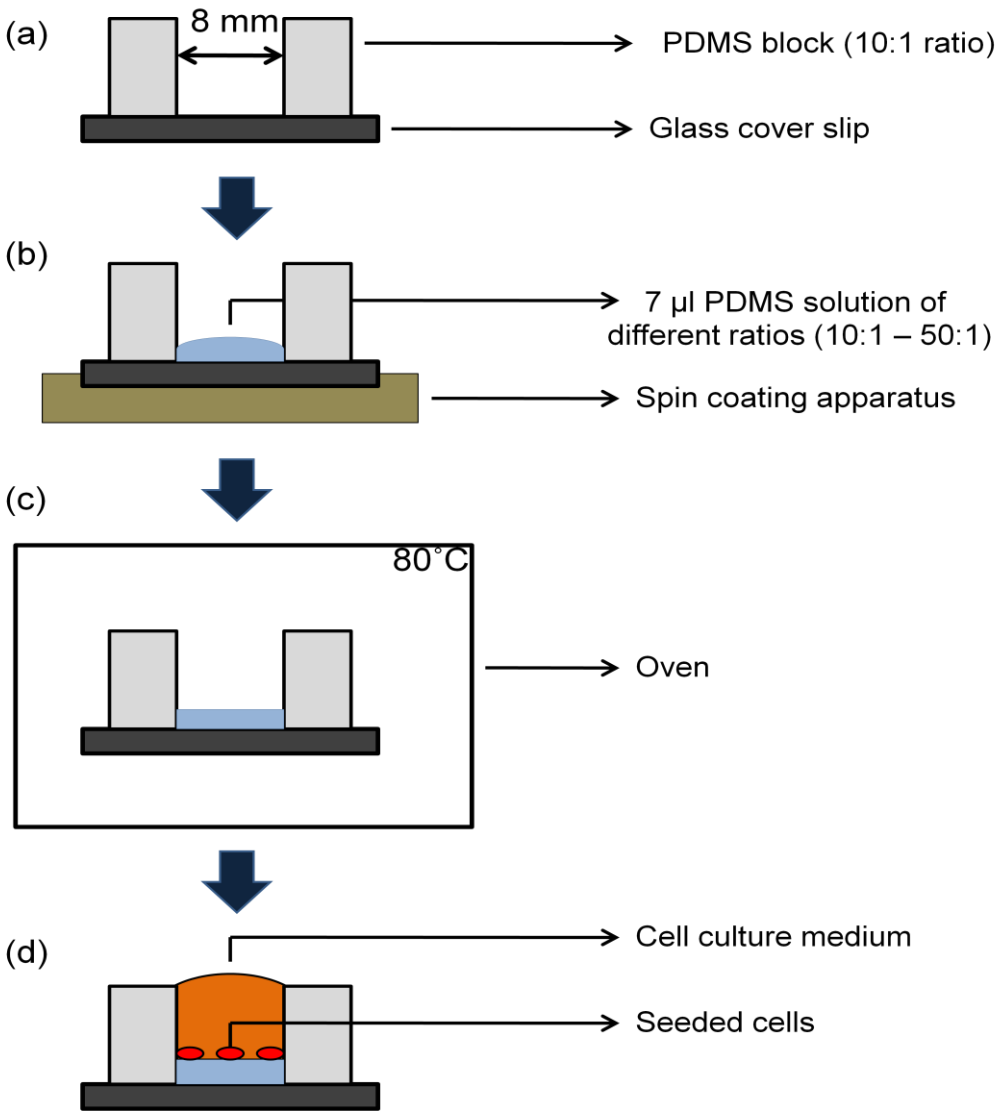


Figure 2.1 Schematic of the fabrication procedure of the PDMS device with different stiffness (a) A PDMS block is assembled on a 22X22 mm glass cover slip (b) Add the PDMS solution having different base to crosslinker ratios into the PDMS device and keep on a spin coating apparatus (c) Keep the device in an oven at 80˚C for about 48 hours to let the PDMS cure (d) Sterilize the devices and seed cells and monitor cellular response to different stiffness

For cell culture purposes, these devices were kept in a sterile biosafety cabinet and left under UV light for about 30 minutes. To further ensure the sterility of the devices, the punches

were filled with about 250 µl of 70% ethanol and left for about 20 minutes. They were then washed with sterilized de-ionized water three times and filled with sterilized 1xPBS inside a sterile hood and stored until further use.

2.2 Fabrication of PDMS array of varying stiffness

A PDMS sheet (10:1 mixture) was first made in a manner described above and punches (6mm diameter) were then created into this sheet to fabricate a PDMS array (4 rows and 6 columns). A schematic representation of the fabrication procedure is shown in the Figure 2.2.

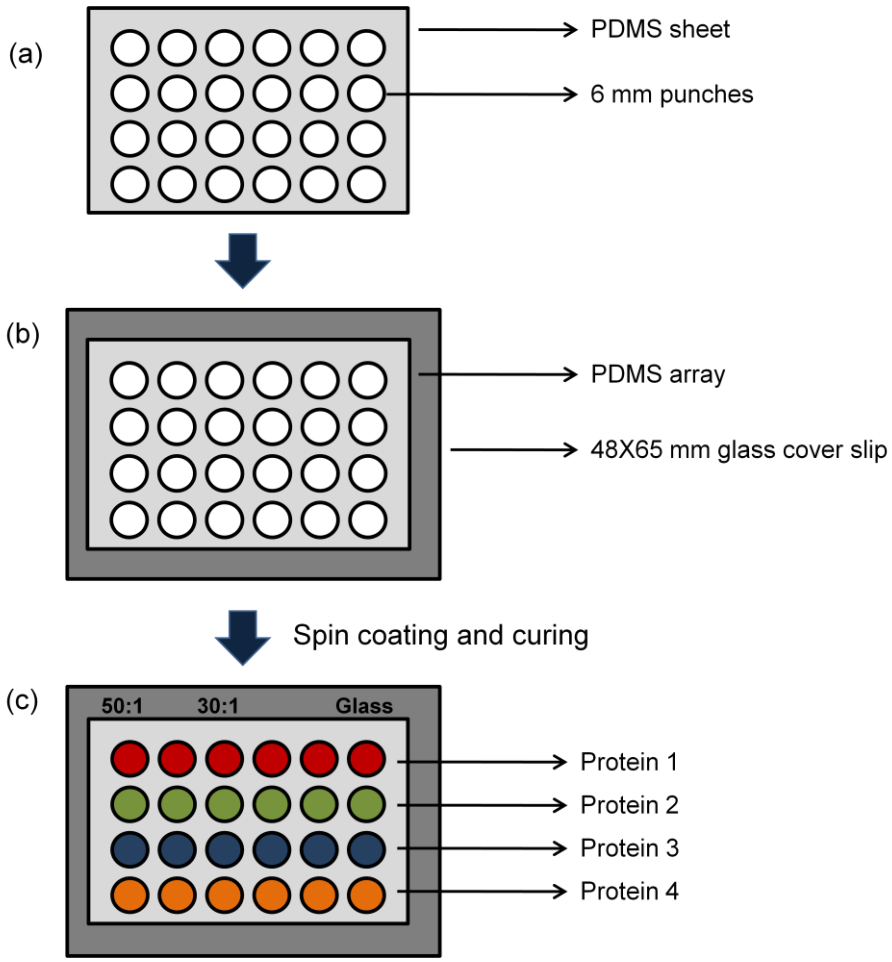


Figure 2.2 Schematic of the fabrication procedure of protein coated PDMS array having different stiffness substrates (a) Punch a PDMS sheet using 6mm punches (b) Assemble on a 48X65mm cover slip to form an array and add PDMS solutions of different ratios in the corresponding

punches (c) Spin coat and cure the polymer and coat each row with different proteins to form a protein coated PDMS array having substrates of different stiffness

The PDMS array was treated with plasma and then assembled on a clean 48x65mm cover glass to ensure there was no leaking of medium when used for cell culture purposes. The PDMS solution (7 µl/each punch) of five different base to crosslinker ratios (i.e. 50:1, 40:1, 30:1, 20:1 and 10:1) were then added into five of the columns whereas the sixth column was left empty for use as the control (i.e., cover glass). This array was then loaded onto a custom built spin coating apparatus and left in an oven at 80°C for 48 hours to ensure curing of the prepolymer in a similar way. Once the prepolymer had completely cured in each of the punches of the array, the device was sterilized by treating it under UV light for 30 minutes. Additional sterilization was done using 70% ethanol and the devices were then washed with DI water. The arrays were then stored in sterile conditions inside a biosafety cabinet.

2.3 Characterization studies

Prior to the fabrication of the device, the stiffness of the varying substrates made by mixing different ratios of PDMS base and curing agent were measured. Once the device was fabricated, the thickness of the underlying substrate was also measured. Also, the amount of protein (laminin) adsorption on glass and that on the PDMS substrates to facilitate protein attachment was quantified and analyzed.

2.3.1 Substrate Young's modulus determination

The stiffness of the PDMS substrates was measured by determining the Young's modulus of the polymer inside each punch, whose initial solution was prepared with varying ratios of the PDMS base to the crosslinker. For the purpose of determining the Young's modulus of the polymer material, a PDMS slab was made from the prepolymer of each of the different compositions of the base and curing agent (10:1, 20:1, 30:1, 40:1 and 50:1) by pouring the prepolymers on glass petri dishes and curing in an oven for 48 hours. These PDMS slabs were then peeled off from the dishes and cut into thin slices of PDMS using razor blades. These slices were attached onto the clamps of a universal mechanical tester and a load of 10 N was

used to measure the Young's modulus of the samples. The stretch speed of the mechanical tester was kept fixed at 50 mm/sec. At least three samples of each of the conditions were used to measure the Young's modulus that was determined by the mechanical testers' plotting of the stress v/s strain graph. The Young's modulus was thus averaged over the measurements of each of the conditions in the linear range of elongation and the stiffness of the varying PDMS ratios was obtained as a measure of its Young's modulus.

2.3.2 Thickness measurements of the PDMS substrate

The thickness of the PDMS substrates was also measured. For this purpose, the outer PDMS block of the entire assembly was carefully peeled off from the cover slip ensuring that the cover slip did not break and that the substrate remained intact, thus only leaving the substrate that was prepared from one of five varying ratios of base to crosslinker polymer solution (i.e., 10:1, 20:1, 30:1, 40:1 and 50:1) on the glass cover slip. A digital vernier caliper was used to measure the thickness of multiple samples of random substrates, which varied in stiffness due to the altered ratios. Finally, the substrate thickness for 15 random samples was recorded.

2.3.3 Comparison of protein adsorption on glass and PDMS substrates

Before the devices were used for cell culture purposes, the amount of protein that could be adsorbed on the hydrophobic PDMS surface was compared with the amount of protein that could be adsorbed on glass. For this purpose, PDMS devices having the substrates of the ratios - 10:1, 30:1 and 50:1 were used along with glass substrates as the control (n=4 for each of the conditions). The PDMS and glass substrates of the device assembly were cleaned, sterilized and treated under air plasma for 10 minutes so as to oxidise and deposit silanol (Si-OH) groups on the PDMS surface. The deposition of these free radicals makes the PDMS hydrophilic for a short time and hence the devices were immediately filled with 0.01% Poly-D-Lysine Hydrobromide (PDL) (Sigma Aldrich) in de-ionized water. The positively charged PLL was allowed to adsorb overnight on the negatively charged surface of plasma-oxidized PDMS to coat the surface of the PDMS substrates. After washing off the unbound PDL the next day with

de-ionized water, 10µg/ml laminin in 1X PBS was added as the model protein to coat the PDL coated PDMS substrates of different ratios. The devices were allowed to be coated with laminin overnight at 37˚C and then washed and filled with sterile PBS the next day and stored for immunochemical analysis.

To determine the amount of laminin that had been coated on the PDMS and glass substrates, immunostaining was performed. Markers for laminin (anti-laminin, Sigma) were used as the primary antibodies and were added to the substrates and left overnight at 4˚C. The next day, the unbound primary antibody was washed and the samples were treated with a secondary antibody to obtain fluorescent images of laminin.

Fluorescent images of the laminin present on the substrates were taken (n=5 for each sample; 4 samples/condition). To quantify the amount of laminin adsorbed, ImageJ was used and the gray scale intensities at 10 points for each of the 5 samples was measured so as to get a total of 50 readings for each sample and 200 readings for each stiffness. The mean values for each of the samples over the different conditions was calculated along with the standard deviations from the mean and plotted on a graph. These values were then analyzed to check if there was any significant difference in the amount of laminin adsorbed on the different conditions (i.e. 10:1, 30:1, 50:1 and glass). This was done with the help of ANOVA using Tukey post hoc test.

2.4 Cell seeding and culture on different stiffness devices

Devices of six different stiffness (i.e. cover glass, 10:1, 20:1, 30:1, 40:1, and 50:1) were used for the culture of primary human glioblastoma multiforme (GBM), mouse derived neural stem cells, and E-18 rat embryo derived cortical neurons. Human primary GBM samples were obtained from consenting patients at the University of Texas Southwestern Medical Center (Dallas, TX) with the approval of the Institutional Review Board. For rat derived cortical neurons and mouse derived neural stem cells, all procedures were performed as per IACUC (Institutional Animal Care and Use Committee) approved protocols.

2.4.1 Substrate modification

The devices that were prepared and sterilized earlier and filled with 1X PBS were used under sterile conditions. To prepare the substrates for cell seeding, the devices having substrates of different PDMS base to crosslinker ratios were kept in an air plasma chamber for about 10 minutes to make the PDMS substrates hydrophilic and ensure that the surface of the PDMS substrate was favorable for protein attachment. After 10 minutes, 200 µl of 0.01% Poly-D-Lysine Hydrobromide (PDL) (Sigma Aldrich) in de-ionized water was immediately poured into the punch and left overnight at room temperature for adsorption. Next day, the punches were washed three times using de-ionized water to remove the unbound PDL. In order to maintain an unbiased approach, the glass substrates that were used as the experimental control were also treated in the exact manner.

2.4.2 Seeding and culture of mouse derived neural stem cells

For seeding mouse derived neural stem cells, the PDMS substrates were additionally coated with laminin on the top of the PDL coating. This was done in order to facilitate and promote the adhesion and improve the migration of cell clusters on the PDMS substrate. 200 µl of 10µg/ml laminin in 1X PBS was added into each of the punches of the PDL coated substrate and allowed to adsorb overnight at 37˚C in a cell culture incubator. The next day, the substrates were washed with 1X PBS to remove the unbound laminin. After laminin coating, the PDMS devices were filled with serum-free medium. The serum-free medium was prepared using Dulbecco's Modified Eagle's Medium/F-12 medium (Cellgro) and 2% B-27 supplement (1x, Invitrogen), with the addition of Insulin-Transferrin-Selenium-X (1x, Invitrogen), gentamycin, murine EGF (20ng/ml, Peprotech) and fibroblast growth factor (20ng/ml, Peprotech). The devices filled with medium were stored in a cell culture incubator until further use.

The mouse derived neural stem cells were drawn from floating populations of these cells that were labeled with Green Fluorescent Protein. 20 µl of the cell cluster population was drawn out using a pipette and added to each of the devices that were previously filled with

medium and allowed to grow in a cell culture incubator at 37˚C and 5% CO_2 for 72 hours. The culture medium was not changed throughout the 72 hour period so that the cell orientation was not disturbed. To facilitate this, the cells were kept alongside a wet chamber i.e. a petri dish containing gauze and DI water so as to maintain the osmolarity of medium and prevent any medium evaporation. A total of 30 devices were used for these experiments (n=5 for each of the six different stiffness). The proliferation and migration of the neural stems cells from the seeded cell clusters was monitored and imaged after 4, 24 and 72 hours at the same locations. After 72 hours, the cells were fixed with 4% paraformaldehyde in 1X PBS.

2.4.3 Seeding and culture of primary human Gliobastoma Multiforme cells

For the culture of human Gliobastoma multiforme (hGBM) cells, the PDMS substrates were treated in a way similar to that for the culture of mouse derived neural stem cells. After additionally coating the substrates with laminin, the devices were filled with serum-free DMEM/F-12 medium aided with the above supplements and growth factors. The primary hGBM cell clusters floating in the medium that were labeled with Red Fluorescent Protein were used for seeding into the devices. Using a pipette, 20 µl of the cell suspension was drawn and added into the devices containing the medium. The cells were kept alongside wet chambers in a cell culture incubator at 37˚C and 5% CO_2 and allowed to grow for a period of 72 hours without any medium change. The cell migration of hGBM from the cell clusters was monitored and imaged after 4, 48 and 72 hours of cell seeding after which they were fixed using 4% paraformaldehyde in 1X PBS.

2.4.4 Seeding and culture of E 18 rat embryo derived cortical neurons

For the culturing of cortical neurons, the devices were filled with serum-free medium after being coated with PDL. This medium consisted of Neurobasal medium (Invitrogen) supplemented with B-27 (1x, Invitrogen), gentamycin, and growth factors Nuurotrophin-3 and Brain Derived Neurotrophic Factor (10ng/ml, Peprotech) as they have shown to contribute

towards neuroprotection. The medium filled devices were later stored in a cell culture incubator until cells were ready to be seeded.

For the collection of E-18 rat embryo derived cortical neurons, the cortical tissues were dissected, cleaned and collected from the rat embryos in L-15 medium. They were then enzymatically dissociated using 0.125% trypsin for 20 minutes. The tissue was then triturated and the gathered cortical neuron cell suspension was used for seeding into the devices. About 200,000 cortical neuron cells were seeded in the punches of each of the 18 devices (n=3 for each of the six stiffness) and allowed to grow in a cell culture incubator at 37˚C alongside wet chambers to maintain the osmolarity of the medium. The ells were left undisturbed for a period of 72 hours after which they were fixed with 4% paraformaldehyde in 1X PBS for quantitative immunochemical analysis.

2.5 Seeding and culture of primary hGBM cells on the multi-protein coated PDMS array

The PDMS array was used for the culture of primary human Gliobastoma Multiforme cells. Prior to the culture of the human GBM cells in the PDMS array, the array was coated with different extracellular matrix (ECM) proteins. This was done to compare the cellular behavior on different stiffness and on different ECM proteins simultaneously. Three PDMS arrays (6 columns X 4 rows) having substrates pre-coated with PDL were used and coated with 5 different ECM proteins. For the first device, the first two rows were coated with fibronectin (10µg/ml) and the next two rows were coated with vitronectin (1µg/ml). The second array had two rows coated with collagen type I (50µg/ml) and two coated with bovine Serum Albumin (50µg/ml). Similarly, two rows of the third device were coated with laminin (10µg/ml). The remaining 2 rows from the third array were not coated with any protein (PDL only) for use as the control. The proteins were incubated overnight at room temperature and the unbound protein was washed off the next day. The arrays were then filled with serum-free media (DMEM/F-12 medium supplemented with B-27 and mEGF and fibroblast growth factor).

20µl of RFP-labeled primary human GBM cell clusters was then drawn out from a floating population of these cells using a pipette and seeded into each of the different stiffness substrates of the multi-protein coated PDMS array to observe differences in cell behavior. Finally, the cells were fixed 48 hours after seeding them using 4% paraformaldehyde in 1xPBS and they were then imaged using a fluorescence microscope.

2.6 Immunostaining of neural stem cells and cortical neurons

Immunostaining was performed on the mouse derived neural stem cells and the E-18 rat embryo derived cortical neurons that had been previously fixed for further analysis. The cells were first blocked for an hour using 4% goat serum in 0.5% triton-X (1X PBS) and incubated overnight with their respective primary antibodies at 4°C, which was followed by treatment with their respective secondary antibodies. For immunostaining of neural stem cells, markers for axons (βIII Tubulin, Sigma) and astrocytes (Glial Fibrillary Acidic Protein, Dako) were used as the primary antibodies. For immunostaining of cortical neurons, the axons were immunostained using monoclonal anti- βIII-Tubulin (Sigma).

2.7 Quantification and analysis

In order to quantify the correlation between substrate stiffness and the differentiation of mouse derived neural stem cells into either neurons or astrocytes, a total of 60 images (10 images/sample, 3 samples/each stiffness; 30 for axons and 30 for astrocytes) were randomly taken using a fluorescence microscope. The axonal area and the area of astrocytes in each of the images were measured using ImageJ (NIH) and the ratio of the area covered by the axons and the area covered by the astrocytes was then calculated. The means of this ratio and the standard deviations from the means were calculated and ANOVA was then carried using the Tukey post hoc test to check for any significant differences in the ratio of differentiation of the stem cells into axons and astrocytes over the six different stiffness. Also, the growth factor ‘k’ was calculated for each of the stiffness (4 samples per stiffness, 6 stiffness conditions) to check for any significant differences in the growth rates on the different substrates. This was done by

measuring the area occupied by the cells in each of the images at the 4 different time points (4, 24 and 72 hours) and plotting them on a graph so as to get the value of the slope (k) from the linear equation of the curve formed.

For quantification of cortical neuron outgrowth on the different stiffness substrates, a total of 30 fluorescent images (10 images/sample, 3 samples/each stiffness) were randomly taken. The area covered by axons was then measured using ImageJ. Statistical significance between groups of substrates with differing stiffness was carried out by ANOVA using a Tukey post hoc test to see for differences in the outgrowth rates on the different substrates.

Finally, the growth factor ‘k’ was also calculated for the migration of human GBM cells in a similar way as used for the neural stem cells. Briefly, the area occupied by the cells on the different substrates at different time points was quantified and plotted on a graph to obtain a linear equation. The slope of this equation gave us the ‘k’ value for the different substrates. The growth factor was calculated for each of the stiffness by analyzing the data from 4 samples of each of the 6 stiffness. ANOVA using a Tukey post hoc test was then carried out to calculate any significant differences in the migration rate of human GBM cells on substrates of different stiffness.

CHAPTER 3

RESULTS

3.1 Fabrication of the PDMS stiffness device and PDMS array

The adjustable stiffness PDMS device was successfully fabricated using PDMS of five different base to curing agent ratios i.e. 10:1, 20:1, 30:1, 40:1 and 50:1 so as to get substrates over a vast range of stiffness to study their effects on cellular behavior. The entire device was assembled on a 22X22 mm glass cover slip and had a cell culture area of 50.42 mm^2.The figure below shows the fabrication procedure and the final device (Figure 3.1 - C) that was used for as the substrate for cell culture after further sterilization.

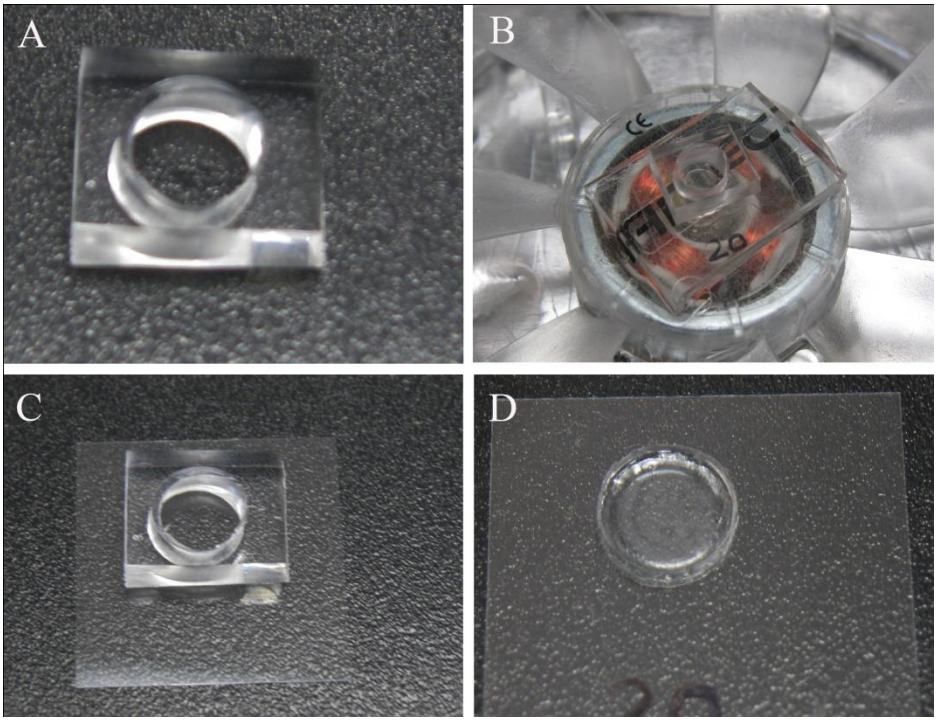


Figure 3.1 Fabrication procedure of the PDMS device having different stiffness (A) A PDMS block punched using an 8 mm biopsy punch. (B) Punched block assembled on a glass cover slip and placed on a custom built spin coating apparatus. (C) Final PDMS device obtained after curing of the prepolymer to form the substrate. (D) Outer PDMS block peeled off to show the PDMS substrate where cells are cultured.

A similar procedure was followed for the fabrication of the PDMS array. However, the PDMS sheet was punched using 6 mm punches to form punches of 6 columns and 4 rows. This entire array was then assembled on a single 48X65 mm glass cover slip. The final array obtained after spin coating the PDMS and curing it to form the substrate, the entire array looked as shown in the Figure 3.2 below. Once the array was fabricated, it was sterilized, coated with the desired proteins and used for cell culture purposes to study cell behavior on different substrates and different ECM proteins simultaneously.

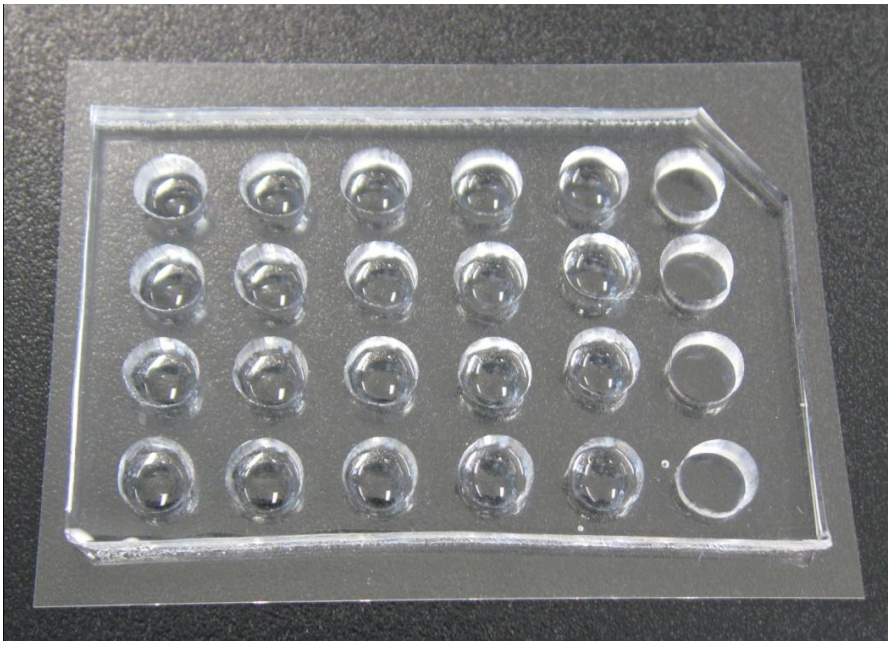


Figure 3.2 Picture of the representative 6x4 PDMS array

3.2 Characterization studies

3.2.1 Young's modulus of the substrates

The stiffness of the PDMS strips used as substrates made from solutions of different ratios of the polymer base to crosslinker were measured using the universal mechanical tester. An inverse relationship was observed between the elastic modulus (Young's modulus) of the PDMS strip (substrate) and the ratio of base to crosslinker from which it was prepared. As the ratio of PDMS elastomer base to crosslinker increased from 10:1 to 50:1, the elastic modulus of the substrate decreased, indicating a decrease in resistance of the substrate to deformation.

The Young's modulus measured for each of the ratios used as the cell culture substrate was as follows - 10:1 = 2200 kPa, 20:1= 600 kPa, 30:1= 200 kPa, 40:1= 60 kPa and 50:1= 20 kPa. The Young's modulus of the glass that was used as the sixth substrate (control) has shown to be in the range of 50 – 100 GPa in previous studies.

3.2.2 Thickness measurements of the PDMS substrate

Once the device was made, the thickness of the underlying cell culture substrate where cells are seeded and cultured was measured. For this purpose, the outer PDMS block was peeled off from the glass thus leaving only the PDMS substrate behind as shown in Figure 3.1 (D). The thickness was measured for 20 such substrate samples as shown in the Table 3.1.

Table 3.1 Thickness measurements of the PDMS substrate

Reading no.	**Thickness (μm)**
1	70
2	40
3	50
4	50
5	40
6	30
7	40
8	30
9	70
10	40
11	60
12	50
13	60
14	70
15	30
16	40
17	40
18	60
19	40
20	40
MEAN	**47.5**
STD. DEV.	**13**

The Thickness of the fabricated PDMS substrate averaged 47.5 ± 13 µm, which is twice thinner than the thickness of a No.1 cover glass (i.e. 130-150 µm). Furthermore, the thickness of this PDMS substrate can be controlled by regulating the volume of the PDMS prepolymer mixture that is poured into the punches of the device to obtain substrates of the desired thickness.

3.2.3 Protein adsorption on glass and PDMS substrates

To compare the amount of protein (laminin) adsorbed on the glass and PDMS substrates of different stiffness, fluorescent images of the adsorbed protein were taken and quantified using ImageJ as shown in Figure 3.3. After measuring the grayscale intensity values for 10 random spots in each of the 5 images for each of the samples (4 samples/ condition, 4 different stiffness conditions), ANOVA was performed to check for any statistical differences between the groups and the following data was obtained.

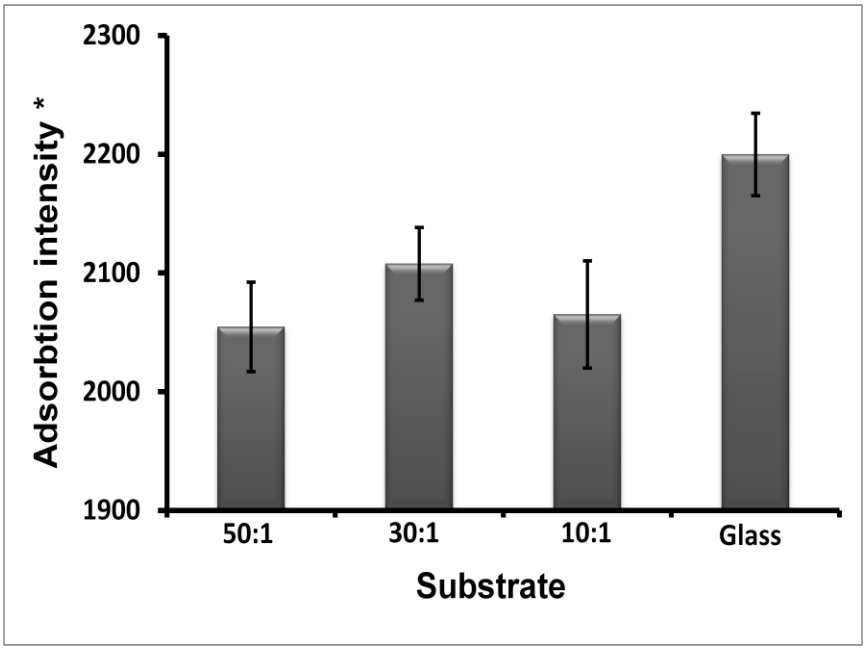


Figure 3.3 Graph showing the comparative protein adsorption on different substrates
*Grayscale value of the adsorption intensity of 16-bit images

From the above data, it can be seen that the average grayscale intensities obtained for the different substrates were as follows – 50:1 = 2054.75 ± 37.62, 30:1 = 2107.85 ± 30.65, 10:1

= 2065.16 ± 45.14 and glass = 2199.89 ± 34.67. The statistical analysis performed using ANOVA showed that there was no statistical difference between any of the PDMS groups or between the PDMS substrates and the glass substrate i.e. there was no significant difference in the amount of protein (laminin) adsorbed on the glass and PDMS substrates.

3.3 Cell behavior on different stiffness PDMS substrates

To investigate how E-18 rat derived cortical neurons, mouse neural stem cells (NSC) and human GBM cells grow on different stiffness substrates, these cells were seeded onto the substrates of the devices and cultured for 72 hours. The NSCs and hGBM cells were plated in a cluster form to observe the cellular migration and differentiation patterns from a cluster.

3.3.1 Mouse NSC proliferation and differentiation

After taking images of the cells in the same place after 4, 24 and 72 hours of seeding them onto the glass and PDMS substrates of different ratios, significantly high numbers of neural stem cells appeared to have proliferated and migrated out from their clusters on cover glass as compared to the PDMS substrates. The NSCs on cover glass appeared to have spread out completely and the clusters appeared to have a flattened morphology as early as 4 hours after cell seeding. For the PDMS substrates, most of the clusters remained as a sphere after 4 hours. Interestingly, most of NSC clusters completely flattened and cells migrated out from the clusters on the cover glass 24 and 72 hours after cell culture, whereas a mixture of flattened and spherical clusters was seen on all PDMS substrates. Even after 72 hours of cell culture, many of the cell clusters on the PDMS substrates remained spherical and very little cell migration was observed on the softer PDMS substrates i.e. PDMS substrates having the ratio 40:1 and 50:1. The Figure 3.4 below shows a pictorial representation of the comparison of the neural stem cells spreading sequence from a cluster at 4, 24 and 72 hours after seeding on different stiffness substrates varying from softest (50:1 ratio of PDMS base and curing agent) to the stiffest (cover glass).

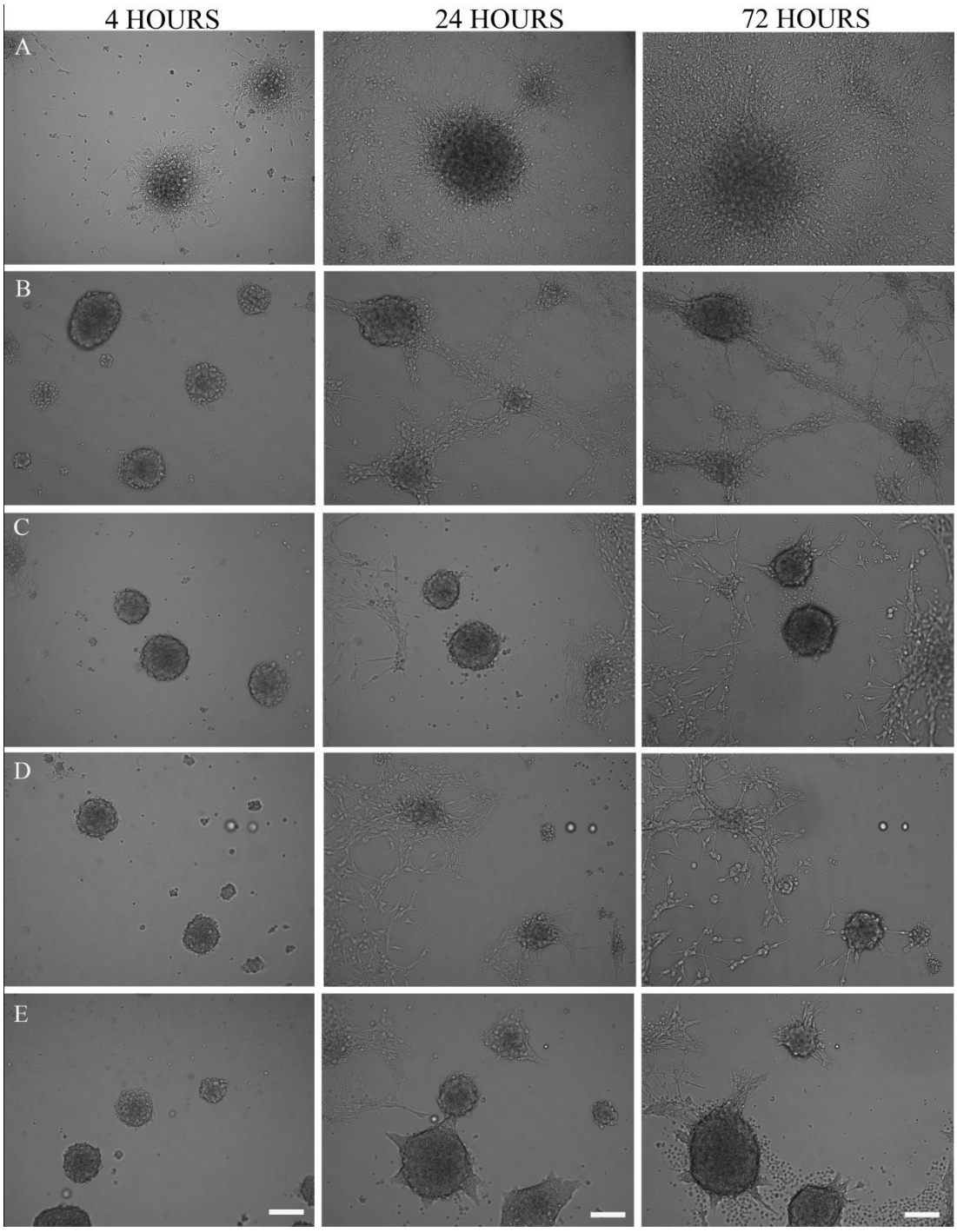


Figure 3.4 Mouse neural stem cell spreading on different substrates. Cell clusters seeded on the PDMS devices having different base to crosslinker ratios (A) 50:1, (B) 40:1, (C) 20:1, (D) 10:1 and (E) cover glass and images taken at 4 hours, 24 hours and 72 hours after cell seeding. Scale bar = 100μm.

72 hours after seeding the mouse neural stem cells in the devices, the cells were fixed and immunostained to understand the effects of substrate stiffness on the differentiation patterns of the NSCs. The figure below shows the representative immunofluorescent images of the NSCs. The cells were immunostained for neurons using the βIII tubulin marker (red) and astrocytes using the GFAP marker (blue) on different substrates as shown in Figure 3.5.

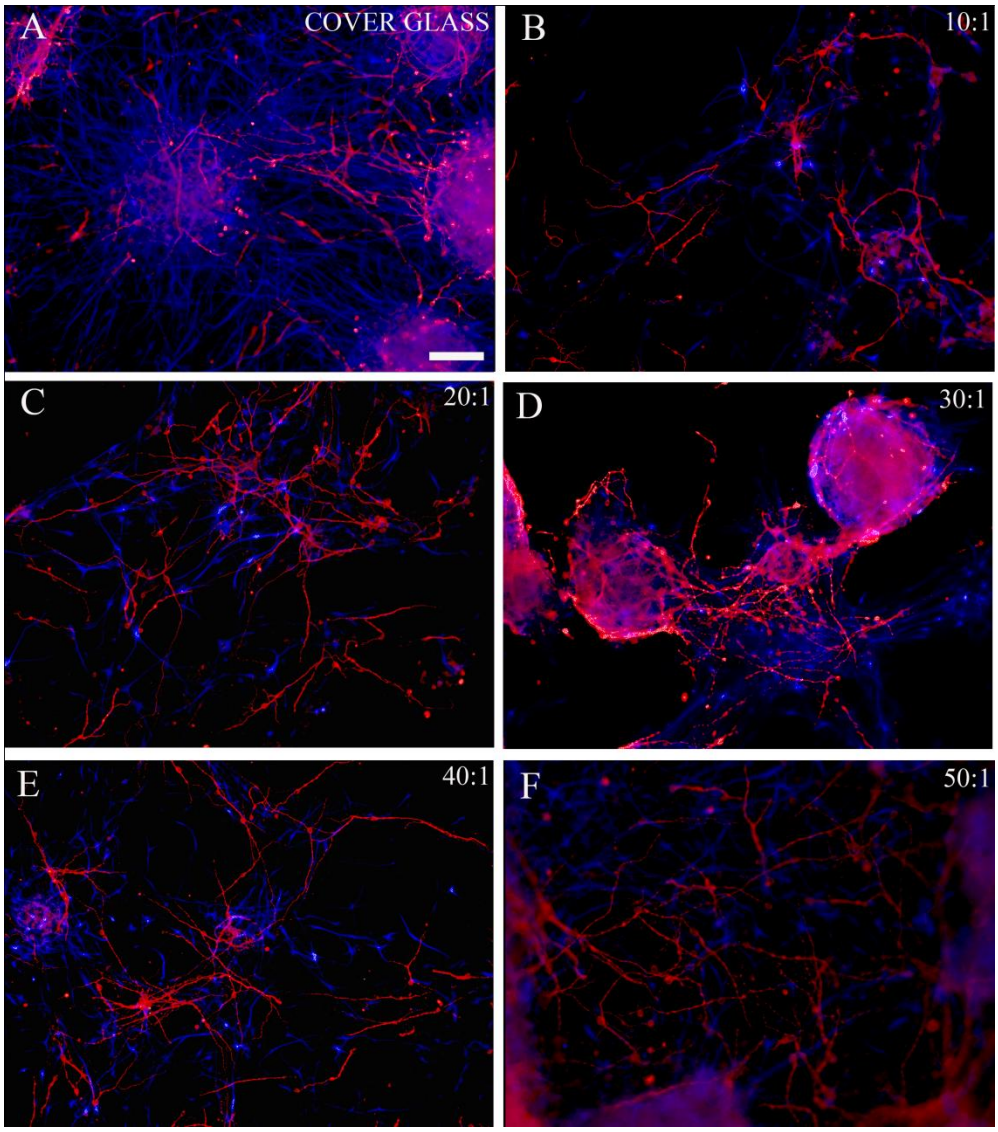


Figure 3.5 Representative immunofluorescent images of mouse neural stem cells on the different substrates. The cells were double immunostained for β-III Tubulin (red, axon) and GFAP (blue, astrocytes) on different substrates (72 hours after seeding). (A) Cover glass, (B) PDMS substrate having base:crosslinker ratio 10:1, (C) 20:1, (D) 30:1, (E) 40:1 and (F) 50:1. Scale bar = 100 µm.

For the quantitative analysis, the axonal area and the area occupied by the astrocytes were measured using the ImageJ software and their ratio was calculated. As seen from Figure 3.6, the results demonstrate that the differentiation of the mouse neural stem cells into either axons or astrocytes was significantly influenced by the substrate stiffness. On the stiffest substrate (cover glass), the number of NSCs that had differentiated into axons was lesser than that compared to those that had differentiated into astrocytes, the ratio being 0.46 ± 0.13. For the PDMS substrates of different base and crosslinker ratios, the ratio of the differentiation of NSCs into axons and astrocytes was – 10:1 = 0.77 ± 0.26, 20:1 = 0.79 ± 0.24, 30:1 = 0.79 ± 0.13, 40:1 = 0.81 ± 0.24 and 50:1 = 0.95 ± 0.25. Using ANOVA, a statistical difference was observed in the ratio of NSC differentiation into axons and astrocytes between the glass substrate and the PDMS substrates. No statistically significant difference was observed in this differentiation ratio amongst the different PDMS experimental groups. This is shown in the Figure 3.6.

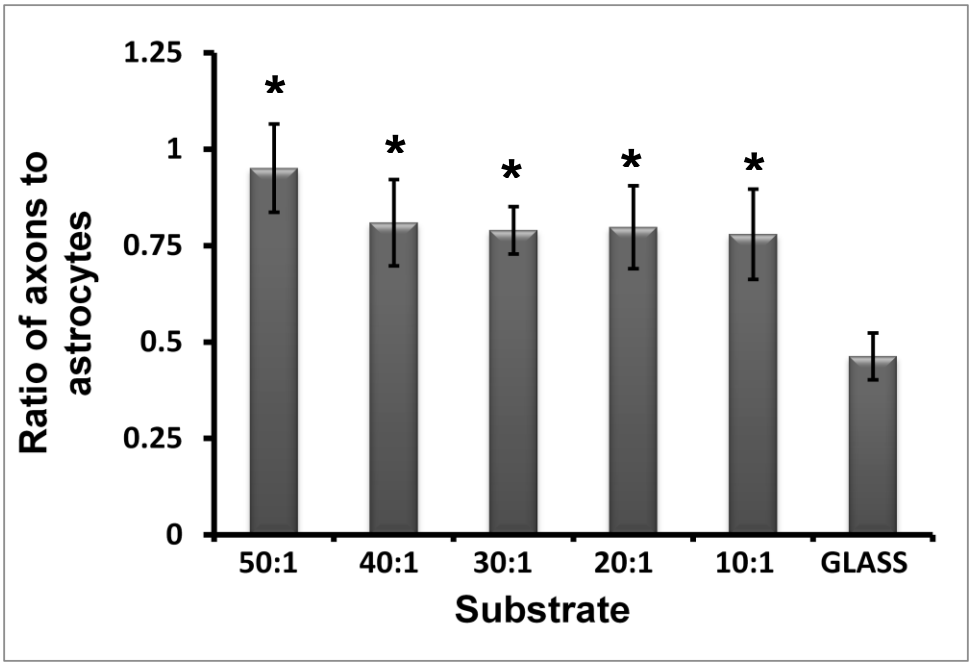


Figure 3.6 Graphical representation of the ratio of NSC differentiation into axons and astrocytes on different substrates. Average ± SEM. *P<0.01 between cover glass and others.

The growth factor ‘k’ was then calculated for each of the different stiffness based upon cell migration images taken at three different time points: 4, 24 and 72 hours for 4 samples of each of the stiffness. The k values obtained from plotting the cell area at different time points for the different substrates to get the slope of the linear equation were as follows – 50:1 = 0.78 ± 0.1, 40:1 = 0.91 ± 0.05, 30:1 = 1.43 ± 0.26, 20:1 = 1.18 ± 0.06 and glass (control) = 5.98 ± 0.31 respectively. Using ANOVA, significant statistical differences were observed in the migration rates of the mouse neural stem cells on cover glass versus all the five experimental PDMS substrates. However, no significant differences in the migration rates represented by the k values were seen amongst the PDMS substrates. This is shown in the Figure 3.7 below.

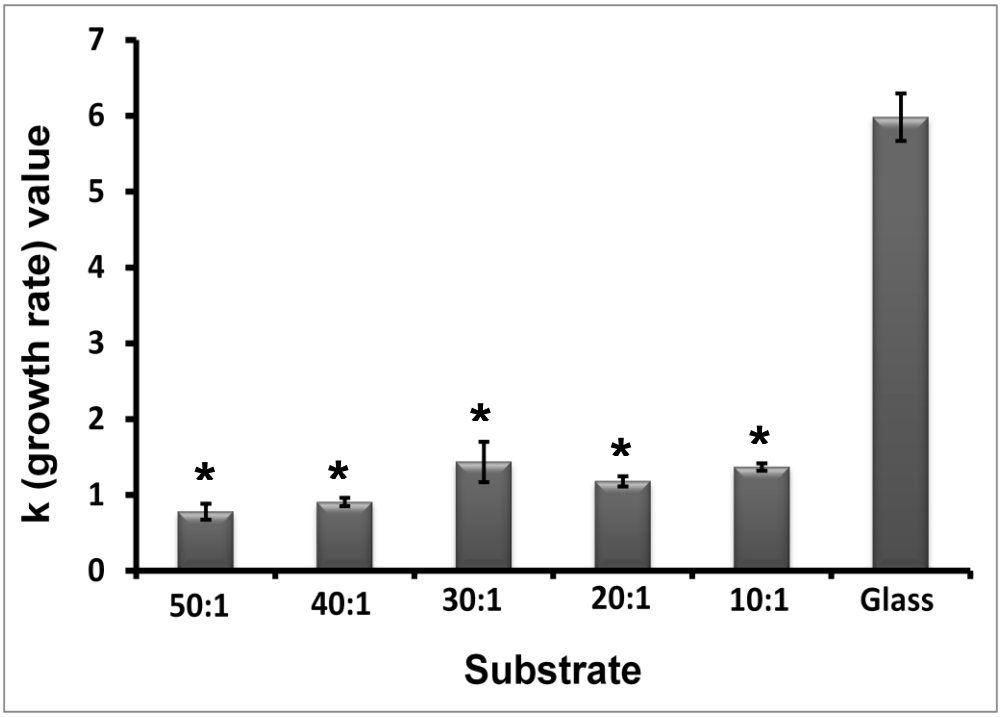


Figure 3.7 Graph representing the growth factor ‘k’ calculated for the migration rates of neural stem cells on the different substrates. Average ± SEM. *P<0.05 between cover glass and other groups.

3.3.2 Primary human GBM cell migration

Primary human GBM cells seeded in the PDMS devices also showed difference in their migration patterns based on the stiffness of the underlying substrate. On the stiffest substrate

(cover glass), like NSC, GBM clusters firmly flattened on the substrate within 4 hours of seeding and the cells robustly migrated out from the cluster. For the same stiffness, 24 hours after cell seeding, most of cells migrated out from the original cluster so that no clusters were observed. In contrast, on all of the PDMS substrates (10:1 to 50:1), the original cell clusters remained as a sphere and low number of cells migrated out within the first 4 hours of cell seeding. 24 hours after seeding, more cells migrated out from the clusters on the stiffer PDMS substrates (10:1 and 20:1) compared to the softer substrates (30:1-50:1). However, a few of the clusters still appeared to be in the form of spheres even on the stiffer PDMS substrates. After 72 hours of cell seeding, on the stiffer PDMS substrates, cells had migrated out extensively and almost no cell clusters were seen as compared to the softer PDMS substrates where the cells migration out from the clusters was limited and most cells still remained in clusters. This suggested that GBM cells prefer to adhere to each other and stay together rather than spreading out as a single cell on such soft substrates. The cellular migration on three different substrates (cover glass, 20:1 and 50:1) is shown in the Figure 3.8.

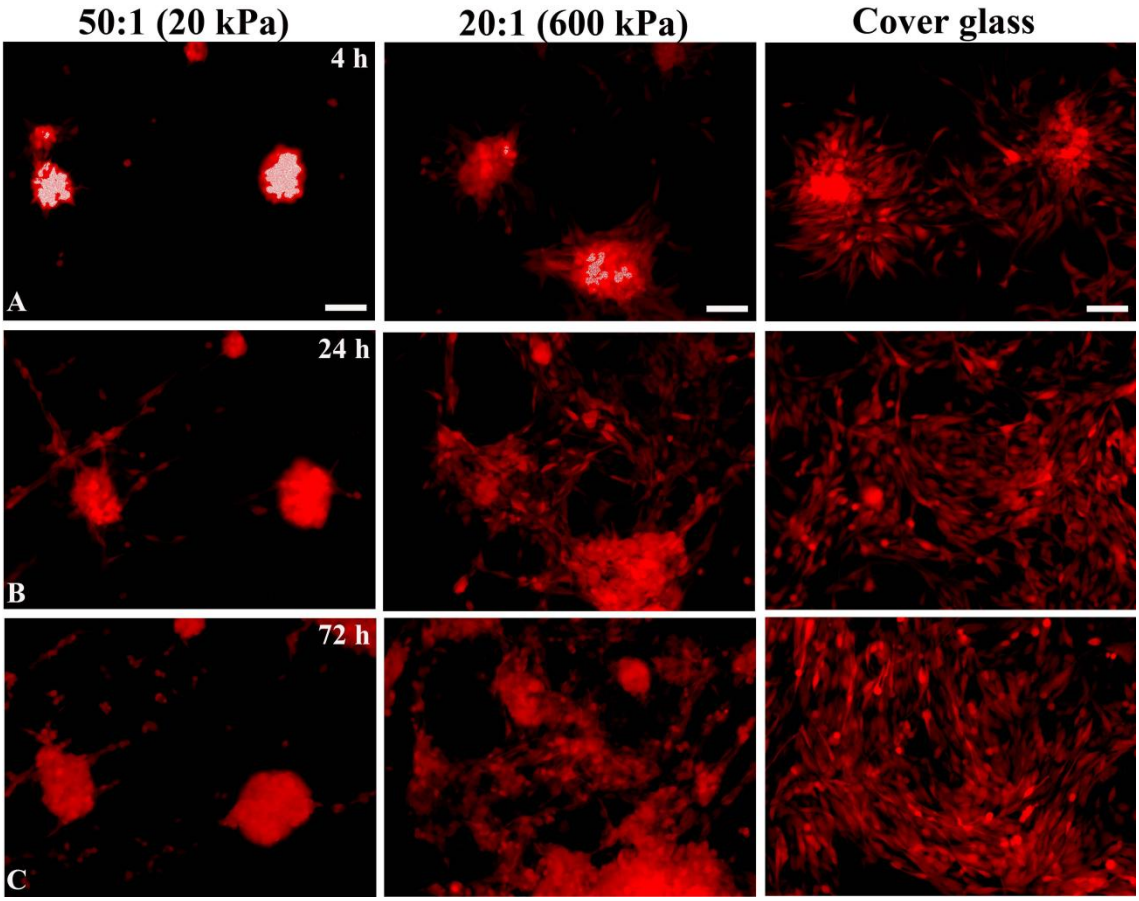


Figure 3.8 RFP labeled primary human glioblastoma multiforme cell migration on different substrates. Cell clusters seeded on PDMS devices having different base to crosslinker ratios of 50:1 and 20:1, and a cover glass as the cell culture substrate. Fluorescent images were taken at (A) 4 hours, (B) 24 hours, and (C) 72 hours after cell seeding. Scale bar = 100µm.

To further validate the observation obtained from the Figure 3.8 that GBM migration rate was higher on the stiffer substrates; the k (growth rate) values were calculated for the different groups and plotted on a graph so as to calculate the migration rates on their respective substrates. After plotting the graph and obtaining the linear equation from the curve, the k value obtained as the slope of the curve for the glass substrate was 1.16 ± 0.03. The values obtained for the PDMS substrates of different prepolymer base to curing agent ratios were – 50:1 = 0.27 ± 0.02, 40:1 = 0.26 ± 0.01, 30:1 = 0.32 ± 0.03. k values obtained for the stiffer PDMS substrates were as follows – 20:1 = 0.42 ± 0.03 and 10:1 = 0.45 ± 0.03. Statistical analysis that was

performed using ANOVA showed that there was a significant difference in the k values representing the migration rates between the hGBM cells seeded on cover glass as the substrate and all of the PDMS substrates. Also, significant differences were seen in the migration rates of the cells seeded on stiffer PDMS substrates (10:1 and 20:1) and the softest substrates (40:1 and 50:1). This is shown graphically in the Figure 3.9 below.

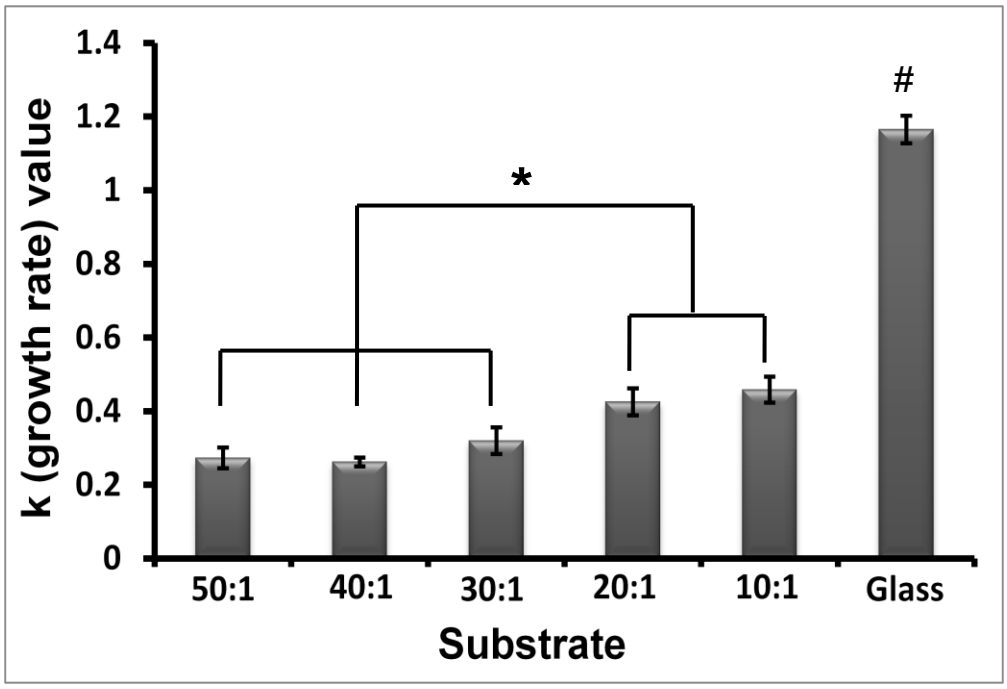


Figure 3.9 Graph representing the growth factor 'k' calculated for the migration rate of human GBM cells on the different substrates. Average ± SEM. *P<0.05 between groups; #P<0.05 between cover glass and other groups.

3.3.3 E-18 rat derived cortical neuron outgrowth

The difference in outgrowth for the cortical neurons on the different substrate stiffness was also studied. Once an equal number of cortical neurons were seeded in each of the devices having different substrate stiffness, they were allowed to attach on the substrates and cultured for 72 hours. After 72 hours, the cells were fixed, immunostained for axons using axonal marker β-III Tubulin, imaged and quantified to compare the axonal outgrowth difference amongst the different stiffness substrates. The cortical neurons appeared to have formed a dense neuronal network on the glass substrate as well as the stiffer PDMS substrates (i.e. 10:1

and 20:1). However, poor axonal network was seen on the softer 40:1 and 50:1 PDMS substrates. Figure 3.10 represents immunofluorescent images of the cortical neuron outgrowth patterns on the different stiffness substrates.

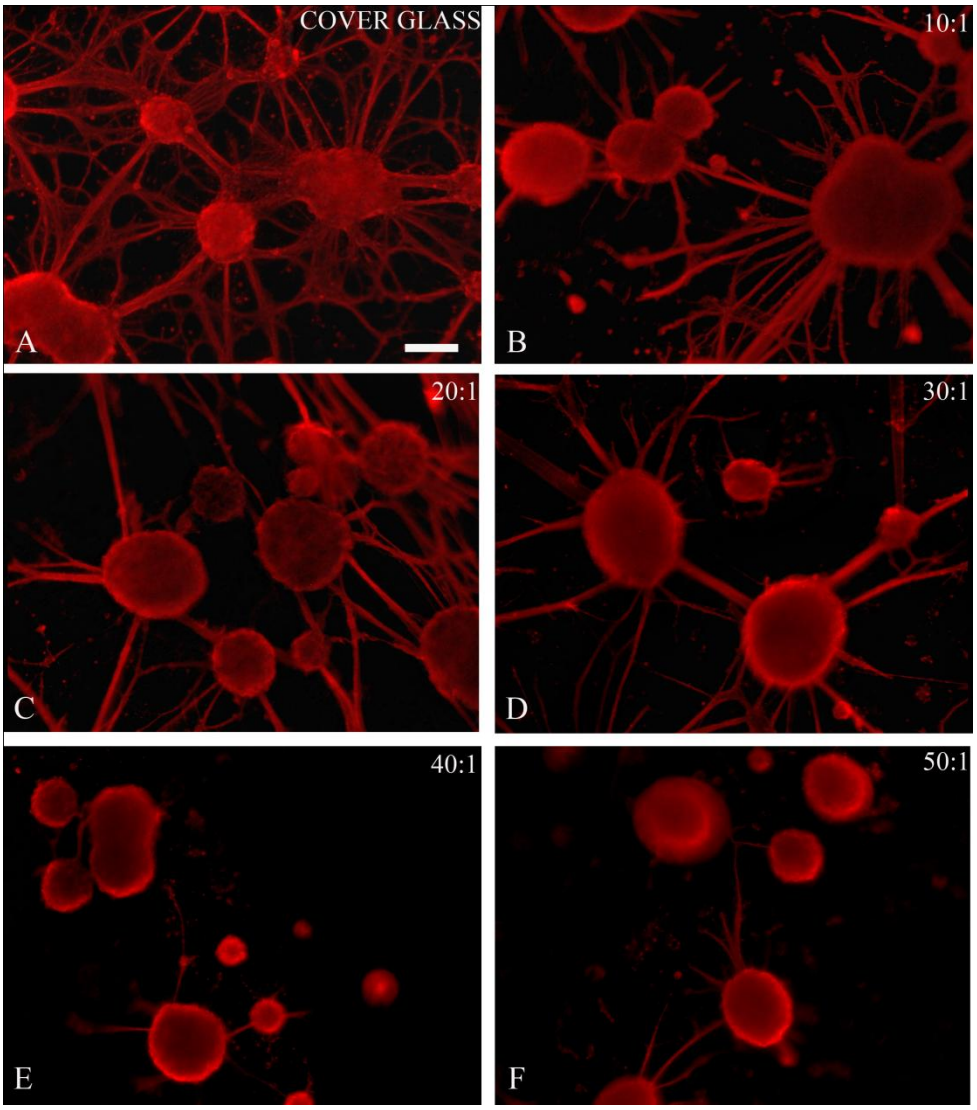


Figure 3.10 Representative immunofluorescent images of E-18 rat embryo derived cortical neurons on the different substrates. The cells were immunostained for β-III Tubulin and imaged 72 hours after cell seeding on (A) PDMS substrate having base to curing agent ratio 50:1 (softest substrate), (B) 40:1, (C) 30:1, (D) 20:1, (E) 10:1 and (F) cover glass (stiffest substrate). Scale bar = 100 µm.

The axonal area was calculated from the images taken for the different stiffness using ImageJ. It was observed that as the substrate stiffness increases, the neuronal network density

on the respective substrate also increases. The axonal area calculated from the images were 25.08 ± 3.47 mm^2 and 25.75 ± 2.51 mm^2 for the comparatively softer PDMS substrates i.e. having the base and curing agent ratios of 50:1 and 40:1 respectively. For the stiffer PDMS substrates i.e. having ratios of 30:1, 20:1 and 10:1, the axonal area measured were 31.87 ± 4.5 mm^2, 36.57 ± 3.66 mm^2 and 35.8 ± 4.33 mm^2 respectively. For the stiffest substrate used for neuronal cell culture i.e. cover glass that was used as the control, the axonal area was calculated to be 67.39 ± 6.25 mm^2. Unlike high dense neuronal network on the cover glass and on the stiffer PDMS substrates, the originally dissociated neurons adhered together, formed a sphere and very low neuronal outgrowth was observed on the soft substrates, specifically PDMS ratios of 40:1 and 50:1 that formed softer substrates. These values were then plotted so as to obtain a graph as shown in the Figure 3.11.

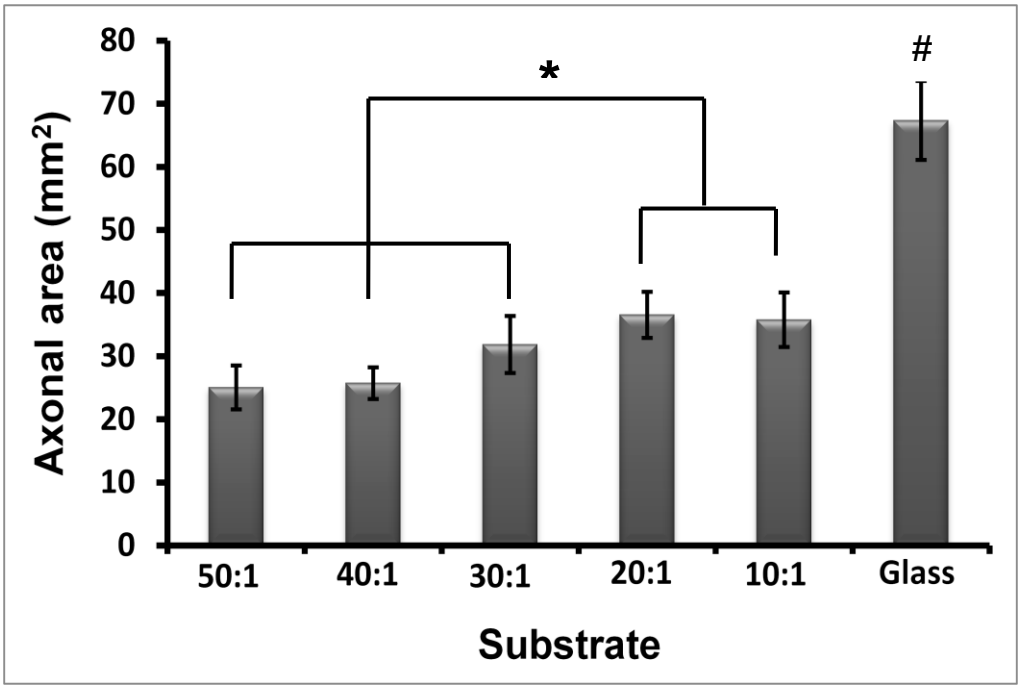


Figure 3.11 Graphical representation of the axonal area of E-18 rat derived cortical neurons on the substrates of different stiffness. Average ± SEM. *P<0.05 between different groups; #P<0.05 between cover glass and other groups.

To check if there was any significant statistical difference in the axonal area over the different stiffness, the values were compared using ANOVA. These tests indicated that there was a statistical difference between the axonal areas of the cortical neurons seeded on the glass substrates and those seeded on all of the PDMS substrates (10:1, 20:1, 30:1, 40:1 and 50:1). There was also a statistically significant difference between the axonal areas of the cells seeded on the stiffer PDMS substrates (10:1 and 20:1) and the softer PDMS substrates (40:1 and 50:1). All the experimental substrate groups (glass and PDMS) that showed a significant different had p values of <0.01. The statistical comparison for the axonal area of the cortical neurons is summarized in the Table 3.1 below.

Table 3.2 Statistical comparison of the E-18 rat embryo derived cortical neuron axonal area on the different substrates. Bold represents statistical difference *P<0.01

P value	50:1 ratio	40: 1 ratio	30:1 ratio	20:1 ratio	10:1 ratio	Cover glass
50:1 ratio	-	0.9998	0.0758	**0.0001***	**0.0009***	**0***
40:1 ratio	0.9998	-	0.1741	**0.0007***	**0.0037***	**0***
30:1 ratio	0.0758	0.1747	-	0.414	0.6622	**0***
20:1 ratio	**0.0001***	**0.0007***	0.414	-	0.9997	**0***
10:1 ratio	**0.0009***	**0.0037***	0.6622	0.9997	-	**0***
Cover glass	**0***	**0***	**0***	**0***	**0***	-

3.4 hGBM cell behavior on the PDMS arrays coated with various ECM proteins

To study the effects of the substrate stiffness and the underlying proteins simultaneously on human Gliobastoma Multiforme cell migration, 3 PDMS arrays (6x4) having substrates of different stiffness were used. After coating the PDMS arrays having substrates of different stiffness with the different ECM proteins, they were seeded with clusters of human GBM cells from a floating cell population and allowed to grow for 48 hours after which their cell migration from the clusters was observed. We observed qualitatively that in the case of the cells that had been seeded on substrates coated with the proteins fibronectin, collagen type I and laminin, the hGBM cell migration was strongly dependent on the substrate stiffness and

behaved in a similar manner as observed in the earlier results and the underlying protein did not alter this behavior i.e. cell migration increased with an increase in the substrate stiffness. However, for the substrates coated with the protein vitronectin, the comparative migration of the cells from their clusters was the highest and seemed independent of the substrate stiffness for all of the stiffness conditions. For the substrates coated with the protein BSA and those that were not coated with any protein and used as the control (PDL only), the primary hGBM cell migration seemed very poor and the clusters appeared to remain in their spherical conformation on substrates of all stiffness even after 48 hours of cell culture. The PDMS arrays having different stiffness and coated with different proteins and the corresponding cell behavior on these substrates is shown in the Figures 3.12 – 3.14.

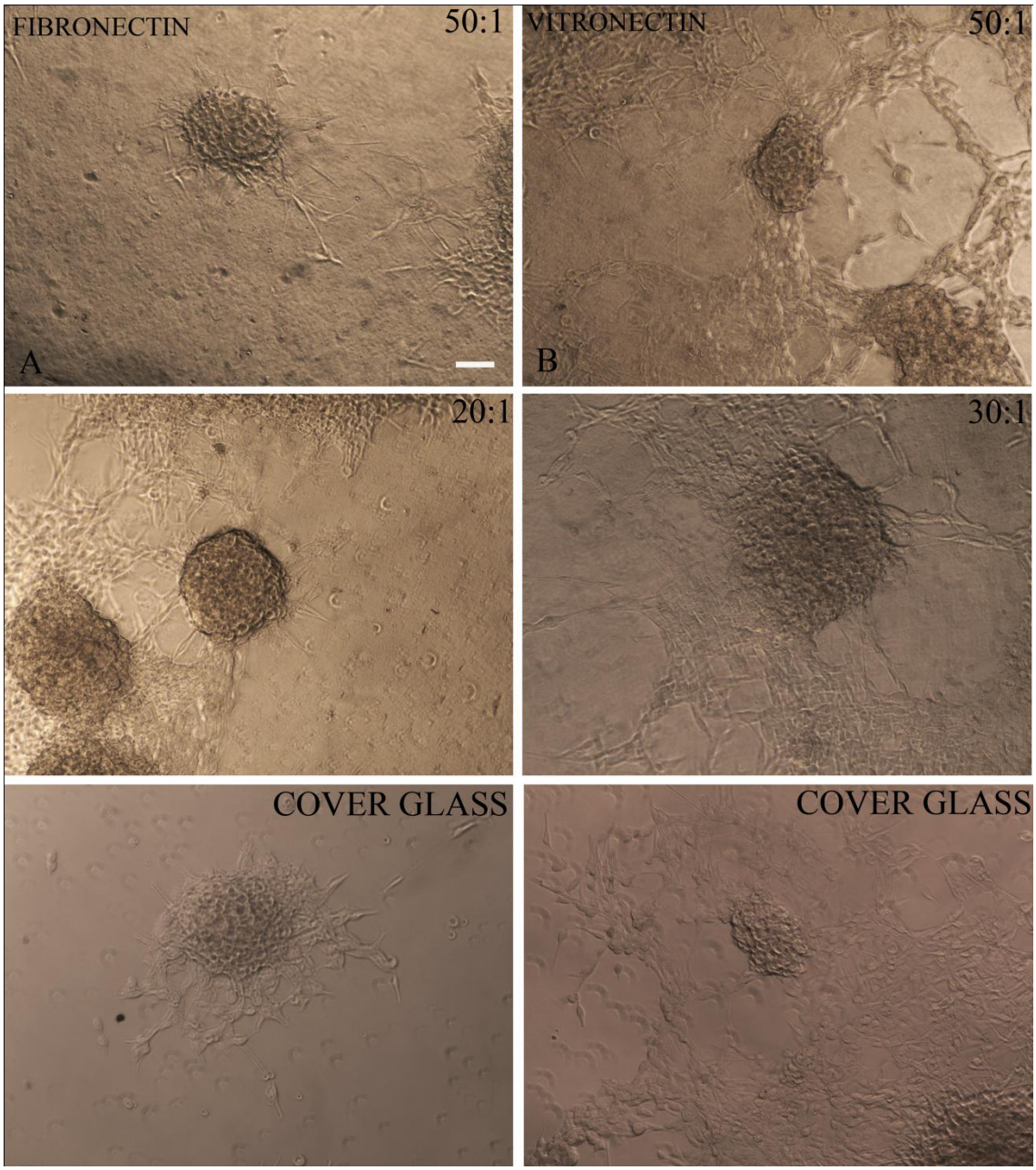


Figure 3.12 Primary human Gliobastoma Multiforme cell behavior the PDMS array having substrates of different stiffness coated with the ECM proteins – (A) Fibronectin and (B) vitronectin. Images were taken 48 hours after cell seeding. Scale bar = 100 μm.

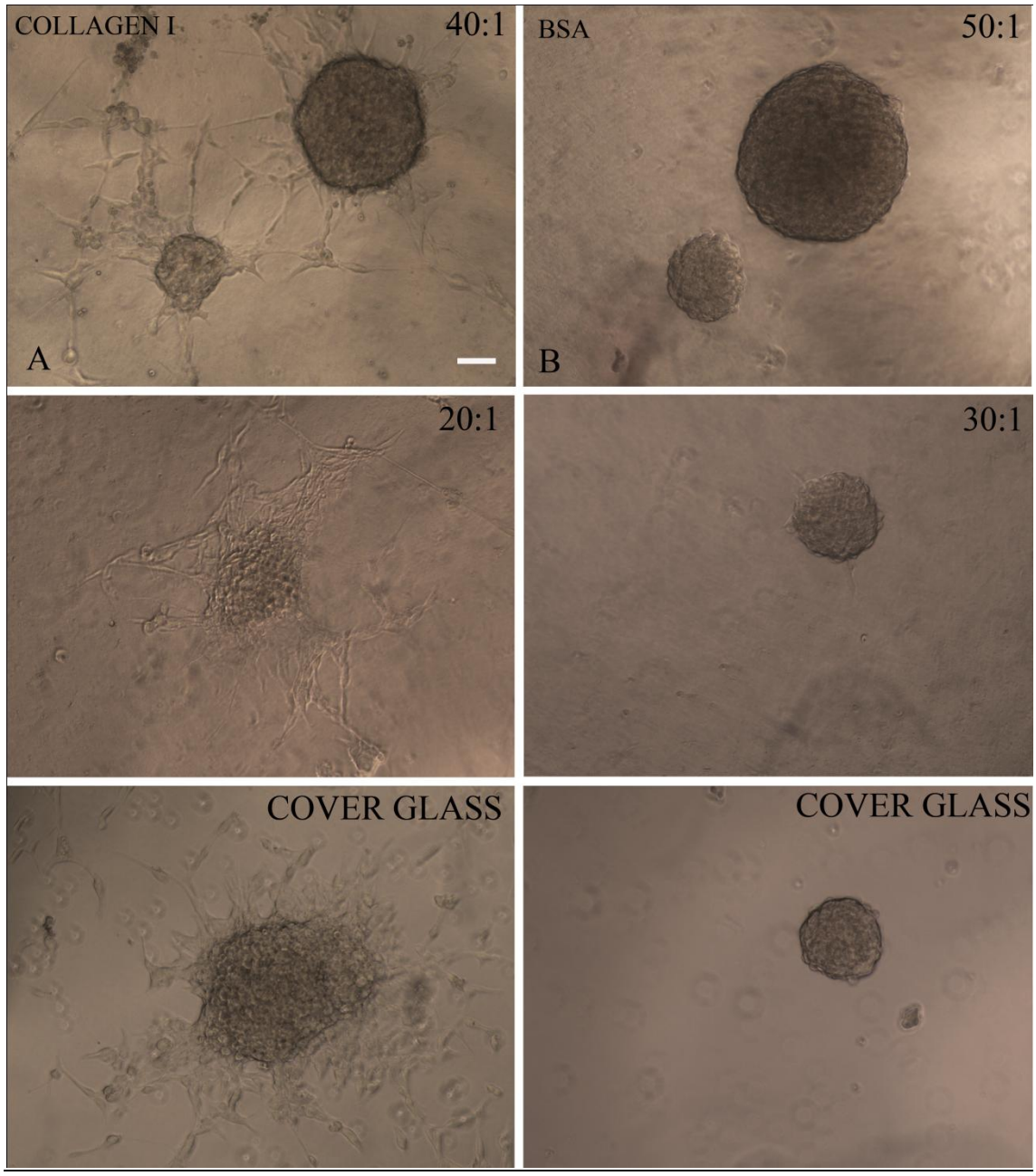


Figure 3.13 Primary human Gliobastoma Multiforme cell behavior on the PDMS array having substrates of different stiffness coated with the ECM proteins – (A) Collagen Type I and (B) Bovine Serum Albumin. Images were taken 48 hours after cell seeding. Scale bar = 100 μm.

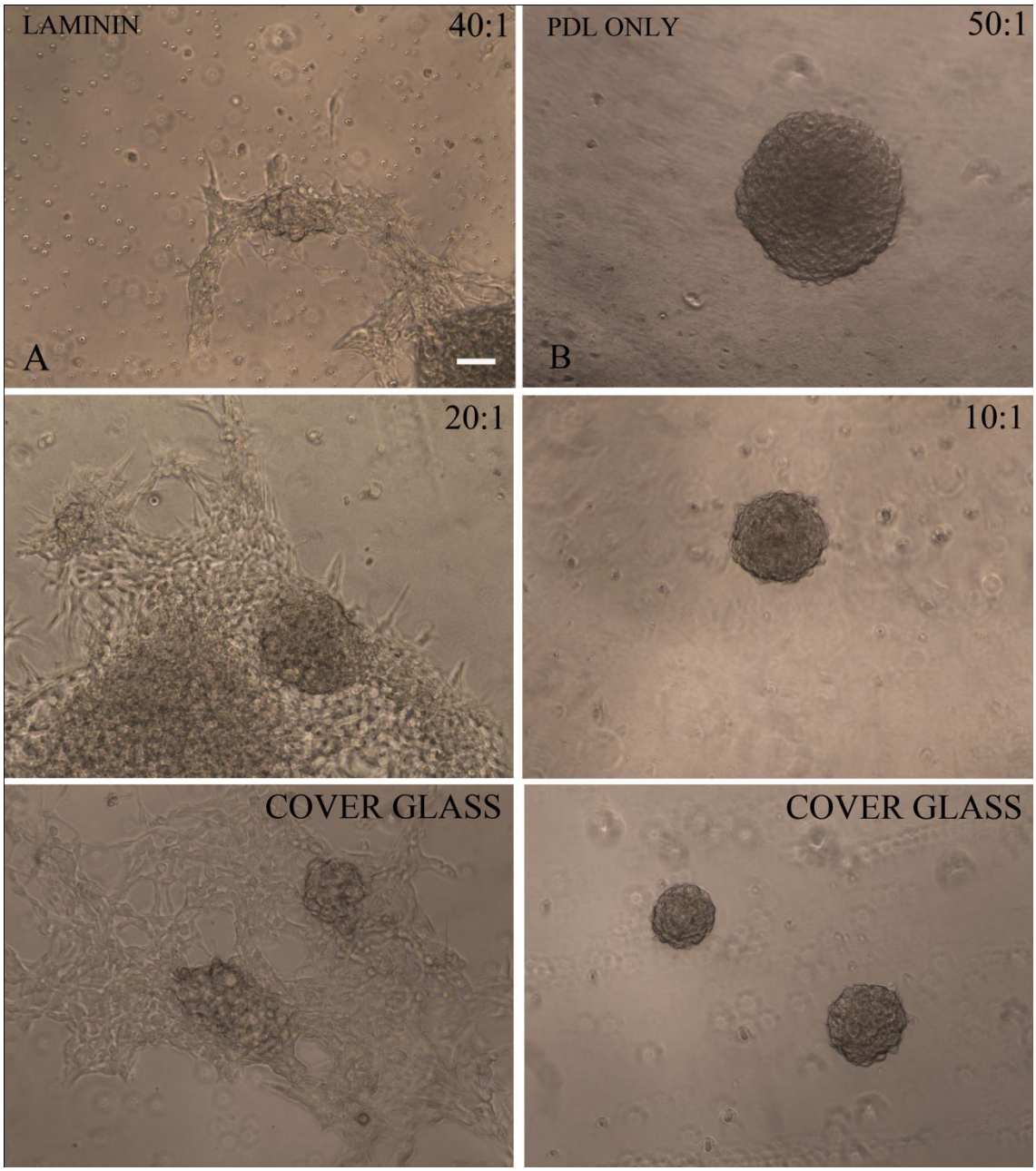


Figure 3.14 Primary human Gliobastoma Multiforme cell behavior on the PDMS array having substrates of different stiffness coated with the ECM proteins – (A) Laminin and (B) No protein (PDL only). Images were taken 48 hours after cell seeding. Scale bar = 100 μm.

CHAPTER 4

DISCUSSION

Most *in vitro* cell culture studies in the past have used hard substrates such as plastic or glass with or without any protein adsorption on the substrate surface. However, most cell types in multicellular organisms in their native microenvironment are attached to either neighboring cells or extracellular matrices whose rigidity (100 Pa - >10,000 Pa) is a lot less compared to the commonly used glass (50 - 100 GPa) or polystyrene plastic (2 - 3.5 GPa) with some surface modification [15,37]. Previous studies have also shown that the matrix stiffness regulates the cellular response and the nature of the adhesion receptor by which the cell binds its substrate [36,37]. The inherent material properties of the matrix has a huge impact on the amount of cellular force that it can resist and thus using stiff substrates like glass and plastic may not provide proper information when using them as substrates for *in vitro* experiments [35]. Thus using glass or polystyrene may not be a true representation of the cellular substrate and researchers have started looking at other options for use as substrates and have experimented with various biomaterials that are capable of culturing cells at various levels of stiffness [41]. These include fibrin, collagen, cross-linked polysaccharides (e.g., alginate and agarose gels), polyacrylamide gels and PDMS [40,45,81]. Among these materials, we have used Poly (dimethylsiloxane) (PDMS) for making different stiffness substrates as its numerous advantages for cell culture purposes over other materials have been shown before [41,62,82]. Different substrate stiffness has been achieved using this inert and biocompatible polymer by using different ratios of the PDMS base and curing agent (10:1, 20:1, 30:1 40:1 and 50:1). As the concentration of the crosslinker decreased, reduced substrate stiffness was observed owing to the presence of unbound and mobile crosslinker molecules. In order to study the cellular behavior of the less understood cells of the nervous system, we have used three different cell

types found in the nervous system and cultured them on PDMS substrates having stiffness ranging from the manufacturer's suggested value (2 MPa) to that of the native elastic modulus of the brain (0.5- 50 kPa) [83]. The different stiffness values obtained from the different ratios of PDMS were similar to those reported in earlier studies [41,81]. This stiffness adjustable device is easy to fabricate and reproduce, and the thickness of cell growing substrate (47 μm) is half the thickness of the commercially used No.1 cover glass. This thin substrate allows one to monitor live cells at higher magnifications which is not possible in previous studies as either the PDMS substrate is too thick or is cured in a polystyrene cell culture well plate. In addition, to increase the number of culture substrates that could be studied at the same time and to reduce the number of devices needed, we have fabricated a PDMS array comprising of substrates of different stiffness that can be assembled on a single cover glass. This array can also be used to study the effects of the substrate stiffness along with other factors such as the underlying protein that may affect cellular behavior simultaneously.

Substrate stiffness has shown to have many effects on cellular functions under conditions where the chemical signals are constant. The stiffness of a cell's environment impacts cell adhesion, proliferation, migration, differentiation and phenotype [47]. This has been shown on an assortment of cell types including epithelial, endothelial, vascular smooth muscle, fibroblasts, neutrophils and stem cells [37,40,56,84,85]. The growth and viability of the cells along with resistance to apoptosis can be regulated by the stiffness [86]. The strength with which the cells adhere to their substrate and their degree of spreading is also influenced by the substrate stiffness [32,33]. It also regulates the degree of cell-matrix adhesion and size of focal adhesions along with the stiffness and tension developed by the cell itself [57]. Previous studies using fibroblasts have shown that cells generate more traction force and develop a broader and flatter morphology on stiffer substrates and they preferentially migrate from a soft to a stiff surface, a mechanism known as durotaxis [87]. Thus, motility and cell alignment can also be associated with the matrix stiffness [88]. A previous study has also shown the fundamental

effects of substrate stiffness on the differentiation of mesenchymal stem cells (MSCs). The results showed that MSCs cultured on substrates having stiffness comparable to the brain tissue differentiated into neurons whereas those cultured on substrates with the stiffness of muscle and bone differentiated into myocytes and osteoblasts [89]. The dependence of various cellular functions on the stiffness of the substrate is thus evident. However, for much softer tissues like those of the nervous system, the dependence of cell behavior on substrate mechanics is less clear [47].

Like these cells, our results clearly demonstrate that the outgrowth, differentiation, or migration of E-18 rat embryo derived cortical neurons, mouse neural stem cell, and brain cancer cells (human Gliobastoma Multiforme) are significantly influenced by the stiffness of the substrate on which the cells are seeded and cultured. Our results clearly show that the differentiation of the mouse neural stem cells into astrocytes increased as the stiffness of the substrate increased from the 50:1 to cover glass (Figures 3.5 and 3.6). A previous study has shown that in the adult neural stem cell differentiation, enhanced neuronal maturity is observed on the substrate having a similar elasticity to that of the native tissue when using a serum-free growth medium as used in our study [28]. NSC differentiation into glial cells (astrocyte) has been seen to increase when the same cells are seeded on stiffer hydrogels substrates such as those made from polyacrylamide [27]. In our results too, we observed that the neural stem cells prefer to differentiate into astrocytes on the harder substrates (i.e., cover glass). When cultured on cover glass, the ratio of axons to astrocytes was found to be the lowest (0.463), but a significant increase in this ratio can be seen for all the PDMS substrates. The softest PDMS substrate (50:1) yields the highest ratio of axons to astrocytes differentiation of NSCs (0.951). This may be because of the nature of neurons in the brain. They do not bear any loads due to their discrete localization and are isolated from the external environment by the cranium [28]. Our results thus indicate that the differentiation of NSCs into either neurons or astrocytes depends upon the substrate stiffness. Like the adult brain that has a mechanically

heterogeneous environment possessing regions of different moduli, the variable stiffness PDMS device used in this study also provides with different stiffness and has also made it possible to study the neural stem cell behavior in such different conditions. Since they have the capability to differentiate into neurons and glia, variation in stiffness can be used as a control parameter to tune their differentiation while performing *in vitro* studies. In the cortical neurons outgrowth, we observed that the axonal outgrowth gradually increased with an increase in the substrate stiffness over the experimental PDMS groups and dramatically increased when the cortical neurons were cultured on the hardest substrate (cover glass) as seen from Figures 3.10 and 3.11. Previous studies have shown contradictory results culturing cortical neurons on different stiffness. Some studies have shown that isolated hippocampal neurons prefer to branch and grow on softer substrates whereas some other study has shown that cortical neurons are not affected by the stiffness of the substrate on which they grow and have attributed this insensitivity to the homogenous environment of the developing cortex [46,90]. However, the qualitative data obtained from our study along with the significantly different values of the axonal area on the different stiffness, it is clear that there exists a difference in the neuronal outgrowth on different substrates i.e. neuronal growth increases with increased substrate stiffness. These discrepancies may be due to a number of reasons such as the differences in signaling or the age and class of the neuron along with the time and length of observation [46]. The variable stiffness device used in this study may thus be further used to get a detailed and unbiased outlook at the neuronal behavioral changes with changes in the substrate stiffness. GBM is a highly invasive and aggressive form of primary brain tumor. The major problem faced during its treatment is that it migrates and infiltrates into the surrounding normal tissue thus evading the treatment [93]. Thus it is important to study their migration in a more physiologically relevant stiffness range while developing anti-invasive strategies. In the human GBM cells migration from a cluster, a significant increase in their migration rate was seen with an increase in the stiffness of the substrate on which they were cultured. From the values of k (growth rate)

obtained that represent the migration rates, it was clear that there exists a significant increase in the rate of migration of GBM cells when cultured on glass and the stiffer PDMS substrates (10:1 and 20:1). A similar kind of response by glioma cell lines has been shown previously as well [91]. The rigidity of the surface has shown to affect the cell spreading, motility, proliferation and other such factors owing to the interaction between the integrin protein family expressed on the glioma cell surface and the substrate [92]. All the above parameters have been shown to have enhanced on rigid substrates and shown to decrease on the more compliant substrates. From our results obtained, it can be thus seen the migration rate (k value) was highest in the case of the glass substrate and significantly higher in the case of the stiffer PDMS substrates compared to the softer ones (30:1, 40:1 and 50:1). This indicates that stiffer substrates facilitate the motility of these cells and that substrate stiffness plays an important role in the migration rates of hGBM cells. We also introduced another factor along with substrate stiffness to see if the cell behavior with respect to the stiffness changed using a PDMS array having substrates of different stiffness such as PDMS made from the ratios of 10:1 – 50:1 and cover glass. Biochemical signals have shown to play an important role in regulating GBM invasion [91]. A number of *in vitro* studies have been performed in the past and have shown the importance of a variety of ECM proteins including fibronectin, laminin, collagen and other such proteins in stimulating migratory phenotype in both GBM cell lines and biopsy explants [93]. For most of the ECM proteins that we used in our study (3 out of 5), it was observed that the cell behavior was dependent on the substrate stiffness irrespective of the ECM protein coating the substrate. However, the cell migration was observed to be the highest in the case when the substrate was coated with the protein vitronectin and appeared to be independent of the effects of substrate stiffness unlike other proteins (Figures 3.12, 3.13 and 3.14). This indicates that the vitronectin could override the effects of the substrate stiffness and result in increased cell migration regardless of the substrate stiffness. The array can thus be further used to understand the mechanics behind the GBM cell behavior when cultured on vitronectin and other proteins.

Based upon our results and literatures, it can be inferred that since a lot of the cellular responses such as cell differentiation, migration, proliferation etc. depend on the mechanical rigidity of the substrate, substrates having different stiffness may be needed for examining the mutual relationship between cells and their micro-environment. The PDMS based device and array reported in this study can be used to achieve substrates of different stiffness and systemically study cellular responses to different substrate stiffness *in vitro* along with additional factors that could affect their behavior simultaneously.

CHAPTER 5

CONCLUSION

We have shown here the fabrication of a simple yet novel PDMS based device and array having an adjustable stiffness that has been used to systemically study the mechanical effects of the cellular substrate on the outgrowth, differentiation, and migration of mouse derived neural stem cells, rat embryo derived cortical neurons and human GBM cells. The results clearly demonstrate that the cells behave differently on substrates of different mechanical stiffness, indicating that while studying cellular properties *in vitro*, the mechanical properties play a vital role in the cellular behavior and thus must be considered. The device and array used here thus incorporate an important mechanical cue i.e. substrate stiffness and can be used to control a variety of cellular activities such as proliferation, differentiation and migration. Also, the fabrication procedure of the device/array can easily be modified to produce substrates having controlled thickness and a wide range of stiffness that can include the broad physiologically relevant range assembled on a single cover glass. Thus, this novel device/ array may be suitable to mimic the *in vivo* mechanical environment of a variety of cells in *in vitro* and additionally be used to study the effects of a number of other physical and chemical cues along with the substrate stiffness on cellular behavior.

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BIOGRAPHICAL INFORMATION

Chetan Bhuwania was born in Bhagalpur, India on 28th May 1986. He graduated with a Bachelor's degree in Biomedical Engineering from Mahatma Gandhi Mission's College of Engineering and Technology, Mumbai, India in June 2009. During the course of his bachelor's degree, he completed his hospital training at Dr. B Nanavati Hospital in December 2008 where he gained technical expertise. After showing immense interests in the field of Tissue Engineering, he moved to the United States to pursue a Master's degree in Biomedical Engineering at the University of Texas at Arlington. He joined the Neuro-engineering lab under the supervision of Dr. Young-tae Kim in September 2009 to acquire knowledge and research skills for developing techniques that would help improve the quality of human life. He thus wishes to pursue a career in research and development that would ultimately lead him to his final goal.