Engineering cell adhesion for applications in biotechnology

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Abstract

The ability to control the placement of mammalian cells is important for many commercial and research applications. Any attempt to constrain normal mammalian cells will have functional consequences and will require an understanding of the basic mechanisms underlying adhesion between cells and their underlying substratum. We describe the current model of cell adhesion and the use of micropatterned surfaces to control cell behavior.

1. Introduction

Living cells have been used for decades for the production of therapeutic and diagnostic materials. Biochemical pathways in simple single-celled organisms like bacteria and yeast have been exploited to yield products including small organic compounds, carbohydrates, and proteins [1]. Recently, for a variety of reasons, some commercial applications have forced the biotechnology industry to turn to using more costly mammalian cells as production agents. Leading the way to the marketplace are protein-based therapeutics manufactured in large-scale mammalian cell culture bioreactors. Most of these processes currently approved by the Food and Drug Administration (FDA) rely upon transformed (cancerous) cell lines cultured in suspension (similar to bacterial and yeast culture) in stirred-tank reactors (see Chu et al, 2001 for review). These homogeneous systems have several salient features, including well-established scaling parameters, and are currently being used to produce drugs like Herceptin™ to treat breast cancer and Wellferon™ to treat hepatitis C [2].

Despite the successes of suspension culture, most applications in biology will require using cells that adhere onto a surface. First, there are intrinsic limitations to using transformed cell lines, and the majority of normal (non-transformed) cells die when cultured in suspension. For example, transformed cell lines exhibit abnormalities in glycosylation and other pathways for post-translational modifications, and are thus unsuitable for the production of many proteins. There are also safety issues associated with using transformed cell lines for cell-based therapies, and tissue engineered products will therefore need sources of normal cells. Second, many applications require spatially heterogeneous systems. Adherent cells can be used to create addressable platforms for

screening and other cell chip applications. Controlling cell adhesion and understanding its ramifications are thus critical to the future of the biotechnology industry.

2. Fundamentals of Cell Adhesion

Mammalian cells have strict requirements for maintenance in culture. Both normal and transformed cells are propagated in specialized culture medias that contain a balance of salts, glucose, amino acids, vitamins, and growth factors. In addition to being maintained at the appropriate temperature (37°C), humidity (100%), and pH (7.2), most cells from solid tissues grow as adherent monolayers, and need to attach and spread out on the substrate before they will start to thrive and proliferate [3]. In fact, normal cells commonly interpret the lack of adhesion as a signal to commit suicide. For culture in the research laboratory, cells are grown attached to the bottom surfaces of appropriately treated glass and polystyrene flasks and dishes. For industrial applications requiring large cell numbers, cells can also be cultured on coated microspheres that are suspended in stirred tanks.

Cell adhesion is mediated by specific receptors on the cell surface that interact with extracellular matrix (ECM) molecules (Fig. 1). In vivo, cells are surrounded or underlain by ECM, which organizes cells into tissues. ECM forms the polymerized scaffolding that holds tissues together, and includes collagen fibers, elastins, proteoglycans, and glycoproteins such as fibronectin and laminin. ECM molecules are found naturally in some media supplements, and are also secreted by cells themselves. In culture, cell attachment and spreading is preceded by the adsorption of ECM onto the substrate surface. Cells are thus unable to attach and spread onto substrates that resist the

adsorption of ECM. In vitro studies have demonstrated that cell-ECM adhesion is not merely to immobilize cells, but also has many functional consequences that include changes in cell survival, growth, metabolism, protein synthesis, and gene expression [4]. The regulation of cell function by cell-ECM adhesion is not completely understood and is an active area of investigation.

Although there are many types of cell surface adhesion receptors, cell-ECM adhesion is primarily mediated by members of the integrin family. Integrins are transmembrane heterodimers composed of α and β subunits. Thus far, 17 α and 8 β subunits have been identified, and there are at least 22 functional heterodimers in mammalian cells. Portions of both subunits form the ECM ligand-binding site, giving the different integrin heterodimers specificity for different ligands. For example, $\alpha1\beta1$ and $\alpha2\beta1$ both bind to collagen and laminin, while $\alpha5\beta1$ binds to fibronectin. Most of the different integrins are expressed in a variety of cell types, and most cells express several integrin heterodimers, allowing them to adhere to many different ECM molecules [5].

Cell-ECM adhesion is both dynamic and tightly regulated. Integrins have relatively low affinities for their ligands ($K_D \sim 8.6 \times 10^{-7}$), but most cells express relatively large numbers of these molecules (5×10^5 receptors/cell) [6]. Because of their positions at the surface, integrins form an important bi-directional link between cells and their environments. That is, cells use integrins to detect changes in the composition of ECM on the culture surface and respond by modifying cell behavior. Cells can also change the levels of integrins displayed at the cell surface to alter cell adhesion. For example, cells must dynamically regulate integrin expression in order to transition from being firmly adherent to highly motile across the substrate.

3. Engineering Cell Adhesion

Many strategies to control cell-ECM adhesion rely on controlling the placement of ECM on the surface. The most commonly used technique is to nonspecifically adsorb the ECM protein of interest from solution prior to plating the cells. The advantage of nonspecific adsorption is that it is a simple and straightforward approach. One disadvantage of nonspecific adsorption is that the process is not well-understood and not easy to control at the molecular level. Most ECM proteins have several different cell-binding regions or epitopes. Proteins can potentially adsorb onto the surface in multiple conformations, masking some epitopes and unmasking others. Therefore, even the simplest case of nonspecific adsorption of a single ECM protein creates a heterogeneous substrate for cell adhesion, and could be considered analogous to the use of an undefined growth medium (such as one containing serum) instead of a mixture of purified, defined ingredients. Another disadvantage of nonspecific adsorption is that the substrates formed are intrinsically unstable since the process relies upon non-covalent interactions between the protein of interest and the surface.

Another technique to control cell adhesion is to covalently immobilize ECM proteins to the substrate. Typically, glass or polymeric surfaces are treated to present an activated functional group that will react with primary amines or other groups present in proteins. The reaction between the ECM protein of interest and the substrate is carried out prior to plating cells. The advantage of covalent immobilization is that it produces a substrate that is more stable than one produced by nonspecific adsorption. However, the specific techniques for covalent immobilization cannot be generalized: The surface

materials are limited to those that can be modified with activated functionalities. Another disadvantage of covalent immobilization is that most ECM proteins are rather large molecules (>350 kDa) and they contain many reactive groups that can form a bond with the activated substrate, so covalent immobilization still produces a heterogeneous surface. To circumvent this problem, a number of investigators have adapted approaches from the enzyme-immobilization industry to tag the ends of proteins with specific moieties (such as His, myc, or GST) and then immobilize the tagged molecule [7]. This approach allows one to specifically control the orientation of the ECM molecule on the surface, but is a costly manufacturing process that produces substrates with low stability. Better immobilization strategies are currently being explored.

Theoretically, controlling the ECM proteins present on a substrate should limit adhesion to cells that express the appropriate integrins. For example, fibronectin-coated surfaces should only support the adhesion of cells that express $\alpha 5\beta 1$ and other integrins that bind to fibronectin. Surfaces of defined adhesivity could thus be used to "pan" for, or selectively capture, specific cells from within a heterogeneous mixture. Practically, however, panning for cells is limited by the complexity and redundancy of the integrin-ECM adhesion system. Unfortunately, there is no single "endothelial cell ECM" or "hepatocyte ECM" that can be used to definitively separate these from other cell types. Most ECM proteins are capable of being bound by several integrins, and most cell types express numerous different integrins. Therefore, while there is some specificity for different cells to adhere to different ECM, there is also significant overlap. In practice, the principal motivation for providing well-defined ECM ligands for cell adhesion is not to capture specific cells, but to control the behavior of cells by appropriately engaging

specific integrins. This strategy is analogous to the need to use specific cytokines to induce desired behaviors in cells. Since the differentiated function of several cell types is controlled by their adhesion to specific ECM molecules [8], specifying ECM on a surface in vitro can be used to recapitulate in vivo phenotypes, to enhance growth rates, or to improve protein production.

While immobilized ECM proteins can be used to control the initial adhesion of cells on a substrate, most surfaces are unstable in the presence of cells and thus remain defined only transiently. Cells can remodel the surfaces on which they are attached over time courses as short as 2 hours [9]. First, most cell types naturally produce and secrete endogenous matrix molecules, which either adsorb onto the surface nonspecifically or bind to the immobilized ECM through specific protein-protein interactions. Second, cells can actively remodel their substrates by secreting proteases that can degrade the immobilized ECM [10]. Therefore, even the most chemically stable immobilization procedure is potentially susceptible to damage by cells. Inhibiting these processes can slow surface degradation, but these interventions can be cytotoxic. For example, pharmacologically blocking protein secretion will inhibit ECM turnover, but will also inhibit other essential secretion-dependent processes. Pharmacological inhibitors of proteases will inhibit active degradation, but cells can simultaneously upregulate the expression of many distinct proteases. Controlling cell adhesion is thus a multifaceted issue that must take into account effects from the cells themselves.

4. Designer Surfaces

While bulk populations of adherent cells are commercially desirable for the production of protein-based therapeutics and diagnostics, many applications require finer spatial control of cell adhesion. For example, the architecture and organization of cells in space can affect how the cells function as a population. Several microfabrication-based strategies have been developed to pattern cell adhesion at the micrometer scale, and most rely on controlling the placement of ECM molecules or other adhesive moieties on the surface. These microarrayed adhesive regions are surrounded by nonadhesive regions, which typically resist cell adhesion by resisting the adsorption of protein. Cell adhesion is thus confined and limited to the microarrays of ECM.

We have found that one of the most robust methods to pattern cells is to print the ECM protein of interest directly on the surface using an elastomeric stamp (Fig. 2a). The bare regions are then backfilled by coating the substrate in a solution of a nonadhesive protein such as bovine serum albumin (BSA) or nonadhesive polymer such as pluronics. Also called "microcontact printing", stamping of proteins falls into a general class of techniques dubbed "soft lithography", which transfer patterns from a master created by photolithography to the desired substrate using an elastomeric stamp. The stamp is created by casting a liquid prepolymer of polydimethylsiloxane (PDMS) on the master, allowing it cure, and peeling it off the surface. The stamp surface thus forms a negative replica of the original pattern on the master. We then "ink" the PDMS stamp by coating it in a solution of ECM protein, washing it with saline, and drying it under a stream of nitrogen. Placing the inked stamp in conformal contact with the surface of interest transfers the pattern of ECM. Microcontact printing is a relatively quick, convenient, and inexpensive technique. Beyond the microfabrication facilities needed to generate the

initial silicon master (a process which can be outsourced), all steps can be carried out in a typical biological or chemical laboratory. The most significant drawback to this technique is that it is difficult to render a surface completely resistant to the eventual adsorption of protein or degradation from cellular processes. Over time (days to weeks), the patterned cells will break down the nonadhesive material and secrete new ECM proteins whose adsorption facilitates the migration and adhesion of the cells into the nonadhesive regions. We have recently demonstrated that even the most chemically inert surfaces are subject to degradation by cell-dependent processes [10]. Another disadvantage to microcontact printing is that cells cultured on printed ECM often exhibit slightly different behaviors from those cultured on ECM adsorbed from solution.

The converse approach is to first pattern the nonadhesive material and then backfill the ECM protein by adsorbing it onto the bare surface from solution (Fig 2b). This type of a strategy allows one to take advantage of the stability and protein resistance of a number of hydrogels, including agarose and polyacrylamide [10, 11]. Hydrogels can be patterned on a surface using microfluidic approaches. First, a PDMS stamp is sealed against a flat surface, such as a glass slide. Then, a hot solution of agarose or a monomeric solution of acrylamide is perfused through and allowed to gel in the channels created between the stamp and the slide. Removing the stamp from the slide reveals bare regions of glass in the pattern of interest surrounded by walls of nonadhesive agarose or acrylamide. The substrate is then coated in a solution of ECM protein to control cell adhesion. Cells seeded on these substrates will only attach and spread in the wells (Fig. 3a-b). Changing the height of the features on the master allows one to change the height

of the walls of hydrogel. Since cells cannot degrade agarose or acrylamide, covalently coupling the hydrogel to the surface creates very stable patterns.

Why pattern cell adhesion? There are a number of potential advantages and applications derived from controlling cell adhesion. First, tissue engineers can benefit from being able to replicate in vivo structure by a priori defining the placement of cells [12]. Many solid tissues have complex geometries of multiple cell types, and it is clear that for most, the function of the tissue is closely tied to its structure. The relationship between structure and function is apparent even at the level of the individual cell, in that cell shape can direct proliferation (Fig. 3c) and death [13]. Understanding, controlling and patterning the ECM environment is the first step to creating functional tissue in vitro. Second, defining the location of cells by controlling adhesion is useful for positioning cells on the sensing elements of cell-based biosensors. Third, microarrays of cells can be used for drug screening applications. A major multibillion-dollar problem currently facing the pharmaceutical industry is that cells cultured in vitro often respond differently to potential therapies than cells do in vivo, leading to the failure of promising drug candidates once they are put through clinical trials [14]. Future developments for this field will be to create systems to control the placement of multiple cell types into single reactors, and to organize cells in three dimensions. By controlling the ECM, we can drive cells to better approximate their in vivo counterparts and thereby create more robust, high-throughput assays.

5. Concluding Remarks

Combining knowledge of the mechanisms of cell adhesion with microfabrication approaches is a robust strategy to develop systems able to harness cellular processes for commercial applications. By controlling the bio/material interface, engineers can create hybrid systems for tissue engineering, bionics, and diagnostic applications. The eventual design and manufacture of these systems will require further collaborations between engineers and biologists for such products to make it to the marketplace. Finally, knowledge gained from culturing cells in engineered environments has the potential to contribute to the design of better systems for the in vitro study of cell biology.

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Figure 1. Cells adhesion is mediated by interactions between cell-surface receptors and ECM molecules.

Figure 2. Schematic of (A) microcontact printing and (B) microfluidic methods used to pattern cell adhesion.

Figure 3. Phase contrast images of adherent cells on homogeneous surfaces (A) and micropatterned substrates (B). Scale bar = 150 μm. (C) Proliferation rate of endothelial cells as a function of island size.

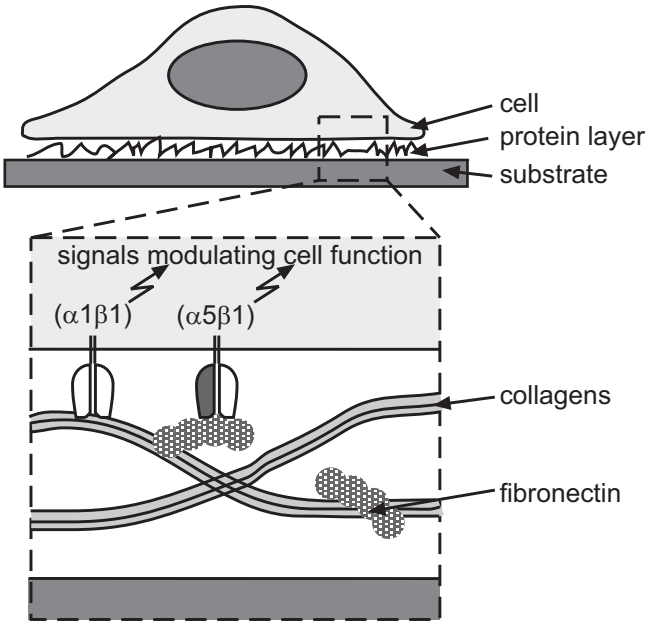
cell
protein layer
substrate
signals modulating cell function
(α1β1)
(α5β1)
collagens
fibronectin

Figure 1

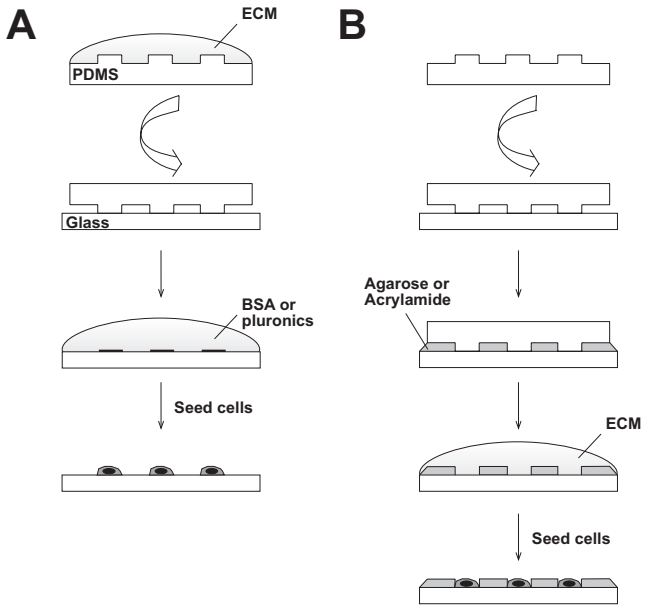
A
ECM
PDMS
Glass
BSA or
pluronics
Seed cells
B
Agarose or
Acrylamide
ECM
Seed cells

Figure 2

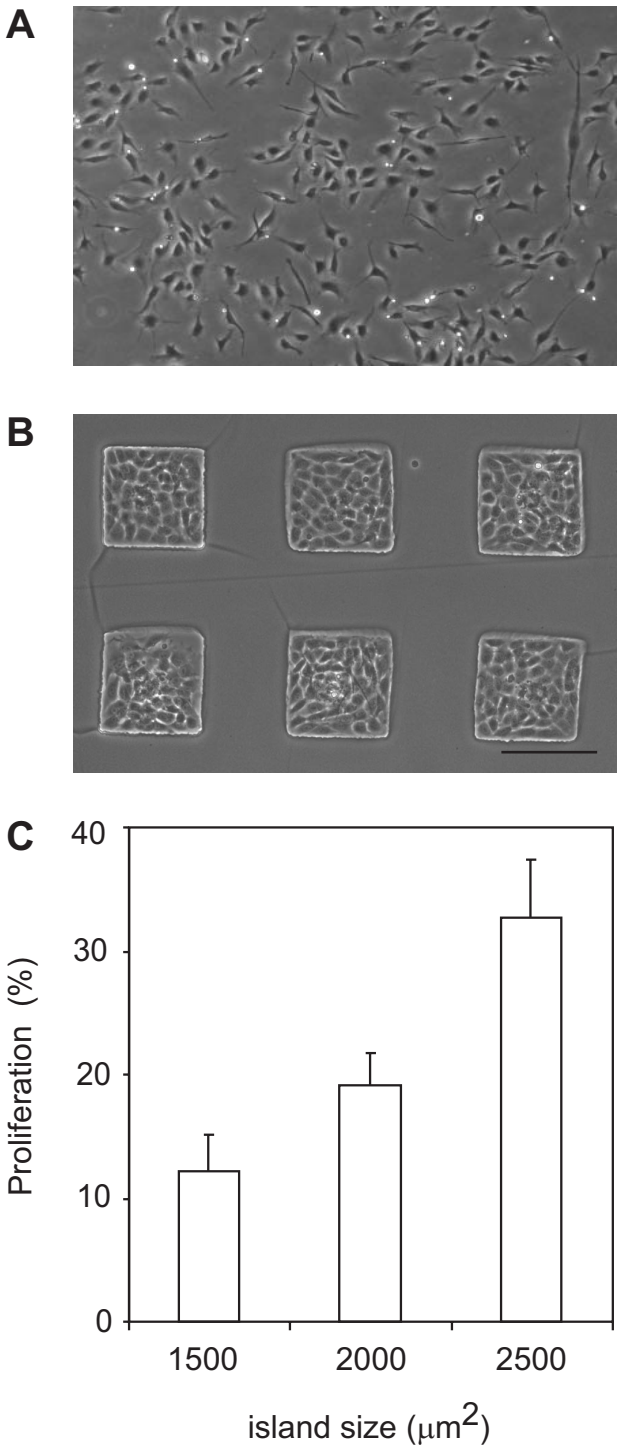
A
B
C
40
30
20
10
0
Proliferation (%)
1500
2000
2500
island size (μm2)

Figure 3