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Engineering cellular microenvironments to improve cell-based drug testing

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Recent progress in the biology of cell adhesion is enabling cell culture models to better reproduce *in vivo* functions. Cues from adhesion to extracellular matrix and neighboring cells are important regulators of cell behaviors. The recent adaptation of semiconductor tools to spatially organize cells and their adhesions has enhanced our ability to engineer cell functions *ex vivo*. By using these tools to create more *in vivo*-like cultures, cell-based drug discovery and target validation could be improved. This review explores the biological advances made by these microfabrication tools and discusses how they could enable high-throughput cell-based assays.

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▼ Recent advances in organic chemistry and applied genomics have meant that the rate-limiting steps in the drug development process have shifted away from compound synthesis and target identification towards target validation. Radical advances in combinatorial chemical synthesis, micro-analytical techniques and predictive computational techniques have exponentially increased chemical synthesis capabilities and have changed the paradigm for drug research from identifying targets and synthesizing molecules to synthesizing molecules and identifying targets [1]. The parallel advances in biological tools to identify, mutate, express, characterize and purify proteins have also led to rapid identification of novel targets associated with disease processes [2]. However, with this rapid progress a new bottleneck has emerged in early phases of drug research: the need for processing large numbers of potential drug candidates and identifying those that have a high probability of becoming marketable products as early as possible [3].

The pathways to drug discovery adopted by different companies vary [4], but a common approach is to first screen a small-molecule library in solution-binding assays against a target protein. Those compounds that bind with a particular stringency are used to build an initial class of lead compounds, which are then progressively winnowed out through a series of toxicity and functional assays in either cell-based or early animal testing. Because the cost of development increases exponentially along this pathway [5], the guiding principle in the drug industry has been to identify poor candidates earlier rather than later. Solution-binding assays, which are used in the earliest screening steps, can quickly identify lead molecules. However, such tests do not always reflect how a drug interacts with a target molecule within the complex milieu of an intact cell. It has therefore been proposed that increased reliance on cell-based validation early in the discovery process will prove economically advantageous [6–9].

In the transition from a simplified solution-binding assay to *in vivo* testing, cell-based testing has proven to be a valuable stepping-stone to quickly weed out toxic and nonfunctional compounds. The low cost and high speed of testing compounds in cell culture, and the obvious advantages of using intact cells as the most expedient first representation of the living patient, have made cell-based testing a key component of drug discovery programs [10]. However, many in the industry would argue that cell models continue to give unsatisfactorily misleading and non-predictive data for *in vivo* responses. We propose that a principal component of this failure results from our lack of understanding of, and inattention to, how to culture cells specifically so that they phenotypically represent their *in vivo* counterparts.

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We believe that the use of miniaturized cell-based systems that are specifically engineered to mimic *in vivo* behavior can reduce costs, add efficiencies and, most importantly, increase predictive accuracy of the drug discovery process. In this review, we describe enabling technologies and an emerging interdisciplinary field combining cell biology with semiconductor manufacturing tools used in making microelectronic circuits, to control the expressed phenotype of cells by manipulating cues in the local cellular microenvironment. We will first provide evidence that the current state of cell-based screening and target validation remains unreliable, and propose that this could in part be because the same cell can behave differently depending on its microenvironment. Under different culture conditions, target-specific signaling pathways might not be active, and when active they might not be wired the same way; therefore, it is probable that the predictability of cell-based assays depends heavily on the quality of the cell culture system. Several examples are reviewed to illustrate the role of microenvironment in controlling cell function. It is proposed that, by engineering cell behavior through the manipulation of their extracellular environment and then replicating these cultures in miniaturized regular arrays, better and more economic *in vitro* models can be obtained for cell-based screening.

Current state of cell-based testing of drugs

Cell-based testing is well established in drug discovery research, with well-described cell lines and models that exist for cancer [11], intestinal absorption [12] and diabetes [13]. As an offshoot of tissue engineering research, a model for *in vitro* skin has thus far shown good correlation with animal testing for ocular irritation [14,15]. A cell-based model that is faithful to its *in vivo* behavior offers obvious advantages, in savings of time, cost and predictability. However, current models fall short of this ideal [16–18].

This failure to predict future efficacy is well illustrated by experience in the field of cancer research. There has been a recent shift in screening for cancer drugs from relying exclusively on tumor cytotoxicity to understanding the signaling context within which the particular molecular target operates [11,17,18]. Experience at the National Cancer Institute with screening for anticancer drugs showed that screens for either a general effect, such as cytotoxicity, or a specific molecular target, such as mitogen-activated protein (MAP) kinase, have resulted in poor chances of therapeutic success when such screens ignore the signaling milieu of the assay [18]. The fact that cell-based screens give different results than later *in vivo* responses suggests that the pathways regulating proliferation and apoptosis are different in different cellular contexts. Understanding cell behaviors in specific contexts highlights the multiple pathways that probably contribute to the regulation of any particular behavior. For example, stimulation of normal cell proliferation by the addition of serum or an increase in cell adhesion results in different patterns of activation of the pathways involved in cell-cycle regulation [19]. Such data suggest that the cell culture context in which the screen is performed could alter the predictive success of any particular cell-based screen.

Similar concerns have been echoed elsewhere [16]. Caco-2 cells are a human colon-derived transformed cell line that is widely used to model intestinal absorption. However, *in vitro* absorption assays have not faithfully predicted *in vivo* bioavailability [20], partly because these cultures exhibit significant differences from the *in vivo* normal intestine phenotype in protein expression and cell morphology. It is not surprising that there has been a call for better *in vitro* models [16] but, in doing so, we must consider both the 'nature' and 'nurture' bases for failure. Perhaps one lesson to be learned is that transformed cell lines, such as Caco-2, which are known to be genetically unstable, have inherent changes in gene expression, proliferative control and signal transduction wiring that make them poor models to begin with. Using a normal, or less transformed, cell line could yield a better model. Equally compelling, however, is the consideration that our current approaches to culture cells *in vitro* are relatively crude. It is well-known that even genetically normal primary cells placed in cell culture quickly lose their differentiated gene expression pattern and phenotype [21]. Thus, even with genetically faithful cell sources, designing better physiologic and pathophysiologic models in this and other cases will involve understanding the fundamental basis of how cellular microenvironment modulates phenotype *in vitro*. It is possible to vary environmental signals in a quantitative fashion to modulate cell functions, such as migration [22], proliferation [23] or differentiation [24]. Developments in culture technologies have reached a stage in which it might soon be appropriate to leverage our experience in cell biology for drug screening.

How the extracellular microenvironment controls phenotype *in vitro*

Cells change their phenotype dramatically depending on their environment, enough so that it is not clear whether the gene expression differences between cell types or differences between phenotypic states of one cell type have a greater dynamic range. Thus, even though any particular cell type isolated from an animal might have a restricted subset of genes that it expresses compared with the whole expressible genome, the specific proteins that are expressed within that subset in response to a given environment can

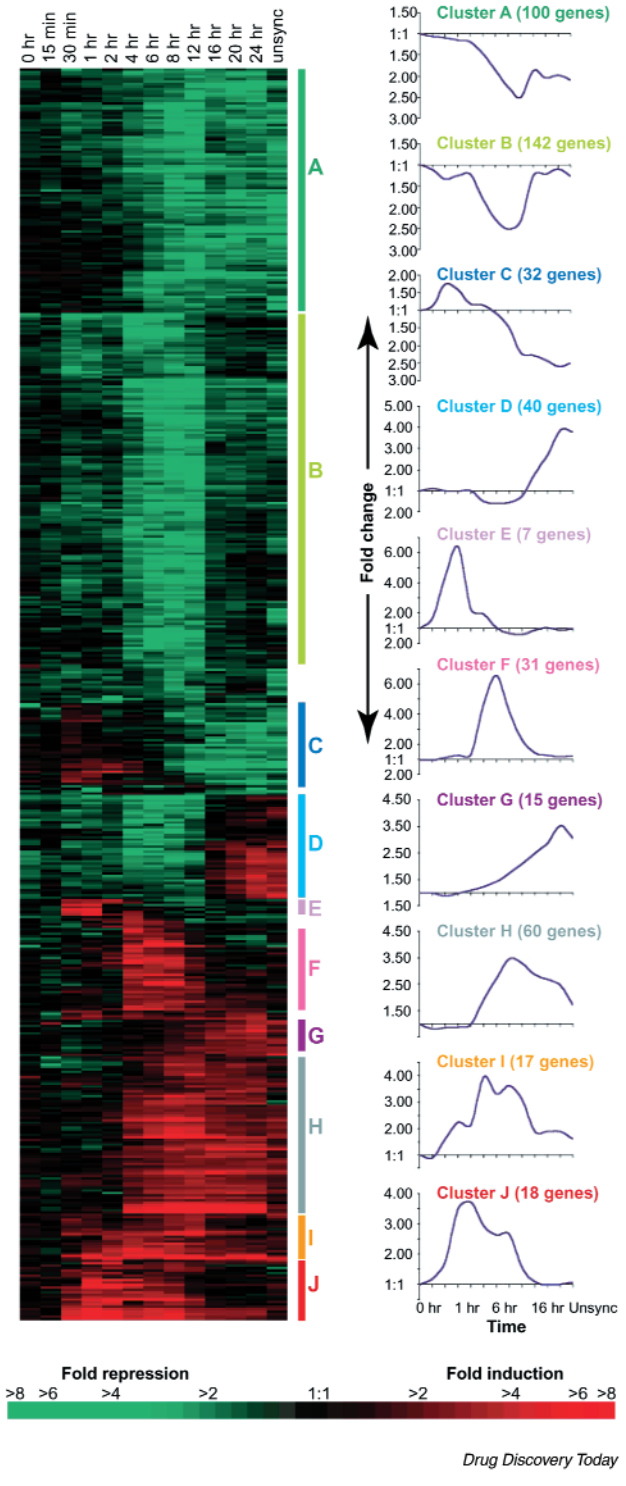

Figure 1. The transcriptional program activated by serum stimulation of fibroblasts. The tiled image represents the expression pattern of 517 genes, each one represented as a horizontal strip. The graphs represent the average expression profiles for clusters created from the 517 genes, each of which shares the same activation profile. Reproduced, with permission, from Ref. 25.

change dramatically. For example, the simple addition of serum to fibroblasts activates a genetic program that unfolds a complex tapestry of functional gene clusters activated in time (Fig. 1) [25]. This program resembles, in part, that of the wound healing response, presumably because fibroblasts see serum *in vivo* only in the context of a wound [25]. How many other cues exist within the healing wound that should also be mimicked to ultimately make the *in vitro* cellular response to an intervention predict its response *in vivo*, and how does a scientist decide which culture condition would best mimic that of a particular cell type or cellular behavior *in vivo*?

Cellular microenvironment in growth and differentiation

Many workers have focused on this exact question, and have learned that not only does the growth factor milieu control cell behavior but also cues emanating from the extracellular matrix, direct cell–cell contacts and mechanical forces [26–28]. In the context of growth control as a model for wound healing, the addition of serum or specific growth factors is not the only modulator of cell-cycle progression. It is equally well known, and often overlooked, that the proliferative response is also affected by the presence of extracellular matrix ligands adsorbed to the culture dish and the density of the cell culture [29]. Specific binding of integrin receptors to the extracellular matrix enables the sustained activation of many of the same signals activated by growth factor receptors in the presence of serum [30–32]. However, simultaneous ligation of integrin and growth factor receptors is insufficient for cell-cycle progression [32], suggesting that other cues are also necessary. The growth arrest that occurs when cells are cultured to confluence has been attributed to both a decrease in cell spreading and an increase in cell–cell contacts as cells crowd into the available space [33]. We and others have demonstrated that each of these two physical cues (cell spreading and intercellular contact) provide distinct inputs to regulate cell proliferation [34,35]. Using a specialized set of semiconductor manufacturing tools, we have made cell-culture substrates that are micropatterned with micrometer-scale islands coated with extracellular matrix ligands surrounded by nonadhesive regions. Culturing endothelial cells or hepatocytes on such substrates, single cells were made to attach on each island such that cells spread to the size and shape of the engineered islands (Fig. 2). Using such a system, we and others have demonstrated that cell spreading itself, in the absence of cell–cell contact and in constant growth factor concentrations, can switch cells between quiescence and proliferation (Fig. 2) [23,36]. If pairs of cells are placed on such islands, such that cell–cell contact is introduced

without changing cell spreading, contact induces proliferation [33]. In the context of microvascular endothelial cell proliferation as a model of angiogenesis, one must ask whether the three different stimulatory signals for proliferation – growth factors, cell spreading and intercellular contact – stimulate proliferation through different pathways, and if so, which form of stimulation best models the *in vivo* angiogenesis process. Evidence would suggest that the stimulatory pathways are indeed different, as growth factor stimulation is known to upregulate cyclin D expression, whereas cell adhesion and spreading downregulate the cyclin-dependent kinase inhibitor, $p27^{kip1}$ [19,37]. It therefore remains to be determined which *in vitro* model would best mimic a particular *in vivo* situation, but the data are clear: different *in vitro* conditions result in differences in the cellular processes being studied. If we are to improve the predictability of *in vitro* testing, the linkage between cues within the *in vitro* microenvironment and appropriate models of cellular behavior must be solidified.

The previous evidence shows the importance of extracellular matrix, cell adhesion and intercellular contact in regulating cell proliferative responses, and perhaps should be taken into consideration in improving predictability for wound healing and angiogenesis. Is the cellular microenvironment important in other processes? In the context of drug discovery, perhaps the least progress has been made in developing *in vitro* models of cells in their quiescent, unactivated or ground state. Such states would be crucial in modeling natural tissue properties, such as permeability of the blood–brain barrier, metabolism of compounds by the liver and the anticoagulative nature of healthy microvessels. In one differentiation model, mammary epithelial cells cultured on attached collagen gels spread on the gels and dedifferentiated, but when the gels were released from attachment to the culture dish the tension in the cells contracted the gels, decreased cell spreading and increased differentiated function, as assayed by mammary cell-specific gene expression, including that of the milk protein β-casein [38,39]. The capacity of prolactin to induce β-casein gene expression and to enhance the stability of the protein was also dependent on reduced cell spreading on the released collagen gels [40,41]. Further refinement of mammary epithelial cell culture has been shown by the addition of the laminin-containing matrix of Englbreth–Holm–Swarm sarcoma cells [42]. Thus, it appears that there also exists a complex interplay between adhesive cues, growth factors and cell shape in regulating cell differentiation.

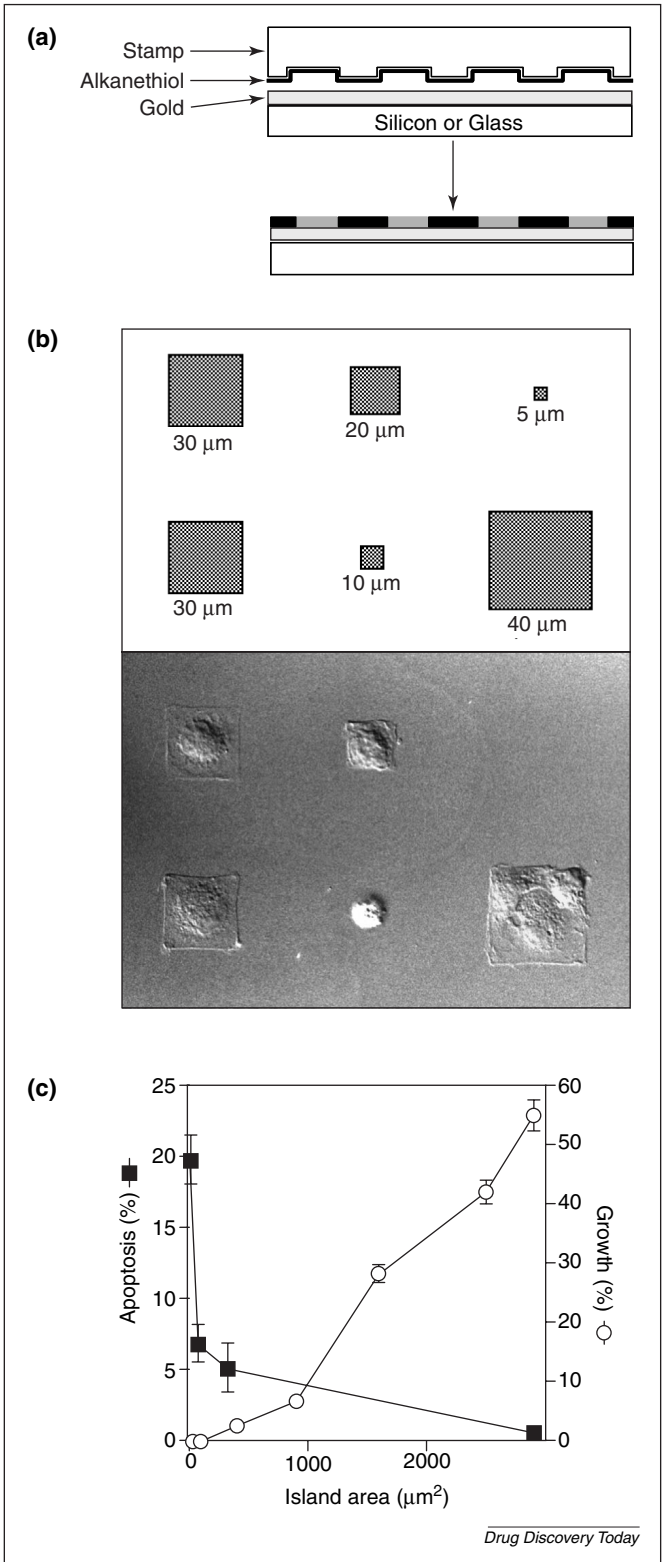

Figure 2. The effect of cell shape on cell fate. **(a)** Outline of the microcontact printing method. **(b)** Schematic of the pattern used to create adhesive islands on which cells were plated (top), and (below) the micrograph shows patterned cells conforming to the shape of the islands. **(c)** Proliferation and apoptosis as a function of cell spreading. Adapted, with permission, from Ref. 23.

Culture models: endothelial cells and hepatocytes

Two specific cell types in which controlling cellular differentiation is particularly relevant to drug discovery are

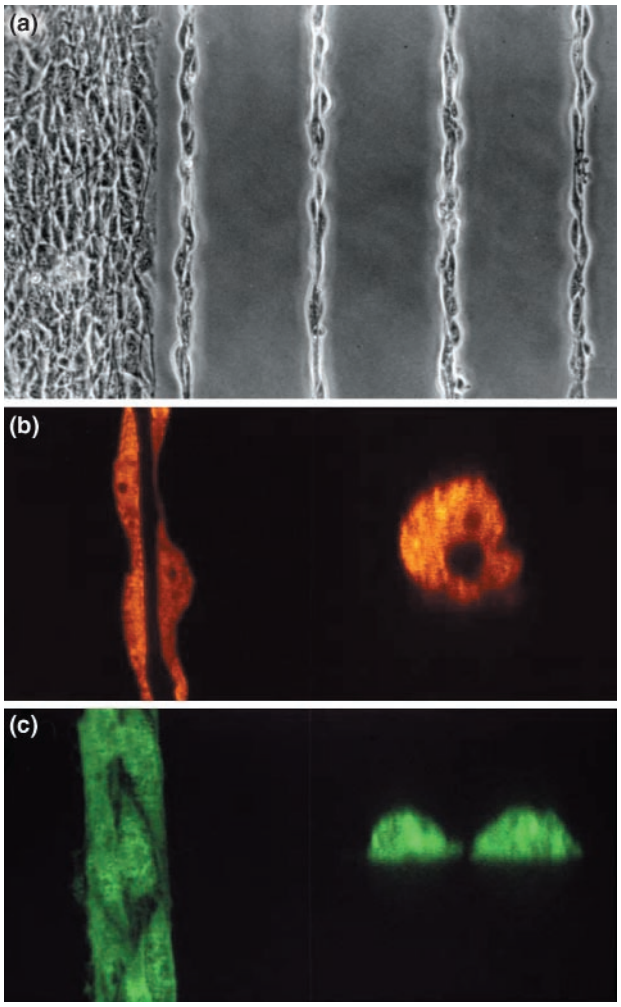

Figure 3. *In vitro* capillary formation using microfabricated substrates. **(a)** Phase contrast image showing capillary endothelial cells forming tube-like structures on 10 μm wide lines coated with fibronectin. **(b)** Confocal images of these tubes showing a central lumen. **(c)** Confocal images of endothelial cell monolayers on 30 μm wide lines. Adapted, with permission, from Ref. 24.

endothelial cells and hepatocytes. Endothelial cells present an attractive target for both the promotion and inhibition of angiogenesis in a variety of clinical settings [43]. However, the formation of capillary networks *in vitro* remains a poorly characterized art. Similarly, hepatocytes have a major role in drug metabolism and toxicity but, because current methods for hepatocyte culture do not produce cells that function as they do *in vivo*, predictive preclinical metabolism and toxicity studies are confined to animal studies [44]. Perhaps the use of adhesive cues and cell shape can improve current methods to manipulate both endothelial and hepatocyte cell function.

Several model systems exist to study endothelial cell differentiation into capillaries. One widely used system for *in vitro* angiogenesis is the culture of endothelial cells either on or within gels of collagen, fibrin or Matrigel – a basement membrane secreted by a mouse sarcoma. In the presence of phorbol esters [45], basic fibroblast growth factor (bFGF) [46] and vascular endothelial growth factor (VEGF) [47], cells form interconnected networks that are reminiscent of capillary branches. Nonetheless, it remains unclear how gel-cultures promote network formation, whether such networks are physiologically relevant and how to better define the culture system. Another way to form capillary networks has been shown to be by modulating extracellular matrix (ECM) coating density on plastic surfaces. Endothelial cells stimulated by bFGF spread well on a high density of fibronectin (Fn) on petri dishes and undergo DNA synthesis, whereas cells on intermediate Fn densities form capillary-like networks [29]. These findings suggested that limiting the adhesive signals by preventing cell spreading can promote endothelial cell differentiation. Because intercellular contact is also a demonstrated differentiation-promoting signal [48], we micropatterned capillary endothelial cells onto thin lines of Fn to simultaneously prevent cell spreading and to enable cell–cell contact. Cells cultured on wide lines (>30 μm) formed flat, monolayer ribbons of cells that spread well and underwent proliferation [24]. Cells cultured on narrower (10 μm) lines were less well spread, entered a quiescent state of neither growth nor apoptosis and reorganized to form multicellular cylinders containing an apparent central lumen running down the center of the cylinder (Fig. 3). Hence, subtle changes in the adhesive microenvironment resulted in completely divergent phenotypic responses in these cells. As such culture models are further characterized, predictive assays for the microvascular effects of drug candidates will be testable in cell-based systems.

In contrast to endothelial culture, hepatocyte culture remains less well understood. When cultured in monolayers, hepatocytes rapidly lose their differentiated function [21]. The challenge in applied hepatocyte research has been to preserve liver cell function for extended periods of time both for tissue-engineered devices [49] and for drug testing [44]. As with other cell types, cell spreading is correlated with changes in hepatocyte function [21,36]. When constrained to remain rounded on a low density of Fn or on malleable collagen gels, hepatocytes are growth-arrested and exhibit enhanced differentiated function, as assayed by the secretion of liver-specific proteins, such as albumin, transferrin and fibrinogen [21,50]. When allowed to spread on a high density of Fn, the same cells proliferate and rapidly downregulate liver-specific genes [21].

In hepatocytes, additional adhesive factors other than cell spreading can enhance differentiated function.

Changing hepatocyte microenvironment by overlaying collagen gels on monolayer cultures upregulates liver-specific function for weeks [51] to months [52]. Metabolic function, as well as cytochrome p450 activity, is upregulated in such cultures and is associated with the presence of structures resembling *in vivo* bile canaliculi that exist in the apical side of liver plates [53]. The introduction of intercellular contacts between hepatocytes also has a role in liver-specific functions. Hepatocytes cultured to form solid multicellular spheroids show microvilli-lined channels that contain bile [54] and maintain liver-specific functions, such as high albumin secretion, urea excretion and cytochrome p450 activity over a period of several weeks, whereas non-aggregated hepatocytes do not [48]. Importantly, intercellular contact between liver parenchyma and stromal-like cells further enhances hepatocyte function. Using a micropattern-ing technique to co-culture islands of hepatocytes with fibroblasts, Bhatia and colleagues found that the fibroblasts modulate and stabilize hepatocyte liver-specific function through direct intercellular contact [55]. Although it is clear that manipulating the geometry and form of adhesive cues can improve hepatocyte culture, we are only beginning to elucidate the underlying principles that drive these behaviors.

Thus, the importance of extracellular matrix, cell morphology, intercellular contact and multicellular organization have been recognized for many years, but the tools to engineer and manipulate these cues *in vitro* have only now begun to emerge.

Novel microfabrication technologies to interface cells with drug discovery

Although specific culture environments to mimic various physiologic and disease states are continuing to improve, others are developing the foundations for how such environments might be miniaturized. We believe that such microfabrication-based approaches, which are being used to engineer the cell-culture environment, can be easily transformed to improve cell-based testing in the pharmaceutical industry. The ability to manipulate cell function *in vitro* using such approaches holds several inherent properties that are useful to drug testing:

- better control of cellular environments might decrease the biological cell-to-cell and culture-to-culture variability that has thus far defined the lower limit of the size of cell-based experiments to no less than 1000 cells;
- more predictive responses of *in vitro* cultures could dramatically improve the economics of cell-based testing;
- the low cost of fabrication in these manufacturing-ready techniques can bring designer culture techniques to affordable levels; and
- the miniaturization, patterning and replication of cell cultures in large arrays – currently more than 10,000 cultures per square inch – could enable parallel screening of candidate compounds in cell-based assays, analogous to the solution-binding assays of current HTS systems.

Below, we provide a brief overview of the technologies that are currently being developed to control, at the micrometer scale, cell adhesion, cell–cell contact, co-cultures and the soluble environment. These tools provide a glimpse of what might secure the future of high-throughput cell-based screening.

Technologies for the future

Similar to DNA arrays, one can pattern cellular arrays; however, in the case of cells, controlling cell spreading and relative position is used to engineer cell function as well as to code the position of the cells. Numerous methods have matured that enable such patterning. We have used a simple method based on microcontact printing of alkanethiol 'inks' onto gold-coated coverslips [56]. The technique relies on the use of a silicone rubber stamp that contains a bas-relief pattern of posts; upon printing, the posts on the stamp print the ink onto the gold as islands. When ECM proteins are adsorbed on such a surface, they preferentially bind to the printed alkanethiol pattern, hence creating an adhesive island for the cells [56,57]. Investigators have recently shown that it is possible to directly pattern proteins onto substrates by adsorbing the protein as the ink directly onto elastomeric stamps [58,59]. Photolithographic approaches have also been used successfully to pattern proteins and cells in a process similar to how the semiconductor manufacturers pattern metals and insulators on microchips [60]. Photochemical coupling techniques in which heterobifunctional light-activatable linkers can immobilize macromolecules on surfaces have been used to pattern antibodies [61] and protein gradients [62] and to control cell attachment and shape [63].

Several mask-based techniques have been developed to enable the miniaturization and production of patterned co-cultures of multiple cell-types. One approach has been to use an elastomeric membrane with holes the size of individual cells. The membrane is sealed against a culture surface to serve as a mask to prevent cell attachment to certain regions of substrate. Plating one cell-type results in cells cultured only in the holes of the membrane. Removing the mask to attach the second cell-type leads to selective placement of the second cell-type next to the first [57]. Another way has been to pattern surfaces with different degrees of adhesivity for cells. Then, two different cell-types with high and low adhesivity for the surface can be segregated based on stringency onto the two types of

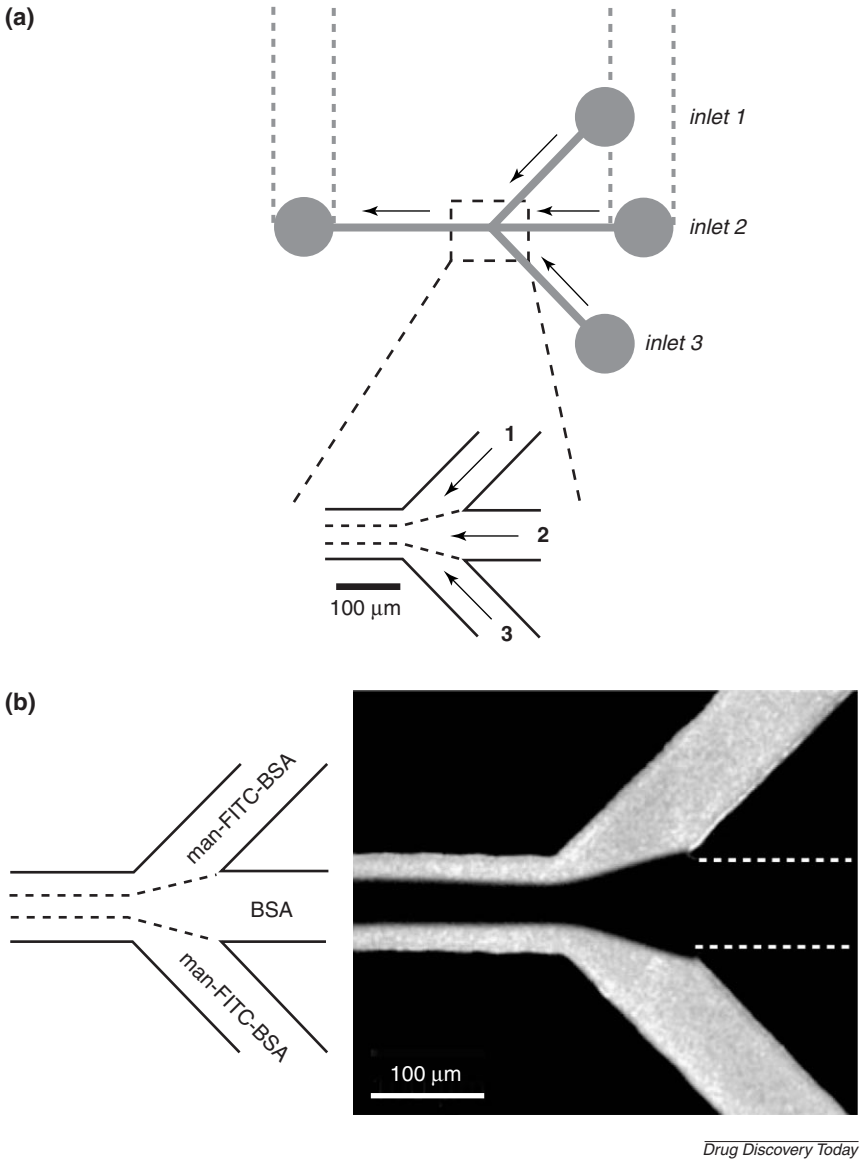

Figure 4. (a) Schematic top-view of the fluidic channels used for micropatterning. **(b)** Micrograph of adsorbed fluorescein isothiocyanate-coupled bovine serum albumin (man-FITC-BSA) or nonfluorescent BSA created by flowing the proteins in the fluidic channels. Adapted, with permission, from Ref. 69.

regions of the surface [55]. A powerful new approach involves the development of surfaces that can be switched between nonadhesive and adhesive states by electrochemistry, thereby creating an electroactive mask [64–66]. Switchable regions of a surface could then be activated sequentially to capture specific cells to that region.

Techniques also exist to control the solution-based environment. For some time investigators have been engaged in developing fluid handling techniques for HTS that are directly applicable to cell-based screening. This work has been reviewed elsewhere [67]. One fluid-based technique of note has been used specifically to pattern both cells and protein on the micrometer scale that takes advantage of the property of laminar flows in microchannels [68]. The small dimensions of these surfaces permit low Reynolds number laminar flows, permitting essentially no bulk fluid mixing. Takayama *et al*. have used such a technique to pattern proteins and cells in parallel laminar flow in the same microchannel (Fig. 4) [69]. The uniqueness of this technique is in permitting micropatterning of the fluid space as adjacent flows do not mix, which could, for example, enable neighboring cells to be exposed to different cytokines.

Although each of the microfabrication-based approaches is being integrated successfully with standard cell biological approaches, the challenge for the future is clear. The integration platform that will enable the coupling of culture of multiple cell-types, or large arrays of reproducibly identical microcultures of one cell-type, to high-throughput fluidic delivery and assay systems remains to be seen.

Conclusions

Our understanding of the importance of many types of cues in the environment that regulate cell function has led to the development of new technologies to manipulate those cues. In particular, the use of microfabrication technologies to control cell adhesion, cell shape, intercellular contact and co-culture interactions are enabling the development of culture systems that might induce cells to model their *in vivo* gene expression and functional states more faithfully. Such culture models could prove useful in the early phases of drug discovery in which the predictive power of cell-based assays is sorely needed.

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