Integrin-independent Cell Adhesion Substrates: Possibility of Applications for Mechanobiology Research

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Cells can mainly sense mechanical cues from the extracellular matrix via integrins. Because mechanical cues can strongly influence cellular functions, understanding the roles of integrins in the sensing of mechanical cues is a key for the achievement of tissue engineering. The analyses to determine the roles of integrins in the sensing of mechanical cues have been performed by many methods based on molecular- and cell-biological techniques, atomic force microscopy, and optical tweezers. Integrin-dependent cell adhesion substrates have been also used for this purpose. Additionally, the cells can adhere on several substrates via integrin-independent mechanisms. There are two types of integrin-independent cell adhesion substrates; 1) the substrates immobilized with ligands against the receptors on cell surface and 2) the substrates suppressing protein adsorption. Cells can exhibit specific functions on these substrates. Here, the examples of integrin-independent cell adhesion substrates were reviewed, and their possible applications in mechanobiology research are discussed.

Keywords Integrin, cell adhesion, extracellular matrix, receptor, protein adsorption, signal transduction

(Received June 30, 2016; Accepted September 1, 2016; Published November 10, 2016)

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1 Introduction

The extracellular microenvironment, particularly the extracellular matrix (ECM), is one of the most important points of study in the fields of tissue engineering and regenerative medicine because cells can sense information contained in ECM and modulate their functions in cell proliferation, migration, morphogenesis and differentiation.1-2 There have been many efforts to regulate cell function by biochemical cues in the past few decades. ECM proteins and cell-adhesive peptides (e.g., the Arg-Gly-Asp peptide) have been frequently immobilized to cell culture substrates to provide biochemical cues to the cells.3-6 In addition to biochemical cues, the ECM can provide mechanical cues to the cells to regulate cell function.7-9 Durotaxis is one of the first phenomena to be reported as a cell function regulated by mechanical cues from the ECM.7 Durotaxis is a mode of cell migration regulated by the elasticity of the ECM; that is, most normal cells prefer to migrate onto stiffer ECM. More importantly, the differentiation of stem cells is strongly influenced by the stiffness of the ECM.9 Therefore, mechanical cues have been a focus of studies in tissue engineering and regenerative medicine.

It has been reported that cell adhesion sites to ECM are highly important for sensing the biochemical cues provided by the ECM. Cells mainly adhere to the ECM via receptor proteins on the cell membrane called integrins. Integrins are heterodimers composed of α and β transmembrane subunits that have extracellular domains that can bind to many ECM proteins.10 The binding of integrins to ECM proteins leads to the clustering of integrins on the cell membrane. This clustering leads to the accumulation of many intracellular proteins (e.g., focal adhesion kinase (FAK), talin, paxillin, vinculin and p130Cas) and actin fibers close to the intracellular domains of integrins for the transduction of biochemical cues provided by the ECM.10-12 There are many types of α and β subunit associations which can activate different intracellular signaling pathways and induce different cell functions.10,13

In addition to biochemical cues, mechanical cues from ECM are also sensed by integrins and intracellular signaling proteins at integrin-ECM adhesion sites although mechanical cues from extracellular milieu can be sensed by many receptors such as cadherin and ion channels.14-17 The cells adhere to the substrates and exert traction forces. When the cells adhere to substrates with different stiffnesses, they exert traction forces with different strengths.18 The exertion of traction forces with different strengths leads to changes in the conformation, accumulation and phosphorylation levels of intracellular signaling proteins at integrin-ECM adhesion sites in response to the mechanical cues provided by the ECM.10-22 Integrins seem to play central roles in the transduction of the mechanical cues. Therefore, integrins should be studied for understanding how the mechanical cues provided by ECM are transduced into the cells.

To understand the mechanisms of cell function regulation by biochemical cues, the mechanisms by which integrins and intracellular signaling proteins at the integrin-ECM adhesion sites transduce the biochemical cues provided by the ECM into the cells have been actively examined.13,23-25 There are several methods for modulating integrin-dependent cell adhesion that have been used to determine these mechanisms. Molecular- and cell biology-based methods have been frequently used for this purpose (Table 1). For example, integrin-dependent cell adhesion can be promoted by the induction of integrin over-expression. On the other hand, integrin-dependent cell adhesion can be inhibited by the knock-out or knock-down of integrins and by the use of function-neutralizing antibodies against integrins.6,26-28 In addition to these molecular- and cell biology-based methods, integrin-specific ligands immobilized to the substrates have also been frequently used to promote integrin-dependent cell adhesion. These substrates have been applied for the analyses of the roles of integrins in the transduction of biochemical cues provided by the ECM (Table 1).13,23,29 There are also several substrates that allow integrin-independent cell adhesion. These approaches that use substrates have also been used for the analyses of the roles of integrins in the transduction of biochemical cues provided by the ECM (Table 1).30,31

In contrast to the analyses of the roles of integrins in the transduction of biochemical cues, the methods are limited for the analyses of the roles of integrins in the transduction of mechanical cues provided by the ECM. For this purpose, the analyses have been performed using molecular- and the cell biology-based methods (e.g, genetic mutation technique and Förster resonance energy transfer (FRET)-based molecular tension sensors)32,33 and mechanical cell manipulation methods (e.g, cell manipulations with atomic force microscope and optical tweezer techniques).34,35 In addition to these approaches, the substrates were developed to measure traction forces exerted by the cells. Microbeads embedded elastic substrates have been used to measure the force exerted by the cells to the substrates, which is called traction force microscopy.36 Additionally, the substrates possessing micropillars on the surface have been also used to measure the force exerted by the cells.37 These substrates allow the cells to interact and adhere through integrin-dependent mechanism. The substrates that allow integrin-independent cell adhesion have been hardly used. Using substrates that allow integrin-independent cell adhesion might pave the way toward developing a new method for determining how integrins transduce mechanical cues from the ECM. In this review, we summarize examples of the substrates that allow integrin-independent cell adhesion after a brief discussion of the substrates that promote integrin-dependent cell adhesion. The possible applications of using substrates that allow integrin-independent cell adhesion in mechanobiology research are also discussed. The conceptual figure of integrin-independent cell adhesion substrates is shown in Fig. 1.

| Table 1  | Methods for the modulation of integrin-dependent cell adhesion |
|-----------------------------------------------|-----------------------------------------------|
| **Promotive modulation**                        | **Inhibitory modulation**                        |
| Molecular- and cell biology-based methods       | Knock-down or -out mutations                   |
| Modulation methods with substrates              | Function-neutralizing antibodies               |
| Over expression of integrins                    | Immobilization of ligands specific for cell membrane receptors |
| ECM protein immobilization                      | Suppression of protein adsorption onto the substrate surface |
| Cell-adhesive peptide immobilization            |                                               |
| Anti-integrin antibody immobilization           |                                               |

ANALYTICAL SCIENCES  NOVEMBER 2016, VOL. 32
The Substrates that Promote Integrin-dependent Adhesion

The immobilization of integrin-specific ligands to substrates is usually used to promote integrin-dependent cell adhesion. There are mainly three types of the ligands used for immobilization: 1) ECM proteins (e.g., fibronectin and collagen), 2) cell-adhesive peptides derived from ECM proteins and 3) antibodies against integrins. Each method that uses a particular ligand has both advantages and disadvantages (Table 2). The appropriate method should be selected depending on the purpose of each study.

The immobilization of ECM proteins can completely mimic the physiological interaction of the ECM with integrins. However, it is difficult to determine interactions with specific integrin heterodimers because ECM proteins have binding abilities to multiple types of integrin heterodimers. For example, fibronectin can bind to the integrin heterodimers \( \alpha_4\beta_1 \), \( \alpha_5\beta_1 \) and \( \alpha_v\beta_3 \). Several laminins can bind to the integrin heterodimers \( \alpha_3\beta_1 \), \( \alpha_6\beta_1 \) and \( \alpha_6\beta_4 \). Additionally, laminin can bind to non-integrin cell adhesion receptors, such as syndecans and dystroglycan. Therefore, it is difficult to analyze how specific integrin heterodimers transduce the signals provided by the ECM using substrate-immobilized ECM proteins.

Cell-adhesive peptides derived from ECM proteins have also been immobilized to promote integrin-dependent cell adhesion. There are several cell adhesive peptides, which include Arg-Gly-Arg (RGD) and Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER; Hyp and O indicate hydroxyproline). These peptides can somewhat mimic the physiological interaction between integrins and ECM proteins. It is also easy to chemically immobilize these peptides to the substrates in a stable manner. Therefore, these peptides have been widely used. In contrast to these advantages, it has been difficult to determine the interactions with specific integrin heterodimers. For example, integrin heterodimers such as \( \alpha_3\beta_1 \), \( \alpha_5\beta_1 \), \( \alpha_6\beta_1 \), \( \alpha_v\beta_1 \), \( \alpha_v\beta_3 \) can bind to the RGD peptide, which is the most widely used cell-adhesive peptide. Therefore, as with substrate-immobilized ECM proteins, it is difficult to analyze how specific integrin heterodimers transduce the signals provided by the ECM using substrate-immobilized cell-adhesive peptides.

Antibodies against integrins have also been used to promote integrin-dependent cell adhesion. In contrast to ECM proteins and cell-adhesive peptides, these antibodies can bind specific integrin subunits or heterodimers and can activate downstream intracellular signals. Therefore, the substrate-immobilized antibodies against integrins have been used for the analyses of the roles of specific integrin subfamilies and heterodimers in the transduction of signals from the ECM. However, the interaction between integrins and these antibodies is not physiological, although the antibodies can activate intracellular signals by inducing integrin clustering. In the case of signal transduction of mechanical cues from the ECM, conformational changes in proteins are occasionally important. When integrins bind to the ECM proteins, integrin conformation dynamically changes and these changes activate downstream intracellular signals. Therefore, using substrate-immobilized antibodies against integrins might possibly omit the signals activated by conformational changes.

The importance of the substrates used for integrin-independent cell adhesion

To analyze the roles of integrins in the transduction of signals from the ECM, the inhibition of integrin-dependent cell adhesion has been widely performed. Specific integrin-knockdown or

| Immobilized molecules | Example | Advantage | Disadvantage |
|-----------------------|---------|-----------|--------------|
| ECM proteins          | Fibronectin, Laminin, Collagen | Complete mimicking of integrin-ECM protein interaction | Limited specificity to integrin subfamily |
| Cell adhesive peptides | RGD, GFOGER | Easy and stable chemical immobilization onto the substrates | Limited specificity to integrin subfamily |
| Antibodies against integrins | Anti-\( \alpha_5 \) integrin antibody, Anti-\( \beta_1 \) integrin antibody | Specific interactions with the integrin subfamily | Non-physiological interactions with integrins |

Fig. 1 The conceptual figure of integrin-independent cell adhesion substrates.
integrin-knockout cells have been used. The phenotypes and intracellular signal activities of these cells have been observed. These studies showed that integrins play pivotal roles in the transduction of biochemical cues from the ECM.26–28 In addition to integrin knockdown or integrin-knockout cells, cells treated with function-neutralizing antibodies and cell-adhesive peptides (e.g., RGD peptide) that competitively inhibit the interaction between integrins and their ligands have also been used. These treatments suppress integrin-dependent cell adhesion and the transduction of biochemical cues.6,44 However, the above-mentioned methods inhibit cell adhesion to the substrates and most of the cells are kept in suspension. Because mechanical cues from the ECM are sensed during interactions with the ECM, the cells should be adhered to the substrate during the analysis. Therefore, substrates that allow integrin-independent cell adhesion will be helpful for the understanding of the roles of integrins in the transduction of the mechanical cues from the ECM.

To prepare the substrates that allow integrin-independent cell adhesion, there are mainly two approaches: 1) the substrate-immobilization of ligands against the non-integrin receptors on the cell membrane and 2) the suppression of protein adsorption onto the substrate surfaces (Fig. 2). We summarize examples of substrates designed according to the above-mentioned concepts in the sections below.

### 3·2 The substrate-immobilization of ligands against non-integrin receptors on the cell membrane

The use of substrate-immobilized ligands against non-integrin receptors on the cell membrane to allow integrin-independent cell adhesion has been well-studied in biomaterial research. There are many examples of substrate-immobilized ligands against non-integrin receptors on the cell membrane, and the cells exhibit distinct behaviors depending on these substrates (Table 3 and Fig. 2A). There are two types of molecules immobilized on substrates: 1) bioactive molecules that can activate intracellular signals directly and 2) non-bioactive molecules that cannot activate intracellular signals directly. Mouse embryonic stem (ES) cells adhered on an E-cadherin-immobilized substrate in an E-cadherin-dependent but integrin-independent mechanism. On this substrate, the ES cells scattered, and they maintained their stemness.45,46 In contrast, mouse ES cells formed colonies on a gelatin-immobilized substrate, which allowed the cells to adhere via integrin.45,46 Mouse ES cells also maintained their stemness on substrates immobilized with leukemia inhibitory factor (LIF), which allowed the cells to adhere via the LIF receptor.48 Primary hepatocytes maintained their specific functions on substrates immobilized with epidermal growth factor (EGF).49 In addition to cell functions changing, the activities of intracellular signals were also changed depending on the type of substrate-immobilized bioactive molecules. Substrate-immobilized with EGF or LIF can activate extracellular signal-regulated kinase (ERK) for longer periods than soluble forms of EGF or LIF.48,50,51 ERK and Akt were also continuously activated in HepG2 cells that adhered on substrates immobilized with hepatocyte growth factor (HGF) via c-Met, a receptor against HGF.52

In addition to the substrate-immobilization of bioactive molecules, it is also possible to immobilize non-bioactive molecules on substrates to promote integrin-independent cell adhesion. For example, primary hepatocytes adhered on substrates immobilized with galactose via the endocytosis receptor protein, asialoglycoprotein receptor (ASGP-R), which clears old proteins from the blood.56–58 On substrates

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**Table 3** A partial list of substrate-immobilized ligands against non-integrin receptors on the cell membrane

| Immobilized molecule | Cell adhesion mechanism | Cellular function | Reference |
|----------------------|-------------------------|------------------|-----------|
| E-Cadherin           | E-Cadherin              | Maintenance of ES cell stemness | 45, 46    |
| LIF                  | LIF receptor            | Maintenance of ES cell stemness | 47        |
| EGF                  | EGF receptors           | Spheroid formation of primary hepatocytes | 49        |
| HGF                  | c-Met                   | Activation of the ERK signal | 50, 51    |
| N-Cadherin           | N-Cadherin              | Maintenance of neural stem cell stemness | 53        |
| Insulin-like growth factor binding protein 4 | Wnt receptor (the complex of Frizzled 8 and LRP6) | Cardiac differentiation of ES cells | 54        |
| GlcNAc              | Galactose              | Vimentin localized to on the cell membrane | Maintenance of hepatic stellate cells in a quiescent state | 55        |
| Galactose           | Asialoglycoprotein receptor | Maintenance of primary hepatoctye functions | 56, 57    |

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![Fig. 2](image-url) Two approaches for preparing the substrates that allow integrin-independent cell adhesion.
immobilized with galactose, primary hepatocytes can survive and maintain liver specific functions such as albumin secretion.\textsuperscript{56,57} Additionally, primary hepatocytes formed spheroids on substrates immobilized with galactose in the presence of EGF and HGF.\textsuperscript{57,59}

Similar to substrates immobilized with galactose, Ise \textit{et al.} prepared substrates immobilized with N-acetyl glucose by coating poly[N-p-vinylbenzyl-O-2-acetoami-d-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetoamide-2-deoxy-β-D-glucuronamide] (PVGlcNAc) on the substrate surface.\textsuperscript{55,60} PVGlcNAc-coated substrates allowed mesenchymal cells to adhere via intermediate filament proteins such as desmin and vimentin that appeared on the cell membrane surface.\textsuperscript{60} In particular, Hepatic stellate cells can adhere on PVGlcNAc-coated substrates via these intermediate filament proteins. The expression of α-smooth actin, a marker of activated hepatic stellate cells, was suppressed on PVGlcNAc-coated substrates suggesting that hepatic stellate cells can maintain a quiescent state.\textsuperscript{55}

When substrates immobilized with non-bioactive molecules were used, intracellular signals were not activated directly by the immobilized molecules, though many cell functions were altered. Cell survival was especially believed to be maintained by the activation of survival signals followed by the binding of cell-secreted proteins to the ECM proteins.\textsuperscript{61,62} However, cells can adhere on collagen- and fibronectin-coated substrates.\textsuperscript{63} In particular, HepG2 cells were able to adhere on a PMEA-analogous polymer-coated substrates,\textsuperscript{69} which suppress protein adsorption, allowed the cells to suppress interactions with integrins.\textsuperscript{66,71}

3-3 Suppression of protein adsorption onto the substrate surface

Cells are capable of adhering on substrates via electrostatic interaction and other non-specific interactions (i.e., integrin-independent cell adhesion).\textsuperscript{63-65} Although cells can adhere on substrates via non-specific interactions, serum-derived and cell-secreted proteins can be adsorbed onto substrate surfaces. Adsorbed proteins can mask substrate surfaces to inhibit non-specific interactions with substrates and induce the cells to interact via integrins (Fig. 4A).\textsuperscript{66,67} Therefore, it seems that the suppression of protein adsorption can allow the cells to adhere on the substrates via integrin-independent mechanisms (Fig. 4B).

There are several polymers that can suppress protein adsorption onto the surfaces. Polyeethylene glycol (PEG), poly(2-hydroxyethyl methacrylate) (PHEMA) and poly(2-methacyryloxyethyl phosphorylcholine) (MPC) have been frequently used to suppress protein adsorption.\textsuperscript{66,70} In particular, PHEMA-coated substrates are sometimes used in cell culture to suppress interactions with integrins.\textsuperscript{69,71} Primary hepatocytes and a hepatocarcinoma cell line, HepG2, exhibited round shapes when cultured on PHEMA-coated substrates.\textsuperscript{69,71} Additionally, primary hepatocytes maintained their specific functions when adhering on PHEMA-coated substrates.\textsuperscript{69} However, the low numbers of adherent cells cultured on these polymer-coated substrates made the analyses difficult.\textsuperscript{68,70,71}

Recently, we reported that substrates coated with poly(2-methoxyethyl acrylate) (PMEA) and its analogous polymers, which suppress protein adsorption, allowed the cells to adhere.\textsuperscript{71} On PMEA-analogous polymer-coated substrates, the cells can adhere even in the presence of ethylenediaminetetraacetic acid (EDTA), an inhibitor of integrin-dependent cell adhesion. The few focal adhesions formed in cells cultured on PMEA-analogous polymer-coated substrates also indicate that the cells adhered via integrin-independent mechanisms.\textsuperscript{72,73} The cells exhibited different functions on PMEA-analogous polymers-coated substrates compared with conventional culture substrates (e.g., tissue culture polystyrene). In particular, HepG2 cells were able to adhere on a PMEA-coated substrate via integrin-independent mechanisms and form a round shape that increased hepatocyte-specific functions such as albumin expression. Actin monomers were not assembled. Moreover, Yes-associated protein (YAP) was located in a cytosol
in HepG2 cells adhering on a PMEA-coated substrate to increase hepatocyte-specific functions.71

3-4 A comparison of the two types of integrin-independent cell-adhesive substrates

As mentioned above, there are two approaches to prepare the substrates that allow integrin-independent cell adhesion. Each approach has several advantages over the other (Table 4). The interactions between the cells and the substrates with immobilized specific ligands have been clearly determined. In contrast, the interactions between the cells and the substrates that suppress protein adsorption are not clear because the cells might be adhering on these substrates *via* non-specific interactions.72 Many reports have tried to determine the adhesion mechanisms that occur *via* non-specific interactions.73,74 The proposed mechanisms have not been accepted broadly, so far. Therefore, analyses of the signals transduced by the substrates might be easier when using the substrates immobilized with specific ligands than when using the substrates that suppress protein adsorption. However, there are many possible ways to activate intracellular signals using the substrates immobilized with specific ligands and with bioactive molecules, in particular. Therefore, the particular intracellular signals activated by the immobilized ligands should be always considered to elucidate the mechanisms of signal transduction from the substrates. Moreover, it is sometimes difficult to immobilize specific ligands to the substrates. In particular, the availability of bioactive molecules can be easily lost due to conformational changes that might be induced during the immobilization process.

To investigate the roles of integrins in the transduction of signals from the substrates, the level of cell adhesion contributed by integrins should be controlled. To control the level of cell adhesion contributed by integrins, different approaches can be taken depending on which of the two types of substrates will be used. For the substrates immobilized with specific ligands, some integrin-specific ligands could be co-immobilized with non-integrin-specific ligands on the substrate surface to control the level of cell adhesion contributed by integrins.75 For the substrates that suppress protein adsorption (*e.g.* PMEA analogous polymers), the amount of adsorbed protein could determine the level of cell adhesion contributed by integrins (Fig. 4B).

For both types of substrates that allow integrin-independent cell adhesion, the alteration of cell adhesion mechanisms should be considered. Even if the substrates that can suppress protein adsorption are used, the cells can spontaneously recruit ECM proteins that can be deposited at the interface between the cell membranes and the substrate surfaces after a long time in culture. Deposited ECM proteins can bind to integrins and convert the cell adhesion mechanism from an integrin-independent to an integrin-dependent mechanism.66,78,79 If the cells are adhering on the substrates immobilized with specific ligands, then a similar alteration in the adhesion mechanism can also occur.66,80 Therefore, the effects of deposited ECM proteins should be considered to interpret the obtained results after the long-term culture although the integrin dependency of cell adhesion is strictly regulated in the initial period of the culture.

3-5 Perspectives on the application of integrin-independent cell adhesion substrates for mechanobiology research

In mechanobiology research, gels composed of ECM proteins (*e.g.*, collagen) and the Engelbreth-Holm-Swarm (EHS) gel have often been used.31,81 Additionally, polyacrylamide gels immobilized with integrin ligands have been frequently used.32 These gels allow the cells to adhere *via* integrin-dependent mechanisms only. To investigate the roles of integrins in the transduction of mechanical cues from these elastic substrates, several methods have been used (*e.g.* genetic mutation and optical tweezer-based cell manipulations).32,33,37-39 Although these approaches are very effective for analyzing the roles of integrins, unexpected side effects from these treatments can arise. For example, genetic mutations might modulate intracellular signals, which are independent from mechanical stimulation. Optical tweezer-based cell manipulation can basically load the mechanical cues at a limited area. To avoid unexpected side effects, using substrates that allow integrin-independent cell adhesion can provide another analytical method to further examine the roles of integrin in signal transduction activated by mechanical cues from the other viewpoints. With

Table 4 A comparison of substrate preparation approaches that allow integrin-independent cell adhesion

| Advantage | Disadvantage |
|-----------|--------------|
| Immobilization of ligands against non-integrin receptors on the cell membrane | Determined interaction mechanisms |
| Suppression of protein adsorption | Low activation of intracellular signals |
| Activation of intracellular signals by immobilized ligands | Possible loss of availability of immobilized ligands |
| Low cell adhesion (PEG, PHEMA and MPC) | Unclear adhesion mechanisms |

![Fig. 4 Integrin-independent cell adhesion using the substrates suppressing protein adsorption.](image)
integrin-independent cell adhesion substrates, it might also be possible to control the level of cell adhesion contributed by integrins to enable the quantitative analysis of the integrin dependency of the transduction of mechanical cues from the substrates. Therefore, it is expected that using the substrates that allow integrin-independent cell adhesion will pave the way for new methods analyzing the roles of integrins in mechanobiology.

In this review, we focused on integrin to transduce the mechanical cues from ECM. However, there are non-integrin receptors to transduce the mechanical cues from extracellular milieu, for example some ion channel and cadherins. It might be possible to immobilize the ligands against these receptors and channels on elastic substrate surfaces. And these elastic substrates can be used as cell adhesion substrates and might provide mechanical cues to the cells through corresponding receptors in a quantitative manner. We think that these types of integrin-independent cell adhesion substrates can be applied for the research to clarify the mechanisms to respond to mechanical cues sensed by not only integrin but also other receptors.

4 Conclusions

In mechanobiology research, integrins are one of the key proteins studied to understand how mechanical cues from the ECM influence cell functions. Modulation of integrin-dependent cell adhesion is frequently performed by introducing genetic mutations and using function-neutralizing antibodies that control integrin binding activities directly. Other approaches that use cell culture substrates to modulate integrin-dependent cell adhesion were discussed in this review. There are two approaches for preparing substrates that allow integrin-independent cell adhesion: the substrate-immobilization of specific ligands against receptors on the cell membrane and the suppression of protein adsorption onto substrate surfaces. Because both of these approaches have advantages and disadvantages (Table 4), the appropriate method for preparing the substrates that allow integrin-independent cell adhesion should be selected for the analysis. Additionally, these substrates can quantitatively control the level of cell adhesion contributed by integrins. We believe that using substrates that allow integrin-independent cell adhesion might be helpful for analyzing the roles of integrins in the transduction of mechanical cues from the ECM.

5 Acknowledgements

This research was supported by a Grant-in-Aid for Young Scientists (A) (2672016), which was funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. This research was also supported by the Center of Innovation Program of the Japan Science and Technology Agency (JST).

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