Application Note

Cell Analysis

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Dynamic Monitoring of Cell Adhesion and Spreading

xCELLigence real-time cell analysis

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Introduction

The cells that make up the various tissues and organs are held together by specific molecules that essentially serve as “biological glue”. These molecules confer shape, structure, rigidity, or plasticity to the cells. During embryogenesis, these biological molecules, referred to as extracellular matrix (ECM) proteins, serve as “tracks” that direct cells to the appropriate region within the embryo. This is so they can give rise to different tissues and organ systems. ECM proteins also play a prominent role during wound healing and also are involved in directing many important cellular processes such as proliferation, survival, and differentiation. Failure of cells to interact with the appropriate biological surface or molecule can be detrimental to the fate of the cells and can contribute to cancer cell metastases.

The various ECM components, such as fibronectin (FN), collagens (CL), laminins (LM), and vitronectin (VN), interact specifically with different cells through specialized cell surface receptors called integrins. Integrins recognize and bind to specific motifs within the ECM proteins, mediating the ability of cells to specifically adhere to and interact with the appropriate matrix proteins.[1] Integrin receptor interaction with ECM proteins also begins an intracellular signaling cascade that directs cellular processes, such as cell survival, proliferation, differentiation, and migration.[1]

The ECM proteins must be purified from human or animal serum before any biological effects on cells can be studied. The purified ECM proteins are then applied to an appropriate surface, such as a plastic tissue culture dish or a glass surface. When applied to an appropriate surface at low concentrations, the ECM proteins precipitate and coat the surface. Cells can be applied to the coated surface and cellular events, such as cell adhesion and spreading, can be assessed by various cellular and molecular techniques. In general, these adhesion and spreading assays determine:

- If a certain cell type can adhere to a specific adhesive substrate
- Whether the adhesive substrate is capable of supporting spreading (a process that requires both cell adhesion and activation of intracellular signaling pathways)
- Whether cell adhesion and spreading are sensitive to specific reagents that block cell/ECM interaction, interfere with cell signaling pathways, or disrupt cytoskeletal architecture

There are several methods for assessing and quantifying cellular adhesion and spreading on an ECM-coated surface:

1. The most widely used method involves applying the cells to surfaces coated with appropriate ECM components, allowing the cells to attach and adhere for a specified length of time, then washing away the unbound cells. The attached cells are then fixed, labeled with fluorescent reagent, such as rhodamine phalloidin, and pictured using an epifluorescence microscope or an epifluorescence confocal microscope.
2. Alternatively, cells can be labeled with a dye, such as crystal violet, and quantified. Quantification involves either manually counting the cells under a light microscope or measuring the absorbance of the stain after it is solubilized.
3. Cells can also be prelabeled with a fluorescent dye, such as 6-carboxyfluorescein diacetate (CFDA), and then applied to an appropriate ECM-coated surface. The unbound cells are washed off and the bound cells are quantified using a plate reader.
4. A method that is designed to assess the role of integrins and other adhesion proteins. This method involves coating different surfaces with antibodies or peptides, which are specific for the various receptors, then seeding those surfaces with cells that express the appropriate integrin receptors. The interaction of integrin receptors on the cell surface with the antibody or peptide-coated surface allows the cells to adhere and undergo specific morphological and biological changes. These changes can then be assessed using one of the three methods discussed above.

While the assays described above have been informative, they all have limitations. All are endpoint assays, providing only a "snapshot" of the adhesion process. Further, the assays involve labor- and cost-intensive prelabeling or postlabeling of cells. Finally, they all involve fixation and permeabilization, which destroys the cell before it can be analyzed.

The xCELLigence system allows label-free, dynamic monitoring of cell events in real time. It addresses some of the major limitations of the assays described in this application note. For instance, because the technique is noninvasive, it does not require the cells to be fixed or lysed. That means it can be used to monitor biological events that occur after adhesion and spreading, such as proliferation and differentiation.

In this application note, a series of experiments is described to demonstrate that this new impedance-based system is suitable for monitoring cell adhesion and spreading.

Materials and methods

Cells

All the cells used in this study were obtained from ATCC and maintained in a 37 °C incubator with 5% CO_2 saturation. NIH3T3 cells were maintained in DMEM media containing 10% FBS, 1% penicillin, and 1% streptomycin. Jurkat T cells and BxPC3 cells were maintained in RPMI containing 10% FBS, 1% penicillin, and 1% streptomycin.

Cell adhesion assays using impedance technology

The indicated concentration of either FN or the control PLL was added into the wells of 96-well E-Plates, then the plates were incubated for one hour at 37 °C. The protein-coated plates were washed with PBS and incubated with 0.5% BSA solution in PBS for 20 minutes at 37 °C. The wells of the treated plates were washed with PBS before media and cells were added. Cells were trypsinized, spun, resuspended in serum-free media containing 0.25% BSA and adjusted to an appropriate concentration. A 100 µL volume of the cell suspension was transferred to ECM- or PLL-coated wells on E-Plates. The extent of cell adhesion and spreading, measured as changes in impedance with the xCELLigence

system, was monitored every three minutes for 1 to 3 hours depending on the experiment. The assay system expresses impedance in arbitrary Cell Index (CI) units. The CI at each time point is defined as $(R_n - R_b)/15$, where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the media alone.

Treatment with inhibitors

For each inhibitor, cells were pre-incubated for 15 to 30 minutes with the indicated inhibitor concentrations and then added to ECM-coated wells of E-Plates. All other steps were the same as previously mentioned.

siRNA Transfection

BxPc3 cells were transfected with 20 nM of siSRC using siPORTamine at a final volume of 60 µL. Cells were assayed for adhesion function 48 hours after transfection.

Immunofluorescence and light microscopy

Cells were seeded into PLL- or FN-coated 16-well chamber slides. The cells were allowed to attach, and then were fixed with 4% paraformaldehyde at the indicated time points. The cells were permeabilized, stained with rhodamine-phalloidin, then photographed using an epifluorescence microscope connected to a digital camera.

Results and discussion

Dynamic monitoring of cell adhesion and spreading on different surfaces using impedance technology

To assess the extent of adhesion and spreading, E-Plates were coated with either FN or PLL (control). NIH3T3 cells were applied to the coated wells and the extent of adhesion and spreading was monitored using the impedance-based system. Simultaneously, chamber slides were also coated with FN or PLL and the same number of cells were added to each well. To assess cell attachment and spreading, cells were stained with rhodamine-phalloidin and analyzed with an epifluorescence microscope.

As shown in Figure 1A, the Cell Index (CI) increased dramatically when cells are applied to FN-coated wells. In contrast, the CI increased slowly and steadily when cells are applied to PLL-coated wells. Similarly, immunofluorescent images (Figure 1B) showed that cell attachment on FN was accompanied by immediate spreading. The spreading was maximal after one hour. On PLL-coated wells, the cells tend to remain round even two hours after initial attachment.

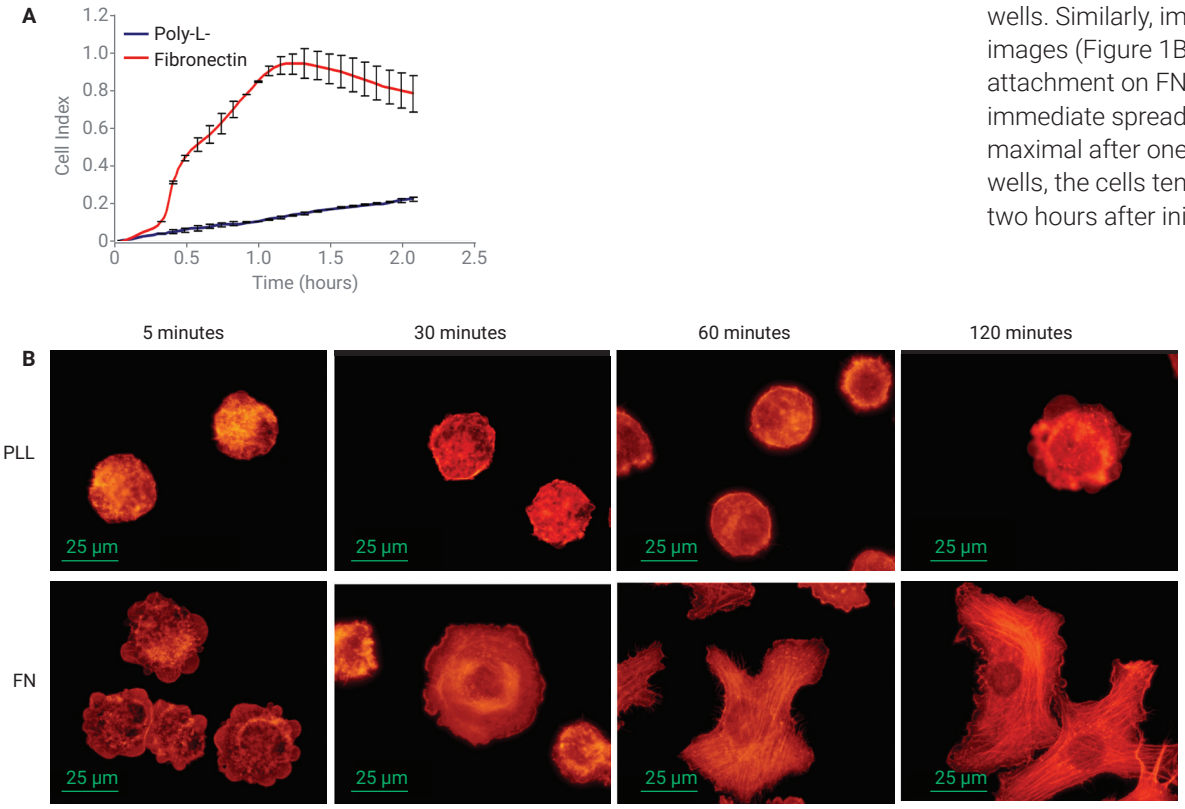

Figure 1. (A) Dynamic monitoring of cell attachment and spreading on PLL- and FN-coated surfaces. (B) The Cell Index correlates with the extent of cell attachment and spreading observed using conventional phalloidin staining of the actin cytoskeleton and immunofluorescence microscopy.

To determine the effect of FN concentration on cell adhesion and spreading, E-Plates were coated with increasing concentrations of FN, ranging from 0 to 20 µg/mL. NIH3T3 cells were added to the wells and the extent of attachment and spreading was monitored using the impedance-based system. As shown in Figure 2A, the CI increased proportionately as the amount of FN coating increases. To demonstrate that CI was proportional to the number of cells adhering to the substrate, the cells were trypsinized at three hours postadhesion and counted manually. As shown in Figure 2B, the raw cell number obtained at three hours for the different FN concentrations was proportional to the CI obtained at three hours.

The above experiments demonstrate that impedance technology can be used to quantitatively assess cell attachment and spreading in real time, under label-free conditions.

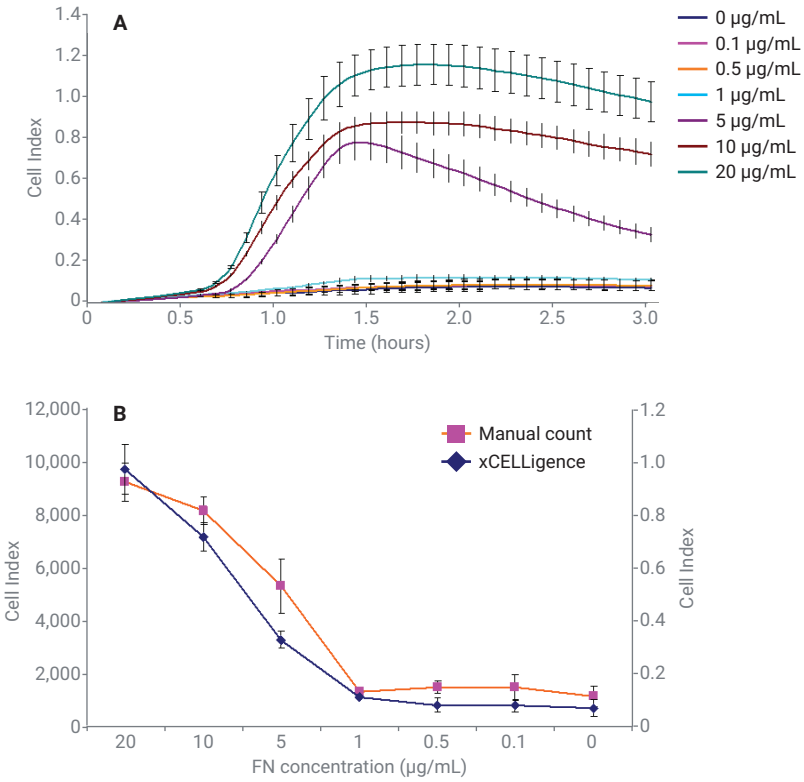

Figure 2. (A) Quantitative, dynamic monitoring of cell attachment and spreading in response to increasing concentrations of FN. (B) Comparison of Cell Index units with manual cell counts obtained for different FN concentrations. Analysis was performed after three hours of treatment.

Inhibition of cell attachment and spreading with peptides that contain RGD

Integrin heterodimers on the cell surface, that bind to FN (for example, α5β1 integrins), recognize a specific motif in FN, namely the arginine-glycine-aspartic acid (RGD) motif.[1] It has been shown that peptides containing the RGD motif can competitively inhibit the binding of these cell surface receptors to FN.[2]

To evaluate the inhibitory effects of RGD-containing peptides on cell attachment to FN, NIH3T3 cells were detached and incubated in the presence of increasing amounts of cyclic-RGD peptides. Treated cells were plated onto FN-coated E-Plates and monitored with the impedance-based system. As seen in Figure 3A, cyclic-RGD peptides blocked NIH3T3 cell adhesion and spreading in a concentration-dependent manner. A control peptide, which lacked the RGD motif, had no effect on cell attachment and spreading. After three hours, the 0.1 and 10 µM concentrations of cyclic-RGD peptides blocked cell adhesion and spreading by 20 and 40%, respectively (Figure 3B). These experiments indicate that the disruption of integrin receptor function can be assessed quantitatively and in real time using impedance-based technology.

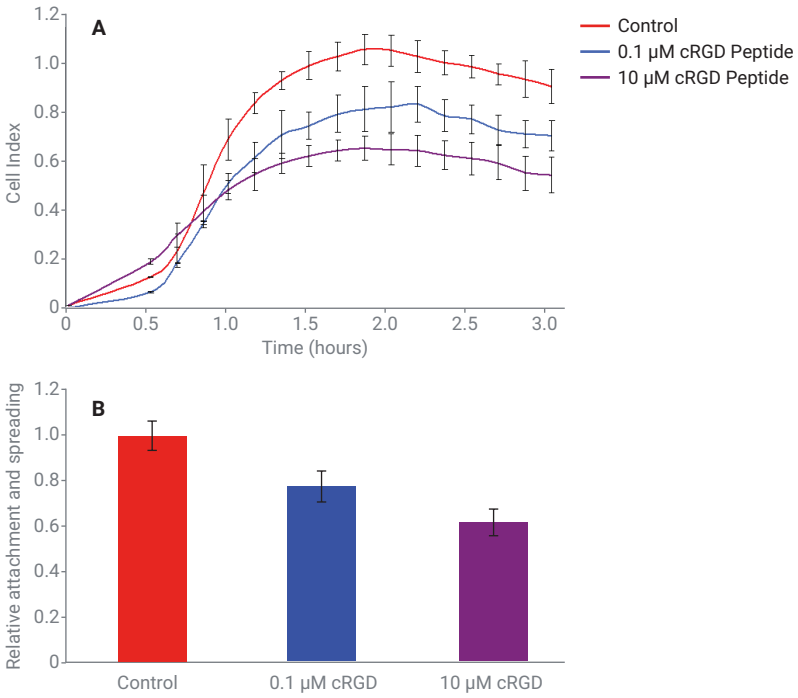

Figure 3. (A) Dose-dependent inhibition of cell attachment and spreading in response to cyclic-RGD peptides. (B) Effect of treating cells with either a control peptide or with cyclic-RGD peptides. Cell attachment and spreading was measured after three hours of treatment.

Inhibition of cell attachment and spreading with actin-disrupting agents or with specific inhibitors of signaling proteins involved in attachment and spreading

Integrin-mediated cell adhesion is known to organize the actin cytoskeleton in a specific manner. The reverse is also true; the actin cytoskeleton helps organize integrins and other intracellular signaling proteins into signaling modules that regulate cell attachment and spreading.[1]

To determine the role of the actin cytoskeleton in cell attachment and spreading, NIH3T3 cells were detached and pre-incubated with increasing concentrations of Latrunculin, a potent inhibitor of actin polymerization. The cells were then seeded onto FN-coated wells in E-Plates and the extent of adhesion and spreading was monitored using the impedance-based system. As shown in Figure 4A, Latrunculin inhibited cell attachment and spreading in a concentration-dependent manner. When cells are analyzed after two hours of treatment, the results clearly demonstrated that Latrunculin is a potent inhibitor of cell attachment and spreading (Figure 4B).

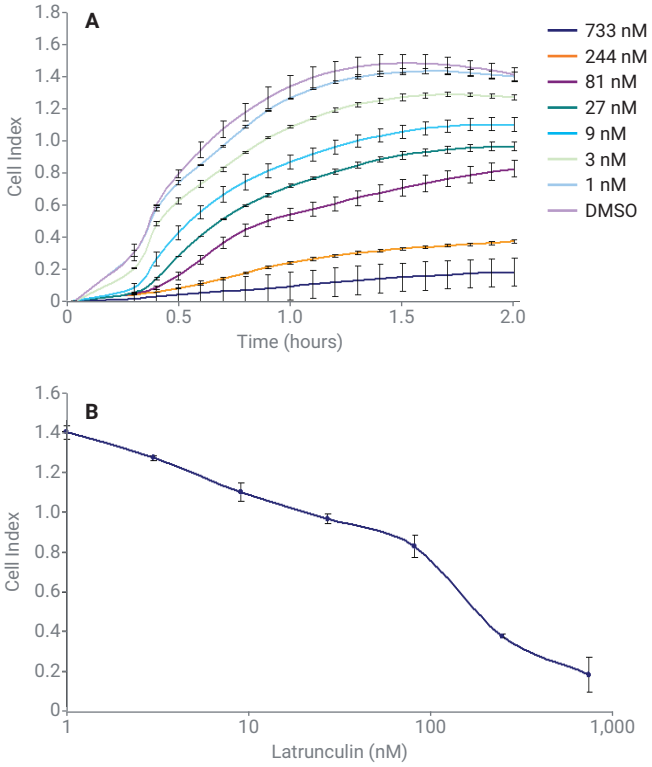

Figure 4. (A) Dynamic monitoring of the dose-dependent effect of Latrunculin on cell attachment and spreading. NIH3T3 cells were pre-incubated with the indicated concentrations of Latrunculin, then seeded onto FN-coated wells. (B) Analysis of the dose-dependent effect of Latrunculin on NIH3T3 cell attachment and spreading, measured two hours after seeding.

The group of signaling proteins that participate in integrin-mediated cell attachment and spreading includes the Src family of nonreceptor tyrosine kinases.[1]

To determine the contribution of Src family kinases to cell attachment and spreading, BxPC3 cells were pre-incubated with the Src kinase inhibitor PP2 and then seeded onto FN-coated wells in E-Plates. The extent of cell attachment and spreading was monitored using the impedance-based system. As shown in Figure 5A, cell attachment and spreading was significantly inhibited by the presence of the Src inhibitor. At two hours after seeding, the cells treated with the PP2 compound displayed approximately 60% less cell attachment and spreading than DMSO-treated cells (Figure 5B). This finding confirmed previous results obtained with conventional methods.[3]

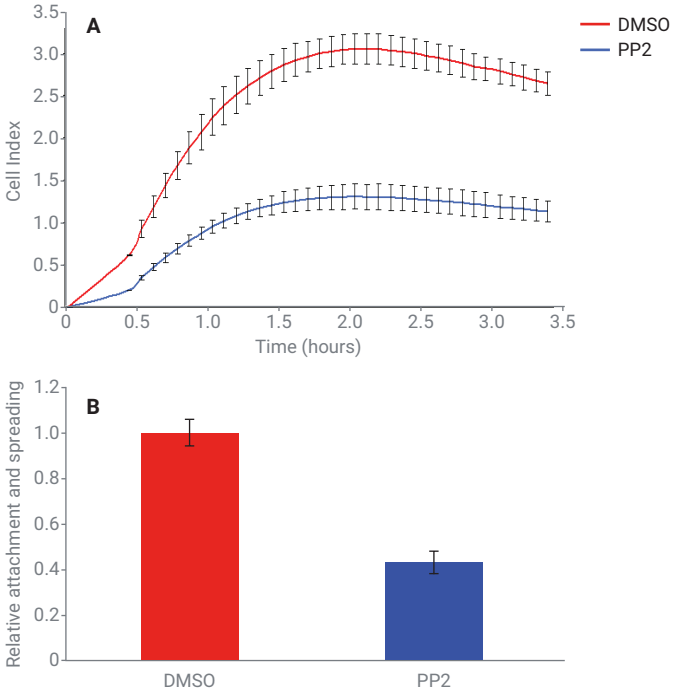

Figure 5. (A) Dynamic monitoring of the effect of the Src inhibitor, PP2, on cell attachment and spreading. BxPC3 cells were pre-incubated with either PP2 or DMSO, then seeded onto FN-coated wells. (B) Comparison of the effect of treating cells with either DMSO or PP2. The extent of cell attachment and spreading on FN was measured two hours after the treated cells were seeded onto FN-coated wells.

An additional impedance-based method for assessing the role of Src kinase in cell attachment and spreading was developed. BxPC3 cells were transfected with either a control siRNA or a siRNA specific for the c-Src mRNA. Forty-eight hours after transfection, the cells were detached and seeded onto FN-coated wells in E-Plates and the extent of cell adhesion and spreading was monitored. As shown in Figures 6A and B, down regulation of the c-Src gene product led to a 30% decrease in cell attachment and spreading within two hours of cell seeding. The disparity between the inhibitory effects of the PP2 inhibitor and the c-Src siRNA can be explained by the fact that PP2 inhibits all Src family members, and the siRNA specifically inhibits c-Src.

In summary, these experiments demonstrate that an impedance-based system can monitor and quantitatively assess cell attachment and spreading in real time. Since the system does not require labor- and cost-intensive cell labeling, it is quicker and more economical than conventional methods. The noninvasive nature of the impedance-based technique allows the user to monitor the effect of matrix proteins on adhesion, spreading, and other biological events, such as differentiation or proliferation, in a single experiment. Traditional methods would require separate experiments to monitor each of these events.

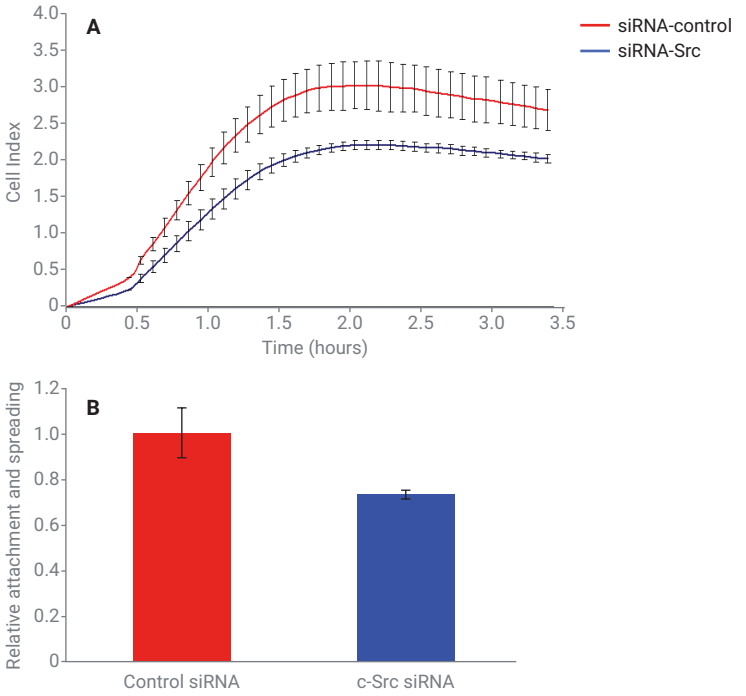

Figure 6. (A) Dynamic monitoring of cell attachment and spreading observed after BxPC3 cells are transfected with either an siRNA specific for c-Src or a control siRNA. (B) Comparison of the effect of c-Src siRNA and a control siRNA. The extent of cell attachment and spreading was measured two hours after transfected BxPC3 cells were seeded onto FN-coated wells.

References

1. Hynes, R. O. *Cell* **2002**, *110*, 673–87.
2. Koivunen, E.; Gay, D. A.; Ruoslahti, E. *J. Biol. Chem*. **1993**, *268*, 20205–10 .
3. Duxbury, M. S. *et al. Biochem. Biophys. Res. Commun*. **2004**, *317*, 133–41.

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