xCELLigence System

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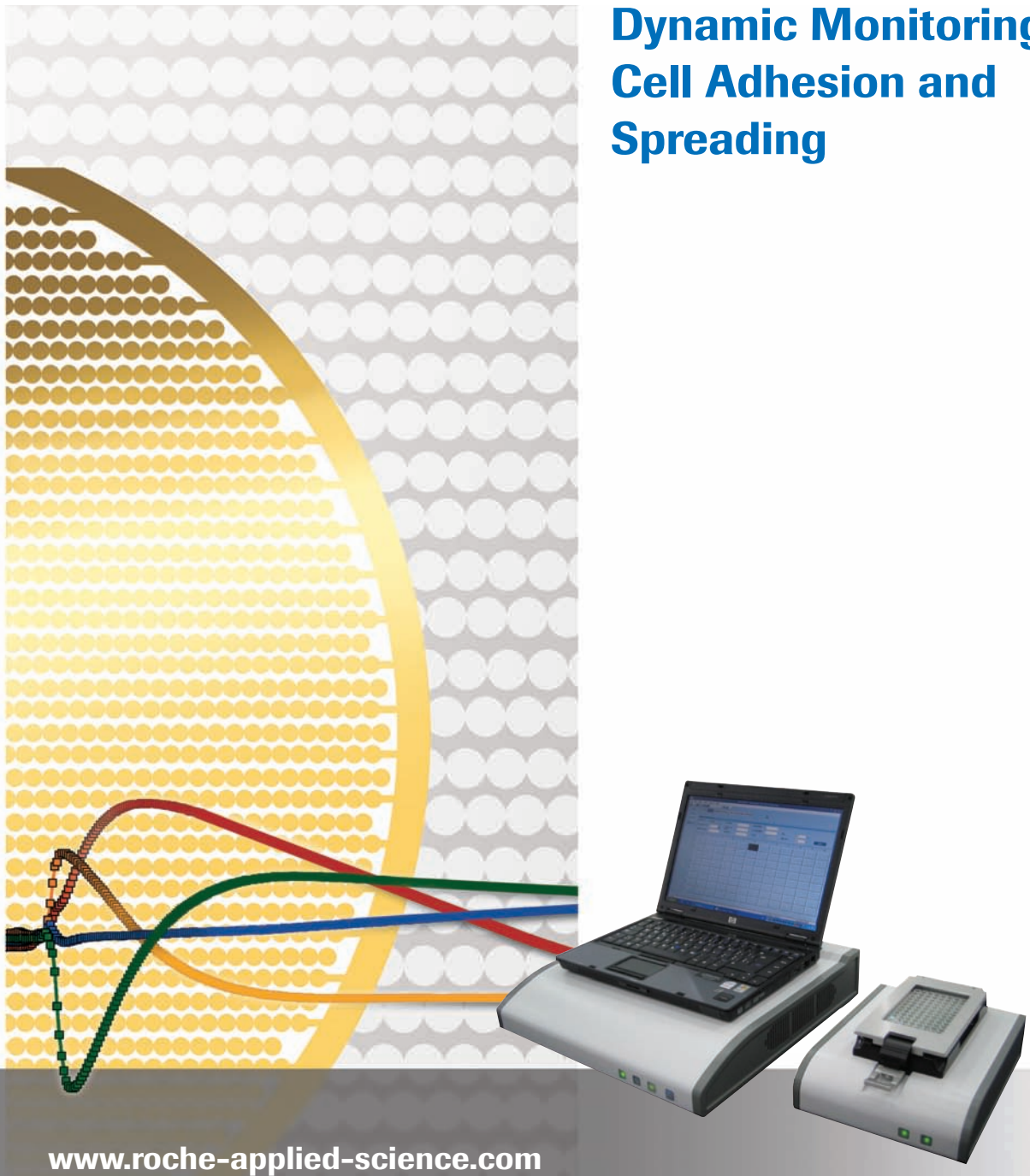
Dynamic Monitoring of Cell Adhesion and Spreading



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Dynamic Monitoring of Cell Adhesion and Spreading

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 so they can give rise to different tissues and organ systems. ECM proteins also play a prominent role during wound healing, and 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, thereby mediating the ability of cells to specifically adhere to and interact with the appropriate matrix protein *(1)*. In addition to mediating cellular adhesion, integrin receptor interaction with ECM proteins also initiates an intracellular signaling cascade that directs cellular processes such as cell survival, proliferation, differentiation and migration *(1)*.

Before the biological effects of ECM proteins on cells can be studied, the proteins must be purified to homogeneity from human or animal serum. 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 that surface. Cells can be applied to the coated surface and cellular events, such as cell adhesion and spreading, can be assessed by means of various cellular and molecular techniques. In general, these adhesion and spreading assays determine:

- whether 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), and
- 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 visualized using an epi-fluorescent microscope or an epi-fluorescent confocal microscope.

2. Alternatively, the 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 pre-labeled 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 specifically designed to assess the role of integrins and other adhesion proteins 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, which can then be assessed using one of the three methods discussed above.

Introduction continued

While the assays described above have been informative, they all have certain limitations. Each of these assays is an end-point assay, which provides only a "snapshot" of the adhesion process. Further, the assays involve labor- and cost-intensive pre-labeling or post-labeling of the cells. Finally, they all involve fixation and permeabilization, which destroys the cell before it can be analyzed.

ACEA Biosciences developed an impedance-based system (RT-CES®) that allows label-free, dynamic monitoring of cell events in real-time. This system is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences. This system addresses some of the major limitations of the assays described above. For instance, because the technique is non-invasive, 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 determine whether 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 to wells on 96X E-Plates, then the plates were incubated for 1 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. One hundred µL 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 RT-CES® system, was monitored every 3 minutes for a period of 1-3 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 $(R_n\text{-}R_b)/15$; 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 15-30 minutes with the indicated inhibitor concentrations and then added to ECM-coated wells of E-Plates. All other steps were exactly as described above.

siRNA Transfection. BxPc3 cells were transfected with 20 nM of siSRC using siPORT*amine* 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 16X 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) increases dramatically when cells are applied to FN-coated wells. In contrast, the CI increases slowly and steadily when cells are applied to PLL-coated wells. Similarly, immunofluorescent images (Figure 1B) show that cell attachment on FN is accompanied by immediate spreading; the spreading is maximal after 1 hour. On PLL coated wells, the cells tend to remain round even two hours after initial attachment.

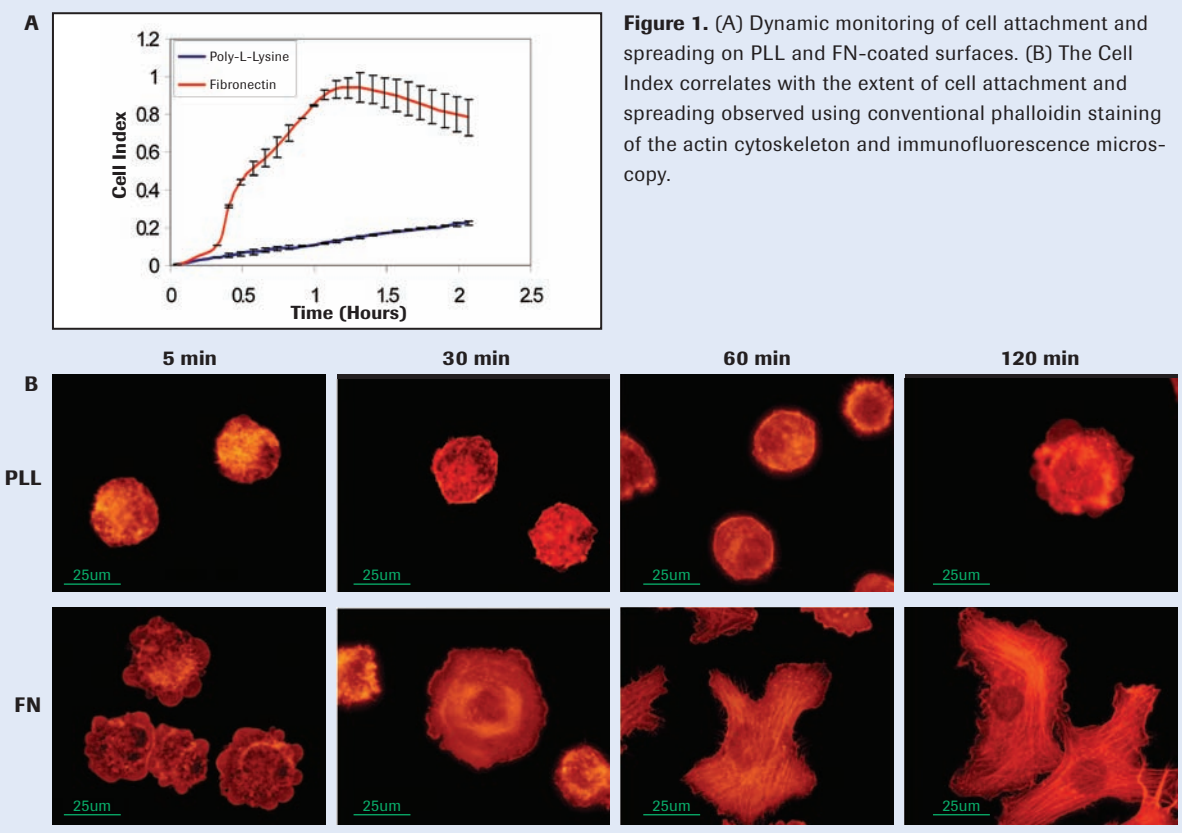

Figure 1. (A) Dynamic monitoring of cell attachment and spreading on PLL and FN-coated surfaces. (B) The Cell Index correlates with the extent of cell attachment and spreading observed using conventional phalloidin staining of the actin cytoskeleton and immunofluorescence microscopy.

To determine the effect of FN concentration on the extent of cell adhesion and spreading, E-Plates were coated with increasing concentrations of FN, ranging from 0 µg/mL 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 increases proportionately as the amount of FN coating increases.
In order to demonstrate that CI is proportional to the number of cells adhering to the substrate, the cells were trypsinized at three hours post-adhesion and counted manually. As shown in Figure 2B, the raw cell number obtained at three hours for the different FN concentrations is 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.

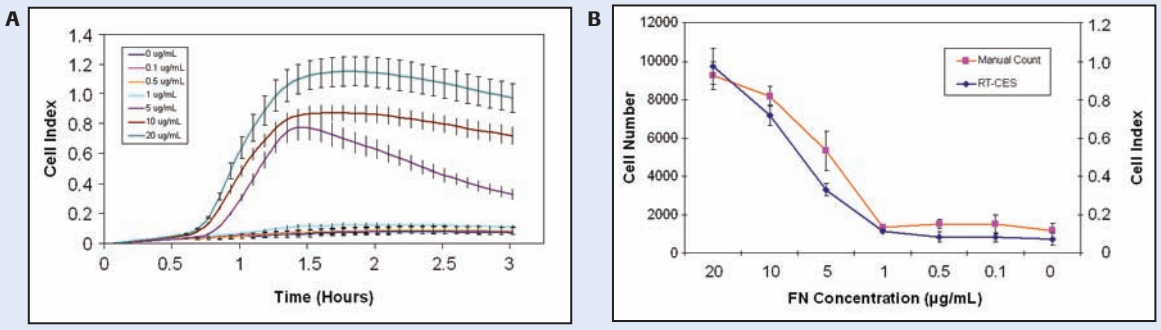

Figure 2. (A) Quantitative, dynamic monitoring of cell attachment and spreading in response to increasing concentrations of FN. (B) Comparison of Cell Index units with manual cell counts obtained for different FN concentrations. Analysis was performed after 3 hours of treatment.

Inhibition of cell attachment and spreading with peptides that contain RGD

Integrin heterodimers on the cell surface that bind to FN, *e.g.* 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 µM and 10 µM concentrations of cyclic-RGD peptides block cell adhesion and spreading by 20 % and 40 %, respectively (Figure 3B).

These experiments indicate that the perturbation of integrin receptor function can be assessed quantitatively and in real-time using impedance-based technology.

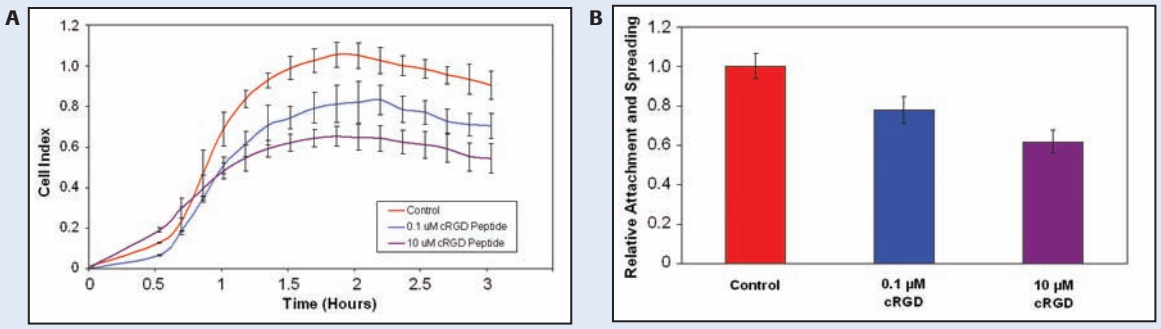

Figure 3. (A) Dose-dependent inhibition of cell attachment and spreading in response to cyclic-RGD peptides. (B) Effect of treating cells with either a control peptide or with cyclic-RGD peptides. Cell attachment and spreading was measured after 3 hours of treatment.

Results and Discussion continued

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, i.e. 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, which is 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 inhibits cell attachment and spreading in a concentration-dependent manner. When cell attachment and spreading are analyzed after two hours of treatment, the results clearly demonstrate that Latrunculin is a potent inhibitor of cell attachment and spreading (Figure 4B).

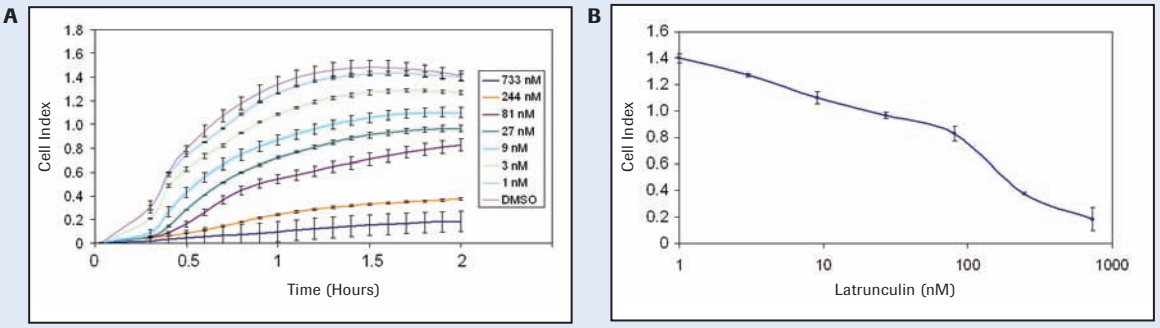

Figure 4. (A) Dynamic monitoring of the dose-dependent effect of Latrunculin on cell attachment and spreading. NIH3T3 cells were pre-incubated with the indicated concentrations of Latrunculin, then seeded onto FN-coated wells. (B) Analysis of the dose-dependent effect of Latrunculin on NIH3T3 cell attachment and spreading, measured 2 hours after seeding.

The group of signaling proteins that participate in integrin-mediated cell attachment and spreading includes the Src family of non-receptor 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 is 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 confirms previous results obtained with conventional methods (3).

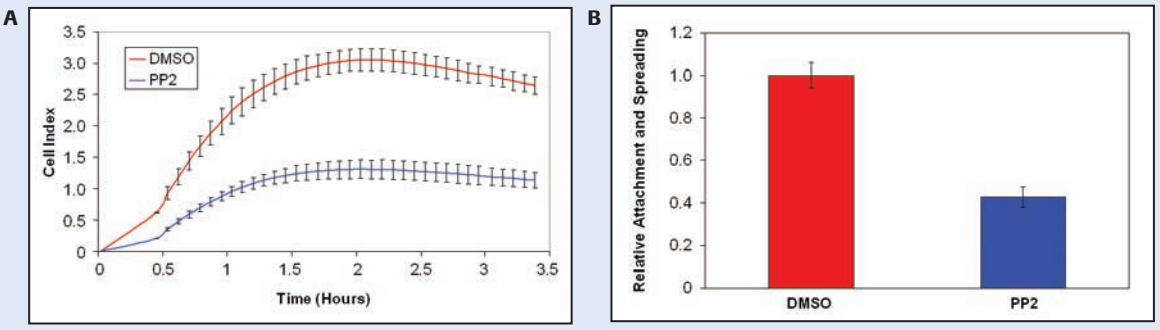

Figure 5. (A) Dynamic monitoring of the effect of the Src inhibitor, PP2, on cell attachment and spreading. BxPC3 cells were pre-incubated with either PP2 or DMSO, then seeded onto FN-coated wells. (B) Comparison of the effect of treating cells with either DMSO or PP2. The extent of cell attachment and spreading on FN was measured 2 hours after the treated cells were seeded onto FN-coated wells.

Results and Discussion continued

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 an 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 Figure 6A and B, down regulation of the c-Src gene product leads to a 30 % decrease in cell attachment and spreading within two hours after 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 be used to 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. Moreover, since the impedance-based technique is non-invasive, the user can monitor the effect of matrix proteins on adhesion, spreading and other biological events, e.g. differentiation or proliferation, in a single experiment. Traditional methods would require separate experiments to monitor each of these events.

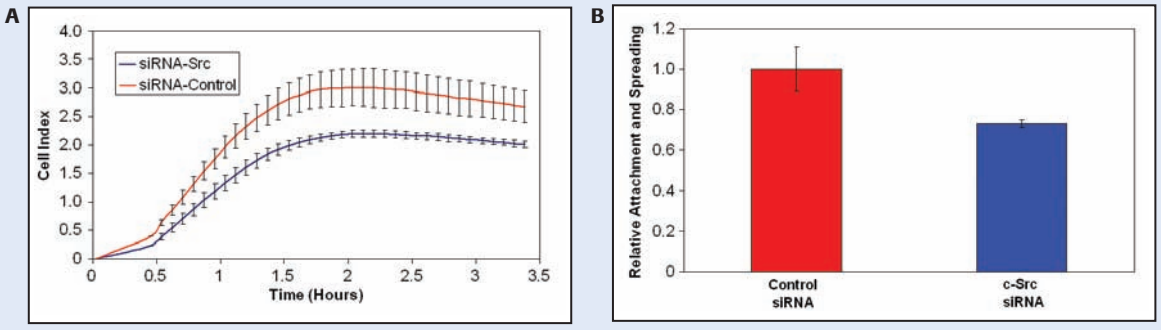

Figure 6. (A) Dynamic monitoring of cell attachment and spreading observed after BxPC3 cells are transfected with either an siRNA specific for c-Src or a control siRNA. (B) Comparison of the effect of c-Src siRNA and a control siRNA. The extent of cell attachment and spreading was measured 2 hours after transfected BxPC3 cells were seeded onto FN-coated wells.

References

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The original impedance-based assay system (RT-CES®), which was used to perform the experiments described in this application note, is the predecessor of the new xCELLigence System jointly developed by Roche Applied Science and ACEA Biosciences. While retaining the advantages of impedance-based technology described in this publication, the xCELLigence System will have improved functionality over the original system.

Intended Use:
The xCELLigence System is intended for life science research only. It is not intended for use in diagnostic procedures.

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