Technical Note

Fibronectin-Replating Experiment: Procedure and Analysis

(integrins / tyrosine kinases / cell adhesion / phosphotyrosine / cytoskeleton)

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Abstract. In this review protocols are described for studying protein tyrosine kinase signalling upon integrin-mediated cell adhesion. We have outlined detailed procedures for fibronectin-replating experiment, biochemical examination of the phosphotyrosine content of cellular proteins by immunoblotting using phosphorylation-specific antibodies or immunoprecipitation and analysis with general phosphotyrosine antibodies. Despite great advances that were made toward optimizing the described procedures, all these methods still remain in many respects an art, given the plentiful of variables and the extent to which the optimum conditions vary from one experimental condition to the other. Examples of performed experiments using the described procedures thus also include notes regarding variability of approaches based on experimental conditions.

Introduction

Cell adhesion to the extracellular matrix causes a number of intracellular events, including increased tyrosine phosphorylation of a number of cellular proteins, elevation of phosphoinositide levels, and increase in intracellular calcium concentrations. A major role in providing a physical connection between extracellular adhesion proteins and intracellular signalling molecules is played by the integrin family of receptors. At the cytoplasmic surface of the integrins, cytoskeletal proteins cluster into focal adhesions. The focal adhesions contain multiple proteins that provide a structural and signalling complex communicating the integrin-mediated signals inside the cell.

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Abbreviations: CAS – Crk-associated substrate, FAK – focal adhesion kinase, PI3K – phosphatidylinositol 3-kinase, SBTI – soybean trypsin inhibitor solution, SH2 – Src homology 2, TBST – Tris-buffered saline containing 0.2 % Tween 20.

Protein tyrosine phosphorylation has been implicated to have a central role in integrin-initiated signal transduction (Guan and Chen, 1996; Schlaepfer and Hunter, 1998; Panetti, 2002). Although integrins do not have any intrinsic enzymatic activity, they influence the activity of cytoplasmic protein kinases either directly or indirectly. At least β1- and β3-containing integrins can activate focal adhesion kinase (FAK) by an incompletely understood mechanism involving clustering of integrins maintained by Rho-mediated contraction of the actin cytoskeleton (Burridge et al., 1997). Upon activation FAK undergoes autophosphorylation at Tyr-397 permitting interactions with a number of different signalling effectors containing Src homology 2 (SH2) domains. Src-family kinases recruited to the Tyr-397 site phosphorylate two FAK-interacting proteins, Crk-associated substrate (CAS) and paxillin, which ultimately results in regulation of Rho-family GTPases contributing to cell motility. CAS phosphorylation, as well as phosphatidylinositol 3-kinase (PI3K) activation resulting from its binding to the FAK Tyr-397 site, have been implicated as downstream FAK signalling events that confer resistance to apoptosis (reviewed in Hanks et al., 2003).

The importance of tyrosine phosphorylation in integrin-mediated signalling brought the requirement for biochemical, immunochemical, and microscopic approaches to measuring protein tyrosine phosphorylation after cell adhesion.

Fibronectin Replating: Experiment

Plating cells on fibronectin coated-surfaces is a valuable approach for determining whether the analysed protein is phosphorylated on tyrosine in response to integrin-mediated adhesion.

A positive result may be of great importance since tyrosine phosphorylation of the studied protein upon integrin-mediated adhesion strongly suggests a signalling role of this protein in regulating cell behaviour resulting from integrin interaction with the extracellular matrix (see above). Moreover, a more extended version of fibronectin-replating experiment makes it possible to determine the kinetics of phosphorylation of the studied protein upon integrin-mediated adhesion.

Experiments with replating cells on fibronectin played a very important role in determining how intracellular

signalling processes are activated at sites of cell-ECM contact. In the early 1990s evidence pointing to a role for protein tyrosine kinases in these processes had accumulated. Early studies revealed rapid and dramatic increases in phosphotyrosine content of a 120-kDa protein after plating mouse NIH 3T3 fibroblasts on fibronectin (Guan et al., 1991), and of 115- to 130-kDa proteins after incubating human KB epithelial cells with antibodies to the P13 integrin component of the fibronectin receptor (Kornberg et al., 1992). On the basis of the results presented in Hanks et al. (1992), it was suggested that FAK is a major component of the phosphotyrosine-containing proteins described in these earlier studies. Fibronectin replating and other experiments led to development of the hypothesis that fibronectin-induced intracellular signalling is initiated by aggregation of the integrin receptors at focal adhesions, with coincident autophosphorylation of the associated FAK molecules. These observations provided a foundation for studies on the role of tyrosine phosphorylation in integrin control of cell behaviour. Later on, other proteins were recognized using similar approaches to be phosphorylated upon integrin-mediated adhesion, e.g. paxillin (Burridge et al., 1992) and CAS (Nojima et al., 1995). Further studies confirmed all these proteins to be key players in transmitting integrin-mediated signalling in the cytoplasm.

Fibronectin Replating: Procedure

The protocol described bellow is optimized for the fibronectin-replating experiment performed with fibroblasts and includes analysis of attached and suspended cells and cells at different times of re-attachment.

Two days before the experiment

Cells of each cell type used for the experiment are split into eight 100 mm Petri dishes in normal cultivation media (e.g. DMEM + 10 % FBS). Cells are diluted so much that they will be just confluent two days later. The dilution usually varies between 1/4 and 1/10 depending on the cell type. For each cell type, one dish will be required for analysis of attached cells, and six dishes for analysis of both suspended and re-attached cells (one dish for further cultivation).

One day before the experiment

Cells are placed in DMEM containing reduced serum (0.5% FBS) overnight. For each cell type at least four fibronectin-coated dishes (two time points of re-attaching, two dishes for each time point) should be prepared. To prepare fibronectin-coated plates, 10 µg/ml fibronectin solution in sterile 1x PBS is prepared to cover the required number of 60-mm Petri dishes (2 ml/dish). If fibronectin used is in the form of powder, it is first reconstituted with 1 ml sterile H_2O/mg of protein. It is allowed to dissolve for at least 30 min at 37 °C. Subsequently fibronectin is diluted in sterile 1x PBS. For coating, it is recommended to always prepare fresh solution; freezing and thawing of reconstituted fibronectin should be avoided as a breakdown of protein will occur. Plates are covered with fibronectin solution and allowed to air-dry for at least one hour at room temperature. It is important to make sure that the entire plate is covered with fibronectin. Plates are stored at 4 °C overnight.

The day of the experiment

The confluence of cells is assessed. It is important that all dishes are confluent, especially the dish used for the analysis of the attached cells.

The fibronectin-coated plates are prepared for use. The remaining fibronectin solution is aspirated, plates are washed two times with 1x PBS and left in 1x PBS until cells are ready for plating.

One confluent dish of cells is analysed as "attached cells". The dish is transferred onto ice for 5 min. After 5 min media is aspirated from the dish and cells are rinsed two times with ice-cold 1x PBS. The PBS is repeatedly aspirated, while making sure to aspirate as much PBS as possible by tilting plates. With the plate still on ice, 1 ml of ice-cold lysis buffer, e.g. modified RIPA buffer (50 mM Tris-HCl, pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 1 % sodium deoxycholate, 50 mM NaF, 1 % aprotinin and 0.1 mM Na_3VO_4) is added to the cells. To achieve full solubilization of proteins, plates are kept on ice for 15 min with periodic rocking. After 15 min the lysate is scraped to the bottom of the tilted plate, collected and transferred to a 1.5-ml microtube. The lysate in the microtube is sheared using 1-ml syringe with a 26 gauge needle until it is significantly less viscous (approximately 6–12 times). The lysate is transferred to –20 °C and kept there until all lysates are made. In this way the lysate from attached cells is prepared.

For suspended and re-attached cells, soybean trypsin inhibitor solution is required. Soybean trypsin inhibitor is reconstituted in PBS to prepare 2 mg/ml solution and allowed to dissolve in a nutator. Approximately 30 ml of solution are needed for six 100-mm Petri dishes with cells. In addition, serum-free DMEM has to be tempered.

The rest of plates are washed two times with ice-cold 1× PBS. PBS is carefully aspirated from the plates and cells are trypsinized at 37 °C by adding 1 ml of trypsin/EDTA solution for as short time as possible for cells to be off the plate (usually cca 1 min). Immediately after trypsinization, 3 ml of serum-free media are added to the assay plates and cells from all dishes are collected into a 50-ml conic centrifugation tube. After cells from all the dishes are collected, the same volume of soybean trypsin inhibitor is added to the cells in the tube. Including the volume of cells it will be approximately 25 ml of cell suspension and 25 ml of soybean trypsin inhibitor solution (SBTI), the final concentration of SBTI will be 1 mg/ml. Cells are immediately centrifuged at 200 *g* for 10 min. Supernatant is aspirated, carefully avoiding the cell pellet. Each cell pellet is re-suspended in 20 ml of serum-free DMEM (8 ml will be needed for detached cells, 6 ml for each time point of re-attaching). Suspended cells are rotated at 37 °C for 30 min in an orbital rotator. In the meantime, fibronectin-coated plates are

washed last time by adding 4 ml of fresh PBS. The last wash is aspirated to dryness and plates are labelled.

After 30 min of rotating, suspended cells of appropriate cell type are retrieved from the incubator and mixed thoroughly. Three ml of cells are added to the labelled fibronectin-coated plates. Twelve ml of suspension will be needed (two time points of re-attaching, two 60-mm dishes for each time point). Timer is set for times of re-attaching (e.g. 15 and 30 min).

The remaining 8 ml of suspension of "detached" cells are transferred into a 15-ml conic tube and centrifuged for 5 min at 200 *g*, 4 °C. Supernatant is carefully aspirated and pellet is resuspended in 12 ml of PBS and centrifuged again for 5 min at 200 *g*, 4 °C. After removing the supernatant, the pellet is re-suspended in 1 ml of PBS and transferred into a 1.5-ml microtube and centrifuged in the same conditions in a refrigerated microcentrifuge. Supernatant is removed and 1200 µl of ice-cold lysis buffer are added to the pellet. The pellet is triturated (using a pipette tip) and resuspended and suspension is incubated on ice for 5 min. After 5 min the lysate is sheared using a 1-ml syringe with a 26 gauge needle until it is significantly less viscous (approximately 6–12 times). Lysate is transferred to –20 °C and kept there until all lysates are made. In this way the lysate from suspended cells is prepared.

At desired time points (e.g. 15 and 30 min), the two fibronectin-coated dishes of particular cell type are retrieved from the incubator. The dishes are transferred onto ice for 5 min. After 5 min media is aspirated from the dishes and cells are rinsed two times with ice-cold 1x PBS. The PBS is repeatedly aspirated, while making sure to aspirate as much PBS as possible by tilting plates. With the plate still on ice, ice-cold lysis buffer is added to the cells. To achieve full solubilization of proteins, plates are kept on ice for 15 min with periodic rocking. After 15 min the lysates are scraped to the bottom of the tilted plate, collected and transferred from both dishes into one 1.5-ml microtube. The lysate in the microtube is sheared using a 1-ml syringe with a 26 gauge needle until it is significantly less viscous (approximately 6–12 times). Lysate is transferred to -20 °C and kept there until all lysates are made. In this way the lysate from re-attached cells, attaching for selected time is prepared.

When all lysates are collected, they are centrifuged at 15 000 *g*, 20 min, 4 °C. Supernatants are transferred into new microtubes, aliquots are taken for determining the protein concentration. Protein concentration is determined using an appropriate method or kit of choice (e.g. DC Protein Assay, Bio-Rad Laboratories, CA).

After all lysates are collected and the protein concentration is determined, it is time to determine the phosphotyrosine content of the analysed protein in the samples.

Fibronectin Replating: Analysis

The next step of the fibronectin-replating experiment depends on the availability of the phosphorylation-specific antibodies for the protein analysed. If such antibodies are available, direct immunoblotting of whole cell lysates could be used to determine changes in the tyrosine phosphorylation status of the protein in response to suspension and re-attachment. If there are no phospho-specific antibodies for the analysed protein, protein can be immunoprecipitated from all samples and subsequently analysed by immunoblotting with general phosphotyrosine antibody.

Both methods (using phospho-specific antibodies and immunoprecipitation to determine the phosphotyrosine content) are general and widely used very important methods in integrin cell signalling and will be demonstrated here on the analysis of the fibronectin-replating experiment.

Variant A (phosphorylation-specific antibody is available)

A valuable approach to biochemical examination of tyrosine phosphorylation of the analysed protein at specific sites is immunoblotting using phosphorylation-specific antibodies. SDS PAGE/immunoblotting is generally the method of choice for the qualitative and quantitative detection of specific signalling protein in a given sample. The lysates are separated by SDS PAGE, transferred onto a membrane, and analysed proteins are then identified using specific antibodies. If the aim of an experiment is limited to the identification and/or quantification of a specific protein in a given sample, SDS PAGE/immunoblotting of a cell lysate using specific antibodies should yield sufficient information. The advantage of separating and blotting the protein extract onto a membrane compared to the simple detection of a protein of interest, e.g., in an ELISA assay, is the additional information about the size of the protein that is detected. This is particularly important when it is not clear whether the antibodies used for detection of the protein cross-react with other proteins in the cell lysate. A wide variety of options have been developed for membranes (mostly nitrocellulose and nylon-based), blocking solutions, and detection strategies including both non-radioactive (via chemiluminescent or colorimetric detection) and radioactive methods (Poxton, 1990). Advances in visualization using high-sensitivity films or imaging instrumentation capable of integrating a range of signals provide more accurate and permanent records of immunoblot data (Fournier et al., 2003).

Based in large part on the successful use of short synthetic peptides to produce epitope-targeted antibodies (Suctliffe et al., 1983), an immunochemical approach became an attractive method for detecting changes in the state of phosphorylation of specific proteins at a specific site. The use of phosphorylation state-specific antibodies takes advantage of the sensitivity and selectivity afforded by immunochemical methodology to greatly increase not only the throughput, but also the quantitative accuracy of phosphoprotein assays (reviewed in Nagata et al., 2001).

To use the phospho-specific antibody for the analysed protein, first, a suitable phosphorylation site has to be

chosen. For example, if one would consider to analyse phosphorylation of FAK kinase, it must be decided whether to analyse tyrosine phosphorylation of the autophosphorylation site (Tyr-397), phosphorylation of activation loop tyrosines (Tyr-576, 577), or other phosphorylated tyrosines (Tyr-861, Tyr-925). This step is individual for each protein analysed and has to be done based on the purpose of the analysis. Next, the availability of suitable phospho-specific antibody must be found out. There are many web resources of suppliers of phosphorylation-specific antibodies, a very comprehensive list can be found at Protein Kinase Resource (http://www.kinasenet.org/pkr). If the suitable phospho-specific antibody is available, it is strongly recommended to learn which methods were used for confirmation of the specificity of the antibody. Western blot analysis results should be provided by the supplier, demonstrating that there are no cross-reacting bands labelled by the antibody in complex samples, such as a homogenate or cell lysate. The second level of specificity should also be shown, proving that the antibody reacts only with the form of protein phosphorylated at a selected site (e.g. western blots with proteins mutated at the phosphorylation site).

Example (analysis of results of fibronectin-replating experiment)

Samples for SDS-PAGE are made. All samples must have the same protein concentration (optimally ca 1 µg/µl). RIPA lysates equivalent to 10–30 µg protein are diluted in 2x SDS-PAGE sample buffer for immunoblot analysis of all cell extracts prepared during the experiment.

The common order of loading the samples on SDS-PAGE gel is: cell extract from attached cells, suspended cells, cells re-attaching for shorter selected time (e.g. 15 min), cells re-attaching for longer selected time (e.g. 30 min).

For immunoblotting, samples are separated on SDS-polyacrylamide gels and transferred to an appropriate membrane – e.g. BioTrace NT membrane (Pall Corporation, East Hills, NY). Non-specific activity is blocked by incubating the membrane for 1 h at room temperature in Tris-buffered saline containing 0.2 % Tween 20 (TBST) and 5 % non-fat dry milk. Membranes are then incubated overnight in primary antibody, extensively washed with TBST, and then incubated for 1 h at room temperature with HRP-conjugated secondary antibody. After extensive washing in TBST, the blots are developed by enhanced chemiluminescence (e.g. ECL, Amersham Biosciences, Piscataway, NJ) and exposed to autoradiographic film.

Note

The washing steps represent a critical element of immunoblotting detection. The number of washes, the duration, and the volumes used for each step are important factors in minimizing general background as well as the appearance of non-specific protein bands. In addition, further improvement of signal-to-noise ratios can be accomplished by systematic adjustments to such variables as the source and percentage of the blocking reagent (e.g., 1–5 %), the amount of extract run on the gel and the amount of primary or secondary antibody used.

Variant B (phosphorylation-specific antibody is not available)

In case the phospho-specific antibody for the analysed protein is not available or if changes in total phosphotyrosine content rather than single phosphorylation site need to be analysed, protein must first be immunoprecipitated. The immunoprecipitate is subsequently analysed with both antibody specific for the immunoprecipitated protein and with general phosphotyrosine antibody. Immunoprecipitation is one of the most widely used immunochemical techniques. In the immunoprecipitation method, the protein from the cell or tissue homogenate is precipitated in an appropriate lysis buffer by means of an immune complex, which includes the antigen (protein), primary antibody, and Protein A-, G-, or L-agarose conjugate or a secondary antibody-agarose conjugate. The choice of agarose conjugate depends on the species origin and isotype of the primary antibody. The choice of the method depends on the specific antigen-antibody system.

Immunoprecipitation followed by SDS-PAGE and immunoblotting can be used in a variety of applications:

- determination of the presence and quantity of the studied protein in the studied sample
- determination of the molecular weight and isoelectric point of the immunoprecipitated protein
- determination of precursor-product relationships
- monitoring of protein post-translational modifications including glycosylation
- studying protein-protein interactions
- determination of specific enzymatic activity.

To determine the phosphotyrosine content using immunoprecipitation a general phosphotyrosine antibody is required. Commonly used antibodies are 4G10, PY-20 or PY-99.

Example (analysis of fibronectin-replating experiment)

Immunoprecipitations are carried out from 0.5 ml of each lysate (cca 500 µg of protein). Lysates are incubated 4 h on ice with primary antibody and immune complexes are collected on Protein A-, G-, or L-agarose conjugate (20 µl 50% slurry; e.g. Zymed, Camarillo, CA). The immunoprecipitates are washed 5 times with 1 ml of ice-cold modified RIPA buffer, resuspended in (usually 50–100 µl) 2× SDS-PAGE sample buffer and processed for immunoblotting as described in Variant A using anti-phosphotyrosine primary antibody.

Notes

The choice of lysis buffer depends on the properties of the immunoprecipitated protein and the purpose of im-

munoprecipitation. RIPA buffer generally gives a lower background, but can denature some kinases. It also has the potential to disrupt protein/protein interactions.

NP40 buffer (the same buffer without SDS and sodium deoxycholate) is less denaturing, but gives a higher background. It is less likely to inhibit kinase activity and disrupt protein complexes.

The choice of immobilized antibody binding the protein depends upon the species that the antibody was raised in. Protein A binds well to rabbit, human, pig and guinea pig IgG as well as mouse IgG2a and IgG2b. Protein G binds strongly to IgG from goat, sheep, horse, rabbit and guinea pig and to mouse IgG1 and IgG3.

In both cases immunoblotting must be done with general antibody against the analysed protein. The other blot should be done with phosphorylation-specific antibody (if available) or general phosphotyrosine antibody.

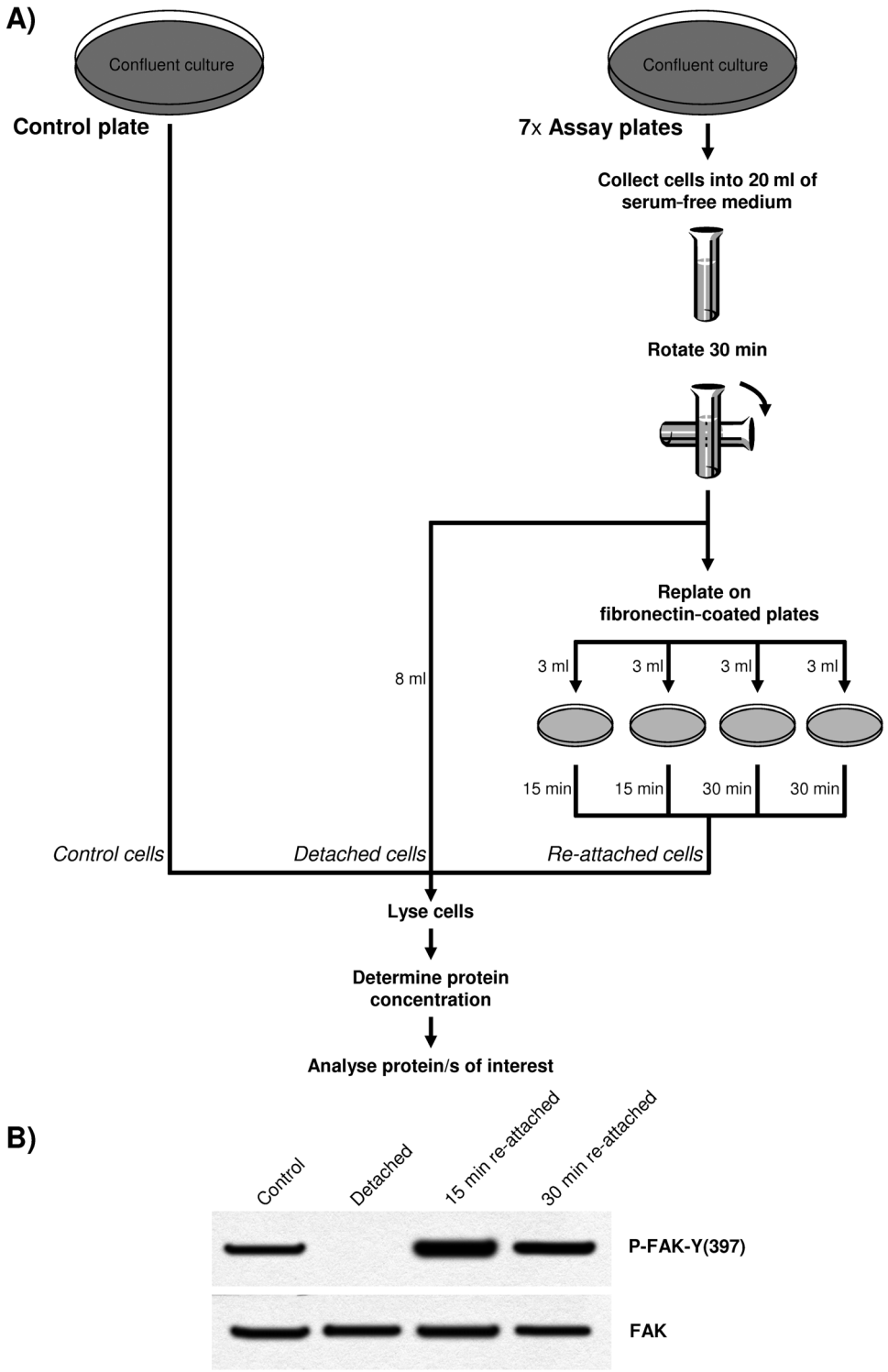

Fig. 1: Fibronectin-replating protocol. **A)** The scheme of fibronectin-replating protocol. **B)** Illustrative results of replating experiment with anti-FAK phosphoTyr-397 and control anti-FAK antibodies.

As a control, the same experiment is done with the only difference that the dishes for re-attaching cells are not coated with fibronectin but with 1 mg/ml poly-L-lysine. This experiment should reveal the degree of background phosphorylation of the analysed protein after re-attaching, which may not be directly related to activation of integrins.

If both experiments are performed carefully, their result should answer the question whether the analysed protein is phosphorylated on tyrosine in response to integrin-mediated adhesion. Next experiments may include more time points to learn about the kinetics of this phosphorylation.

The protocol may also be used with little modifications for studying changes in tyrosine phosphorylation of the protein analysed in response to cell attachment to other ECM proteins that interact with distinct integrin receptors.

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