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Olga Volotskova
*George Washington University*

Mary Ann Stepp
*George Washington University*

Michael Keidar
*George Washington University*

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# Integrin activation by a cold atmospheric plasma jet

**Olga Volotskova**[1,3], **Mary Ann Stepp**[2] **and Michael Keidar**[1,3]

[1] Department of Mechanical and Aerospace Engineering, The George Washington University, SEAS, 801 22nd Street NW, Phillips Hall 735, Washington, DC 20052, USA

[2] Department of Anatomy and Regenerative Biology, The George Washington University, SMHS, 2300 I Street NW, Ross Hall 226, Washington, DC 20037, USA

E-mail: olyanv@gwu.edu and keidar@gwu.edu

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**Abstract.** Current breakthrough research on cold atmospheric plasma (CAP) demonstrates that CAP has great potential in various areas, including medicine and biology, thus providing a new tool for living tissue treatment. In this paper, we explore potential mechanisms by which CAP alters cell migration and influences cell adhesion. We focus on the study of CAP interaction with fibroblasts and corneal epithelial cells. The data show that fibroblasts and corneal epithelial cells have different thresholds (treatment times) required to achieve maximum inhibition of cell migration. Both cell types reduced their migration rates by ∼30–40% after CAP compared to control cells. Also, the impact of CAP treatment on cell migration and persistence of fibroblasts after integrin activation by $MnCl_2$, serum starvation or replating cells onto surfaces coated with integrin ligands is assessed; the results show that activation by $MnCl_2$ or starvation attenuates cells' responses to plasma. Studies carried out to assess the impact of CAP treatment on the activation state of $\beta 1$ integrin and focal adhesion size by using immunofluorescence show that fibroblasts have more active $\beta 1$ integrin on their surface and large focal adhesions after CAP treatment. Based on these data, a thermodynamic model is presented to explain how CAP leads to integrin activation and focal adhesion assembly.

[3] Authors to whom any correspondence should be addressed.

## Contents



## 1. Introduction

The migration of cells within tissues plays an important role in many physiological and pathological processes, including embryonic development, wound repair, angiogenesis and metastasis. Various signaling pathways control these processes. Integrins (single-pass type I transmembrane proteins) are a major family of metazoan cell-surface-adhesion receptors playing a key role in the signaling and mechanotransduction mechanisms. Their functions include maintaining cell adhesion, tissue integrity, cell migration and differentiation [1–3]. Previous studies revealed that integrins could be affected by the application of cold atmospheric plasma (CAP) [4, 5]. Recent studies of CAP jets have shown that they have great potential for use in biomedical applications. Their distinguished physical and chemical properties are defined by the uniqueness of non-thermal non-equilibrium plasmas. Depending on their configuration they can be used for wound healing, sterilization, targeted cell/tissue removal and cancer treatment [6–9]. It was found that CAP jets can slow down cell migration and change integrin expression on the cell surface, i.e. treatment of cells with CAP jet resulted in a decrease of $\beta 1$ and $\alpha$v-integrins $\sim$24 h after treatment [10]. In addition, the localization effect of cold plasma treatment on cell migration was shown [11]. The plasma effect is determined by the size of the plasma jet and does not alter cell migration outside the treated area. The CAP effect has not only spatial but also temporal dependence since it persists for at least 33 h. Despite the progress made in studying CAP interaction with the living tissue, the question remains: what causes the motility and cell adhesion of cells to change in response to plasma treatment? The answer to this question needs to be obtained to allow application of cold plasma in aiding patients with wound healing [1, 12].

The goal of this study is to understand the mechanism by which the CAP jet alters cell migration and influences adhesion. Although the main focus here is on the study of the

interaction between the CAP jet and fibroblasts, we also present data comparing fibroblast responses to those of epithelial cells.

## 2. Materials and methods

### *2.1. Cell culture*

Wild-type tertiary passaged mouse fibroblast cells (WTDF 3, the procedures used to obtain cells from mice were authorized and approved by the GWUMC Institutional Animal Care and Use Committee; wt mice (BALB/c) obtained from NCI-Frederick [13]) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen Corp., Carlsbad, CA) enriched with 5% serum, 1% NEAA, 1% L-glutamine, 1% Pen.-Strep. Diluted cells (30% confluence) were plated on multi-well plates or double-well glass slides and treated with plasma on the third day in culture. The human corneal limbal epithelial cell line (HCLE; Dr Ilene Gipson, Harvard Medical School and described in [14]) was grown in HCLE medium (500 ml of Keratinocyte Serum Free Medium with added 1.2 ml of Bovine Pituitary Supplement, 4l $\mu$l of Epidermal Growth Factor, 0.17 mM $CaCl_2$ and 5 ml of Pen.-Strep, Invitrogen Corp., Carlsbad, CA). HCLE cells were used on the second day of culture with a confluence of about 30–40%. During plasma treatment, plates with cells were kept on a heating plate (Boekel Scientific, model 240000, Feasterville, PA) to maintain the temperature of the medium at 37 °C. Serum starvation experiments were performed on WTDF 3 cells and placing the cells in DMEM with 1% serum ∼24 h before the plasma treatment. For studies using $MnCl_2$ to activate integrins, 0.5 mM $MnCl_2$ was added to DMEM with 5% serum immediately after cells were subjected to cold plasma treatment.

Some cold plasma treatments were conducted on cells that had been placed in plates that had been pre-coated with extracellular matrix proteins. Coating of tissue culture plates was performed as described in [12]. Fibroblasts were either placed onto plates pre-coated with 10 $\mu$g ml$^{-1}$ fibronectin (FN; BD Bioscience, Pasadena, CA, catalog no. 354008)/1% collagen (CN) type I (Advanced BioMatrix, San Diego, CA, part no. 5005) or with 10 $\mu$g ml$^{-1}$ vitronectin (VN) (BD Bioscience, catalog no. 354238). Plasma treatment was performed 8 h after replating.

### *2.2. Cold atmospheric plasma (CAP) jet*

The cold plasma jet (see figure 1) is the dielectric barrier discharge in helium that is described elsewhere [4, 15]. The output voltage is about 4.5–5 kV, the frequency is ∼13 kHz, the helium flow rate ∼11–12 liter min$^{-1}$ and the average jet power ∼4 W. The distance between the jet outlet and the culture plate/slide was always kept at about 20 mm. The depth of the medium was maintained at ∼2 mm. Dermal fibroblasts and epithelial cells were prepared and equivalent numbers were plated onto the culture plastic (for time-lapse studies) or glass slide (for immunostaining) and maintained in DMEM supplemented with 5% serum (fibroblasts) or HCLE medium (epithelial cells). Fresh medium or fresh medium with $MnCl_2$ was added to cells following plasma treatment.

### *2.3. Time-lapse studies*

Time-lapse studies were conducted on an Olympus IX81 research microscope (Olympus America, Center Valley, PA) equipped with a Proscan motorized stage (Prior Scientific Instruments Ltd, Rockland, MA) and placed in a temperature- and $CO_2$-controlled chamber

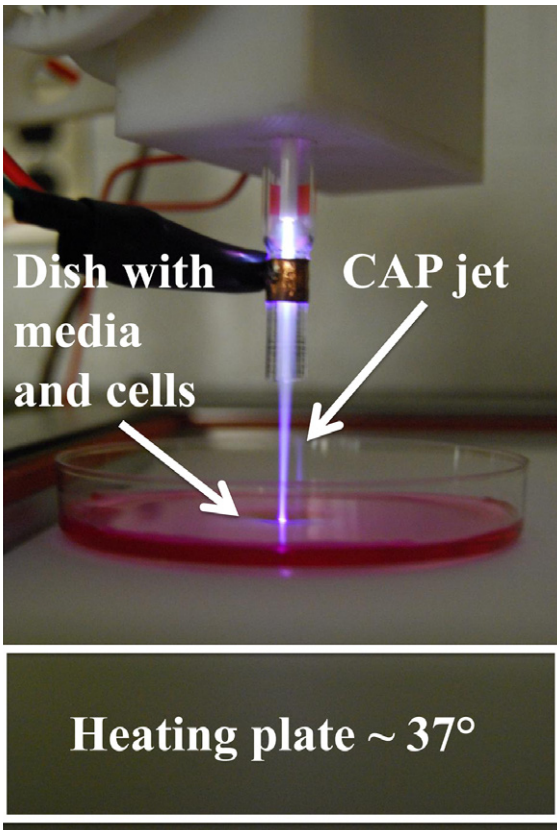


**Figure 1.** The CAP jet. A schematic representation of CAP treatment of the living cells is shown.

(LiveCell Incubation System, Neue Biosciences, Camp Hill, PA). Using relief-contrast optics, images were taken every 10 min for 16 h 40 min (100 images). Images were transferred to a workstation equipped with Metamorph image analysis software (Molecular Devices Corporation, Chicago, IL) where velocities of ten cells were calculated using the track cell module in each tracked location. A more detailed description can be found elsewhere [13]. At least 80 cells were analyzed per conditional change, such as the time point and/or treatment. The tracking experiment was started immediately after treatment.

### *2.4. Immunofluorescent studies*

For experiments involving immunofluorescent staining of cells, cells were plated onto two-well glass chamber slides for 24 h prior to CAP treatment. For cold plasma treatment, chamber slides containing cells were placed on the heating plate to maintain the temperature and treated with cold plasma as described above. Fresh medium was added and cells were returned to the 37 °C $CO_2$ incubator for 3 h. The medium was discarded and cells were fixed in 4% paraformaldehyde (product no. 28908, Thermo Scientific, Rockford, IL) in PBS for 10 min and then rinsed in PBS. Non-specific staining of cells was blocked by incubating the cells for 15 min in blocking buffer (1% bovine serum albumin in 1× PBS). Cells were incubated in an appropriate dilution of each of the primary antibodies below for 1 h, followed by washing (15 min in PBS) and incubation in

secondary antibodies. When the primary antibodies used were generated in different species, cells were stained with multiple antibodies simultaneously. For studies of active and total surface integrins, cells were used for immunostaining without permeabilization. However, for studies of vinculin, and the $\alpha$v receptor within focal adhesions, cells were permeabilized by incubating in 0.1% Triton X-100 for 10 min and then incubated in PBS. Permeabilization is needed because vinculin is an intracellular protein and antibodies to detect it cannot penetrate the cell unless it is permeabilized. Total b1 integrin was detected with the Hamster derived CD 29 monoclonal antibody clone Ha2/5 (BD Bioscience, catalog no. 555004) at a dilution of 1:200. For activated b1 integrin, the rat-derived monoclonal antibody against activated CD 29, clone 9EG7 (BD Bioscience, catalog no. 553715), was used at a dilution of 1:200. $\alpha$V integrin was visualized using the rat-derived monoclonal antibody against CD51 RMV7 (BD Bioscience, catalog no. 552299) at a dilution of 1:200. Focal adhesions were visualized by staining for the focal adhesion protein vinculin using the mouse-derived monoclonal antibody clone VIIF9 (7F9) at a dilution of 1:200 (catalog no. MAB3574, Chemicon Int., Temecula, CA). Nuclei were visualized using DAPI, dilution 1:2000 (D21490; Invitrogen Corp.). Images were acquired at 20$\times$ magnification using a Nikon fluorescent microscope equipped with an RT-Slider SPOT camera (Melville, NY).

### *2.5. Data analysis*

Adobe Photoshop 7.0 was used to manage the images. For quantification analysis Image Pro Plus, version 6.2 software (Media Cybernetics Inc.) was used. Data were subjected to statistical analyses using InStat software (Graphpad Software, Inc., San Diego, CA). The differences between groups were determined by two-tailed unpaired *t*-tests and data were considered significant for $p$-values $< 0.05$. When standard deviations between groups were not equal, significance was determined using Welch corrected unpaired $t$ tests and when the distributions of values within a data set were not Gaussian, significance was determined using the Mann–Whitney test. Histograms were generated using mean and standard error of the mean (SEM) values [13].

## 3. Results

### *3.1. Dermal fibroblasts and human corneal epithelial cells reduce their migration in response to CAP*

While we had reported that dermal fibroblasts migrate more slowly after CAP treatment, we are yet to evaluate a different type of cell, epithelial cells, to determine their response to CAP. Presented in figure 2(A) are relief contrast images of the tracks taken (red lines) by migrating fibroblasts (WTDF 3) and epithelial (HCLE) cells with and without CAP treatment over a 16 h and 40 min time period. The tracks of the cells after plasma treatment (fibroblast cells were treated for $\sim$60 s and epithelial cells for $\sim$100 s) are significantly shorter than untreated cells (control). Additional experiments were conducted to assess the impact of increasing plasma treatment times on cell migration rates of fibroblasts and epithelial cells, i.e. to determine the threshold of treatment (threshold here is determined as the treatment dose (duration of treatment) after which the change becomes statistically non-significant (i.e. $p > 0.05$ between two neighbor points) and no other changes occur). In figure 2(B), cell migration rates are

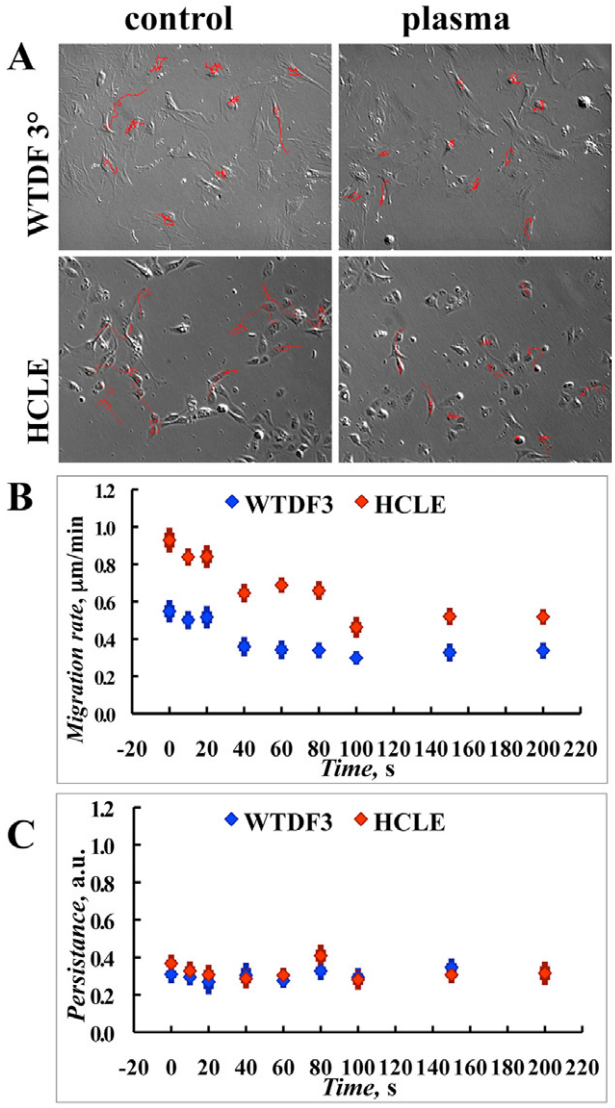


**Figure 2.** Temporal dynamics of the cells' response to CAP (plasma) treatment. (A) Relief-contrast images of the WTDF 3 and HCLE cells with their tracks: control (not treated) and plasma (CAP treated, WTDF 3 ∼ 60 s, HCLE ∼00 s). (B, C) The data are shown for ∼16.5 h of tracking. Error bars indicate the standard error of mean for the presented data. At least ∼80 cells were analyzed per time point. The migration rates and the persistence of the cells are assessed as a function of the length of plasma treatment time: fibroblast cells are shown in blue and epithelial cells are shown in red.

shown as a function of the duration of CAP treatment. Fibroblasts show a maximum drop in the migration rate at about 40 s; increasing treatment times ranging from 40 to 200 s do not alter cell migration rates. The impact of CAP on the migration of epithelial cells is more graded; migration rates of epithelial cells decrease with increasing treatment times from 10 to 100 s; treatment times between 100 and 200 s do not alter epithelial cell migration rates. The decrease in cell migration rates for both cell types is about 30–40%. It was shown earlier that

helium treatment alone does not affect the cell migration without additional conditions such as serum starvation or $MnCl_2$ activation [10, 11], so only untreated cells were used as the controls. Figure 2(C) shows the persistence of cell motion as a function of the duration of the plasma treatment. The persistence was measured as the ratio between net and total displacements and thus represents the directionality of cell motion. Neither fibroblasts nor corneal epithelial cells show significant changes in persistence as a function of increasing plasma treatment time.

### *3.2. Activating fibroblast integrins reduces their response to CAP*

One of the possible reasons for the decrease in cell migration rate after CAP treatment is the activation of integrins on cell surfaces. Integrins can be activated by adding $MnCl_2$ to their media [1, 16]. This treatment shifts the integrins from a folded state to an unfolded state exposing their ligand-binding sites. Serum starvation of fibroblasts increases their spreading, which induces activation of $\alpha 5\beta 1$ integrin [17]. Figure 3(A) shows the migration rates of fibroblasts: controls (not treated), only helium treated for 100 s and CAP treated (plasma treated for 100 s) followed by incubation in standard medium, medium supplemented with $MnCl_2$ and low-serum medium. In the case of no additional treatment, we found a 30–40% drop in the migration rates of the cells as expected. However, under conditions where integrins were activated, no change in migration rate was observed after CAP treatment. The decrease of cell migration rates for all three conditions is 30–40% of the optimal cell migration rate. These data suggest that CAP treatment activates integrins on cell surfaces.

Plating cells onto surfaces coated with integrin ligands activates integrins engaged by those ligands. $\beta 1$ family integrins bind to FN and CN, whereas $\alpha$v-family integrins bind to VN. Next we assessed whether plating cells onto FN/CNI- or VN-coated surfaces impacts on their ability to respond to CAP. In figure 3(B), cell migration rates and persistence of migration for fibroblasts plated on un-coated surfaces and surfaces pre-coated with FN/CNI or VN are shown for untreated and for CAP (100 s)-treated cells. Data are shown for only the first 6 h of cell tracking, i.e. right after cells adhered to the surface. There are no significant differences in cell migration rates between cells plated onto uncoated compared with FN/CNI- and VN-coated surfaces for the control cells ($p$-value $> 0.05$). In addition, pre-coating surfaces with FN/CNI or VN did not alter their response to plasma treatment and a drop of about 30% in cell migration rate was seen for the three conditions studied. Statistical analyses of these data showed that there greater statistical significance ($p$-value $< 0.001$) for fibroblasts plated on the un-coated surface rather than on the surface pre-coated with FN/CNI or VN ($p$-value $< 0.01$). No significant changes were found in the persistence of the cells' motion. Note also that helium alone does not effect the cell migration, which was shown earlier [10, 11].

### *3.3. $\beta 1$ integrins are activated by CAP*

We have shown so far that integrin activation by $MnCl_2$ and starvation attenuates the response of cells to CAP but adhering cells to specific integrin ligands had no impact on cells' ability to respond to CAP. To look directly at the impact of CAP on integrin activation, control cells and cells treated with CAP were fixed and used for immunofluorescence studies without permeabilization to assess the ratio of activated to total $\beta 1$ integrin on their surface. The method requires the use of an antibody that recognizes total $\beta 1$ integrin regardless of conformation (Ha2/5) and another that recognizes only the active conformation of the $\beta 1$

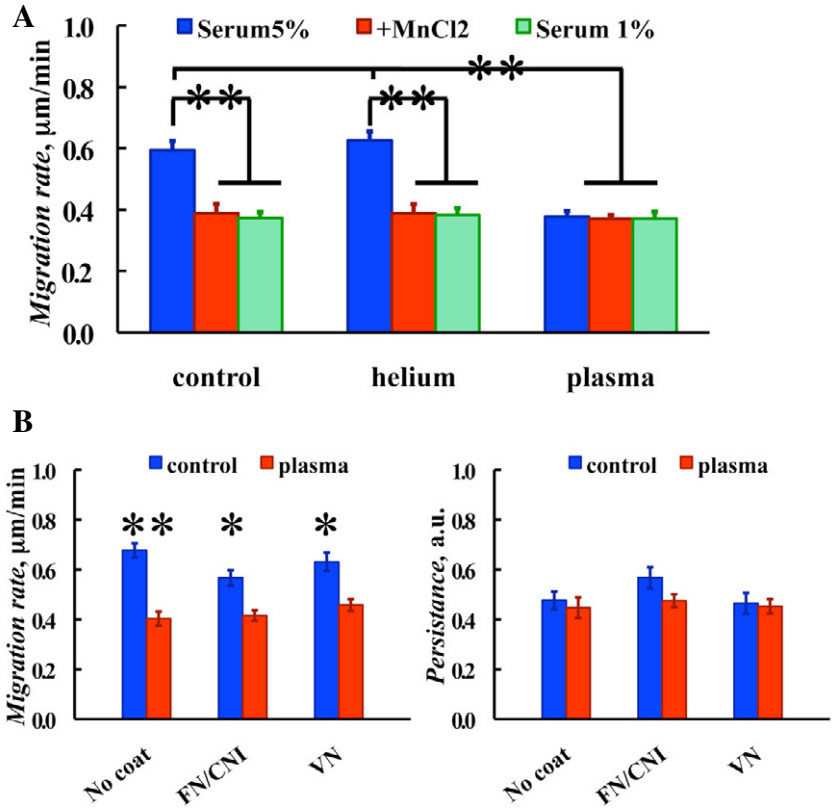


**Figure 3.** Integrin activation of the fibroblasts reduces their response to CAP. (A) The migration rates of the fibroblast cell controls (no CAP treatment and treated with helium only) and cells treated with plasma for ∼100 s are shown under different conditions: blue—no additional pre-treatments; red—treated with $MnCl_2$; and green—serum-starved cells. Data are shown for ∼16.5 h of tracking. (B) The migration rates ($\mu$m min$^{-1}$) and persistence of the cells motion (arbitrary units) of the WTDF 3 cells control (blue)—no plasma treatment and cells treated with plasma for ∼100 s (red) plated on different pre-coated surfaces: no pre-coating, pre-coated with FN/CN type I and with VN. The data are shown for the first 6 h of the cell tracking. The standard error of mean is shown with error bars. At least ∼80 cells were analyzed per conditional change. Double asterisk stands for $p$-value $< 0.001$ and a single asterisk for $p$-value $< 0.01$; otherwise there were no statistical significant differences in the data.

integrin (9EG7). Immunofluorescent images are acquired and subjected to quantitative image analysis as described in section 2. Figure 4 shows the results of immunofluorescence studies to assess the activation state of the $\beta 1$ integrin in the CAP-treated fibroblasts. Untreated cells (control), cells treated with $MnCl_2$ only, CAP (100 s)-treated cells and cells treated with plasma (100 s) and $MnCl_2$ are shown in figure 4(A); figure 4(B) shows an enlargement of the image shown in figure 4(A). Data show that activated $\beta 1$ integrin is present at significantly higher amounts in cells treated with $MnCl_2$, CAP and both CAP and $MnCl_2$, confirming that $\beta 1$ integrin is activated by CAP treatment (see figure 4(C)).

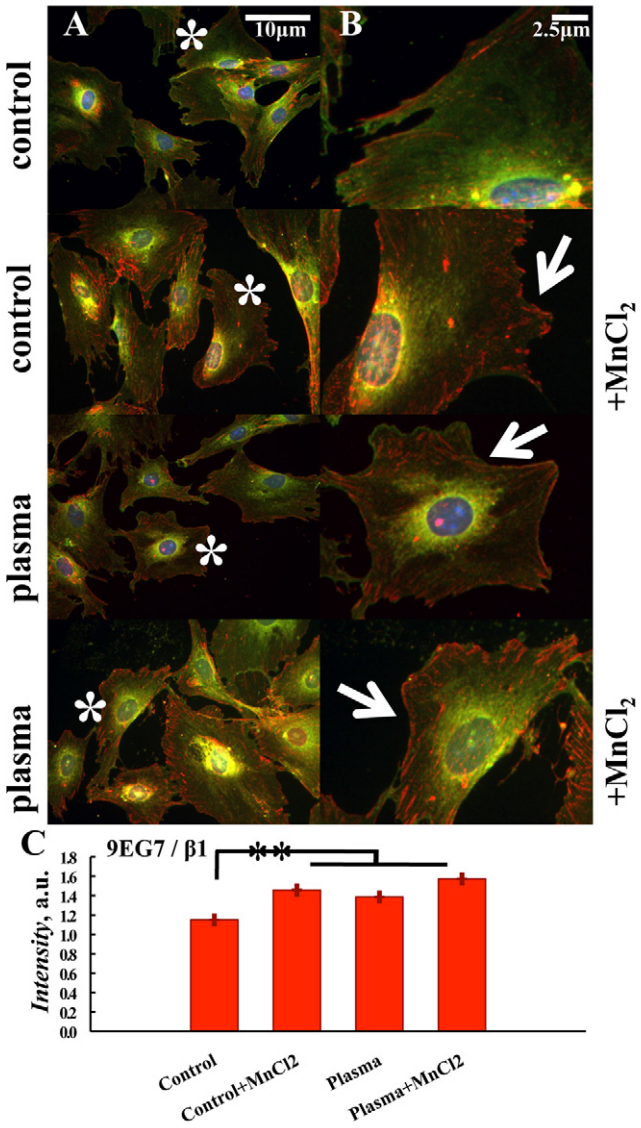


**Figure 4.** Activation of the $\beta 1$ integrin with CAP. The immunofluorescence images of the fibroblast cells with different scales are shown: control (no treatments), control + $MnCl_2$ (treated with only $MnCl_2$), plasma (treated with CAP 100 s) and plasma + $MnCl_2$ (treated with both plasma 100 s and $MnCl_2$). Total $\beta 1$ integrins are shown in green, activated $\beta 1$ (clone 9EG7) in red and nuclei in blue. (A) Images were taken with the magnification 20×. Cells enlarged in figure 3(B) are marked with a white asterisk. (B) Enlarged typical cells are shown. White arrows indicate the increase of the activated $\beta 1$ integrin. (C) The data represent the ratio of 9EG7 and total $\beta 1$ integrin for the peripheral part of the cells; error bars stand for the standard error of mean, ∼20 cells were analyzed per conditional change. The double asterisk shows the statistical significance ($p$-value $< 0.001$) in the increase of the intensity of the 9EG7 over the total $\beta 1$ in the cells treated with $MnCl_2$, plasma or both.

### *3.4. CAP-treated cells have larger focal adhesions*

Activated integrins accumulate within focal adhesions, increasing their size and reducing cell migration rates. To determine whether focal adhesions of CAP-treated cells were indeed larger, control cells and cells treated with CAP were fixed, permeabilized and used for immunofluorescence to detect the presence of vinculin as well as $\alpha$v integrin within focal adhesions; the data are presented in figure 5(A) and quantified in figure 5(B). Vinculin is a focal adhesion protein [1, 3] and the amount of protein engaged in the focal adhesion is proportional to the size of the focal adhesion [18]; thus the intensity of the fluorescence antibody will be proportional to the size of the focal adhesion. CAP-treated cells, cells treated with $MnCl_2$ only or cells treated with both CAP and $MnCl_2$ show an overall increase in vinculin intensity and thus have larger focal adhesions [18], whereas a statistically significant ($p$-value$< 0.001$) increase of $\sim$20% is seen for vinculin intensity at the periphery of cells treated with $MnCl_2$, CAP or both. However, no significant changes were found in the expression of the total $\alpha$v integrin $\sim$3 h after treatments.

## 4. Discussion

The results shown here demonstrate that CAP activates $\beta$1 integrin, decreases cell migration and increases focal adhesion size. The ability of CAP to alter cell migration rates is not specific to fibroblasts but extends to human corneal epithelial cells; both types of cell reduce their cell migration rates by at most 30–40% after CAP treatment. The extent of reduction in cell migration rate after CAP in fibroblasts and epithelial cells was identical to the reduction in cell migration achieved by activating integrins using $MnCl_2$ or by serum-starving the cells.

In both cell types, CAP decreases cell migration as a function of the length of plasma treatment time up to a threshold above which no further change in cell migration is seen. That threshold is reached at 40 s for fibroblasts and at 100 s for epithelial cells. The difference in threshold between cell types suggests that epithelial cell adhesion is mediated by integrins that are more adhesive and more difficult to activate. Epithelial cells but not fibroblasts express $\alpha 6\beta 4$ integrin, which acts like a cellular glue to mediate the tight adhesions needed between the epidermis and underlying dermis [12]. It is likely that the increased adhesion seen in epithelial cells underlies the higher threshold seen in the length of CAP treatment required to reduce epithelial cell migration by 30–40%. It is important to note that CAP treatment of about 40 s leads to discrimination between fibroblast and epithelial cells, thus allowing differential treatment of various cells present in tissue.

$MnCl_2$ treatment is known to activate integrins and to reduce cell migration rates; here we show that $MnCl_2$ alone reduces cell migration rates to the same extent as plasma treatment. The fact that the migration rates of plasma-treated cells and those of plasma-treated cells subsequently treated with 0.5 mM $MnCl_2$ are similar links plasma treatment to integrin activation. If plasma treatment did not activate integrins, incubating cells with $MnCl_2$ would further reduce cell migration rates. One caveat to that interpretation of the data is that there may be a lower threshold to cell migration rates below which cell death occurs.

Serum starvation induces cell flattening and activates integrins via mechanotransduction of stress forces to the cells' focal adhesions. The fact that plasma treatment does not alter the migration rates of serum-starved cells can be interpreted in numerous ways. It is possible that the reduction in cell migration after plasma treatment requires nutrients lacking in medium with

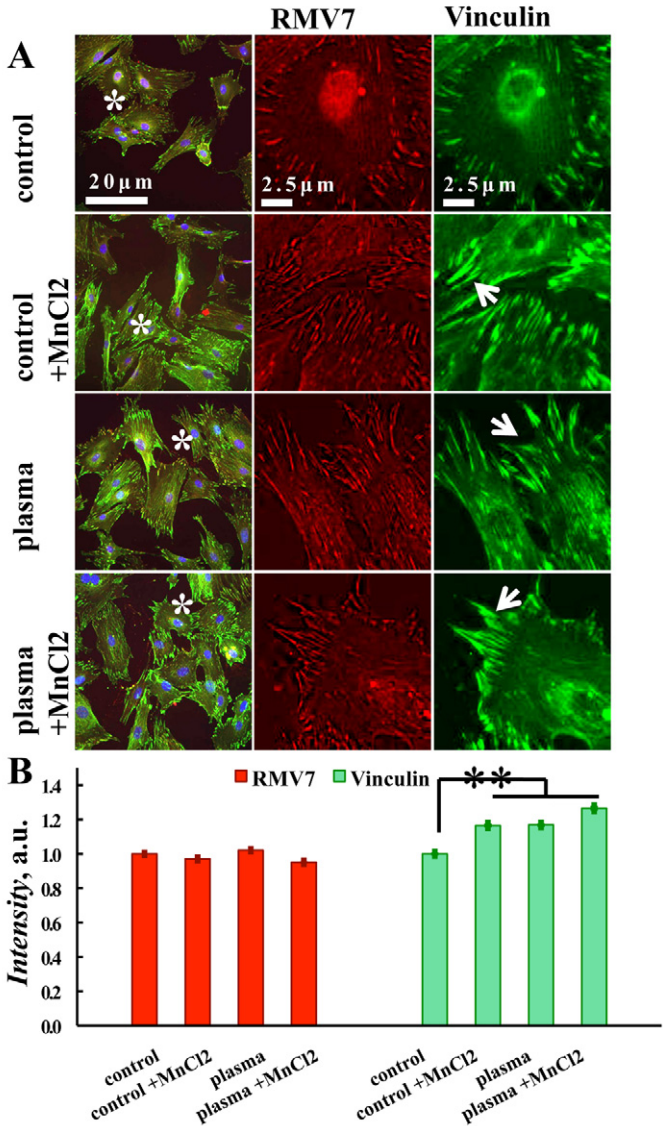


**Figure 5.** CAP treatment induces enlargement of the focal adhesions. (A) The immunofluorescence images of WTDF 3 cells (control—no treatments; control + $MnCl_2$—treated with $MnCl_2$ only; plasma—treated with plasma 100 s only; and plasma + $MnCl_2$—treated with both plasma 100 s and $MnCl_2$) are shown. $\alpha$v (RMV 7) integrins are shown in red, focal adhesion protein vinculin in green and nucleiin blue. The first column shows the images taken with the magnification 20×, the white asterisk indicates the cells enlarged in the second and third columns: the second one shows only $\alpha$v (red) and the third one—only vinculin (green). The change in size of the focal adhesions is shown with white arrows. (B) Statistical data of the pixel intensities (arbitrary units) of the RMV 7 and vinculin (normalized to the controls) are shown. The statistically significant increase (∼20%) in the vinculin intensity is shown with the double asterisk ($p$-value $<0.001$). The error bars indicate the error of the mean for the presented data.

1% serum; this is unlikely since rapid cell migration demands energy and nutrients. It is also possible that cell migration in 1% medium is reduced to a level that cannot be reduced further without loss of cell viability. Although cell migration rates did not change after CAP treatment of serum-starved cells, it is remarkable that the most observed starved cells did not show an increase in cell death in response to CAP treatment (the analyzing software gives information about the slopes of the velocity change and by the positive slopes we can confirm that ∼90% of the observed cells were alive; data not shown).

Integrins are expressed on cell surfaces as $\alpha\beta$ heterodimers. There are two different classes of integrins on fibroblasts: those that adhere primarily to FN and CNs and are primarily $\beta1$-containing integrins ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha7\beta1$, $\alpha8\beta1$, $\alpha9\beta1$ and $\alpha11\beta1$) and those that adhere to VN, which include the $\alpha$v-containing integrins ($\alpha v\beta3$ and $\alpha v\beta5$) [19]. Epithelial cells are more complex, having an additional integrin heterodimer ($\alpha6\beta4$) to mediate attachment to laminin [12, 19].

Tissue culture plastic is charged. When cells are placed in dishes with medium containing 5% serum, low concentrations of glycoproteins with charged sugar moieties on their surface that are present in the serum stick to the uncoated tissue culture plastic. FN and VN, two integrin ligands, are among the proteins present in serum that stick to tissue culture plastic. This yields a low concentration of those ligands on uncoated tissue culture plastic and supports cell adhesion and migration by both $\beta1$ and $\alpha$v integrins. When cells adhere to high concentrations of FN–CN type I (FN/CNI), $\beta1$ integrins cluster, which induces their activation; adhering cells to VN clusters and induces activation of $\alpha$v integrins. When cells migrate on FN/CNI- or VN-coated surfaces, they migrate using primarily $\beta1$- or $\alpha$v-integrins, respectively.

The purpose of the replating studies was to determine whether cells using primarily $\beta1$ integrins to migrate respond to plasma treatment differently than cells using primarily $\alpha$v integrins or cells using both $\beta1$ and $\alpha$v integrins on uncoated plastic. If plasma treatment acts by increasing $\beta1$ integrin activity, then plating cells on FN/CNI would reduce or eliminate the impact of plasma treatment on cell migration rate because $\beta1$ integrins would be active prior to plasma treatment; in addition, if plasma treatment acts by increasing $\beta1$ integrin activity, plating cells on VN would enhance the plasma effect. Putting cells on VN reduces the cells' surface ligated and activated $\beta1$ integrin compared with cells on uncoated plastic. There would be less active $\beta1$ integrin present on cells on VN than on cells on uncoated plastic and therefore there would be an increase in the ability of plasma to alter cell migration if plasma operates by increasing $\beta1$ integrin activation alone. As shown in figure 3, there is a reduction in the ability of plasma to alter cell migration when cells are plated on FN/CNI compared to uncoated plastic and VN-coating dishes. These results show that plasma treatment operates primarily through $\beta1$ integrins but that $\alpha$v integrins are also involved because plating cells on VN did not increase the ability of plasma treatment to reduce cell migration.

The data obtained for $\beta1$ integrin activation using immunofluorescence (see figure 4) confirms that $MnCl_2$ treatment, as expected, activates $\beta1$ integrin but the data were only statistically significant for $\beta1$ integrin at the cell periphery where the ratio of active to total integrin increased from 1.1 to 1.4; we did not see an increase in $\beta1$ integrin activation at the cell center. The failure to demonstrate $\beta1$ integrin activation at the center of cells after $MnCl_2$ likely reflects the limitations of 9EG7, which binds to its activation-state epitope more readily when the integrin is both ligand bound and active [20]. Because we did not see integrin activation at the cell center of control cells treated with $MnCl_2$, we focused our assessment on whether $\beta1$ integrin was activated by plasma treatment on changes seen at the cell periphery. The data

presented in figure 4 show that without plasma treatment the ratio of active to inactive is 1.1, whereas after plasma treatment it is 1.38, a value that is statistically significant; treating cells with plasma followed by $MnCl_2$ increases the ratio of active to inactive $\beta 1$ integrin to 1.5. It is also worth noting that there is a statistically significant difference in the presence of the activated $\beta 1$ integrin between cells treated with only plasma and only $MnCl_2$ and between only plasma and $MnCl_2$ and both. This interesting observation can be the subject of future study, since it is revealing about the different mechanisms involved in integrin activation. The data describing the cells treated by both plasma and $MnCl_2$ suggest that most likely these two processes run independently of each other and show a cumulative effect.

Integrin activation is one of the conditions required for the formation of focal adhesions [21, 22]. The activation of integrins leads to an increase in the size of focal adhesions. The data presented in figures 5(A) and (B) show that plasma-treated cells and cells treated with $MnCl_2$ both show an increase in the size of the vinculin-positive focal adhesions measured in pixel intensities at the cell periphery of $\sim$20%. Treating cells with plasma followed by $MnCl_2$ further increased the size of the focal adhesions, but the increase was small and not mathematically significant. Ligated $\alpha$v integrin also localizes to focal adhesions. After plasma treatment, focal adhesions increase in size, and activated $\beta 1$ integrin accumulates within these larger focal adhesions and yet $\alpha$v integrin does not increase within focal adhesions. If the ratio of $\alpha$v integrin to vinculin is determined, it is clear that plasma and $MnCl_2$ treatments both reduce the localization of $\alpha$v integrin within focal adhesions. Taken together with the cell migration rate data presented in figure 3(B) and the $\beta 1$ integrin activation data in figure 4, these data show that plasma treatment increases $\beta 1$ integrin activation, leading to increased accumulation of $\beta 1$ integrin and reduced accumulation of $\alpha$v integrin within focal adhesions. These events increase focal adhesion size. The increased size of focal adhesions resists disassembly during cell migration and impedes cell migration rates. Interestingly, the migration rate reduction we see by plasma and $MnCl_2$ is about $\sim$30–40%; the low migration rate value seen after these treatments may represent some universal lower threshold for cell migration rate. Besides, the presented data suggest that the decrease in cell migrations induced by plasma should be quite transient ($\sim$2–3 h) compared to the total surface integrin change reported earlier ($\sim$24 h) [10].

Let us discuss a possible physical mechanism leading to integrin activation and thus decrease in cell motility. We propose a mechanism based on the thermodynamic model of mechanotransduction developed by Shemesh *et al* [23]. This model is based on the thermodynamic argument that the pulling force leads to self-assembly of molecules into an aggregate, increasing the number of focal adhesions in order to decrease the stresses induced on the cell membrane.

This paper considers cold plasma jet interaction with the cell layer. Several mechanisms such as reactive chemical species, charged species or charging can possibly effect cell migration [5–7, 24]. In the following model, we have demonstrated that cell membrane *charge change* can affect the formation of the focal adhesions. The change in charge that is caused by the plasma at the cell might lead to conformational changes in the extracellular domain of the integrin ligands [25] and therefore integrin activation. While our model is based on the membrane charge change, there are several effects that CAP can lead to [7]. Consider the direct charge transfer from the plasma to the cell. Recall that while cells are covered by media, plasma jet action leads to the removal of the liquid above the treated cells. However, there are other mechanisms related to intracellular activity that are triggered by the CAP [7].

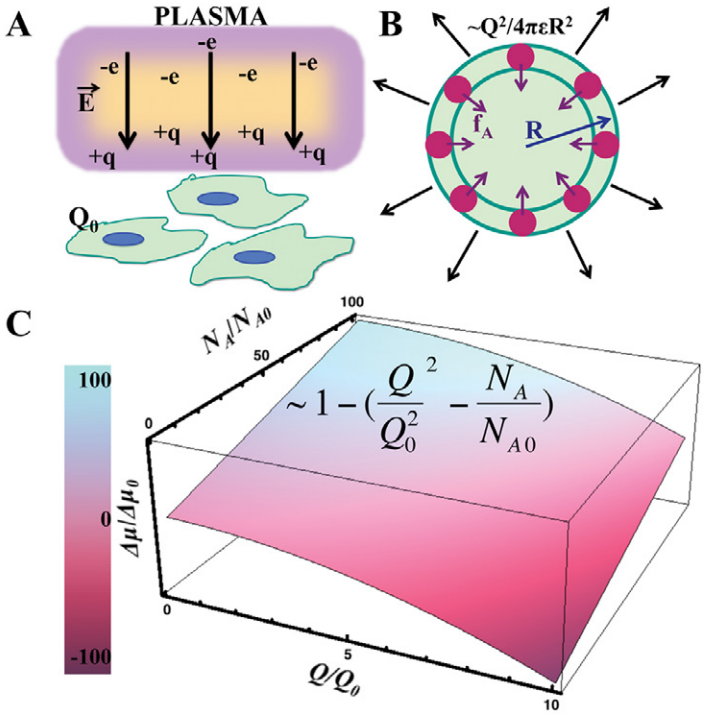


**Figure 6.** (A) Schematic representation of the cold plasma interaction with a cell layer is shown. (B) Spherical approximation of the cell with induced charge $Q$ is shown: the resulting resistance forces $f_{\mathrm{A}}$ arise in the points of the formation of the focal adhesions (red circles). (C) The qualitative plot of the chemical potential $(\Delta\mu/\Delta\mu_0)$ as a function of the charge change at the cell $(Q/Q_0)$ and the change of the number of focal adhesions $(N_{\mathrm{A}}/N_{\mathrm{A0}})$ is shown. One can see that as charge increases the chemical potential becomes negative.

Charge that is changed at the cell can be calculated based on the floating potential argument [26] $Q \sim 10^4 \cdot e$ (for Debye length $L_{\mathrm{D}} \sim 10^{-4}$ m, cell radius $\sim 10^{-5}$ m and $e = 1.16 \times 10^{-19}$ C). The charge change will lead to the creation of repulsive forces and thus the elastic stress will be formed along the cell membrane. According to the thermodynamic argument the aggregation (i.e. focal adhesion centers formation) occurs if the chemical potential in the aggregate is smaller than without focal adhesions. One can estimate the force due to the formed focal adhesions as $N_{\mathrm{A}} f_{\mathrm{A}}$, where $N_{\mathrm{A}}$ is the number of focal adhesions and $f_{\mathrm{A}}$ is the resisting force of the anchor. Thus, the change in the thermodynamic potential can be expressed [23] as

$$\Delta\mu(Q, N_{\mathrm{A}}) = \Delta\mu_0 - \left(\frac{Q^2}{4\pi\varepsilon R^2} - f_{\mathrm{A}} N_{\mathrm{A}}\right),$$

where $\Delta\mu(Q, N_{\mathrm{A}})$ is the change in the chemical potential and $\Delta\mu_0$ is the change in the chemical potential in the absence of induced charge. It can be seen that an increase in the number of focal adhesions leads to a decrease in the chemical potential. Calculations based on this model are presented in figure 6. One can see that according to this model induction of the extra charge at the cell surface will lead to the formation of new focal adhesions (see figure 6). This can explain the decrease of cell motility after plasma treatment that is observed experimentally.

## 5. Conclusions

In this paper, we show that CAP treatment decreases fibroblast cell migration by increasing integrin activation. While we see an activation of $\beta 1$ integrin by CAP in fibroblasts, both fibroblasts and epithelial cells reduce their migration rate by ~30–40%. However, it takes longer plasma treatment times for epithelial cells to achieve maximum reduction of migration rate; thus differential treatment is possible. No significant changes in the persistence of the cell motion were found. A thermodynamic model is presented that predicts that CAP treatment leads to an increase in focal adhesion size, leading to a decrease in the cell motility in accordance with the experimental results obtained.

## References

[1] Hynes R O 2002 Integrins: bidirectional, allosteric signaling machines *Cell* **110** 673–87
[2] LaFlamme S E, Nieves B, Colello D and Reverte C G 2008 Integrins as regulators of the mitotic machinery *Curr. Opin. Cell Biol.* **20** 576–82
[3] Harburger D S and Calderwood D A 2009 Integrin signalling at a glance *J. Cell Sci.* **122** 159–63
[4] Shashurin A, Keidar M, Bronnikov S, Jurjus R A and Stepp M A 2008 Living tissue under treatment of cold plasma atmospheric jet *Appl. Phys. Lett.* **93** 181501–3
[5] Haertel B, Wende K, von Woedtke T, Weltmann K-D and Lindequist U 2011 Non-thermal atmospheric-pressure plasma can influence cell adhesion molecules on HaCaT-keratinocytes *Exp. Dermatol.* **20** 278–96
[6] Keidar M, Walk R, Shashurin P, Srinivasan P, Sandler A, Dasgupta S, Ravi R, Guerrero-Preston R and Trink B 2011 Cold plasma selectivity and the possibility of a paradigm shift in cancer therapy *Br. J. Cancer* **105** 1295–301
[7] Stoffels E, Sakiyama Y and Graves D B 2008 Cold atmospheric plasma: charged species and their interactions with cells and tissues *IEEE Trans Plasma Sci.* **36** 1441–57
[8] Vandamme M *et al* 2012 ROS implication in a new antitumor strategy based on non-thermal plasma *Int. J. Cancer* **130** 2185–94
[9] Morfill G E, Shimizu T, Steffes B and Schmidt H-U 2009 Nosocomial infections—a new approach towards preventive medicine using plasmas *New J. Phys.* **11** 115019
[10] Shashurin A, Stepp M A, Hawley T S, Pal-Ghosh S, Brieda L, Bronnikov S, Jurjus R A and Keidar M 2010 Influence of cold plasma atmospheric jet on surface integrin expression of living cells *Plasma Process Polym.* **7** 294–300
[11] Volotskova O, Shashurin A, Stepp M A, Pal-Ghosh S and Keidar M 2011 Plasma-controlled cell migration: localization of cold plasma–cell interaction region *Plasma Med.* **1** 85–92
[12] Clark R A F (ed) 1996 *The Molecular and Cellular Biology of Wound Repair* 2nd edn (New York: Plenum) pp 311–38
[13] Jurjus R A, Liu Y, Pal-Ghosh S, Tadvalkar G and Stepp M A 2008 Primary dermal fibroblasts derived from sdc-1 deficient mice migrate faster and have altered $\alpha$v integrin function *Wound Rep. Reg.* **16** 649–60
[14] Gipson I K, Spurr-Michaud S, Argueso P, Tisdale A, Fong Ng T and Leigh Russo C 2003 Mucin gene expression in immortalized human corneal–limbal and conjunctival epithelial cell lines *Invest. Ophthalmol. Vis. Sci.* **44** 2496–506
[15] Shashurin A, Shneider M N, Dogariu A, Miles R B and Keidar M 2010 Temporary-resolved measurement of electron density in small atmospheric plasmas *Appl. Phys. Lett.* **96** 171502
[16] Mould A P, Askari J A, Barton S, Kline A D, McEwan P A, Craig S E and Humphries M J 2002 Integrin activation involves a conformational change in the alpha 1 helix of the beta subunit -domain *J. Biol. Chem.* **277** 19800–05

[17] De Toni-Costes F, Despeaux M, Bertrand J, Bourogaa E, Ysebaert L, Payrastre B and Racaud-Sultan C 2010 A new alpha5beta1 integrin-dependent survival pathway through GSK3beta activation in leukemic cells *PLoS One* **23** 5 e9807

[18] Humphries J, Wang P, Streuli C, Geiger B, Humphries M J and Ballestrem C 2007 Vinculin controls focal adhesion formation by direct interactions with talin and actin *The J. Cell Biol.* **179** 1043–57

[19] Stepp M A 2006 Corneal integrins and their functions *Exp. Eye Res.* **83** 3–15

[20] Bazzoni G, Ma L, Blue M L and Hemler M E 1998 Divalent cations and ligands induce conformational changes that are highly divergent among b1 integrins *J. Biol. Chem.* **273** 6670–8

[21] Danen E H J, Van Rheenen J, Franken W, Huveneers S, Sonneveld P, Jalink K and Sonnenberg A 2005 Integrins control motile strategy through a Rho–cofilin pathway *J. Cell Biol.* **169** 515–26

[22] Byron A, Morgan M R and Humphries M J 2010 Adhesion signalling complexes *Curr. Biol.* **20** R1063–7

[23] Shemesh T, Geiger B, Bershadsky A D and Kozlov M M 2005 Focal adhesion as mechanosensors: a physical mechanism *Proc. Natl Acad. Sci. USA* **102** 12383–8

[24] Kong M G, Keidar M and Ostrikov K 2011 Plasmas meet nanoparticles—where synergies can advance the frontier of medicine *J. Phys. D: Appl. Phys.* **44** 174018

[25] Weisel J W, Nagaswami C, Vilaire G and Bennett J S 1992 Examination of the platelet membrane glycoprotein IIb–IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy *J. Biol. Chem.* **267** 16637–43

[26] Keidar M, Beilis I I, Boxman R L and Goldsmith S 1995 Nonstationary macroparticle charging in an arc plasma jet *IEEE Trans Plasma Sci.* **23** 902–8