Fibronectin fibrillogenesis influence on nuclear dynamics in polarized fibroblasts

Author: Joan Triadú Galí
Facultat de Física, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain.
Institute for Bioengineering of Catalonia (IBEC),
The Barcelona Institute for Science and Technology (BIST), 08028 Barcelona, Spain.

Advisors: Dr. Amy E. M. Beedle (IBEC), Prof. Jaume Casademunt Viader (UB)

Abstract: In this project we studied the influence of fibrillar adhesions formation on nuclear dynamics and cell migration in human fibroblasts. We first show that their formation is a migration-dependent process that is not observed when fibroblasts are confined. Then, we present significant evidence that in absence of fibrillar adhesions nuclei align with the long axis of the cell less effectively. Moreover, we present promising data that show that nuclear positioning and cell migration are also altered in these conditions. In conclusion, we establish that fibrillar adhesions formation is a potentially important parameter of nuclear and cellular dynamics in migrating human fibroblasts.

I. INTRODUCTION

Fibroblasts are large, flat, and elongated cells that play a key role in the formation of the fibrous matrix that supports and connects tissues in the body. Therefore, they are crucial for processes such as wound healing [1].

Fibronectin is an example of the proteins that are produced by fibroblasts and constitute this matrix. A remarkable feature of fibronectin is that it can transition from a single molecule form to a fibrillous higher-order structure when self-assembled. This conformational change is highly relevant for cell-matrix interactions [2]. In this sense, fibroblasts not only secrete fibronectin, but they promote the creation of the fibronectin fibrils through a force-dependent process[3][4].

The creation of fibronectin fibrils (fibrillogenesis) starts in a complex of proteins that fibroblasts have at their distal parts that allow them to attach to the substrate, exert forces, and migrate. These structures are called focal adhesions (fig. 1.a), and their main component are integrins, a type of proteins that link the cell to the outer proteins such as fibronectin (fig. 2). There is one particular integrin, $\alpha 5\beta 1$, that is dragged by actin filaments (cell contractile motors) from focal adhesions of the cell periphery towards the centre of the cell. $\alpha 5\beta 1$ integrins, along with other proteins, accumulate below the nucleus forming elongated cell-matrix contacts, the fibrillar adhesions (fig. 1.b and 2).

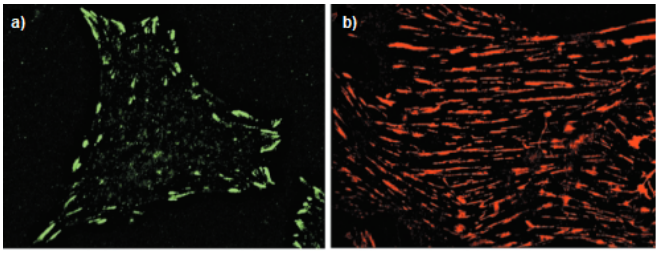

FIG. 1: a) Focal adhesions, located at the cell periphery.b) Fibrillar adhesions, elongated, thread-like structures distributed at the centre of the fibroblast [4].

The key point is that the dynamic tension that leads to the formation of fibrillar adhesions, in parallel, unfolds fibronectin and unmask cryptic self-assembly sites that mediate fibrillogenesis [5][6].

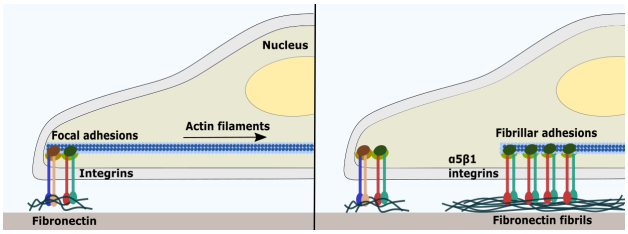

FIG. 2: Fibrillar adhesions formation and fibrillogenesis due to $\alpha 5\beta 1$ slide from the cell periphery to the central region.

Fibrillar adhesions are located beneath the nucleus and are linked to actin filaments. Thus, it is tempting to speculate that their absence can affect nuclear dynamics.

Nuclear dynamics regulation in fibroblasts is a dramatically complex process where many parts of the cell intervene [7]. In particular, it has been already proven that some integrins are coupled to the nucleus through the cytoskeleton [8] and actin complexes beneath the nucleus can alter nuclear positioning[9]. However, there are still some controversial aspects about the regulation of the position and the orientation of the nucleus, and figuring out the exact mechanisms of this process would be of high relevance since nuclear anomalous dynamics can alter cell behaviour and may lead to disease[7][10], inflammation or wound healing defects[11].

Whether fibrillar adhesions formation and fibrillogenesis have a direct effect on nuclear dynamical stability is a question that has not been assessed.

We will investigate it, first, by seeding the fibroblasts into different rectangular patterns. This will allow us to decouple cell migration and nuclear dynamics. Then, we will study the nuclear position, trajectory, and orientation in migrating cells, with and without fibrillar adhesions. Finally, the cell trajectory will be also analysed to evaluate the influence of fibrils on cell migration.

II. METHODOLOGY AND ANALYSIS

Fibronectin-coated glass-bottom Petri dishes were used in all experiments. The cells that were used are Telomerase Immortalized Foreskin Fibroblasts (TIFFs).

A. PRIMO micropattering

The first approach consists in using PRIMO micropatterning, a high-precision photolithography technique that can produce a very particular cell microenvironment. After a strict substrate treatment[12], this tool allows the user to accurately decide which regions are available for the cells to attach.

Two $1800\mu m^2$ rectangular patterns with 1:3 aspect ratio were designed where single cells can fully spread. The first one, for the control condition, is a full rectangular pattern. The second one is a hollow rectangle where cells can fit in and its borders can attach, but the central part of the cell cannot (fig. 3). The aim of the second pattern is to block fibrillar adhesions formation.

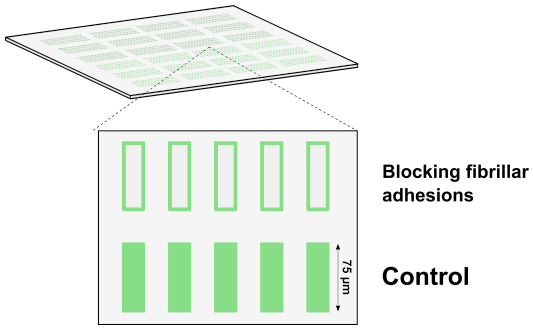

FIG. 3: Sketch of designed micropatterns. The regions in light green are the ones where cells can attach.

Images of the cells within the patterns were taken with the cells fixed and with the DNA and fibrillar adhesions stained. An optical inverted microscope (Nikon Eclipse Ti) with 60X objective was used.

B. 1D migration

To further assess nuclear dynamics with or without fibrillar adhesions, nuclear tracking on one-dimensional cell migration was performed. Fibrillar adhesions formation and fibrillogenesis were blocked using PUR4/FUD peptide, and for the control group, a scrambled version of the latter was applied. To force cells to migrate in straight lines, we used the micro-contact printing technique.

Similarly to PRIMO, this technique allows to pattern the substrate in which the cells will be seeded, but it uses pre-fabricated stamps to print to the Petri dishes, instead of laser light. Cells were seeded onto fluorescent fibronectin-coated 20 μm wide lines.

Images were taken with an inverted optical microscope (Nikon Eclipse Ti) using 20X objective in brightfield and DNA fluorescence, every 15 minutes for 10 hours.

C. 2D migration

Cell tracking in 2D migrating fibroblasts was performed to study the nucleus behaviour within the cell with a higher resemblance to physiological conditions. Fibrillar adhesions blocking and image obtaining processes were identical to 1D migration experiments.

D. Images and data analysis

All the images were analysed with ImageJ software[13]. For the migration analysis, images of the nucleus were analysed with custom-written Macros for ImageJ, and for the segmentation of the brightfield images the *Canny Edge Detector*[14] plug-in was used.

To compute the angle of the cell and the nucleus, their contour was fitted into an ellipse[15] (fig. 4.a). The angle between the major axis and the horizontal was taken as the cellular and nuclear orientation (θ_{cell} and θ_{nuc} respectively). In general, the nucleus is an ellipsoid, in contrast, the cell on some occasions can exert lateral protuberances which can distort the fitting and bias the approximation (fig. 4.b). In addition, some evidence shows that the nucleus only aligns with the long axis of the cell when the fibroblast is migrating[16], and not polarized cells do not migrate effectively.

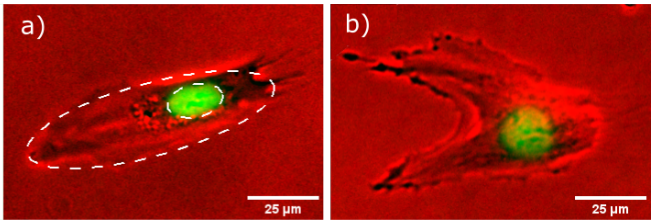

FIG. 4: a) Polarized fibroblast with nuclear and cellular ellipse fitting. b) Not polarized fibroblast.

To overcome these issues, a reasonable threshold was established to determine the conditions in which we should perform our analysis. Only migrating cells with more than 50% of the time-points with at least 1:3 aspect ratio were analysed. This way we have an objective threshold to apply in both conditions that ensured that we would have polarized and migrating cells.

We calculated the angular difference between nuclear and cellular orientation as:

$$|\Delta\theta| = |\theta_{nuc} - \theta_{cell}| \tag{1}$$

To evaluate the positioning of the nucleus within the cell, the centroid of the cell and the nucleus were obtained ($\vec{r}_{cell}$ and $\vec{r}_{nuc}$) for each time-point by averaging

the coordinates of their contour. Then, we calculated the relative position between the two (eq. 2).

$$\vec{r}_{rel} = \vec{r}_{nuc} - \vec{r}_{cell} \tag{2}$$

However, cells are not always aligned with the axis of the image, and it is vital to have a common frame of reference for all cells. To correct this, we rotated the $\vec{r}_{rel}$ with θ_{cell}. Still, there is ambiguity since $\theta_{cell} \in [0, 180)$ degrees, where 0 corresponds to the horizontal of the image. To solve this, the central derivative of the y-coordinate of cell trajectory was taken to determine the direction of migration (eq. 3).

$$f'(y) = \frac{f(y + \Delta t) - f(y - \Delta t)}{2\Delta t} \tag{3}$$

For this purpose only the sign matters to break the ambiguity that θ_{cell} generates, the absolute value is not relevant.

With the nuclear coordinates within the cell, the mean-squared displacement (MSD) for each time-point n was computed as:

$$MSD(n) = \frac{1}{N-n+1} \sum_{i=0}^{N-n} [(x_{(i+n)} - x_i)^2 + (y_{(i+n)} - y_i)^2] \tag{4}$$

In this context, MSD is a good measure of the surface area explored by the nucleus over time. There are different ways to calculate it, we chose this one because it is more precise when data are limiting [17] and it is widely used in experimental biophysics papers [11][18][19].

Nevertheless, for $n > 1$ there is overlapping between the displacements and successive displacements are usually not independent, which can be problematic. In addition, for high n values there is a decrease in the number of coefficients available for averaging, therefore, the MSD only was calculated for the first half of the time-points.

Finally, the cellular trajectories were studied using $\vec{r}_{cell}$ and also the mean-squared displacement of eq. 4.

III. RESULTS

A. Fibroblasts inside rectangular patterns

The objective of these experiments was to obtain a pattern with fibrillar adhesions and another one without them. This way, we would be able to compare the two different conditions and see the effect of fibrillar adhesions on nuclear dynamics.

However, fibrillar adhesions were not seen in any pattern, while in wild-type fibroblasts they are remarkably noticeable (fig. 5).

As we found that migration is required for fibrillar adhesions formation, we did the rest of our experiments on migrating cells.

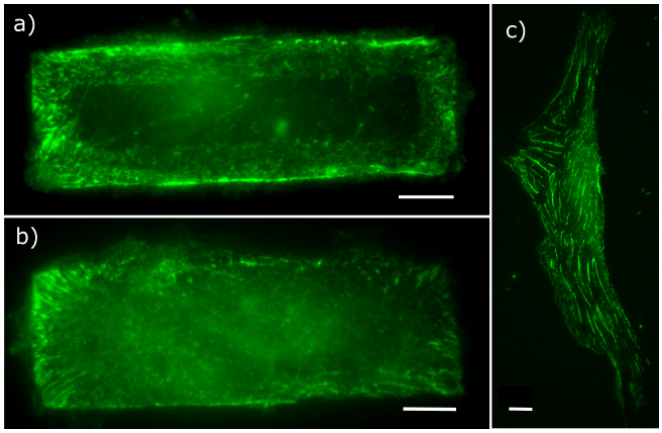

FIG. 5: Fluorescence marks fibrillar adhesions. Bar is $10\mu m$. a) Hollow rectangular pattern. b) Full rectangular pattern. c) Not confined wild-type fibroblast (Image from A. Beedle).

B. Nuclear orientation during 1D & 2D migration

To understand whether the absence of fibrillar adhesions affect nuclear orientation, we calculated the angle between the long axis of the cell and the long axis of the nucleus in migrating fibroblasts, during 10 hours.

The results reveal that the cells and the nuclei are less aligned in the absence of fibrillar adhesions, or what is the same, the angle between the major axis of the cell and the major axis of the nucleus is significantly greater in the PUR4 group (fig. 6).

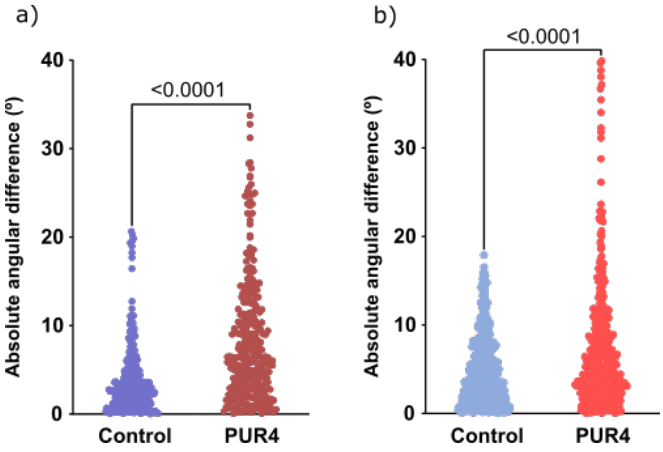

FIG. 6: Absolute angular difference between the major axis of the cell and the major axis of the nucleus. a) 1D migration. $n = 7, 8$ cells from control group and PUR4 respectively. Results from 1 experiment. $N = 288,315$ time-points. b) 2D migration. n=15, 14 cells from 2 independent experiments. $N = 472,426$ time-points. Significance bars obtained with the Mann-Whitney test.

C. Nuclear positioning in 2D migration

Then, to see the effect of fibrillar adhesions on nuclear positioning we computed the average positions of the nuclei within each cell. The results show that the rearward positioning is achieved in both conditions (fig. 7). This was an expected result since it has been proven that rearward positioning is necessary for fibroblasts to migrate effectively [7]. However, the average positions of the nuclei in the PUR4 group are slightly closer to the cell centroid.

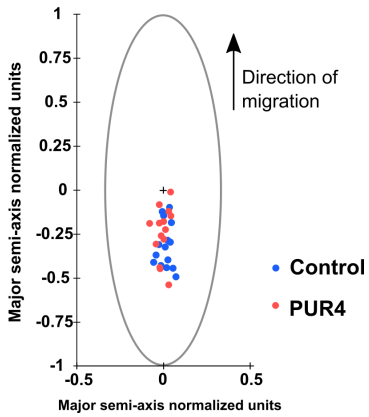

FIG. 7: Average nuclear position of each cell during 2D migration. There is plotted an orientative outline of a cell in grey. Standard deviations are comparable to the size of the dots. $n = 15, 14$ cells from two independent experiments.

D. Nuclear trajectory in 2D migration

Nuclear trajectory within the frame of reference of each cell was qualitatively analysed to explore if in the absence of fibrils the nucleus has a more unstable location.

However, the trajectory of each nucleus did not start at the same point inside the cell, so we have plotted the trajectory of each nucleus relative to its initial position. The results show that the PUR4 nuclei seem to be less confined than the control group ones (fig. 8).

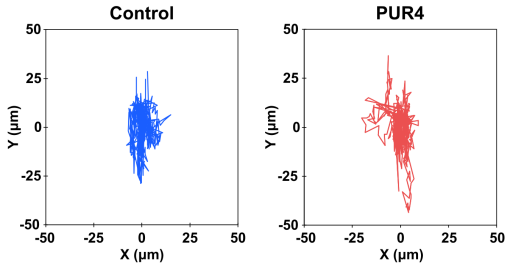

FIG. 8: Nuclear trajectories in the frame of reference of the cell and relative to their initial position, along 10 hours. $n = 13, 12$ cells from two independent experiments.

Nevertheless, the MSD curves over time for these same trajectories, evidence that the surface area explored by the nuclei inside each cell is very similar in both conditions, in most of the cells (fig. 9).

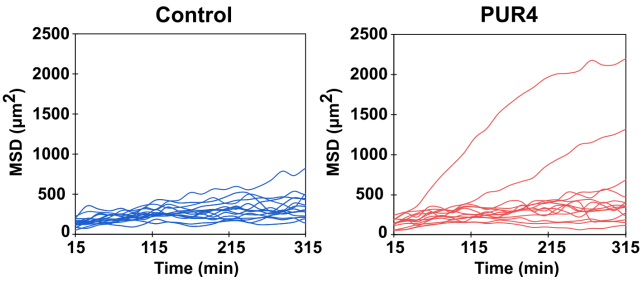

FIG. 9: MSD of the nuclei within the cell along the first 5 hours. $n = 15, 14$ cells from two independent experiments.

E. Cellular trajectory in 2D migration

Finally, the analysis of the trajectory of the cells in 2D migration revealed that the cells exposed to the peptide migrate less effectively than cells with fibrillar adhesions (fig. 10 a). The MSD curves also indicate a qualitative difference between the two groups (fig. 10 b).

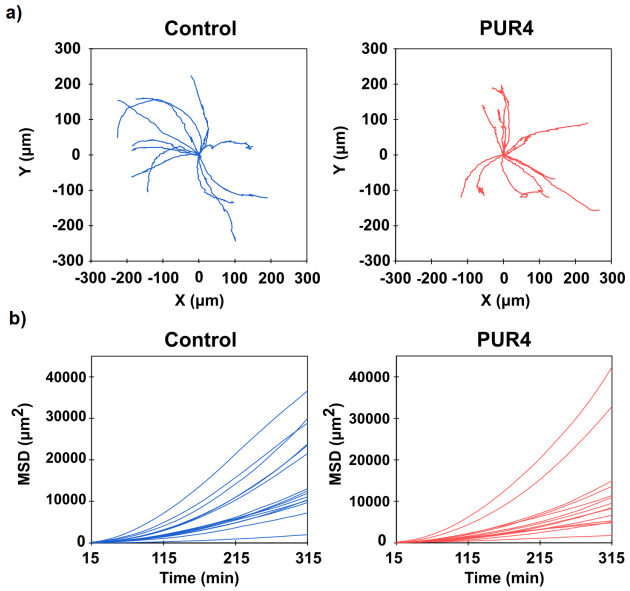

FIG. 10: a) Cellular trajectories relative to their initial position along 10 hours. $n = 13, 12$ cells from two independent experiments. b) MSD of the cells along the first 5 hours. $n = 15, 14$ cells from two independent experiments.

Interestingly, the 2 outliers in the PUR4 group at the MSD plot, are the same cells that have the nucleus located the furthest from the centroid in fig. 7.

IV. DISCUSSION

- Fibrillar adhesions formation and, by extension, fibrillogenesis, are migration-dependent processes that can not be observed in confined fibroblasts.
- Blocking the formation of fibrillar adhesions in migrating fibroblasts can provoke defects on the orientation of the nucleus and deviate the nuclear positioning towards the cell centre. Thus, we conclude that fibrillar adhesions are a potentially relevant element in the regulation of nuclear dynamics in fibroblasts.
- As we have not observed significant differences, more experiments from different approaches should be performed to clarify the effect of fibrillogenesis on the area explored by the nuclei within the cell.
- The fact that the outliers of different figures came from the same cells suggests that the PUR4 peptide was not totally effective in the experiments. Although this peptide is well characterized and widely used [20][21], it has been already reported that fibronectin fibrils can be occasionally observed after its application[22].
- The anomalous positioning of the nucleus and the loss of migrating capacity that most of the PUR4 cells show, could be caused by defects in the forward protuberance that guides the cell while migrating. If this hypothesis was confirmed, it would explain the two anomalous behaviours.
- Time was limited in this project, we would have liked to perform more experiments to have more consistent results.
- Possible future approaches to this question could include direct mechanical stimulation of the nucleus and the study of nuclear dynamics on 3D migrating fibroblasts.

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