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Elasticity, adhesion and tether extrusion on breast cancer cells provide a signature of their invasive potential

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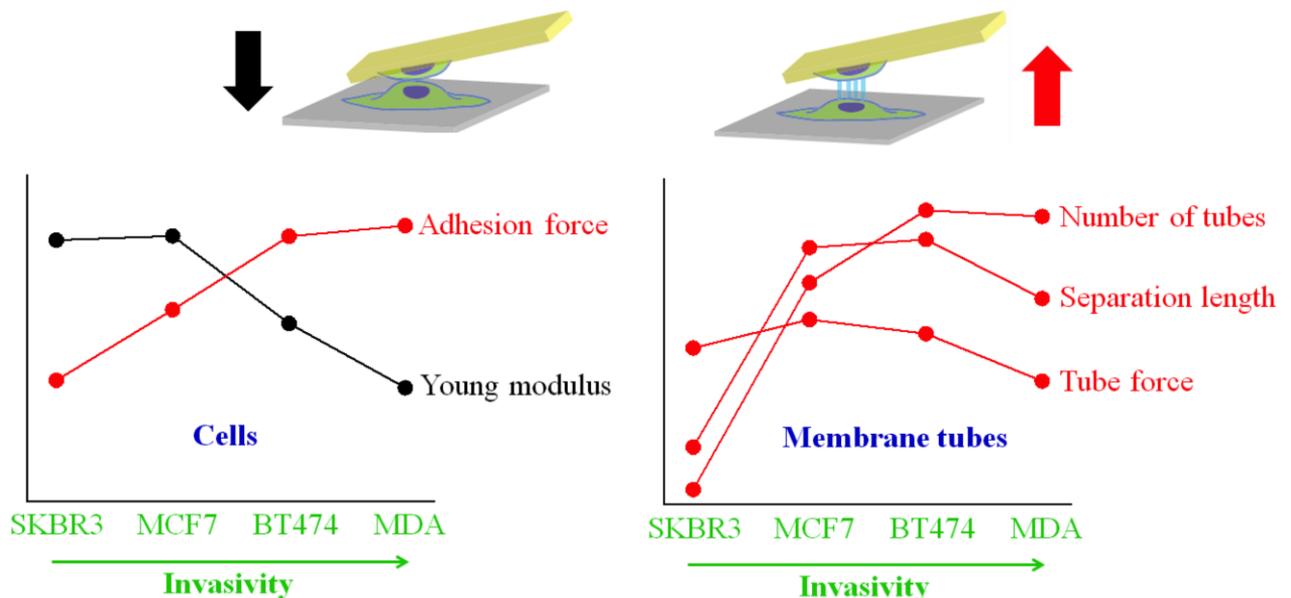
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Keywords: Cancer, AFM, Tethers, invasivity, metastasis, Single Cell Force Spectroscopy, Adhesion, Nanomechanics

Table of contents graphic



Abstract

We use single cell force spectroscopy to compare elasticity, adhesion and tether extrusion on four breast cancer cell lines with an increasing invasive potential. We perform cell attachment/detachment experiments either on fibronectin or on another cell using an Atomic Force Microscope. Our study on the membrane tether formation from cancer cells show that they are easier to extrude from aggressive invasive cells. Measured elastic modulus values confirm that more invasive cells are softer. Moreover, the adhesion force increases with the invasive potential. Our results provide a mechanical signature of breast cancer cells that correlates with their invasivity.

The mechanical properties of living cells affect their fate in normal and pathological situations. The malignant transformation induces perturbations in tissue architecture that finally leads to metastasis formation¹. As metastasis is the main cause of cancer patient death, new biomarkers discovery is a major challenge to develop new diagnostic tools.

Mechanical measurements have gained increasing attention as novel biomarkers in the context of cancer diagnostic and prognostic. A review of the experimental techniques allowing the assessment of the elastic properties of cancer cells is provided by Suresh². Among these techniques, Atomic Force Microscopy (AFM) proves to be suitable to study nano-mechanical properties. In particular, it was demonstrated, both on cell lines³⁻⁶ or on patient cells,⁷⁻⁸ that normal cells were less deformable than cancer cells. The disorganization of the actin cytoskeleton may be responsible for tumors cells softening and their dissemination properties.^{3,5} Furthermore, cell stiffness has been evaluated as a biomarker of invasive potential.^{4,5}

For examining cell adhesion strength on ECM substrate or other cells, different methods have been developed;⁹⁻¹⁰ however AFM single cell force spectroscopy (AFM-SCFS) is the most versatile method that allows to measure adhesive interaction forces of cells with other cells, proteins and surface.⁹ A cell is attached to the AFM cantilever and used to probe a specific substrate (Fig. 1A) or another cell (Fig. 1B). The interaction between the cell and the sample induces a deflection of the cantilever monitored by the deflection of a laser beam. To achieve the separation of interacting cells, large vertical (z) range scanner (at least 50 μm) is necessary to reach complete separation between the cell and the surface. A coupled, inverted optical microscope allows the visualization of the setup (Fig. 1C, D). The cantilever approach-retract cycle provides force curves, from which multiple mechanical parameters are derived (Fig. 1E): the approach part provides the Young modulus, the retraction part gives access to the adhesion force and the parameters of membrane tethers. These tethers are stable cylinders with a radius of a few tenths of nanometers that are formed when the cell membrane is submitted to a point force.¹¹ The force to maintain a membrane tether is $f_0=2\pi(2\kappa(\sigma+W))^{1/2}$,¹² κ is the membrane bending elasticity ($\approx 10 \text{ k}_B\text{T}$) and σ its mechanical tension (from 10^{-6} to 10^{-3} N/m). W accounts for the adhesion energy between the membrane and the underlying cytoskeleton. Therefore, measuring the force to form tethers provides information on the membrane properties and on its interaction with the cytoskeleton¹³. A decrease in membrane-cytoskeleton interaction affects the type of motility used by cancer cells and therefore the measurement of W is a biologically relevant observable in the context

of cancer diagnostic. With the AFM, the study of membrane tether formation has been reported on human cells as MIN6 cells,¹⁴ and brain tumor and endothelial cells¹⁵. To our knowledge, no correlation of the tube force with the invasive potential of breast cancer cells has been reported.

Our goal is to identify new mechanical markers of cancer in cell adhesion assay, using the AFM technique. The only AFM measurements, which compare the interaction forces between cell lines of different invasive potential, relate to a study on breast cancer cell lines (MCF7, T47D, MDA-MB231). The forces of homotypic cell interactions are lower for the most invasive cells that might be due to changes in the expression N and E-cadherin.¹⁶

Our work focuses on four parameters: the Young's modulus, cell interaction forces with one adherent cell or matrix substrate, and tether extrusion. These settings were deduced from cell attachment/detachment of model breast cancer cell lines that represent increasing stages of cancer invasivity. We measure their elasticity when two cells are brought in contact. Then we study the maximal detachment force when cells are detached from a substrate coated with fibronectin (FN), or from another cell of the same type. The most novel aspect of this work is the analysis of the tethers formed during cell detachment experiments. Altogether, the mechanical parameters provide a mechanical signature that can be used to discriminate breast cancer cells lines on the basis of their invasive potential.

Invasive breast cancer cells are softer and more adhesive

We study the elasticity and adhesion properties of breast cancer cell lines with increasing invasive potential. First, we measure the Young modulus of SKBR3, MCF7, BT474 and MDA-MB231 cells by fitting the force curves resulted from cell-cell contact with a Hertz model¹⁷ (Fig.1E, approach curve and blue crosses). Young modulus decreases from 630 ± 310 Pa (mean \pm SD) for SKBR3 cells to 270 ± 90 Pa for MDA-MB231 cells (Fig. 2A). The values are in agreement with those published for MCF7 and MCF10A cells.³ Moreover, we observe a decrease of Young modulus for more invasive mammary cells, also in agreement with previous results¹⁸.

When we measured the maximal adhesion force (Fig. 2B, blue arrow) for the detachment of cells from a FN-coated substrate, we do not observe any difference as a function of the cell type and obtain a value close to 400 pN. On the opposite, for the cell-cell contacts, the adhesion force clearly increases from 370 ± 140 pN for SKBR3 cells to 840 ± 190 pN for

MDA-MB231, showing that more invasive cells are more adherent to cells from the same line (Fig 2B).

The cell adhesion is influenced by their rigidity, as well as by specific biochemical character of their surface. Softer cells should be more adhesive, because at constant applied force and for a short contact time (2s), the deformation of softer cells is higher, providing larger contact area and consequently higher adhesion. We indeed observe higher cell-cell adhesion forces for softer cancer cells. In the same time, supposing that adhesion is proportional to the contact area, the Hertz model yields $F_{adh} \sim E^{-2/3}$. The variation of adhesion should thus be smaller compared to the modulus variation, while we observe the contrary. So there is an additional contribution from the specific surface receptors. Cell adhesion changes are thus not only due to the change in cell rigidity.

If the cell adhesion to substrate or cantilever increases, obviously the effective cell radius increases as well. In such a case, the modulus decreases (Fig. 2A) taking into account $E \sim R^{1/2}$.

The most invasive breast cells show the higher homotypic interaction capacity. Previous studies have also demonstrated, for highly metastatic cell lines from different cancer types, an enhanced homotypic aggregation *in vitro* and their ability to form more metastases *in vivo*, compared to weakly or not metastatic cell lines.¹⁹⁻²⁰ In most carcinomas, initial cell disseminate from primary tumor mass to form metastasis with an associated loss of E-cadherin-mediated cell-cell adhesion.²⁰ Thus, individual cells detach from tumor to migrate.²¹ Numerous studies over the years give arguments in favor of this dissemination mechanism.²² An alternative dissemination process exist where tumor cells keep homotypic interactions to use collective migration to detach from primary tumor mass²¹. They also stay in groups during their dissemination in the blood, to form multicellular tumor, which promote their implantation in the microcirculation at higher efficiencies.²³ A study with female nude mice has demonstrated that TGF β signaling reversibly switches breast cancer cells from cohesive to single cell movement.²⁴ Hence, alternating between the two types of migration could depend on microenvironment. As cell-cell interaction forces increased as a function of invasive potential, our results could suggest that a more marked aggressiveness could be related to their ability to regroup in a particular environment.

Membrane tethers are easier to extrude from invasive cells

In cell adhesion studies to matrix substrate or on adherent cells, the detachment-force curves record two types of small de-adhesion events. The first type involve cell adhesion receptors anchored to the cytoskeleton, referred as jumps; the second type is preceded by a long plateau of nearly constant force and involve receptors not linked to the actin cortex; it corresponds to the formation of a membrane tether.²⁵⁻²⁶

We explore the properties of such membrane tethers formed during the detachment of cells, from FN-coated substrates and other cells of the same type. We measure three parameters: the force per individual tether (Fig. 3A, inset), the number of formed tethers per cell (Fig. 3B, inset) and the maximal separation length (Fig. 3C, inset).

We discriminate tether rupture events from molecular links rupture by ensuring that i) the separation between the cells (at least 5 μm) is larger than the total cells deformation and that ii) the slope of the force curve before the tether rupture is small contrary to what happens for molecular link rupture.^{15,25} We observe that for our four cell lines, the tether rupture force is similar in cell-FN and cell-cell adhesion (Fig. 3A). On the other hand, tether rupture force values are close for SKBR3, MCF7 and BT474 cells on FN (40.7 ± 9.6 , 43.4 ± 9.3 and 34 ± 8.6 pN respectively) and on cells (33.2 ± 9.4 , 39.1 ± 4.8 and 38 ± 7.4 pN respectively) whereas they decrease for the more invasive MDA-MB231 cell lines (25.2 ± 6.7 pN on FN and 26.2 ± 2.3 pN on cells). These forces are in the order of magnitude of published values for tethers pulled from cells,¹⁴⁻¹⁵ even though never measured on breast cell lines.

For the four cell lines, very few tethers are formed in cell adhesion experiments to FN substrate (Fig. 3B). Contrarily, we observe during cell-cell detachment a larger number of tethers that increases from 0.7 ± 0.3 for SKBR3 cells, to 10.7 ± 5.6 , 14.2 ± 6.9 and 13.9 ± 2.2 for MCF7, BT474 and MBA-MB231 cells respectively, showing that invasive cells tend to form more tethers.

We study the separation distance at which the last tethers breaks (Fig. 3C and inset, blue arrow). In cell-cell detachment, it increases from 7.7 ± 2.2 μm for SKBR3 cells to 34.7 ± 7.6 μm , 35.8 ± 4.8 μm and decreases to 25.7 ± 2.3 μm for MCF7, BT474 and MBA-MB231 cells respectively. MDA-MB231 cell line seems an exception from the general trend, probably because these most adhesive cells are better adherent to the substrate or cantilever sides, so their membrane is better fixed and its ability to elongation is decreased. As for the cell-FN contact, for the most of cell lines (except for SKBR3 cells) the separation distance is approximately two times lower compared to the cell-cell case, probably because the tubes may be drawn only from one side in the cell-FN case.

When we compare detachment of four tumor cell lines on fibronectin, for a contact time of 2 seconds, the number of tubes formed is very low (<1 on average) compared to the number of jumps (data not shown). This result suggests that the adhesive events observed correspond mainly to the rupture of adhesive links connected to the actin cytoskeleton. These interactions probably involve integrins²⁶, which are the main adhesion receptors for biological matrices.

In homotypic cell-cell interactions, the less invasive SKBR3 cells having the best organized cytoskeleton (Fig. 4), form very few tethers. On the contrary, the more invasive cells, which have a disorganized cytoskeleton (MCF-7, BT474 and MDA-MB231 cell, Fig. 4) form an increased number of tethers correlated with the invasive potential. Moreover, the tether force tends to decrease for the most invasive cells. This is coherent with the previously mentioned equilibrium description of membrane tethers, where the force to maintain a tether depends on membrane bending rigidity, membrane tension and on the interaction between the membrane and the underlying cytoskeleton. Accordingly, when the cytoskeleton is destabilized by the use of drugs or a decrease of plasma membrane–cytoskeleton coupling, the tether force decreases¹⁵ and the probability of pulling tethers rises.²⁷ Conversely, with the strengthening of the integrin-cytoskeletal linkage, a decrease in the number of tethers is observed.²⁸

We obtained a coherent evolution of physical parameters for the most invasive cells, as obtained in homotypic interactions between activated β cells, compared to non activated cells¹⁴; they are more adhesive between them and more deformable, with an increased number of tethers. The properties of homotypic interactions result from a combination of receptors expressed on cell membrane. The increase of adhesive forces between invasive cells could be due to quantitative and/or qualitative differences in adhesion receptors expression. Numerous studies report that initial cell dissemination (single or collective) from primary tumor mass to form metastasis is associated either with a loss or reduction of E-cadherin-mediated cell-cell adhesion, that is accompanied, in several cancer types, by *de novo* expression of mesenchymal cadherins such as N-cadherin and cadherin-11.²² However, different studies show that SKBR3 and MDA-MB231 cells do not express E-cadherin, unlike MCF7 and BT474 cells.²⁹⁻³⁰ Moreover, the expression of N-cadherin is not detectable for these four cell lines.²⁹⁻³⁰ These data disqualify their involvement in the increased homotypic interactions correlated to invasive potential that we observe. Studies on breast cancer tumor sections highlight other cell adhesion receptors that may have a prognostic value. Receptors such as

ICAM1, ALCAM or L1-CAM could be good candidates. For more details, see Supporting Information file. (S-6)

Cytoskeleton organization

We probe the actin cytoskeleton organization of breast cancer cells (Figure 4). The less invasive and well spread SKBR3 cells exhibit very structured actin stress fibers, which are well aligned and distributed along the long axis of the cell. For more invasive tumor cells (MCF7, BT474, MDA-MB231 cells), the actin cytoskeleton is disorganized. For MCF7 and BT474 cells, we observe a dramatic decrease of stress fibers number and the presence of patches of aggregated F-actin (Figure 4). For well spread MDA-MB231 cells, stress fibers are randomly distributed throughout the cell, and are unequal in length and thickness. Our results confirm the disorganization of the actin cytoskeleton in tumor cells (MCF7, BT474, MDA-MB231 cells), as previously demonstrated for different types of cancer cell lines.^{3,5} This actin disorganization is associated with increased deformability of tumor cells compared to normal cells. In contrast, the less invasive SKBR3 cells present a well organized actin cytoskeleton, not far from what is observed in normal cells.

In Supporting Information file, (S-6) we discuss the difficulty to correlate the cytoskeleton disorganization and the invasiveness of tumor cells.

We have demonstrated that membrane tethers are easier to extrude from invasive cells. We have shown that the number of tethers per cell, as well as the separation distance, increases with invasive potential. In its turn, the tether rupture force display a slight decrease against invasiveness. We have observed that cell-to-cell adhesion force increases with the invasive potential of cancer cell lines. We confirm that Young modulus for invasive breast cancer cells decrease with invasive potential.

The study of the membrane tether formation from cancer cells, as well as determining other cell mechanical parameters, could be a new marker for cancer diagnostic and cell phenotype determination.

Associated content

Supporting information

The supporting information is available, providing the Materials and Methods part, in particular details about cell culture, AFM experiments (cells preparation and data acquisition) and actin labelling, as well as statistical tests applied for obtained values. Additional points of discussion were also specified.

Acknowledgement

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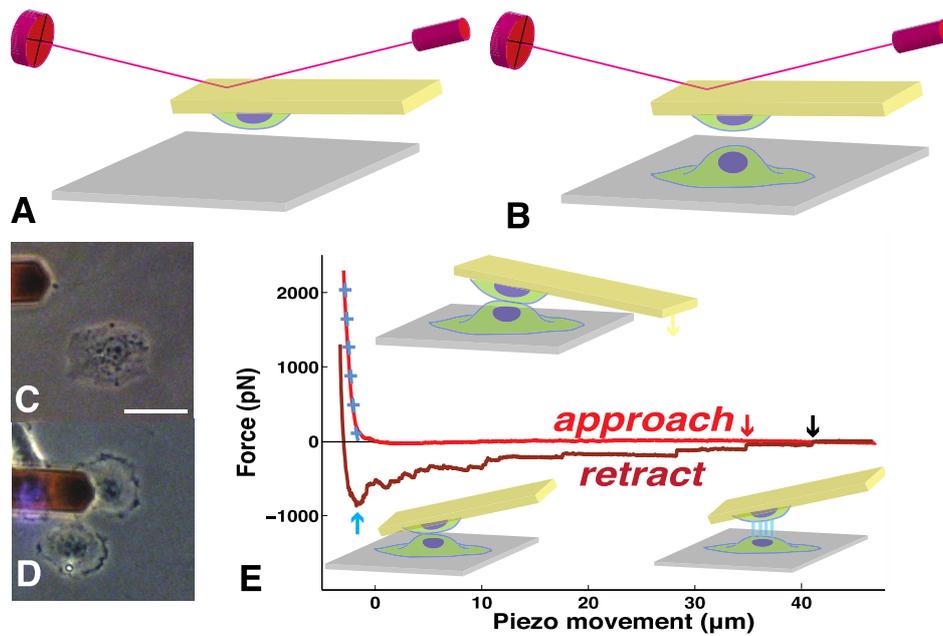


Fig. 1 Principle of living cell mechanical properties measurements from detachment experiments A) Sketch of the setup: a cell is attached to a tipless AFM cantilever coated with Concanavalin A (in yellow) driven by a wide-range (100 μm) piezo element. This cell is brought in contact with fibronectin coated on plastic (A) or another cell (B). The force on the cantilever is monitored by the deflection of a laser beam focused on the cantilever end C-D) Optical micrograph of the setup showing the cantilever, the light of the detection laser and a spread cell (C) one SKBR3 cell, D) MDA-MB231 cells, scale bar: 10 μm E) Characteristic curve showing the force on the cantilever vs. piezo displacement during a cell-cell detachment experiment: during approach (red), the cell on the cantilever is brought into contact at constant velocity until the force setpoint of 2300 pN is reached (upper scheme), then the cell is retracted (brown, lower scheme). The approach curve is further fitted by a Hertz model (blue crosses) to obtain the cell Young modulus.

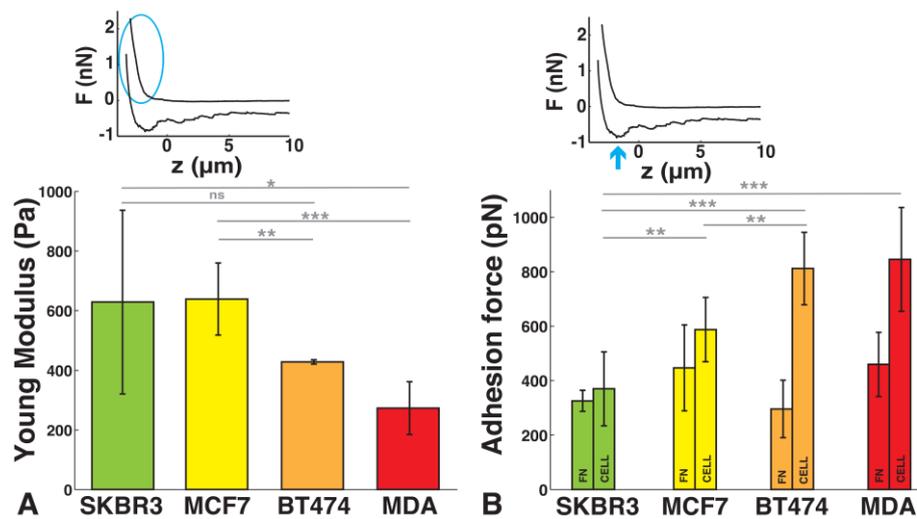


Fig. 2 Mechanical parameters of tumor cells extracted from force curves: In the following, breast cells lines are ranked from left to right in ascending order of their invasive character. A) Young modulus measured in the approach sequence (circled in blue in the insert) B) Maximal cell adhesion force measured in the retraction sequence (blue arrow on the insert) on Fibronectin-coated plastic and on spread adherent cells. Error bars depict standard deviation of the different measurements performed. For every condition, at least 3 cells were immobilized on cantilever, and at least 5 cells immobilized on substrate were probed with each cell immobilized on cantilever. Statistical analysis was performed using 1-way ANOVA test; * $p \leq 0.1$; ** $p \leq 0.05$; *** $p \leq 0.001$

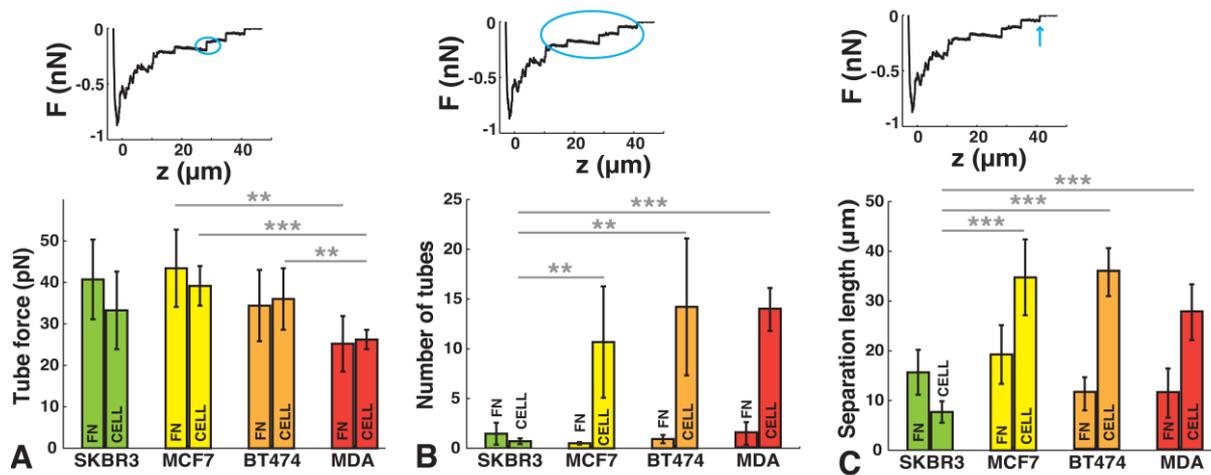


Fig. 3 Mechanical parameters of membrane tethers extracted from tumor cells: In the following, breast cell lines are ranked from left to right in ascending order of their invasive character. A) Force steps corresponding to a single membrane tether, measured in the retraction sequence (circled in blue in the insert) on Fibronectin coating plastic or on a cell B) Average number of membrane tethers in a single detachment experiment, measured in the retraction sequence (circled in blue in the insert) C) Separation length measured in the retraction sequence (blue arrow in the insert). Statistical analysis was performed using 1-way ANOVA test; * $p \leq 0.1$; ** $p \leq 0.05$; *** $p \leq 0.001$

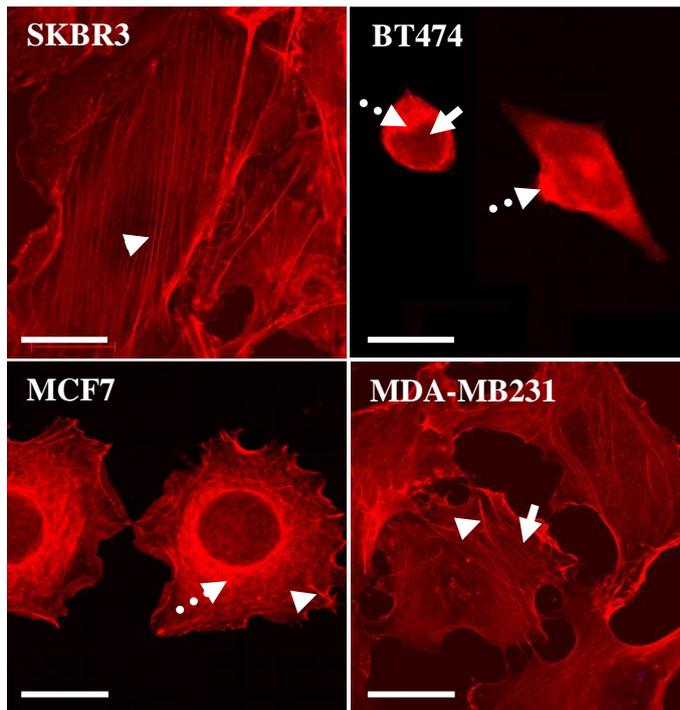


Fig. 4 Cytoskeletal organization in tumor cells cultured on fibronectin-coated glass for 48h. In SKBR3 cell, actin filaments were well organized in stress fibres along the long axis of the cell for the less invasive tumor cells (→). MCF7 and BT474 cells, which grown in islet, exhibited, for isolated cells, a disorganized actin network mostly reduced to patches of aggregated F-actin (- -→) and short stress fibers. The most invasive cells, MDA-MB231 ones, possessed stress fiber that were distributed randomly throughout the cell and appeared to be unequal in size and thickness (thick →; thin →→), to be reduced to single filaments of actin. Actin microfilaments are stained by phalloïdin-TRITC. Bars = 20 μm