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Nanomechanical Investigation of Soft Biological Cell Adhesion using Atomic Force Microscopy

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Abstract

Mechanical coupling between living cells is a complex process that is important for a variety of biological processes. In this study the effects of specific biochemical treatment on cell-to-cell adhesion and single cell mechanics were systematically investigated using Atomic Force Microscopy (AFM) Single Cell Force Spectroscopy. Functionalized AFM tipless cantilevers were used for attaching single suspended cells that were brought in contact with substrate cells. Cell-to-cell adhesion parameters, such as maximum unbinding force (F_{max}) and work or energy of detachment (W_D), were extracted from the retraction force-displacement ($F-d$) curves. AFM indentation experiments were performed by indenting single cells with a spherical microbead attached to the cantilever. Hertzian contact model was applied to determine the elastic modulus (E). Following treatment of the cells with neutralising antibody for epithelial (E)-cadherin, F_{max} was increased by 25%, whereas W_D decreased by 11% in response to a 43% increase in E . The results suggest that although the adhesion force between cells was increased after treatment, the energy of adhesion was decreased due to the reduced displacement separation as manifested by the loss of elastic deformation. Conclusively, changes in single cell mechanics are important underlying factors contributing to cell-to-cell adhesion and hence cytomechanical characterization is critical for cell adhesion measurements.

Key words: AFM-SCFS, Elasticity, beta-(β)-cells, Indentation, Cell mechanics.

Introduction

Measuring cell adhesion is of paramount importance for monitoring physiological and pathological processes including cell growth, differentiation, cancer proliferation, diabetic development and many others. Furthermore, interactions between the cells and the extracellular matrix (ECM) contribute in the maintenance of the structural integrity of tissues²¹. In addition, the adhesive behaviour of cell with other surfaces is crucial for the biocompatibility of implants⁷. Cell adhesion is a dynamic process, which is controlled by the binding of adhesion molecules on the cell surface²³. However, as a living system, cells have a highly complex organisational architecture that exhibits complicated mechanical and adhesive behaviors. Unlike classical mechanics, special considerations needs to be taken into account in characterizing the mechanical properties of biological cells, since their 3-D structure, is distinguished by their complex mechanical and interfacial behaviors³⁰. For example, the microtubule, a major component of cell cytoskeleton, exhibits a fibre-like structure with a diameter less than 25nm, and is normally subjected to complex chemical and mechanical environment, including cell-cell and cell-ECM interactions. Therefore, the cytoskeleton (CSK) is the principal factor that determines the deformation behaviour of a single cell¹. In addition, the deformation behavior of cells and tissues is a result of combined interaction between cytoplasmic elements such as the CSK and the ECM. Therefore, mechanical and adhesive properties are affected simultaneously both at molecular and cellular scale^{26, 2}.

The cell is a dynamic system that continuously interacts with its external environment, i.e. other cells or the extracellular matrix. This interaction is controlled by the plasma membrane. Interaction of cells with their immediate environment is partly regulated by cell adhesion molecules (CAMs). However, CAM proteins do not simply tether cells to each other or the ECM, but they also relay information about the local extracellular microenvironment, and can affect the intracellular structure/function of the cell. Essentially, CAMs are transmembrane molecules that are linked to cytoskeletal filaments at the adherens junction. Connection of

the extracellular domain to the cytoskeleton results in an intricate interplay of the mechanical and adhesive properties, which may lead to alterations in the elastic deformation of the whole cell²⁶. The predictive, diagnostic and therapeutic role of adhesion molecules has been addressed in cardiovascular¹⁷ and Alzheimer's disease³⁴. Puech *et al.* measured the adhesive properties of single cells from zebra fish to coated substrates and concluded that extracellular binding affects intracellular signalling²⁵. In addition, Bershadsky *et al.* highlighted that focal adhesion points act as mechanosensors responsible for the signalling cascade within the cell³. Thus, there is no doubt that cell adhesion events are important in controlling various cellular functions such as growth, wound healing and metastasis^{4, 26}. Investigation of cell-to-cell adhesion is important as a mediator of mechanotransduction¹⁶. However, adhesion between cells is related to mechanical deformation through CAMs. Changes in deformation provide important information about the normal and diseased state of the cell¹. Any changes of the resistance of the cell to elastic deformation can be measured and expressed as changes in the Elastic modulus E or Young's modulus.

Several advanced techniques have been developed for quantitative assessment of cell adhesion, such as micropipette aspiration, AFM, reflection interference contrast microscope (RICM) and optical tweezers; their merits and disadvantages have been reported in detail elsewhere^{20, 35}. Among these, AFM has been one of the most prevailing tools to study cell adhesion. The unique advantages of the technique include its force range (from pN to nN), which allows studies down to single ligand receptor level with excellent displacement accuracy (nanometer resolution). Latest development of long pulling distance AFM (up to 100 μ m) has facilitated the force measurements of the mechanical deformation during the separation of two adhered cells. In AFM single cell force spectroscopy (SCFS) a single cell is attached to a tipless, chemically modified cantilever or alternatively cells can be cultured directly on the cantilevers⁸. During the pulling or separation process, cytoskeletal components are inevitably deformed. The measured detachment energy of the cells will

therefore be a function of both the energy of cell deformation, as well as the energy required to unbind the contact surface between the two cells³¹. In the current study, the changes in functional cell-to-cell adhesion and single cell mechanics after blocking the adhesion molecule E-cadherin, were systematically investigated using SCFS. The cohesive results between cell adhesion parameters and single cell elasticity have facilitated the investigation of the effects of mechanical deformation on cell-to-cell adhesion. In this study we apply AFM-SCFS to measure elasticity and adhesion force/energy of clonal MIN6 β -cells that were treated with anti-E-cadherin-neutralising antibody, for elucidating the complex interplay between single cell mechanics and cell adhesion.

Materials and Methods

Tissue Culturing Materials

MIN6 cells were obtained from Dr. Y. Oka and J.-I. Miyazaki (Univ. of Tokyo, Japan). Fibronectin, Dulbecco's Modified Eagles Medium (DMEM), glutamine, penicillin-streptomycin and phosphate buffered saline (PBS) and anti-E-cadherin were from Sigma–Aldrich (Poole, Dorset, UK). Tissue culture plastic-ware was from Invitrogen Life Technologies (Paisley, UK).

Maintenance of MI6 cells

MIN6 cells (passage 35-40) were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air in DMEM supplemented with 15% FCS, glutamine (2mM) and penicillin/streptomycin (100U/ml/0.1mg/ml). Prior to treatment, cells were seeded onto 40mm petri-dishes and serum starved overnight. Cells were then placed for 48hrs in DMEM containing both low glucose (5mM) and low calcium (0.5mM) +/- the E-cadherin neutralizing antibody¹³ (Sigma UK product code U3254 – antiuvomorulin, raised against a mouse immunogen; final concentration, 68 μ g/ml). Suspended (free) cells were prepared under identical conditions

before being physically scrapped off the T25 flasks with gentle agitation and re-suspended in fresh DMEM.

Atomic Force Microscopy

By operating the AFM in force spectroscopy and by selecting cantilevers with a low spring constant (Arrow TL1, Nanoworld AG, Switzerland) the instrument was used to characterise single cell elasticity and cell-to-cell adhesion. With certain modifications of the AFM cantilever, SCFS with an extended effective displacement range provided the core instrumentation to perform the long distance force spectroscopy experiments, required for this research. Prior the experiments the cantilever was mounted on specially designed cantilever holder that is chemically inert as well as polished on its top and bottom surfaces to allow the transmission of light to the objectives. The actual spring constant of the cantilever was determined before experiments by using the manufacturer's software based on the thermal noise method. The assumptions and conditions of these calculations are described by Hutter & Bechhoefer¹⁵. Calibration curves were performed on the same petri-dishes used for cell culturing, as well as the same experimental conditions i.e. temperature and fluid media. The cantilever's deflection was converted into force, by using the second resonance peak of the thermal noise method. Since the resonance of soft cantilevers in fluid is much lower and very susceptible to noise, a correction factor of 0.251 was used³¹.

The AFM head was integrated optically with a microscope. Experiments were performed using the CellHesion@200 module (JPK Instruments, Berlin, Germany) that was installed on an Eclipse TE 300 inverted microscope (Nikon, USA). During each experiment, cells were maintained at a physiological temperature (37°C) by incorporating the BioCell™ temperature controller (JPK, Berlin, Germany) into the AFM stage. Phase microscopy images were acquired using a CCD camera (DFK 31AF01 Firewire, The Imaging Source, Germany) connected on the side port of the microscope. The whole AFM-FS set-up with the CCD camera was driven by JPK's CellHesion200 software. Images were captured using a 20x

magnification lens. Since such force measurements are extremely sensitive and susceptible to noise, vibrations and environmental conditions were well controlled. The entire optical microscope and AFM head set-up was supported on an anti-vibration table (TMC 63-530, USA). Changes in the temperature of the room were less than 0.5-1.0 °C during the experimental measurements.

Functionalization of Cantilevers

Tip-less cantilevers were chemically functionalised so that a single suspended cell could be attached. Initially the cantilevers were sterilised by UV treatment (10mins). Next, they were incubated in poly-L-lysine (25µg/ml in PBS) for 30mins in room temperature (RT). Subsequently, the cantilevers were transferred in fibronectin solution (20µg/ml in PBS) and they were incubated for 2h at 37°C. After functionalization cantilevers were immersed in PBS solution at 4°C and used within 3 days.

Cell adhesion experiments

The cells were gently separated from the flask via a low-force sweeping motion using a sterile cell scraper with a rubber blade. Then the scrapped cells were transferred using a pipette on a sterile flask for centrifugation. After harvest, the medium was removed and replaced with sterile DMEM. The cells were re-suspended by gentle agitation to break up the cell pellet and provide give a uniform distribution of the non-adherent cells in the flask. Cells were allowed to recover for 5 mins before being introduced to the testing petri-dish. Suspended cells were introduced into the petri dish using a pipette. Since free cells tend to stick on the substrate quickly, the cell-cantilever attachment procedure was performed rapidly (2min). Once a single cell was attached to the cantilever, it was left to recover for at least 5 mins⁹). Then, the cantilever-attached cell was brought in contact with another cell attached on the substrate, until a pre-set contact force was reached (0.5nN). The two cells remained in contact for a set period of time (5secs). During this time bonding between cells was formed. The cantilever was then retracted at a constant speed (5µm/sec) and force

versus displacement was measured until the two cells were completely detached (pulling length 40-70 μ m). The procedure was repeated three times for each tested cell, with 30s intervals between individual retractions.

Single Cell indentation experiments

Colloidal probes were prepared by gluing a 10 μ m polystyrene microsphere (Polybeads[®], Polysciences, Eppelheim, Germany) on a tipless TL-1 cantilever. The bead and the cantilever beam of the AFM cantilever beam were visualized by using the inverted optical microscope during the whole procedure. AFM indentation was conducted by force-controlled, in which the cell is indented according to a pre-set force value. The height of each tested cell was determined by performing a F - d curve with low set point of force (0.2nN) on the surface of the cell, and the height was calculated by displacement difference from the substrate³¹. Each cell was indented 5 times with an interval pause of 60secs, while force versus displacement was recorded simultaneously. All cells were indented on the area directly above the nucleus. Since, the indentation depth was pre-determined for each cell, displacement-controlled or simply height indentation experiments were performed. The approach and retraction speeds were kept constant for all experiments at 5 μ m/sec to avoid hydrodynamic forces acting on the cantilever. Approximately 10 cells per petri dish were tested, while only the extended part of the F - d curve was used for analysis in order to minimize the influence of the adhesion between the tip and the living cell.

Hertz model

The Hertz model is commonly used to extract the elastic or Young's modulus from a force-displacement curve acquired by indentation measurements. Although this model is widely used for biological samples, there are several assumptions that need to be considered in order to match certain experimental conditions. Different indenter geometries lead to different radius of contact area α , therefore different extensions of the original model must be used. As shown schematically in Figure 1, for spherical probes, loading force F is related to indentation depth δ as follows,

$$F = \frac{E}{1-\nu^2} \left[\frac{a^2 + R_s^2}{2} \ln \frac{R_s + a}{R_s - a} - aR_s \right] \quad (\text{eq.1})$$

$$\delta = \frac{a}{2} \ln \frac{R_s + a}{R_s - a} \quad (\text{eq.2})$$

where E and ν are the Young's Modulus and Poisson's ratio of the cell respectively, α is the radius of probe-cell contact circle, and R_s is the radius of the spherical probe.

Hertz theory approximates the sample as a linear, homogeneous sphere. However, soft biological cells are characterized by non-linearity and inhomogeneity. Therefore, the Poisson parameter does not describe such complex material response and has to be approximated. Consequently, the Poisson's ratio of incompressible materials like rubber was assumed as 0.5²². Hertz theory assumes indentation on an infinitely extending space. This means that the depth of indentation is negligible compared to the height of the sample and that the deformation of the sample induced by the indentation is very small in contrast to the extremely thick sample. However, since cells have a very limited thickness and Hertz theory assumes that the sample occupies an infinite half-space, it is very important to define the

indentation depth before experiments. The Hertz model is only valid for indentations up to 10% of the samples height, where substrate effects are considered insignificant ⁶. For this reason, all curves were fitted rigorously with the restriction that the maximum depth of indentation is equal to or less than 10% of the height of each cell.

Data Analysis

To process all force-displacement curves the JPK Data analysis software was used. To signify statistical differences data were evaluated using a paired t-test. Data are expressed as mean \pm SEM and 'n' shows number of experiments. $P < 0.05$ was taken to indicate statistical significance.

Results

Surface blocking of E-cadherin ligation has an inverse effect on adhesion forces

In order to investigate the effects of the antibody on the E-cadherin mediated β -cell-to- β -cell adhesion, the maximum unbinding forces between two cells, which were brought in contact, were measured. F_{max} was calculated by detecting the minimum negative value of force with respect to the baseline of complete separation (Figure 2(b)). Adhesion forces is the most common indicator of cell coupling, since the downwards deflection of the cantilever when being retracted from the sample signifies binding between the adherent cells. Mechanical contact was established above the central region of the cell, which normally corresponds to the area where the largest element of the cell, the nucleus (Figure 2(a); purple colour), resides. The cytoskeleton (Figure 2(a); red colour) is the element of the cell that is principally responsible for maintaining its shape.

The retraction measurements of control (Ca^{2+}) versus treated (Ca^{2+} +anti-E-cad) MIN6 cells are shown in Figure 2(c). To determine the adhesion forces 86 retraction F - d curves were analysed and the results indicate that F_{max} was increased by 25% ($p < 0.001$) after treating the cells with the neutralising antibody. To further assess the changes in surface ligation the number of unbinding events that occurred during the retraction process was investigated. The retraction F - d curve of Figure 2(b) illustrates the number of tether rupture events (TREs) as detected by the step fitting function. The antibody increased the maximum unbinding forces, in correspondence to an increase of rupture bindings during the separation process. Number of TREs can be detected by identifying sharp steps of force that correspond to bond ruptures³¹. During the retraction phase of the cell-to-cell adhesion curves, only upward steps are anticipated, hence only positive steps were selected to avoid drifting errors of the cantilever. Analysis of 34 retraction F - d curves showed that number of tether ruptures was increased by 18% ($p < 0.001$) (Figure 2(d)), when the cells were treated with the antibody.

Treatment with E-cadherin neutralizing antibody increases the rigidity of single cells

Indentation testing was performed to investigate the elastic properties of single cells. The microbead was indented according to a predetermined indentation depth based on cell height. Figure 3(a) shows an extension curve of a control cell with a height of $4.5\mu\text{m}$ and the elasticity values at higher depths than the 10% of the cell's height, using the Hertz model. The contact point is defined as the point where cantilever deflection starts to rise and in fact accurate determination of the contact point is crucial for a reliable calculation of the elastic modulus. By fitting discrete parts of the extension curve to the model, the point where the probe is in contact with the plasma membrane can be identified. The E modulus histogram of control cells is shown in Figure 3(b). On average, treated cells showed to have a higher value of E modulus ($\sim 633\text{Pa}$) than control cells ($\sim 444\text{Pa}$). Figure 3(c) shows the changes in elasticity between the two groups of cells, resulted from the processing and analysis of than 30 curves for each treatment, obtained from 2 separate AFM indentation experiments. The

results indicated that the anti-E-cad increased the E modulus by 43% ($p < 0.001$) when compared to the control cells, suggesting that treated cells became more rigid.

Effects of loss of the elastic deformation on adhesion energy (during the separation process)

In order to assess the effects of changes of the E modulus on cell-to-cell adhesion, the total energy of adhesion before complete separation of the adhered cells was determined. A $F-d$ curve from the retraction movement of the cantilever during a cell-to-cell measurement is shown in Figure 4(a). In the flat region of the curve (phase 1), there was no deflection of the cantilever to the photodiode since there was no contact between the cantilever-cell and the substrate-cell. As the cantilever moved downwards towards the substrate cell, phase 2 was reached where the two cells were in contact and the cantilever deflected according to the predetermined force value (0.8nN). The piezo-actuator remained static in that position for the set contact time (5secs). Then the cantilever was retracted (phase 3) and the ligation of the two cells caused bending of the cantilever. As the cantilever was retracted further away from the sample tethering was disrupted, until phase 1 was reached in which the cells were completely detached from each other. The energy that is consumed during the pulling process (phase 2) until the two cells were completely detached was calculated by the integration of the retraction $F-d$ curve. To determine the work or energy of detachment, 86 retraction $F-d$ curves were analysed and the results are shown in Figure 4(c). The data indicated that W_D was decreased by 11% after treating the cells with the neutralising antibody.

Figure 4 (a) and (b) shows retraction $F-d$ curves of control and treated β -cells respectively. It is noticeable that as the pulling distance is increased, an area is reached where the rupture events are preceded by a displacement plateau. This plateau is due to the deformation of the cell and as signified by the arrows of Figure 4 (a) & (b), it is considerably higher in the control cells. The displacement plateau resulted in an increased pulling length, which in turn affected the distance of complete separation. To determine d_s the pulling length from the

highest negative deflection of the cantilever and the point of complete detachment was calculated. The results are shown in Figure 4 (d) and indicate a decrease in the distance of complete separation by 21% ($p < 0.007$) when the cells are treated with the E-cadherin neutralization antibody.

Discussion

In this study AFM-SCFS was used to detect changes in functional tethering between two individual β -cells. However, cell-to-cell adhesion is a complex process that is controlled by the surface ligation and the interactions between the surface receptors and the F-actin CSK³¹. Living cells are dynamic systems that respond to biochemical stimuli by changing their structure and/or protein expression. Primarily, the focus of this research was to investigate single cell mechanical changes after biochemical treatment, and their effects on adhesion parameters such as the maximum unbinding force and the energy of complete detachment. Analysis of retraction F - d curves, obtained from cell-to-cell adhesion SCFS, provide important information regarding the mechanical coupling between the cells, such as the energy or work of complete detachment, the maximum unbinding force, the distance of complete separation and the number of unbinding events^{10,11, 12, 13, 14, 33, 36}. The viscoelastic nature of surface ligation and its effects on cell adhesion has been demonstrated in various studies^{28, 36}. Schmitz *et al.* highlighted the significance of receptor anchorage mechanics on cell-substrate adhesion²⁸. In the current research the impact of cells' increased rigidity after E-cadherin blocking, on cell-to-cell adhesion was investigated.

Our results suggest that the elastic deformation of the cell plays a major role on mechanical coupling between cells and neutralization of E-cadherin has discrete effects on the adhesion parameters. Despite a significant increase in F_{max} , the value of W_D decreased by 11%. This indicates that although the surface molecular binding was increased, the energy consumed during the separation process was nevertheless decreased. Therefore it would be reasonable to suggest that other underlining cell components integrated with surface

molecular, such as the F-actin, have an important contribution on adhesion parameters. In beta-cells, E-cadherin is the most well characterised adhesion protein ²⁴ which, along with the catenins, form the adherens junction that connects the extracellular domain with the intracellular cytoskeleton. For this reason, it would be reasonable to hypothesize that chemical modification at the surface of the cell could result in cytoskeletal reorganization through catenins⁵. Moreover, the changes in the calculated E modulus after treatment could reflect changes in the cytoskeletal reorganization, since the indentation depths were strictly limited to the 10% of the cell height ^{18, 27, 32}. Therefore, it is suggested that the changes in elasticity revealed changes in the re-arrangement cytoskeleton after blocking the E-cadherin surface adhesion molecule. The average calculated value of E modulus for the control cells was 443 Pa while for the treated cells was 633Pa, indicating that the anti-E-cadherin treatment increased the elastic modulus by 43%. This resulted in increased cell rigidity upon treatment and in the loss of elastic deformations of the treated cells during the separation process. Retraction $F-d$ curves indicated that treated cells have responded with an increase in tether rupturing events, followed by a significant increase of maximum unbinding force. On the contrary, the work of detachment was decreased when the cells were treated with anti-E-cadherin. This reduction was accompanied by a notable decrease in the distance of complete separation and could be partly explained by the increase in cell rigidity of treated cells as manifested by the remarkable increase in E modulus. The higher values of E modulus in our experimental system, could suggest that potentially the CSK was localised on the periphery of the cell, and hence cytoskeletal components were inevitably deformed as the pulling distance between cells was increased ^{19, 33}. Besides, since the number of unbinding events and adhesion forces was increased, our results suggest that the decrease in work of detachment was mainly contributed by the less energy required to completely detach the cells due to loss of cell deformability.

A main concern when studying cell-to-cell adhesion is the difficulty to control the expression of the adhesion proteins at the surface of the cell³⁶. Cells are complex and dynamic by nature and cell adhesion, apart from surface ligation, is contributed by cadherin-cytoskeletal interactions as well as cytoskeletal reorganization²³. In fact, altering the expression of a candidate protein does not necessarily imply that the function of the cell will correspond. Indeed, in the current study, although that the surface E-cadherin ligation was blocked by a specific antibody, the cell responded with an increase in adhesion forces and a decrease in work of adhesion. The systematic investigation of various adhesion parameters and single cell elasticity using AFM-SCFS, suggests that the work of detachment of treated cells was impacted by the decreased displacement plateaus, as manifested by the decrease in the distance of complete separation due to increased cell rigidity, rather than the energy contributed by the increased number of early unbinding events.

Conclusion

In the current study the adhesive and mechanical properties of β -cells treated with anti-E-cadherin were investigated using AFM-SCFS. Chemical modification of the cells led to significant changes of the surface molecular binding in the extracellular domain, while at the same time affected the intracellular domain by increasing the elasticity of individual cells. As a result, adhesion parameters, such as work of detachment and distance of separation, were altered due to the rigidity of treated cells. It is therefore important to take under consideration any alterations of single cell mechanical properties on cell-to-cell adhesion when biochemical effects are investigated.

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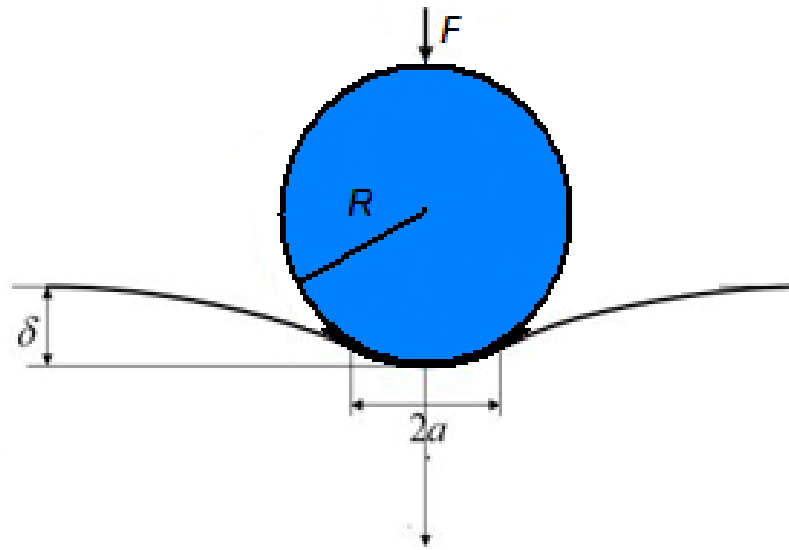
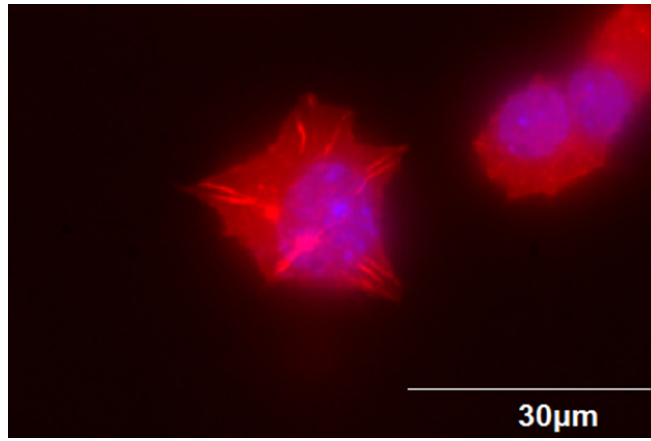
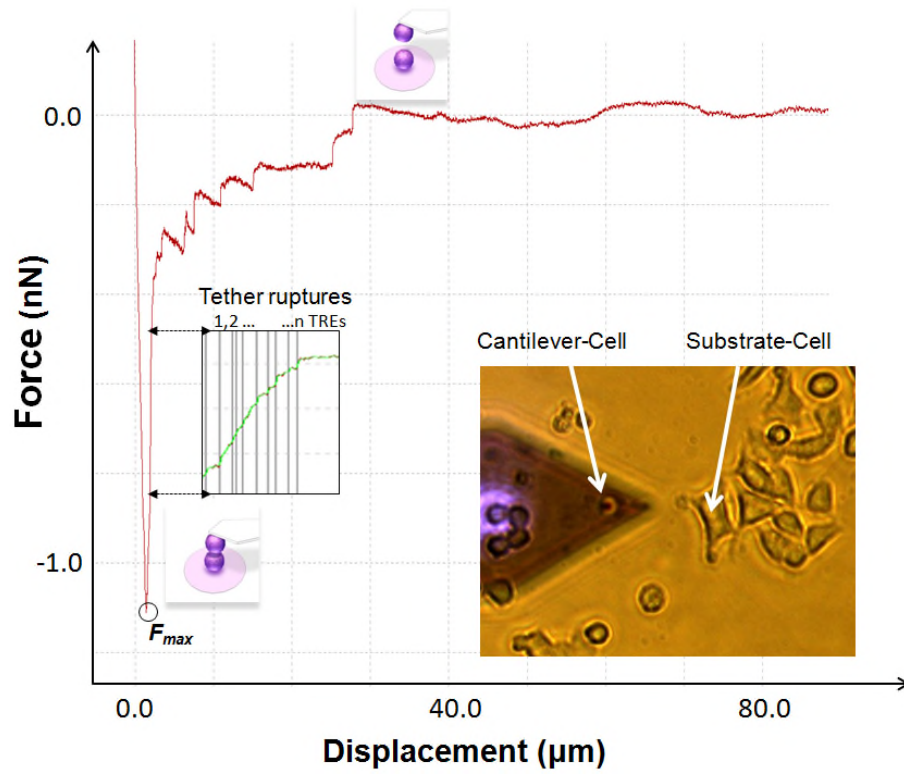


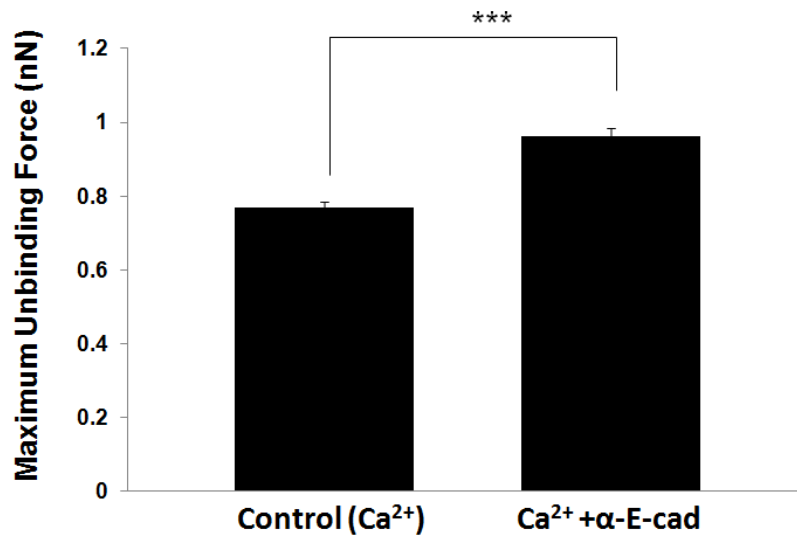
Figure 1: Indentation of a single cell using spherical indenter, where δ is the indentation depth, a is the radius of the contact area between the probe and the plasma membrane, R is the radius of the probe and F the loading force.



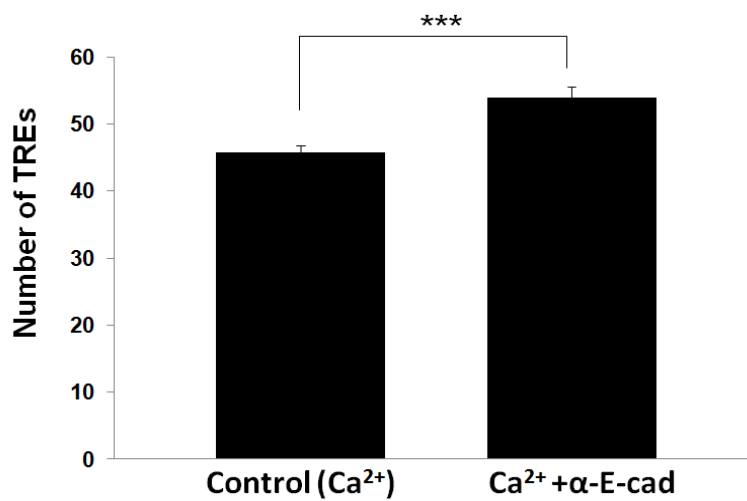
(a)



(b)

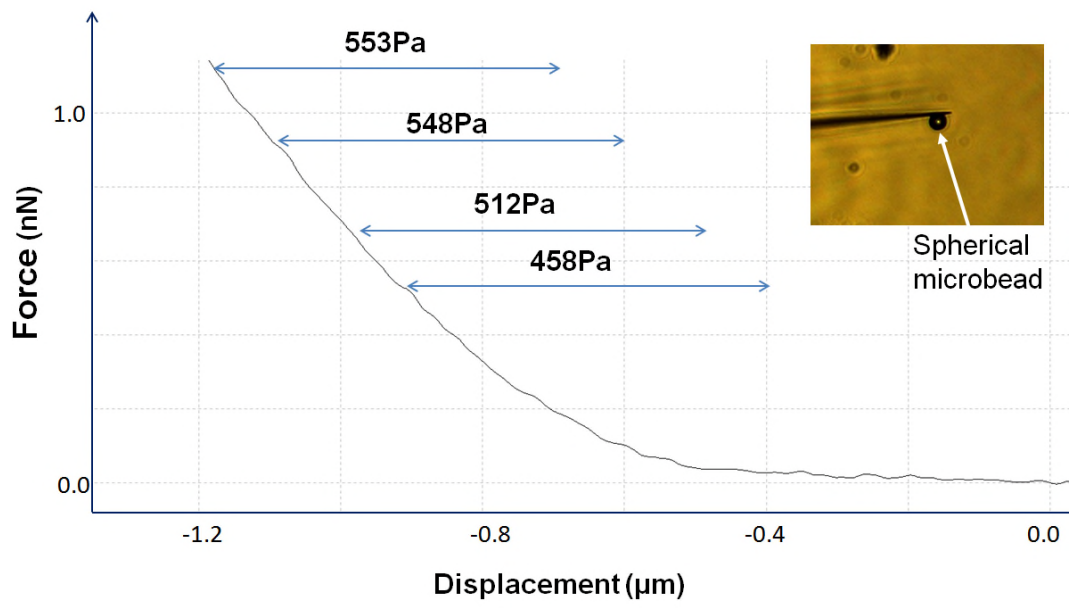


(c)

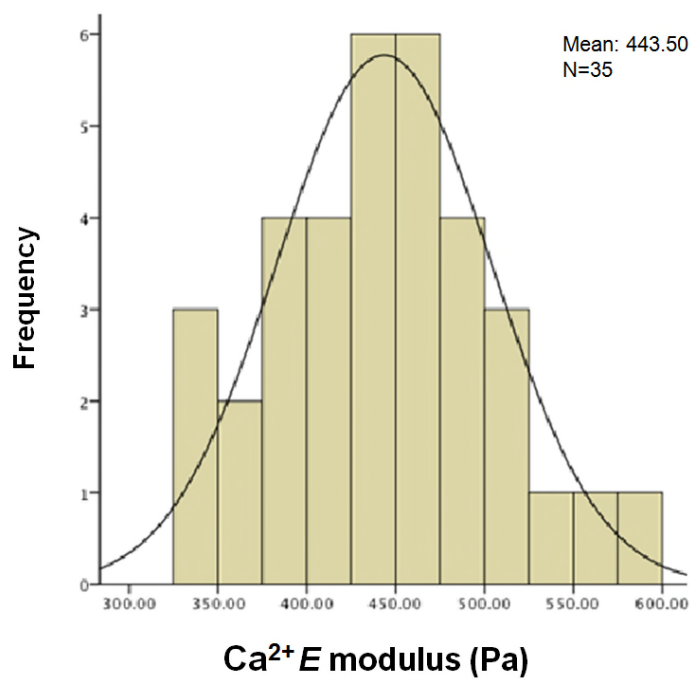


(d)

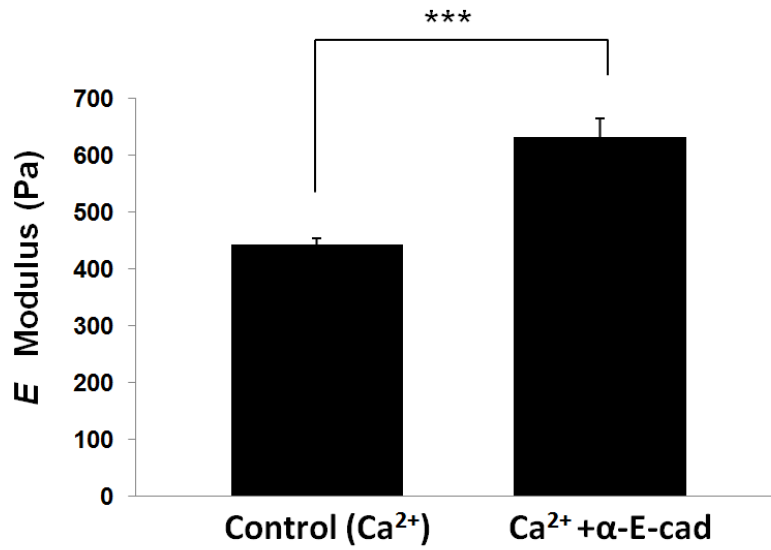
Figure 2: (a) A fluorescence microscopy image showing the morphology of a MIN6 β -cell cultured in low extracellular calcium (0.5mM). (b) A retraction F - d curve of a MIN6 cell treated with the anti-E-cadherin. F_{max} is the difference between the minimum negative force value and the baseline of complete separation. By zooming in the displacement axis the detection of tether rupturing events (TREs) is shown. (c) The maximum unbinding force F_{max} increased by 25% (>25 cells from 3 separate experiments). (d) The number of tethering rupture events (TREs) increased by 18% (10-12 cells from 3 separate experiments). Data are expressed as mean \pm SEM, where key significances are shown, *** p <0.001.



(a)

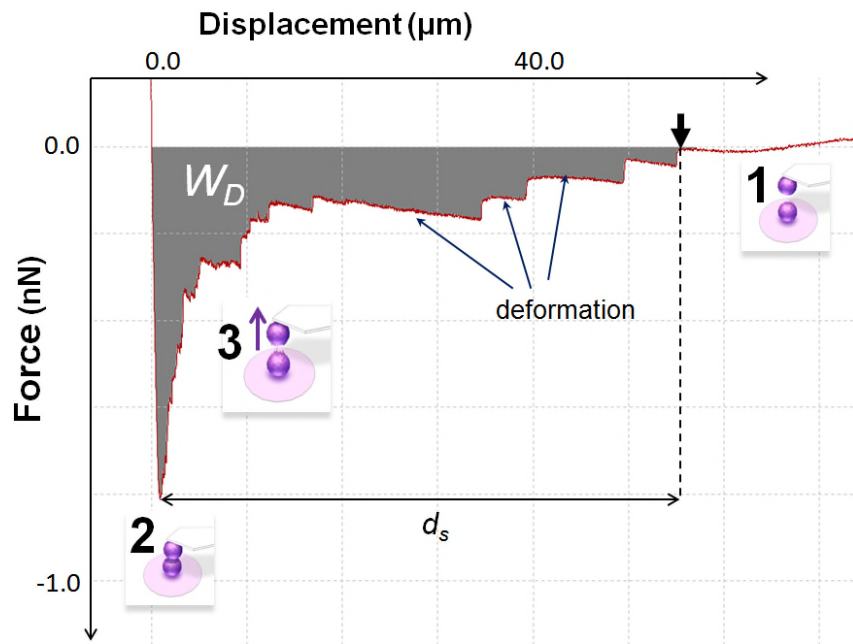


(b)

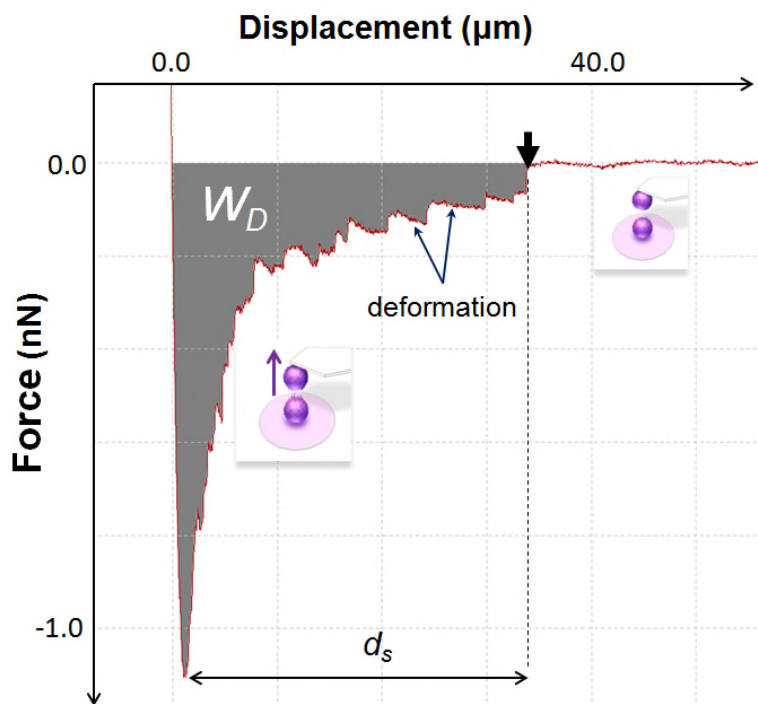


(c)

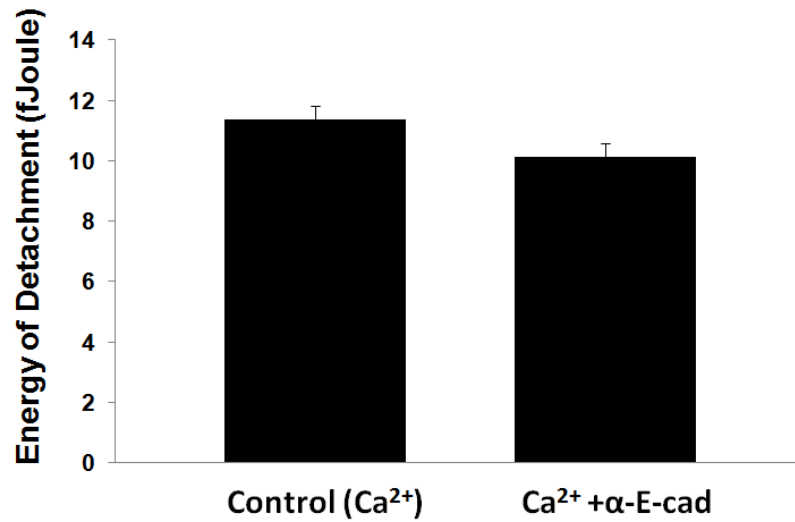
Figure 3: (a) An extension $F-d$ curve obtained by a single cell nanoindentation measurement. A microbead of $10\mu\text{m}$ was glued at the end of the cantilever's tip. Elasticity was calculated by fitting Hertz model into the extended part of the curve, in which adhesion is negligible. (b) A histogram of elastic modulus E obtained from the $F-d$ curve measurements of control MIN6 cells that were indented on a central region. (c) The effects of anti-E-cad on the elastic modulus (increased by 43%) (7-9 cells from 2 separate experiments). Data are expressed as mean \pm SEM, *** $p < 0.001$.



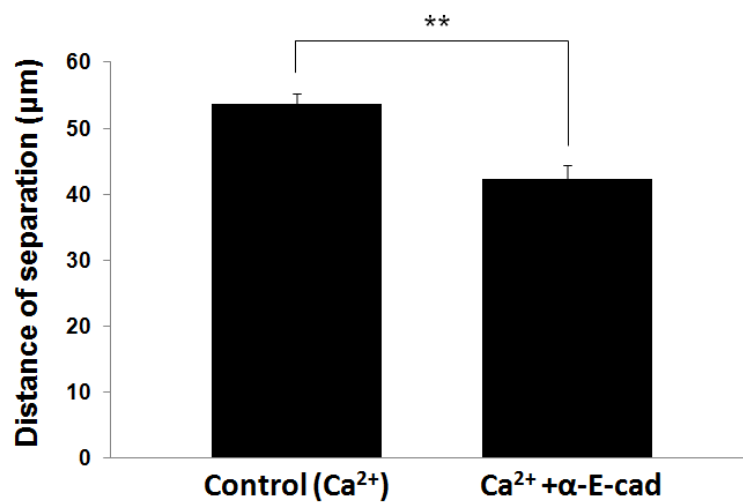
(a)



(b)



(c)



(d)

Figure 4: (a) A retraction $F-d$ curve of a control MIN6 cell. (b) A retraction $F-d$ curve of a MIN6 cell treated with the anti-E-cad. The grey region is the integral of the continuous area under the baseline of complete separation, from which the energy of complete detachment can be determined. d_s can be determined by the difference of displacements between the points of maximum negative force value and that of complete separation. (c) The effects of anti-E-cad on the energy of detachment W_D (decreased by 11%, $p=0.053(>0.05)$). (d) The distance of complete separation decreased by 21% ($p<0.007$). Data are expressed as mean \pm SEM (>25 cells from 3 separate experiments), where key significances are shown, $**p<0.01$.