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Morphometry and Modeling of Label-Free Human Melanocytes and Melanoma Cells

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Abstract A combination of light-microscopy and image processing was applied to investigate morphology of label-free primary-melanocytes, and melanoma cells. A novel methodological approach based on morphology of nuclear body was used to find those single-cells, which were at the same phase of cell-cycle. The area and perimeter of melanocytes and melanoma cells were quantified. We found that there was a significant difference between area and perimeter of adendritic-shaped melanocytes with melanoma cells and the reason(s) of this finding was speculated. Finally, a theoretical model based on losing dendrites was proposed, which was in agreement with our experimental data.

Keywords Morphometry · cell shape · image processing · melanocyte · melanoma

1 Introduction

Melanoma is considered as a deadliest form of skin cancer, which develops from melanocyte lineage. Melanocytes are localized in the stratum basale layer of epidermis, the middle layer of eye, the inner ear, leptomeninges, bones, anogenital tract, and heart of humans [1, 2]. During epithelial to mesenchymal transition of a melanocyte

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to a melanoma cell, adhesion molecules (e.g., CD44, cadherin and integrin), and biochemical cascades (e.g., cadherin and integrin pathways) are changed [2–4]. Then, intracellular signaling pathways are regulated by autocrine and paracrine factors [5, 6], and a new cell-shape would be modulated by rearrangement of cytoskeleton [7, 8]. To study cytoskeleton and morphological features of melanocytes and melanoma cells, microscopic methods e.g., confocal scanning laser microscopy [9–12], atomic force microscopy [13–16], and holographic microscopy [17] are applied.

We selected the phase-contrast microscopic method, and directly addressed the question whether the difference between shape of a single-melanocyte, and a single-melanoma cell can be quantified or not. To answer this question, morphology of human-epithelial-melanoma cells, and primary-human-epithelial-melanocytes were investigated. Cells were prepared with minimum cell-manipulation (not labeling, not fixing, and not synchronizing), and experimented at an identical biophysical condition. Projected area and perimeter of melanoma cells, and phenotypes of melanocytes were quantified. A significant difference between perimeter and area along phenotypes of melanocytes and melanoma cells were found. Based on losing dendrites, “shape-transition” among phenotypes of melanocytes was modeled, which is in good agreement with our experimental results.

2 Material and Methods

2.1 Culturing of primary melanocytes and melanoma cells

Primary-human-epithelial-melanocytes were obtained during circumcision of an infant (4 years old, foreskin) with no known dermatological, genetic or other diseases. The sample was harvested in accordance with the ethical committee approval process of the Skin Research Center of Shahid Beheshti University of Medical Sciences and Health Services (in accordance with the Declaration of Helsinki Principles). The parents of the patient (child) gave their informed consent.

The skin specimen was washed by phosphate-buffered-saline (PBS) and sterilized distilled water and was kept in Dulbecco’s modified Eagle medium (DMEM), which was supplemented with 10% penicillin-streptomycin-fungizone solution (Gibco) for 24 h, at 4 °C. Next, the skin specimen was kept in 0.25% Trypsin-EDTA solution (Gibco) another 24 h, at 4 °C. Then, the skin specimen was washed by DMEM to stop effect of Trypsin-EDTA solution, and was cut into small strips. The final suspension was filtered through a 70 μ m pore size cell strainer (BD Falcon), and centrifuged at 180 $\times$ g for 5 minutes at 25 °C. The obtained cells were resuspend in medium 254 (M254, Gibco), which was supplemented with human melanocyte growth supplement (HMGS, Gibco) and seeded at density of $\simeq 5 \times 10^5$ cells per flask into a 25 cm² non-treated tissue culture flask (SPL Life Sciences) at 37 °C, in a humidified atmosphere containing 5% (vol/vol) CO₂.

HMGS contains phorbol 12-myristate 13-acetate (PMA), transferrin, hydrocortisone, insulin, bovine pituitary extract, basic fibroblast growth factor (bFGF). It is well understood [1, 2, 18] that both factors, absence of keratinocytes and presence of PMA, affect on number and length of dendrites in a melanocyte, while presence of

bFGF in HMGS increases tyrosinase activity. Having all together in a cell culture, a fine balance between melanocyte proliferation, and change in shape of melanocytes (differentiation) were resulted.

Around 24 h after plating, cells were attached to the flask. The primary cell culture flask was let to reach 70% confluency, while medium was changed twice each week. At room temperature, cells were trypsinized for few minutes, and centrifuged (2000 rpm for 4 min). The supernatant was resuspended in the growth medium (M254 and HMGS), and approximately counted by a hemocytometer. Cells at density of 1×10^5 per flask were plated into a 25 cm² non-treated tissue culture flask (SPL Life Sciences) at the same experimental condition and medium that primary cell culture was experienced. Medium was changed twice each week. About 4-6 h after third passages, cell attachment was checked by a microscope and imaging was started. Imaging was done at 0, 2, 7, 8, 9, 10, and 14 days after seeding of third passages.

In melanoma study, two kinds of melanoma cell-lines were used: human-epithelial-melanoma A-375, and murine-epithelial-melanoma B16-F10. Both human and murine melanoma cell-lines were purchased from the national cell bank of Iran (Pasteur Institute of Iran). Cells at density of 2×10^5 cells/cm² were seeded into a 25 cm² non-treated tissue culture flask (SPL Life Sciences) in DMEM, supplemented with 10% (vol/vol) fetal bovine serum (Gibco), 100 U ml⁻¹ of penicillin (Gibco), and 100 µg ml⁻¹ of streptomycin (Gibco). Cells were kept in an incubator at 37 °C in a humidified atmosphere containing 5% (vol/vol) CO₂ to reach 70% confluency. Then, replicate subcultures were prepared and frozen. For experiments (growth curve and imaging) each subculture was defrosted, and continuously passaged for 5 times. This provided us the replicates that were just multiple cultures of the same reference stock.

Growth-curves of melanoma cell-lines were prepared by dissociating cells with trypsin at 24 h, and 48 h after cell-seeding. The viable cell-number per unit area was counted without cell centrifuging with a hemocytometer (Trypan blue assay). Viable cell counting was done from three biological replicates (independent experiments), and data was expressed as mean $\pm$ standard error of mean (SEM).

To study transition of cells from suspended-state to adherent-state and also imaging of single-melanoma-cells, cells were dissociated by trypsin and at density of 2×10^5 cells/cm² were transferred into a 25 cm² non-treated tissue culture flask (SPL Life Sciences). Imaging of the cell culture flask was done within several hours after cell seeding (sub-confluent cell culture).

2.2 Imaging and image processing

Cell observation was done on an inverted microscope (Nikon, TS100) with CFI Achromat ADL 40 $\times$, 20 $\times$ and 10 $\times$ objectives. Images were captured by a digital-camera (Nikon, DS-L2) in the TIFF mode, consists of 1280 $\times$ 960 pixels (1 $\times$ 1 binning). By considering sensor size of the camera and magnification of the objective (40 $\times$), mapped size of one pixel was about 104 $\times$ 115 nm², which was measured by a Neubauer chamber.

Cell border was found by image processing and steps of image processing were explained in the Supplementary Information file. Nucleolus border detection was

Phenotypes	super-polydendritic shaped cells	polydendritic shaped cells	tridendritic shaped cells	bidendritic shaped cells	adendritic shaped cells
Image #	14	17	3	9	9
Single-cell #	14	17	3	14	14
Single-cell-G1 #	8	4	3	9	9

Table 1 Number of images and single-melanocytes. Number of selected images containing at least one single-cell (Image #), number of single-cells (Single-cell #), and number of single-melanocytes, which were at the G1-phase of cell-cycle (Single-cell-G1 #), based on phenotypes of super-polydendritic-shaped cells, polydendritic-shaped cells, tridendritic-shaped cells, bidendritic-shaped cells, and adendritic-shaped cells.

done by using code of Guberman *et al.* [19], which was based on choosing a specific binarization threshold in binarizing the original image. Image processing and data analysis were done by MATLAB (The MathWorks, Natick, MA).

2.3 Statistical Analysis

Statistical analysis of area and perimeter was done by MATLAB. Statistical significance was defined at p -value less than 0.01 using the one-way analysis of variance. The p -value was found much less than $\ll 0.0001$, which indicates that the data set is statistically highly significant in differences.

3 Results

3.1 Selecting single melanocytes

Primary melanocytes were isolated (see Methods) and the phase-contrast microscopic method was used to investigate morphology of melanocytes (Fig. 1-a,b). We captured images of melanocyte culture at 0, 2, 7, 8, 9, 10, and 14 days after seeding of third passages. By considering number of dendrites, phenotypes of melanocytes were categorized as super-polydendritic-shaped cell (Fig. 1-c), polydendritic-shaped cell with more than three arms (Fig. 1-d), tridendritic-shaped cell with three arms (Fig. 1-e), bidendritic-shaped cell with two arms (Fig. 1-f), and adendritic-shaped cells (Fig. 1-g). The super-polydendritic-shaped cell was named to a melanocyte, whose dendrites mostly started from other dendrites (ramification). This is unlike a polydendritic-shaped melanocyte, whose dendrites directly started from cytoplasm. It seemed likely that a super-polydendritic-shaped cell transited to a polydendritic-shaped cell, and then, either to a tridendritic-shaped cell, or to a bidendritic-shaped cell, and even directly to an adendritic-shaped cell.

From collection of images that were captured at 0 (9 images), 2 (11 images), 7 (76 images), 8 (77 images), 9 (15 images), 10 (20 images), and 14 (33 images) days after seeding of third passages, we selected those images that contained at least one "single-cell". The term of single-cell was used for a cell, which was not at any contact with other neighboring cells. Number of selected images, which contained at least one single-cell, and number of single-cells were reported in Table. 1.

3.2 Selecting single melanoma cells

To prepare single-melanoma cells, we needed to find proper time of imaging regarding the seeding time. We prepared growth curves (Fig. 2-a) of human-melanoma cells (A-375), and murine-melanoma cells (B16-F10), where murine-melanoma cells was used as a control sample. Using microscopy, the proper time that melanoma cells needed to transit from “suspended-state” to “adherent-state” was found. In every new cell culture flask (for new cell culture flask see Methods), and after dissociating using trypsin (or de-freezing), melanoma cells looked like spherical-shaped cells (Fig. 2-a-I). After time intervals of t_{sti} and t_{spr} , majority of cells were adherent and spread out, respectively (Fig. 2-a-II and III).

The time interval that a human-melanoma cell (A375) required to spread out after plating was quantified about $t_{\text{sti}} + t_{\text{spr}} \approx 4 - 4.5$ h. By choosing a proper cell-seeding density (see Methods) and imaging within $\tau = 2.5$ h after cells were fully spread out, chance of having neighboring cells (see neighboring cells in Fig. 2-a-IV inside the dashed circles) in a cell culture flask was decreased. Finally, 27 images of A-375 melanoma cells were captured, which contained at least one single-cell in each image.

3.3 Selecting single melanocytes and melanoma cells at early G1-phase

During the cell-cycle, shape, and size of a cell varies [20–24] and therefore, perimeter and area of those melanocytes and melanoma cells must be compared, which were at the same phase of cell-cycle. To prepare cells at the same phase of cell-cycle, we did not use routine methods of cell-cycle synchronization since treating a cell culture with serum-starvation, or other chemicals [25, 26] might affect on cell shape [22, 27].

Alternatively, the nucleolar-cycle (number and shape of nuclear bodies) was used to chose those cells, which were at the same phase of cell-cycle. In nucleolar biology, assembly of chromatin-associated nuclear bodies is regulated by concentration of different kinds of proteins together other factors like nuclear lamin, and possibly nuclear actin in nucleoplasm, when concentration of proteins is reached to a certain level [24]. It has been known that concentration of proteins in nucleoplasm varies during the G1-phase and size of a cell at the early-G1-phase is similar to the size of a cell at the end of mitotic-phase.

Hence, those single-cells (both melanocytes and melanoma cells) whose cell-cycle were at the early-G1-phase, were selected for data analysis. To do that, a cell (Fig. 3-bI) with more than two nuclear bodies (Fig. 3-bII) was assumed as a cell, which was at the early-G1-phase [20, 28]. Fig. 3-a shows the area of nuclear bodies based on phenotypes of melanocytes and melanoma cells. The point that should be noted here is, the reported values were belonged to those nuclear bodies that could be detected by image processing, which were not all nuclear bodies. Those nuclear bodies that were too close to each other were not separable in image processing, while they were still distinguishable by eye. Based on number of nuclear bodies, 33 single-

melanocytes and 10 single-melanoma cells were selected to compare their perimeter and area.

3.4 Morphometry of melanocytes and melanoma cells

After selecting single-melanocytes and single-melanoma cells (at the same phase of cell-cycle), border of cells were found (see Supplementary Information file), and perimeter, and projected area of cells were measured. As Fig. 4 shows, perimeter and projected area of melanocytes were decreased by losing dendrites, in the manner that adendritic-cells had the smallest perimeter and cell-area. Furthermore, area and perimeter of melanoma cells were significantly increased in comparison to adendritic-shaped melanocytes. This finding raises a question about probable factors that could cause this macro-scale difference between cytoskeleton (cytoskeleton is associated with cell-area) of melanoma cells and adendritic-shaped melanocytes.

The increase in area of melanoma cells relative to adendritic-shaped melanocytes could be because of increase in stress fibers of melanoma cells. It was found that Rho, which increases in melanoma cells promotes the pathway of actin polymerization, and also, the pathway of increasing activity of myosin II motors [4, 29]. Both pathways are resulted in an increase in stress fibers of melanoma cells in compare to melanocytes [4, 29]. The point that should be noted here is *in vitro* culturing of both melanoma cells and melanocytes might cause an increase in stress fibers because of the interaction between cells and stiff plastic. Another probable reason on why melanoma cells had a greater surface than adendritic-shaped melanocytes could be because of size of nucleus. It would be interesting in further studies to investigate more on size of nucleus and stress fibers in melanocytes and melanoma cells.

To understand what is going on in “shape-transition” among phenotypes of melanocytes and probable factors that could cause this macro-scale difference between cytoskeleton of melanoma cells and adendritic-shaped melanocytes, we developed a theoretical model based on losing dendrites.

3.5 Modeling of shape transition

To model variation in area and perimeter of phenotypes of melanocytes, we started from microtubules in a melanocyte. Number of microtubules in a melanocyte is summation over number of microtubules for carrying melanosomes, $N^{\text{Tub-Mel}}$, and number of microtubules for physicochemical process, $N^{\text{Tub-Gen}}$. Number of microtubules per dendrite is different along a dendrite and only few microtubules rich tip of a dendrite. Those parts of a dendrite that has a larger width η , contains more microtubules, and by getting toward tip, width of a dendrite becomes narrower and number of microtubules per local area decreases. Therefore, let us assume cell area, A , is proportion to the number of microtubules as:

$$(N^{\text{Tub-Mel}} + N^{\text{Tub-Gen}}) \propto A. \quad (1)$$

Based on our observation, shape-transition was started mainly from a super-polydendritic-shaped cell. Therefore, change in cell area can be normalized to the area of a super-polydendritic-shaped melanocyte, A_{Sup} , as:

$$\frac{A_i}{A_{\text{Sup}}} = \frac{N_i^{\text{Tub-Mel}} + N^{\text{Tub-Gen}}}{N_{\text{Sup}}^{\text{Tub-Mel}} + N^{\text{Tub-Gen}}}, \quad (2)$$

where A_i is the area of a melanocyte and i denotes one of the phenotypes of melanocytes such as: super-polydendrite cell, polydendrite cell, tridendrite cell, bidendrite cell, and adendrite cell.

During shape-transition from a super-polydendritic-shaped cell to one of the mentioned phenotypes (polydendritic-shaped cell, tridendritic-shaped cell, bidendritic-shaped cell, and adendritic-shaped cell), relative change in area of a melanocyte is obtained through:

$$\frac{\Delta A_{\text{Sup} \rightarrow i}}{A_{\text{Sup}}} = \frac{\Delta N_{\text{Sup} \rightarrow i}^{\text{Tub-Mel}}}{N_{\text{Sup}}^{\text{Tub-Mel}} + N^{\text{Tub-Gen}}}, \quad (3)$$

where, $\Delta A_{\text{Sup} \rightarrow i}$, and $\Delta N_{\text{Sup} \rightarrow i}^{\text{Tub-Mel}}$ are relative change in cell area, and number of microtubules, which were lost during shape-transition, respectively.

Next, we assume perimeter of a melanocyte, P , is merely equal to the perimeter of cortical-actin network, which is uniformly (roughly) distributed between a microtubule, and plasma membrane, along cell periphery. This assumption is supported by F-actin filaments staining in a melanocyte [30, 31].

We defined a building block between a microtubule, and the corresponded plasma membrane with length ℓ^{Act} , along side of plasma membrane in a dendrite, as Fig. 4-c shows. Based on definition of the building block, perimeter of a melanocyte would be proportion to the number of building blocks N_i^{Act} , and the length-scale of ℓ^{Act} (see Fig. 4-c) as:

$$P_i \approx N_i^{\text{Act}} \ell^{\text{Act}}, \quad (4)$$

where P_i is perimeter of a melanocyte and i denotes one of the phenotypes of melanocytes (super-polydendrite cell, polydendrite cell, tridendrite cell, bidendrite cell, and adendrite cell).

By normalizing perimeter of a melanocyte to the perimeter of a super-polydendritic-shaped melanocyte P_{Sup} , relative change in perimeter of a melanocyte (during shape-transition) is obtained through:

$$\frac{\Delta P_{\text{Sup} \rightarrow i}}{P_{\text{Sup}}} = \frac{\Delta N_{\text{Sup} \rightarrow i}^{\text{Act}}}{N_{\text{Sup}}^{\text{Act}}}, \quad (5)$$

where, $\Delta P_{\text{Sup} \rightarrow i}$, and $\Delta N_{\text{Sup} \rightarrow i}^{\text{Act}}$ are the relative change in perimeter, and the number of F-actin-blocks (cortical-actin-blocks that were scaled with the length of ℓ^{Act} along cell membrane), which were lost during shape-transition from a super-polydendritic-shaped cell to one of the mentioned phenotypes of melanocytes (polydendritic-shaped cell, tridendritic-shaped cell, bidendritic-shaped cell, and adendritic-shaped cell).

We can approximate the perimeter and area of a single dendrite as $2\Delta N^{\text{Act}} \ell^{\text{Act}}$, and $\eta \Delta N^{\text{Act}} \ell^{\text{Act}}$, where η and ΔN^{Act} are width, and number of F-actin-blocks with length ℓ^{Act} . From the other side, number of microtubules in a single dendrite $\Delta N^{\text{Tub-Mel}}$, is proportion to the area of a single dendrite, which is $\eta \Delta N^{\text{Act}} \ell^{\text{Act}}$. By defining σ as a number of microtubules per area of a dendrite then, $\Delta N^{\text{Tub-Mel}} = \frac{1}{\kappa} \Delta N^{\text{Act}}$, where κ is $1/\eta \sigma \ell^{\text{Act}}$. Rearranging Eq. (3) and Eq. (5), we get a prediction for change in perimeter of phenotypes of melanocytes (during shape-transition) as:

$$\frac{\Delta P_{\text{Sup} \rightarrow i}}{P_{\text{Sup}}} = \kappa \left[\frac{N_{\text{Sup}}^{\text{Tub-Mel}} + N_{\text{Sup}}^{\text{Tub-Gen}}}{N_{\text{Sup}}^{\text{Act}}} \right] \frac{\Delta A_{\text{Sup} \rightarrow i}}{A_{\text{Sup}}}. \quad (6)$$

Therefore, a plot of $\frac{\Delta P_{\text{Sup} \rightarrow i}}{P_{\text{Sup}}}$ vs. $\frac{\Delta A_{\text{Sup} \rightarrow i}}{A_{\text{Sup}}}$ should give a straight line, whose slope is proportional to $N_{\text{Sup}}^{\text{Tub-Mel}}, N_{\text{Sup}}^{\text{Act}}, N_{\text{Sup}}^{\text{Tub-Gen}}$ and κ . We plotted our experimental data and as Fig. 4-d shows, shape-transition in phenotypes of melanocytes consists with Eq. (6). It is likely that, regardless of its phenotype, number of microtubules to cortical-actin-blocks that were lost during losing dendrites is a constant value. One could go further and quantify this constant by staining cortical-actin network, and microtubules in further experiments.

4 Conclusion

We have directly addressed the question whether the difference between morphology of melanocytes and melanoma cells can be quantified or not? To answer this question, we prepared single-human-epithelial-melanoma cells, and single-primary-human-epithelial melanocytes. The nucleolar-cycle was used to select cells, which were at the early-G1-phase. Then, we compared area and perimeter of melanoma cells with phenotypes of melanocytes.

To our knowledge, this is the first single-cell quantification of geometrical features of primary-melanocytes and melanoma cells. Naturity, and simplicity in single-cell-preparation, and single-cell-selection are advantages of the proposed methods.

The approaches outlined here could provide a reasonable starting point for those who wish to initiate time-laps studies of shape-transition in melanocytes. Furthermore, in recent years, investigation on the effect of microenvironment (2D or 3D) on biochemical cascades of malignant cells at both stages of tumor initiation and tumor progression, have been increased. It would be interesting if one could repeat this study on different physical (softness/hardness) substrate, or biochemical substrate. Our approach has a potential application to be used in distinguishing of melanoma cells from melanocytes during confocal reflectance microscopy examination.

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Author contributions

BSG and MMR carried out lab work, partly participated in data analysis. MMR provided medical support from the Skin Research Center of Shahid Beheshti University of Medical Sciences and Health Services for the epidermal sample. ST carried out image processing, statistical analysis, and modeling. HNM and ST conducted the study. HNM supervised and provided the financial support for the conduct. The manuscript was drafted by ST with help of others. All authors gave the final approval for publication. ST and BSG contributed equally to this work.

Declaration of Competing Interest

The authors declare no competing financial, and non-financial interests.

Data Availability

The authors declare that all the data and MATLAB code supporting the findings of this study are available within the article and its supplementary information, or upon request from the corresponding author.

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Fig. 1 Phenotypes of melanocytes. a-b) Images of primary-human-epithelial-melanocyte culture 7 days after seeding ($10\times$). c) A super-polydendritic-shaped cell 2 days after seeding ($20\times$). d) A polydendritic-shaped cell several hours after seeding. e) A tridendritic-shaped cell 8 days after seeding. f) A bidendritic-shaped cell 7 days after seeding and g) an adendritic-shaped cell 8 days after seeding. d-g) Images were captured by a $40\times$ objective. Scale bar = $10\text{ }\mu\text{m}$.

Fig. 2 Growth curve of melanoma cells. a) Growth curve of human-epithelial-melanoma cell culture (A-375), and murine-epithelial-melanoma cell culture (B16-F10) during 48 h. Inset: a-I) An adhered melanoma cell. a-II) and a-III) Melanoma cells, which were fully spread out. a-IV) Two couples of melanoma cells, which were highlighted by dashed circles. t_{sti} and t_{spr} are the time intervals that melanoma cells need to stick and spread. Imaging was done during the time interval of τ . Data were expressed as mean $\pm$ SEM of three replicates. All images were taken of A-375 cell line and scale bar indicates $10\text{ }\mu\text{m}$.

Fig. 3 Nuclear body's area of melanocytes and melanoma cells. a) Nuclear body's area of phenotypes of primary-melanocytes and melanoma cells (A-375), where SP, P, T, B, A and M, denote the phenotypes of super-polydendritic-shaped cell, polydendritic-shaped cell, tridendritic-shaped cell, bidendritic-shaped cell and adendritic-shaped cell, and melanoma cell, respectively. Inset: b-I) A single-melanoma cell (A-375). Scale bar = $10\text{ }\mu\text{m}$. b-II) A nuclear body of a melanoma cell. Scale bar = $1\text{ }\mu\text{m}$.

Fig. 4 Perimeter and area of melanocytes, and melanoma cells. a) Perimeter and b) area of phenotypes of primary-human-epithelial-melanocytes, and human-epithelial-melanoma cells (A-375), where SP, P, T, B, A and M, denote the phenotypes of super-polydendritic-shaped cell, polydendritic-shaped cell, tridendritic-shaped cell, bidendritic-shaped cell and adendritic-shaped cell, and melanoma cell, respectively. c) A polydendritic-shaped melanocyte several hours after seeding. Inset: The length-scale ℓ^{Act} along the plasma membrane, and η is the thickness of dendrite. d) The relative change in perimeter $\frac{\Delta P_{\text{Sup} \rightarrow i}}{P_{\text{Sup}}}$ of cells (data of panel a) based on the relative change in area $\frac{\Delta A_{\text{Sup} \rightarrow i}}{A_{\text{Sup}}}$ of the same cells (data of panel b). Data were expressed as mean $\pm$ standard deviation of data.