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Profiling TRA-1-81 antigen distribution on a human embryonic stem cell

Dengli Qiu,

Department of Biological, Chemical and Physical Sciences, Illinois Institute of Technology, Chicago, IL 60616

Jialing Xiang,

Department of Biological, Chemical and Physical Sciences, Illinois Institute of Technology, Chicago, IL 60616

Zhaoxia Li,

Department of Biological, Chemical and Physical Sciences, Illinois Institute of Technology, Chicago, IL 60616

Aparna Krishnamoorthy,

Department of Biological, Chemical and Physical Sciences, Illinois Institute of Technology, Chicago, IL 60616

Liaohai Chen, and

Argonne National Laboratory, Biosciences Division, Lemont, IL 60439

Rong Wang*

Department of Biological, Chemical and Physical Sciences, Illinois Institute of Technology, Chicago, IL 60616

Abstract

Human embryonic stem (hES) cells hold great promise in regenerative medicine. Although hES cells have unlimited self-renewal potential, they tend to differentiate spontaneously in culture. TRA-1-81 is a biomarker of undifferentiated hES cells. Quantitative characterization of TRA-1-81 expression level in a single cell helps capture the “turn-on” signal and understand the mechanism of early differentiation. Here we report on our examination of TRA-1-81 distribution and association on a hES cell membrane using an atomic force microscope (AFM). Our results suggest that aggregated distribution of TRA-1-81 antigen is characteristic for undifferentiated hES cells. We also evaluated the TRA-1-81 expression level at ~17800 epitopes and ~700 epitopes per cell on an undifferentiated cell and a spontaneously differentiated cell, respectively. The method in this study can be adapted in examining other surface proteins on various cell types, thus providing a general tool for investigating protein distribution and association at the single cell level.

Keywords

Human embryonic stem cell; TRA-1-81; affinity mapping; protein distribution

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*Corresponding author. Fax: 312-567-3494; e-mail: E-mail: wangr@iit.edu (R. Wang).

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Introduction

Human embryonic stem (hES) cells play central roles in tissue engineering, drug discovery and regenerative medicine, thus hold great promise for cures of many hard-to-treat diseases, such as spinal cord injury, stroke, heart diseases and diabetes due to their pluripotency [1,2]. Although hES cells have unlimited self-renewal potential, they tend to differentiate spontaneously in culture. Understanding the molecular mechanism and identifying factors that inhibit the differentiation at the early stage is essential for maintaining well-defined populations of undifferentiated stem cells. Successful identification of cell population in early differentiation was achieved by statistical measurements of biomarker protein expression from a collective number of cells [3–5]. Since cells behave differently, heterogeneity exists in both cell cycle and population even within the same colony. It is imperative to quantify protein expression level and to characterize protein distribution and association at the single cell level. In this work, we report on an atomic force microscopic (AFM) approach which offers the high resolution power for such studies.

Typical marker proteins of undifferentiated hES cells include Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-81 and TRA-1-60 [6,7]. Among them, TRA-1-81 antigen is a keratan sulfate proteoglycan [6,8], which is expressed in undifferentiated cells and dramatically down-regulated in differentiated cells. It was suggested that TRA-1-81 and TRA-1-60 may react with the same protein which has sialidase-sensitive epitopes [9]. The function of TRA-1-81 is uncertain, however, there is no doubt that the expression and distribution of TRA-1-81 are closely regulated during early development. Resolving the distribution and association of TRA-1-81 on a hES cell membrane will not only provide the essence of understanding the protein function, but also offer the opportunity of sorting individual undifferentiated cells from initially differentiated cells.

AFM has become a powerful tool in biological research. Besides the capability of high resolution topographic imaging, a singular advantage of AFM is the ability of identifying protein species via specific interactions. This principle has been adapted in studies of biomimic surfaces and living cells [10–14]. Here we apply this method in revealing the distribution and association of TRA-1-81 on undifferentiated and differentiated hES cells at the single cell level. The work laid the foundation for investigating the dynamic behavior of hES cells at the initial state of differentiation. The same methodology can also be applied in identifying additional surface proteins on progenitor cells, which remains to be a great challenge and an urgent need in advancing stem cell biology.

Materials and Methods

hES cell culture

Human embryonic stem cell line H9 was from WiCell (Madison, WI). Cells were routinely cultured on feeder cells derived from mitotically inactivated mouse embryo fibroblast (MEFs). Cells were maintained in DMEM/F12 (Mediatech, Herndon, VA), containing 20% knockout serum replacement, 1% nonessential amino acid, 1 mM l-glutamine, 100 μ M beta-mecaptoethanol, and 4 ng/ml bFGF (Invitrogen, Carlsbad, CA). The media were changed everyday and cells were passaged every 5 days. All cultures were monitored with hES cell undifferentiated markers staining against SSEA-3, SSEA-4, TRA-1-81 and TRA-1-60, as well as RT-PCR for Oct-4 and Nanog. Alkaline phosphatase staining kit, antibodies against TRA-1-81, TRA-1-60, and SSEA-3 were purchased from Chemicon (Temecula, CA). To prepare spontaneously differentiated cells, the hES cells were trypsinized to obtain single-cell suspension, then directly passaged onto culture dish containing DMEM medium supplemented

with 10% Fetal Bovine Serum (Hyclone, Logan, UT) and cultured for 7 days prior to experimentations.

Preparation of the cell samples for AFM analysis

Sterilized plastic slides ($1 \times 1 \text{ cm}^2$), cut out from cell culture dishes, were coated with 0.1% gelatin overnight in a 6-well plate, then incubated with Matrigel (BD Biosciences, Franklin Lakes, NJ) for 2 hours at 37°C . To culture hES cells on the slides, individual hES cell colonies with typical undifferentiated morphology were manually picked up from the feeder layer by thin-tip pipette under a microscope, and re-plated onto Matrigel coated slides for feeder-free culture with MEFs-conditioned medium plus freshly added 4 ng/ml bFGF.

Immunofluorescent staining

hES cells were grown on feeder-free plastic slides in conditioned medium. The cells were fixed and permeabilized with pre-cold methanol and washed with PBS, then incubated with primary monoclonal antibody against TRA-1-81 (1:40) for one hour at room temperature, followed by the incubation with Alexa Fluor 488 fluorescent conjugated second antibody for another hour. The nuclei were stained with Propidium Iodide (PI) and visualized with a Nikon TE-U 2000 fluorescence microscope.

AFM imaging

After 48 hours feeder-free culture on a pre-cut $1 \times 1 \text{ }\mu\text{m}^2$ plastic slide, the cells were rinsed with PBS buffer for two times. Topographic imaging was performed on live cells, and affinity mapping was performed on cells fixed by cold methanol for 7 min. PBS buffer (pH=7.4) was used as the medium in both experiments.

All AFM measurements were carried out at room temperature using a multimode Nanoscope IIIa AFM (Veeco Metrology, Santa Barbara, CA), equipped with a J-scanner in a commercially available fluid cell. Si_3N_4 tips at a thermal resonance frequency of 8–10 kHz were used in the studies. The spring constant of the tips was $0.07 \pm 0.01 \text{ N/m}$, as calibrated by using reference cantilevers with known spring constants [15].

The AFM was operated in fluid tapping mode for topographic imaging. Affinity maps were collected in fluid contact mode using a functionalized tip. The tip functionalization was the same as we routinely performed in the lab [16,17], in which a cross linker N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, purchased from Molecular Probes, Eugene, OR) was used to conjugate the antibody against TRA-1-81 to a gold coated tip. When the tip scanned on a cell surface, a force spectrum was recorded at each pixel upon the tip approaching and retraction. A strong adhesion peak appeared in the retraction curve, quantitatively revealing the unbinding of antigen-antibody specific interaction. A total of $32 \times 32 = 1024$ force curves were collected (regardless of the scan size) to construct a force volume (FV) image. Thus the FV image (so-called affinity map) illustrates the distribution of TRA-1-81 on the cell membrane. The z-scan rate of the measurements was 1.33 Hz, and the z-ramp size was typically 2 μm .

Results

Immuno-fluorescence analysis of hES cells

Undifferentiated hES cells usually form round-shaped colonies with clear boundaries. In the preparation of undifferentiated cells for AFM study, we manually picked up such colonies from feeder layer supported culture, as shown in Figure 1A. The hES colonies were gently broken into small clumps containing about 10 to 50 cells and seeded onto a Matrigel coated plastic slide. Cells were cultured in the MEFs-conditioned medium for 36 to 48 hours to attach slides

as a small group. This allows us to analyze a desired hES colony with minimal contamination of MEFs cells. The slide samples were prepared in duplicates, one set for AFM analysis, and the other set for biological characterization, such as immunofluorescent staining with the undifferentiated hES cell surface markers or Alkaline phosphatase staining.

Figures 1B and C show the immuno-fluorescent staining of hES cells (48 hours after re-seeding) with the antibody against TRA-1-81. Almost all cells in the re-seeded clumps were strongly positive for TRA-1-81, indicating that the hES cells from this “pick up and re-seed” procedure well maintained their undifferentiated characters within the experiment time setting (48 hours). The cells were then cultured under the conditions for spontaneous differentiation. We observed the gradual drop of fluorescence intensity of TRA-1-81 in the subsequent days. At day 7, only a small trace of TRA-1-81 fluorescence was detectable, and most cells changed remarkably in geometry (shown in Figs 1E–G), indicating that they were committed to differentiated cells. While the fluorescence imaging data are qualitative, they provide a good reference for quantitative analysis of TRA-1-81 by AFM at the single cell level.

Topographic imaging of hES cells

Cell samples similarly prepared as shown in Fig. 1A were studied by AFM to resolve features on individual cells. Figure 2A shows a $50 \times 50 \mu\text{m}^2$ AFM image, illustrating a small group of eight live cells. These cells are spherical in shape, with a dimension of 10–20 μm in diameter and $2.5 \pm 0.3 \mu\text{m}$ in height, representative of undifferentiated cells as reported by others [18]. When imaging an individual cell (Fig. 2B), we found that the cell surface was rather rough, with a roughness of $\sim 200 \text{ nm}$.

The cell morphology changed dramatically after 7 days spontaneous differentiation. Figure 2F shows an optical phase contrast image of differentiated cells showing variable cell geometry. Such cells were also individually examined by the AFM as shown in Figs 2C–E. Majority of the cells were deviated from the spherical shape, well spread on a substrate, were much greater in dimension, and were frequently found as individual cells away from cell clusters. Occasionally, we found spherical cells (Fig. 2D) even after 7 days spontaneous differentiation. While majority of the spherical cells barely expressed TRA-1-81 antigen according to immuno-fluorescent staining, to our surprise, some spherical cells expressed the antigen at a rather high level (data not shown). Thus topographic characterization alone is not sufficient to determine the status of a hES cell.

Affinity mapping against TRA-1-81 antigen on hES cells

Identification of the status of a hES cell relies on the quantification of marker protein expression level. Such measurement was carried out by affinity mapping (FV imaging). As illustrated in Fig. 3A, affinity mapping utilizes the antigen-antibody specific interaction to probe desired protein species on a cell membrane, thus to address the protein distribution [19]. In our study of TRA-1-81 expression, tips modified with its antibody were used to raster scan on cell membranes.

Figure 3B is an $8 \times 8 \mu\text{m}^2$ affinity map collected on an undifferentiated hES cell. Figure 3E shows three typical force curves collected at the arrow-pointed pixels in Fig. 3B. The dark (M), brown (N) and bright (P) pixels correlate to high (532 pN), medium (160 pN) and weak ($< 5 \text{ pN}$) adhesion forces, respectively. Thus the bright-dark contrast in a FV image highlights the high affinity binding sites. For representative comparison, we also purposely examined spherical cells after seven days differentiation, as shown in Fig. 3C. In the control experiments, we carried out similar measurements on PC3 cells, on which TRA-1-81 antigen is absent, and on undifferentiated cells which were pre-incubated with the antibody against TRA-1-81 to abrogate the specific interaction on the cell surface. A total of 1024 force curves were collected

on each $8 \times 8 \mu\text{m}^2$ affinity map, and the results derived from multiple cells (two to three cells prepared separately) were summarized in the histogram (Fig. 3D) to present a statistic measure. On undifferentiated hES cells, 59% of the adhesion forces are less than 80 pN; we frequently observed forces higher than 80 pN, and forces as high as 580 pN were observed. In contrast, more than 96% of adhesion forces are less than 80 pN on PC3 cells, and a significant drop of the force occurrence at more than 80 pN is evident. Due to the lack of TRA-1-81 antigen on a PC3 cell, the non-specific adhesion forces are attributed to tip convolution, cell surface roughness, and the weak interaction of the antibody with other cell surface species. These non-specific interactions are common on all cell samples. Thus we evaluate 80 pN as the non-specific interaction level in our measurements at the $8 \times 8 \mu\text{m}^2$ scan range. Typical specific interaction between a single pair of antigen-antibody is 90 to 130 pN [20]. The non-specific interaction force as high as 80 pN may impede the acquisition of single-pair specific interactions. Reduced non-specific interaction level was achieved on smaller scale affinity maps, as delineated below, for more accurate measurement of specific interactions.

The dark contrast at the highlighted area in Fig. 3B indicates high local concentration of TRA-1-81. To further resolve TRA-1-81 distribution and association, we sequentially collected affinity maps at the highlighted region at scales of $4 \times 4 \mu\text{m}^2$ to $500 \times 500 \text{ nm}^2$. Figure 4A shows a $2 \times 2 \mu\text{m}^2$ affinity map, and the corresponding histogram of adhesion force distribution is shown in Fig. 4B. A negligible number of adhesion forces were observed in the range of 30–50 pN, followed by a sharp increase of force occurrence at more than 60 pN. Thus the non-specific interaction level of a $2 \times 2 \mu\text{m}^2$ affinity map is estimated at less than 30 pN. A much less non-specific interaction level was concluded from the $500 \times 500 \text{ nm}^2$ affinity map. According to the height images, cell surface is smoother at smaller scales. We speculate that the relatively smooth surface at the small area of a cell and the lack of other cell surface species at the selected area greatly diminished the level of non-specific interaction.

Since the $2 \times 2 \mu\text{m}^2$ affinity map has a proper size to include more representative protein distribution information, we choose this affinity map for quantitative analysis. By Gaussian fitting [21], we identified five peaks in the histogram, corresponding to 104 pN, 186 pN, 285 pN, 393 pN and 520 pN, respectively, which appear to be integer multiples of a quanta unit $100 \pm 10 \text{ pN}$. We estimate that $100 \pm 10 \text{ pN}$ is the force of a single-pair antigen-antibody interaction, in consistent with the results from literatures [22]. From the histogram in Fig. 4B, multiple-pair interactions (corresponding to the formation of two, three, four and five specific bonds) are more frequently observed than single-pair interactions. This will be further analyzed in the Discussion section.

Discussion

The TRA-1-81 expression level was quantified with the analysis below. According to the SEM images of a gold-coated probe (radius of tip apex is 35 nm), and according to the dimensions of a cross linker SPDP (~0.7 nm in length) and an antibody (IgM, 24 nm in length) [23], the diameter of the tip contact area is ~120 nm, which correlates to approximate one pixel (125 nm) in a $4 \times 4 \mu\text{m}^2$ affinity map. The antibody modified tip scans pixel by pixel to achieve an affinity map. When the tip dimension is less or greater than the pixel size, some surface proteins may be either ignored or be addressed for multiple times. Since the tip dimension and the pixel size are approximately the same in a $4 \times 4 \mu\text{m}^2$ affinity map, a $4 \times 4 \mu\text{m}^2$ affinity map suites our purpose of analyzing the protein distribution. Based on the $100 \pm 10 \text{ pN}$ force between a single pair of antigen-antibody, a total of 676 epitopes of TRA-1-81 were estimated at the $4 \times 4 \mu\text{m}^2$ area according to the corresponding affinity map. The studied cell has an estimated surface area of $\sim 420 \mu\text{m}^2$. Because the $4 \times 4 \mu\text{m}^2$ area is a selected area with higher TRA-1-81 density, we estimate the total number of TRA-1-81 epitopes on this cell is no more than 17800. A similar estimation was made on a differentiated cell, on which the total number of TRA-1-81

is less than 700 epitopes. This estimation is rather rough. However, it provides the basis of a more accurate and achievable method, in which multiple $4 \times 4 \mu\text{m}^2$ regions of a cell are scanned individually to quantify the total number of epitopes per cell.

It should be noted that TRA-1-81 expression level differs remarkably on distinct undifferentiated cells. While the high expression level of TRA-1-81 was popular in cells in a “healthy” colony, cells with significantly lower level of TRA-1-81 were observed occasionally within the same colony. This observation coincides with the observation by Stewart et al. [5]. In their report, both SSEA-3 positive and SSEA-3 negative cells were identified by screening and isolating single cells within an undifferentiated cell colony using flow cytometry. Both cell types were found to initiate pluripotent hES cell cultures, however, they possessed distinct cell-cycle properties, clonogenic capacity and expression of ES cell transcription factors. It was suggested that the co-existence of the two cell types within a colony be necessary in coordinating the self-renewal of undifferentiated cells [5]. In a separate paper [4], Mantel et al. also reported their observation of a small population of TRA-1-60 negative cells in an undifferentiated cell colony. Accordingly, we speculate that the wide-range variation of TRA-1-81 expression level within an undifferentiated cell colony can be a similar requirement for hES cell self-renewal. It should be noted that, while the flow cytometry analysis is effective in screening and sorting single cells, bias might be introduced when cells were dissected from a colony. The AFM approach can be a complementary method in such studies in unveiling cell populations in their native states.

We observed from the affinity maps (Fig. 3B, 3C, Fig. 4A) that TRA-1-81 antigens frequently localized as aggregates, showing heterogeneous distribution on undifferentiated cells. These are in contrast with the random distribution of the occasionally observed antigens on differentiated cells. With considerations of the tip and the antibody dimensions, a maximum of seven antibodies can anchor on a tip. If the antigens are closely associated on the cell membrane, forces of multiple interactions can be collected. The histograms allow quantitative assessments of the heterogeneous distribution pattern of TRA-1-81. Unbinding forces of 1, 2, 3, 4 and 5 pairs of specific interaction are evident in Fig. 4B. Based on the fitting, 34.9% of TRA-1-81 antigens exist as single proteins, whereas 38.2% of the antigens co-localize in pair, and the rest 26.9% co-localize in larger groups.

Similar heterogeneous distribution of TRA-1-81 antigen is evident in the histograms from $8 \times 8 \mu\text{m}^2$ affinity maps of undifferentiated hES cells, regardless of their pre-incubation with the antibody against TRA-1-81 (Fig. 3D). However, TRA-1-81 antigen aggregation was not observed in differentiated hES cells, neither in the affinity map (Fig. 3C) nor in the histogram (Fig. 3D). It suggests that the aggregated distribution of TRA-1-81 antigens is characteristic in undifferentiated hES cells, and the function of TRA-1-81 may require such close protein association. The heterogeneous distribution pattern of TRA-1-81 may be used as a physical marker to identify the hES cell status.

In conclusion, we examined the difference in protein expression level between undifferentiated cells and cells after seven days differentiation. To probe the initial state of differentiation, time-dependent investigation of cells along the path of differentiation with the established method is under the way. The method developed here is applicable to the study of any cell surface protein. The information derived from such studies can help us correlate the protein distribution with protein function and cell status. While profiling an individual surface protein at the single cell level is important, collective information from profiling an array of surface proteins will significantly impact the mechanistic understanding of hES cell differentiation, which will help establish strategies for preventing spontaneous differentiation and advancing the directed differentiation to specific cell lineage.

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Figure 1.

A) Scheme of preparation of undifferentiated hES cells for AFM studies; B,C,D) Immunofluorescent staining of undifferentiated cells with the antibody against TRA-1-81 (B), nucleus staining with PI (C), and image overlap (D); E,F) Immunofluorescent staining of spontaneously differentiated hES cells with TRA-1-81 (E), PI staining (F); G) phase contrast optical image of the same field as (E) (F).

Figure 2.

AFM images of hES live cells. A) A $50 \times 50 \mu\text{m}^2$ image of an undifferentiated cell colony; B) A $20 \times 20 \mu\text{m}^2$ image of an undifferentiated cell picked from a cell colony shown in A; C,D,E) Images of various spontaneously differentiated single cells at day 7; F) A large scale optical image of differentiated cells. Arrow-pointed are the types of cells imaged in C-E. Scale bars: (A, C, E) $10 \mu\text{m}$, (B) $4 \mu\text{m}$, (D) $3.2 \mu\text{m}$.

Figure 3.

A) Scheme of identifying TRA-1-81 antigen on a cell membrane using an antibody-modified AFM tip; B and C) $8 \times 8 \mu\text{m}^2$ affinity maps of an undifferentiated (B) and a differentiated (C) hES cell; D) Probability histogram of unbinding forces from $8 \times 8 \mu\text{m}^2$ affinity maps of undifferentiated hES cells (red), undifferentiated hES cells after pre-incubation with antibody against TRA-1-81 (green), differentiated hES cells at day 7 (blue) and PC3 cells (cyan); E) Retraction curves collected at the pixels as marked in (B).

Figure 4.

A) A $2 \times 2 \mu\text{m}^2$ affinity map of the undifferentiated hES cell at the highlighted area in Fig. 3B; B) Probability histogram of unbinding forces from all 1024 data points in (A). The histogram is fitted with a Gaussian.