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Mechanical Characterization of Mouse Embryonic Stem Cells

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Abstract — **Current cell detection techniques are antibody staining of specific protein markers, morphometric parameters and transgenic markers. These assays are often qualitative and do not quantitatively define the outcome of a cell progression during differentiation. Consequently, we propose to characterize the mechanical behavior of embryonic stem cell, which will predict its stage of differentiation during lineage differentiation. Using the atomic force microscope, we have performed several experiments on mouse embryonic stem cells (mESC) roughly 7 – 17 μm in diameter and height at the interphase stage of the cell cycle process. Specifically, we conducted single indentation studies on undifferentiated and early differentiating (6 days under differentiation conditions) mESC with a cell indentation range of 2 – 2.5 μm. The data was used to analyze various contact models that can accurately model the geometry of the AFM tip and mESC interaction. With the choice of appropriate contact model, we can determine the accurate modulus of the cell membrane. The experimental results confirmed our research hypothesis that the mechanical property of undifferentiated mESC is different from differentiating (6th day) mESC.**

I. Introduction

Stem cells have the unique capability to differentiate into specialized cell types leading to effective regenerative therapies [1]. Human embryonic stem cells (hESC) have strong potential for therapeutic use in treatment of disease (e.g., heart disease, Parkinson's and spinal cord injuries). Conventionally, differentiating precursor and differentiated cells are distinguished from undifferentiated stem cells by: (a) antibody staining of specific protein markers [2]. This method is time consuming and expensive, (b) morphometric parameters (cell shape/structure), which aid in detecting terminally differentiated cells but not precursor cells [3], and (c) transgenic markers involve cell specific promoters which control the expression of marker proteins. This method requires genetic modification of cells [4, 5]. Thus, generation of pure populations of defined cell types remains a challenge for stem cell biologists. For clinical applications, efficient cell characterization is needed as ESC (both human and mouse) consist of mixed population of cells (differentiated and undifferentiated). Thus, we propose to quantify the mechanical behavior of stem cells as they differentiate into different cell types, such as cardiac cells, neuronal cells, etc.

Atomic force microscope (AFM) is a promising tool to measure forces in nN - pN range [6]. It has been used extensively to study the mechanical properties of biological materials [7-9]. Many researchers [10-13] have used Hertz model to characterize the mechanical property of cells using AFM. Originally, Hertz [14] solved the problem of contact between two spheres. JKR and DMT models [15] take into account the adhesion between the two elastic spheres. These theories predicted the mechanical behavior of cells characterized by significant adhesion force [16, 17]. Apart from solid models, Lulevich et al [18] proposed capsule model for estimating the mechanical behavior of cells indented by AFM.

Force modulation microscopy (FMM) detected the variations in mechanical properties of human mesenchymal stem cells (MSCs) [19]. However, FMM imaging technique is not quantitative. It can detect only relative and qualitative elastic modulus differences between different cell surfaces. To our knowledge, there had been no studies to predict whether there exists any difference in mechanical behavior of mouse embryonic stem cells (mESC) at various stages of differentiation towards a particular cell lineage. Hence, we propose to conduct studies on undifferentiated and early differentiating (6 days under differentiation conditions) mESC using atomic force microscopy (AFM) system. We have evaluated the solid as well as capsule models and determined the appropriate model to characterize the cell stiffness.

II. Materials and Methods

A. Experimental set up

The Atomic Force Microscope (MFP-3D-BIO^{TM}, Asylum Research) system consists of a scan head integrated with a phase contrast module and an inverted microscope (Model: TE2000U, Nikon, Inc). The entire set up was mounted on an active vibration isolation table manufactured by Herzan (see figure 1). The phase contrast module enables imaging low contrast, transparent cells in fluid. XY stage (manual) allows the user to position the cell beneath the cantilever tip of AFM. The AFM set up was enclosed in an acoustic isolation chamber to prevent acoustic noise from interfering with the AFM measurements. The x and y-axes range of the scan head is 90 μm. The z-axis scan range is 40 μm. The AFM also has the capability to measure forces in the range of pN-nN. A silicon nitride (k = 0.06 N/m, Novascan Technologies, Inc) and a silicon cantilever (k = 1.75 N/m, Novascan Technologies, Inc) with a spherical probe (5 μm in diameter)

was used to indent live and fixed mESC respectively. The AFM system was used to obtain force and cell indentation data from biological samples. The mathematical details are described in [20, 21].

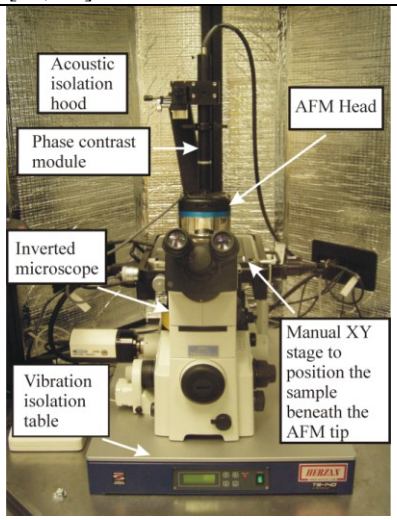

Fig. 1. Experimental setup for mESC indentation studies.

B. *Analytical model for mESC*

The Hertz and the capsule model assume that the two spheres in contact are: (a) linear elastic, isotropic and homogeneous, and (b) no friction or adhesion occurs at the tip-sample interface. According to Hertz theory [15], the force (F) versus indentation (δ) relationship is given by:

$$F = \frac{4}{3} E^* R^{1/2} \delta^{3/2} \quad (1)$$

where, E^* is the combined modulus of the sample (E_1) and the probe (E_2) given by:

$$\frac{1}{E^*} = \frac{1-\nu_1^2}{E_1} + \frac{1-\nu_2^2}{E_2} \quad (2)$$

R is the relative curvature of the sample (R_1) and the probe (R_2):

$$\frac{1}{R} = \frac{1}{R_1} + \frac{1}{R_2} \quad (3)$$

The elastic moduli of cells are in the range of kPa [22]. Hence the assumption is that the probe used for mESC indentation is infinitely stiff compared to mESC ($E_2 >> E_1$).

For a capsule model [18], the relationship between the loading force (F) and the relative deformation (ε) is given by:

$$F = 2\pi \frac{E}{1-\nu} h R_0 \frac{\left[1 + R_0/(2R_s)\right]^2}{\left(1 + R_0/R_s\right)^4} \varepsilon^3 + \frac{\pi}{2(2)^{1/2}} E h^2 \varepsilon^{1/2} \quad (4)$$

The first and the second term in (4) represent stretching and bending of the cell membrane. R_0 and R_s is the radius of cell (undeformed) and the probe respectively. E, ν, and h represent the elastic modulus, Poisson's ratio, and the thickness of the cell membrane. In (4), the bending deformation term can be neglected for $\varepsilon > 0.15$ [18] and is applicable in the mESC indentation studies.

The Hertz and capsule models were compared based on the force-indentation data obtained from the experiments to choose an appropriate analytical model for mESC. The force (loading and unloading) versus time profiles are shown to determine whether force of adhesion exists between the tip and the sample. Previously, large variation in elastic modulus of mESC was observed which could be due to variation in the stage of the cell cycle at the time of measurements [20]. Hence, the single indentation studies were conducted on 40 mESC in interphase stage of the cell cycle process: 20 live and 20 fixed cells (10 undifferentiated and 10 sixth day differentiating). In all the experiments the cell indentation range was 2-2.5um.

1) Live mESC: The force-indentation profiles for live undifferentiated, and 6th day differentiating mESC are shown in figure 2 and 3 respectively. The R^2 value obtained with the Hertz fit is greater than the capsule fit.

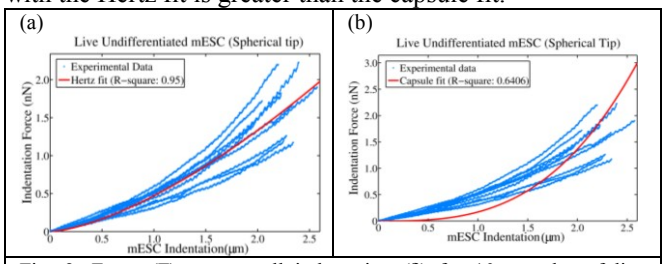

Fig. 2. Force (F) versus cell indentation (δ) for 10 samples of live undifferentiated mESC: (a) Hertz fit; (b) Capsule fit. The R^2 value obtained with Hertz fit (0.95) is greater than capsule fit (0.64).

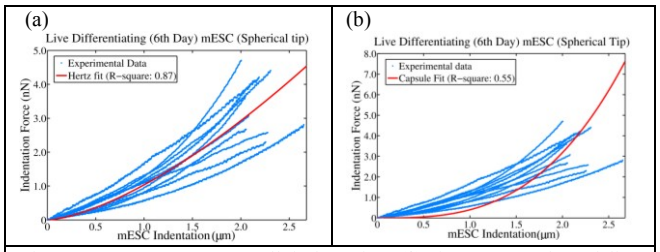

Fig. 3. Force (F) versus cell indentation (δ) for 10 samples of live differentiating (6^{th} day) mESC: (a) Hertz fit; (b) Capsule fit. The R^2 value obtained with Hertz fit (0.87) is greater than capsule fit (0.55)

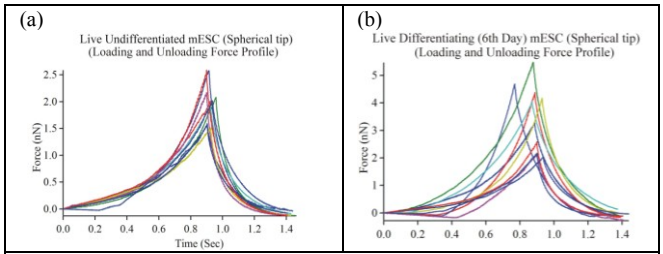

Fig. 4. Force (loading and unloading) versus time profile obtained for 10 samples of live: (a) undifferentiated and, (b) 6^{th} day differentiating mESC.

Figure 4 shows the force (loading and unloading) versus time profiles for live undifferentiated, and 6th day differentiating mESC. The force of adhesion does not exist between the probe and the sample. Thus, the JKR and DMT theories are not applicable. Hence, we infer that Hertz model appropriately describes the mechanical behavior of live mESC.

2) Fixed mESC: The force-indentation profiles for fixed undifferentiated, and 6th day differentiating mESC are shown in figure 5 and 6 respectively. Similar to live mESC, the R^2 value obtained with the Hertz fit is greater than the capsule fit.

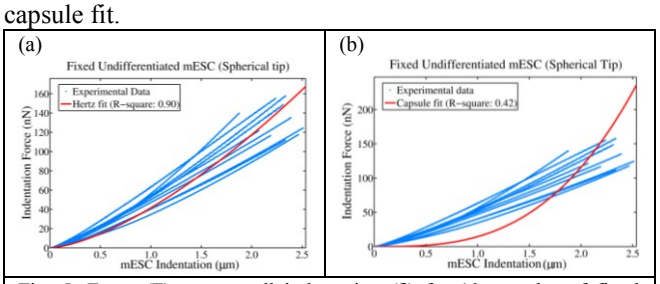

Fig. 5. Force (F) versus cell indentation (δ) for 10 samples of fixed undifferentiated mESC: (a) Hertz fit; (b) Capsule fit. The R^2 value obtained with Hertz fit (0.90) is greater than capsule fit (0.42)

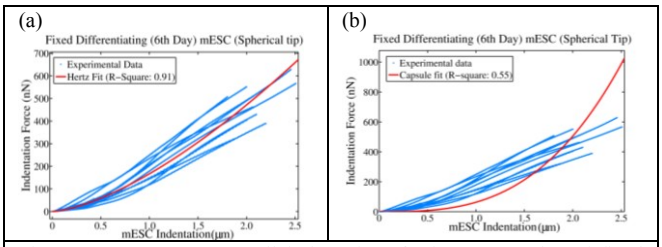

Fig. 6. Force (F) versus cell indentation (δ) for 10 samples of fixed differentiating (6^{th} day) mESC: (a) Hertz fit; (b) Capsule fit. The R^2 value obtained with Hertz fit (0.91) is greater than capsule fit (0.55).

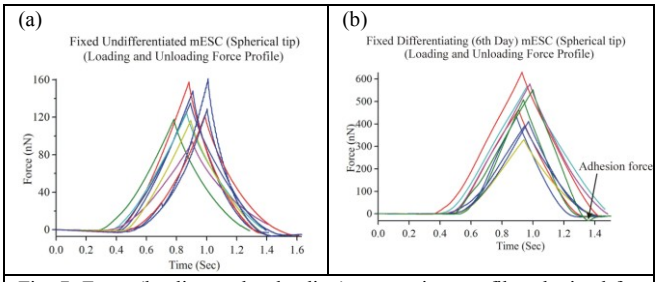

Fig. 7. Force (loading and unloading) versus time profiles obtained for 10 samples of fixed: (a) Undifferentiated, and (c) 6^{th} day differentiated mESC. The force of adhesion exists for only one sample.

Figure 7 shows the force (loading and unloading) versus time profiles for fixed undifferentiated, and 6th day differentiating mESC. The adhesion force (16 nN) exists for only one sample of differentiating mESC and is negligible compared to the peak force (~550 nN) observed by others for the JKR and DMT theories to be applicable [12, 23]. In addition, the adhesion could be due to the wear of the spherical probe [16]. *Hence, we infer that Hertz model appropriately describes the mechanical behavior of live and fixed mESC compared to capsule model.* One of the reasons could be that the mESC (both live and fixed cells) mechanics is influenced by the compression of the cell interior (e.g. cytoskeleton) rather than that of the cell membrane (capsule model considers the stretching and the bending of the cell membrane).

III. Results

Hertz model was used to compute the elastic modulus of each live and fixed mESC sample from the force (F) versus indentation (δ) obtained from the experiments by assuming Poisson's ratio of 0.5. The experiments were performed on R1 cell line and differentiation was induced by LIF removal. We needed to confirm that the variation in mechanical properties of mESC between undifferentiated and early differentiating state is not an artifact and does occur for any independent culture of mESC. Thus, we repeated the indentation studies on a second independent culture of undifferentiated (10 samples), and 6^{th} day differentiating (10 samples) mESC for both live and fixed cells.

A. Live mESC

Figure 8 (a) shows the average elastic modulus of live mESC from two independent cultures. The average elastic modulus was found to be 0.265 kPa, and 0.579 kPa for 20 samples of live undifferentiated and differentiating (6^{th} day) mESC respectively as shown in figure 8 (a). The result shows that the live undifferentiated mESC is supple compared to differentiating mESC. Kruskal-Wallis test was performed on the elastic modulus values for fixed undifferentiated and differentiating mESC. The p-value obtained was 0.0002. Thus, the mechanical property of live undifferentiated mESC differs from differentiating (6^{th} day) mESC.

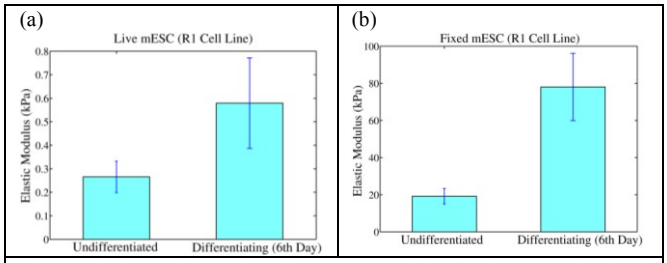

Fig. 8. Elastic modulus of: (a) live mESC and (b) fixed mESC calculated from the Hertz contact model along with the error bar.

B. Fixed mESC

Figure 8 (b) shows the average elastic modulus of fixed mESC from two independent cultures. The average elastic modulus was found to be 19.18 kPa, and 78.05 kPa for 20 samples of fixed undifferentiated and differentiating (6^{th} day) mESC respectively as shown in figure 8 (b). The result shows that the fixed undifferentiated mESC is supple compared to differentiating mESC. Further, Kruskal-Wallis test computed the p-value as 0.0002. Thus, the mechanical property of fixed undifferentiated mESC differs from differentiating (6^{th} day) mESC.

C. Fixed mESC: D3 Cell Line

The results obtained in sections III A and B could be a characteristic oddity of R1 mESC line. Thus, it is crucial to validate the findings with another cell line. Hence, further indentation studies were conducted on two independent cultures of D3 mESC line and the method of differentiation was LIF removal. Sections IIIA and IIIB, show that the results obtained with live cells parallel with fixed cells i.e. undifferentiated mESC is supple compared to 6^{th} day

differentiating mESC for both live and fixed cells. Therefore, studies were conducted only on fixed D3 mESC line to confirm our research hypothesis.

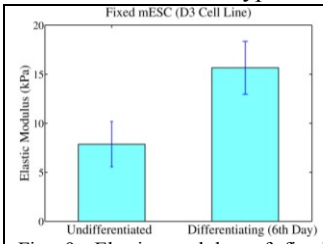

Fig. 9. Elastic modulus of fixed mESC (D3 cell line) calculated from the Hertz contact model along with the error bar.

Figure 9 shows the average elastic modulus of fixed mESC (D3 cell line) from two independent cultures. The average elastic modulus was found to be 7.866 kPa, and 15.658 kPa for 20 samples of fixed undifferentiated and differentiating (6^{th} day) mESC respectively as shown in figure 9. Similar to R1 cell line, fixed undifferentiated D3 mESC is supple compared to fixed differentiating D3 mESC. The p-value obtained was 0.0004. Thus, the mechanical property of fixed undifferentiated mESC differs from differentiating (6^{th} day) mESC, irrespective of the cell line (R1 or D3).

IV. Discussion

In this paper, we have characterized the mechanical behavior of mouse embryonic stem cells (mESC). We conducted indentation studies with an atomic force microscope (AFM) on undifferentiated and early differentiating mESC (live and fixed). The analysis of experimental data using solid and capsule models determined that the Hertz theory appropriately characterizes the mechanical property of mESC probed by a spherical tip. The statistical analysis on the elastic modulus of undifferentiated and differentiating mESC showed that the p-value was less than 0.05 for live as well as fixed cells. Hence, we confirmed our research hypothesis that the mechanical property of undifferentiated mESC differs from early differentiating mESC irrespective of the cell culture and cell line (R1 and D3). The system could be used to develop improved methods of targeted cellular differentiation of human embryonic and/or adult stem cells for therapeutic purposes and for the development of new diagnostic procedures.

Acknowledgement

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