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AFM Assessment of the Mechanical Properties of Stem Cells During Differentiation

Jie Zou [1, 2], Weiwei Wang [1], Xianlei Sun [1, 3], Wingtai Tung [1, 3], Nan Ma [1, 2, 4 *] and Andreas Lendlein [1, 2, 3, 4*]

[1] Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Teltow, Germany
[2] Institute of Chemistry and Biochemistry, Free University of Berlin, Berlin, Germany
[3] Institute of Chemistry, University of Potsdam, Potsdam, Germany
[4] Helmholtz Virtual Institute − Multifunctional Materials in Medicine, Berlin and Teltow, Germany

[*] To whom correspondence should be addressed: Prof. Dr. Nan Ma, Prof. Dr. Andreas Lendlein
Email: nan.ma@hzg.de , andreas.lendlein@hzg.de

ABSTRACT

The dynamic mechanical force transmitted through microenvironments during tissue formation and regeneration continuously impacts the mechanics of cells and thereby regulates gene and protein expression. The mechanical properties are altered during the process of stem cells differentiating into different lineages. At different stages of differentiation, stem cells display different mechanical properties in response to surrounding microenvironments, which depend on the subcellular structures, especially the cytoskeleton and nucleus. The mechanical properties of the cell nucleus affect protein folding and transport as well as the condensation of chromatin, through which the cell fate is regulated. These findings raise the question as to how cell mechanics change during differentiation. In this study, the mechanical properties of human bone marrow mesenchymal stem cells (hBMSCs) were determined during adipogenic and osteogenic differentiation by atomic force microscopy (AFM). The cytoskeletal structure and the modification of histone were investigated using laser confocal microscope and flow cytometry. The mechanical properties of cell nuclei at different stages of cell differentiation were compared. The stiffness of nuclei increased with time as osteogenesis was induced in hBMSCs. The H3K27me3 level increased during osteogenesis and adipogenesis according to flow cytometry analysis. Our results show conclusively that AFM is a facile and effective method to monitor stem cell differentiation. The measurement of cell mechanical properties by AFM improves our understanding on the connection between mechanics and stem cell fate.

INTRODUCTION

The mechanical properties of living cells are strongly related to their cellular behavior and the regeneration of defective tissues as well as to some diseases [1, 2]. The mechanical properties of stem cells have been used as a biophysical marker for

distinguishing differences in cell subpopulations, differentiation potential and cell lineages [3]. The change in cell mechanical properties reflects the variation in composition and organization of the subcellular structure, particularly the cytoskeleton. Therefore, probing the mechanical properties of stem cells and differentiated cells is of great importance for evaluating the therapeutic potential of stem cells prior to *in vivo* applications. The mechanical properties of a cell are mainly determined by the actin cytoskeleton [4], which directly links integrin receptors and the nucleus. The actin cytoskeleton can regulate cell morphology and adhesion, and transmits mechanical signals to the nucleus that control cell proliferation and differentiation. As an important factor in influencing gene expression [5], nuclear stiffness can be affected by changes in the mechanical properties of the actin network. During the process of stem cell differentiation, cytoskeleton organization and nuclear mechanics are dynamic [6]. Therefore, monitoring the mechanical properties of stem cells could be an effective approach for evaluating their differentiation potentiation and lineage commitment.

Comparing to the other methods such as magnetic twisting cytometry, cell monolayer rheology, and optical stretching, the Atomic force microscopy (AFM) has the advantages of high mechanical sensitivity, minimal cell damage, and strong environmental adaptability for the measurement of cells' mechanical properties [7, 8]. AFM provides a simple and effective tool for studying cell-cell and cell-material interactions as well as cell mechanical properties at the micro- and nanoscale [9, 10]. The goal of this study was to determine the mechanical properties of the cytoplasm and nuclei of mesenchymal stem cells during the differentiation process, and to establish the relationship between cell mechanics and stem cell fate. Human bone marrow mesenchymal stem cells (hBMSCs) were cultured in growth medium and induction media (osteogenesis or adipogenesis) respectively. At different time points, the elasticity of the cytoplasm and nuclei were determined by AFM. The organization of the actin cytoskeleton and histone modification (H3K27me3) levels were assessed.

METHODS

Cell culture

Human bone marrow mesenchymal stem cells (hBMSCs) (SCC034, Merk Millipore, Darmstadt, Germany) were used in this study. MesenPRO RS™ growth medium (GM) (ThermoFisher Scientific, Waltham, USA) was used for cell maintenance. For the mechanical testing, hBMSCs were seeded on cell culture dishes (35 mm diameter, Thermo Fisher Scientific, Bonn, Germany) at a density of 5000 cells/dish. To induce cell differentiation, the cells were cultured in either osteogenic induction medium (OM) or adipogenic induction medium (AM) (ThermoFisher Scientific, Waltham, USA).

Atomic force microscopy (AFM) single-cell mechanical testing

Living cells nuclei were stained by NucBlue™ Live ReadyProbes™ Reagent (Life Technologies, Darmstadt, Germany) to locate the nuclei during testing. Single-cell morphology and the mechanical properties were determined via an AFM (Nanowizard4, JPK, Germany), which was mounted on an inverted optical microscope (Zeiss, Germany). Spherically tipped cantilevers (CP-qp-CONT-Au-B-5) (NanoAndMore comp. Germany) were used in this study. For the determination of the elasticity of cell, a force–distance curve was obtained in contact mode with a vertical ramp size of 1 μm and maximum indentation force was 10 nN. The force–distance was converted to a force-deformation

curve as described in a previous report [11]. The force-distance curves were collected in the area of the cell nucleus and cytoplasm. In each group, at least 5 separate cells were tested. The obtained force-deformation curves were analyzed according to the Hertz equation: [12]

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

Where E is the Young`s Modulus, F is the applied mechanical force load by the AFM, v is the Poisson ration of tested material (assumed to be 0.5 for cells) [13], R is the radius of the tip (in this study R was set to 4 μm), and δ is the degree of indentation as measured by AFM.

Flow cytometry

Cells were fixed with 4% (w/v) paraformaldehyde (Sigma–Aldrich, St. Louis, MO USA), permeabilized with 0.1% (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA), and stained with Alexa Fluor® 488 conjugated rabbit anti-H3K27me3, Pacific Blue conjugated mouse anti-histone H3 antibodies (Cell signaling technologies, Danvers, USA). The data were recorded by MACSQuant® flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using "Flowjo" software (Tree Star Inc., Ashland OR, USA).

Immunostaining of F-actin

hBMSCs were fixed with 4% (w/v) paraformaldehyde (Sigma–Aldrich, St. Louis, MO USA), permeabilized with 0.1% (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA), and blocked with 3% (w/v) BSA (Sigma–Aldrich, St. Louis, MO, USA). F-actin was stained with ActinGreen™ 488 Ready Probes (Life Technologies, Darmstadt, Germany). Fluorescence images were captured using a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany).

Statistical analysis

The number of replications for quantitative experiments was equal to or larger than three as specified in the figure legends. The data were expressed as arithmetic mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA with post hoc Tukey HSD test. A p value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

hBMSCs were cultured in different media, the single-cell morphology and E-modulus were assessed by AFM. The cell morphology images showed a clear cell topography, and in the area of the nuclei, the height is greater than in other parts of the cell (Fig. 1A). Cell morphology images were combined with the fluorescence image of the nucleus to locate the nucleus and test the E-moduli of nucleus and cytoplasm. Primary hBMSCs (day 0) had low E-moduli of nuclei as well as cytoplasm (Fig. 1B and C). After 7 days of cultivation, the E-moduli of nuclei and cytoplasm was increased, which was found in the cells that were cultured in three media, and the osteogenic committed cells

exhibited significantly higher E-modulus of nuclei than that of cells cultured in AM or GM. For cells cultured in AM or GM for a longer time period (day 14 and day 21), the E-moduli of nuclei and cytoplasm were significantly lower than that of cells cultured in OM (Fig. 1B and C). At day 7, the E-moduli of nuclei and cytoplasm were increased compared to the beginning of cell cultivation, due to the higher cell contractile force, which was caused by focal adhesion and actin polymerization during the process of cell adhesion and spreading. The contractile force resulted in lamin A/C polymerization and deposition on the inner side of the nuclear membrane, which enhanced the stiffness of the nucleus [14]. At prolonged cell culture times (day 14 and day 21), in GM cell spreading was limited by cell proliferation, which led to a decrease in contractile forces. Cells cultured in AM showed decreased cell contractile force during the process of adipogenesis. Therefore, the E-moduli of the cells' nuclei and cytoplasm when cultured in AM or GM decreased. As high cell contractility favors stem cell osteogenesis [15], the cells cultured in OM showed higher contractile forces compared to the cells cultured in AM or GM, which resulted in higher E-moduli of nuclei and cytoplasm of cells in OM than that of cells cultured in AM or GM.

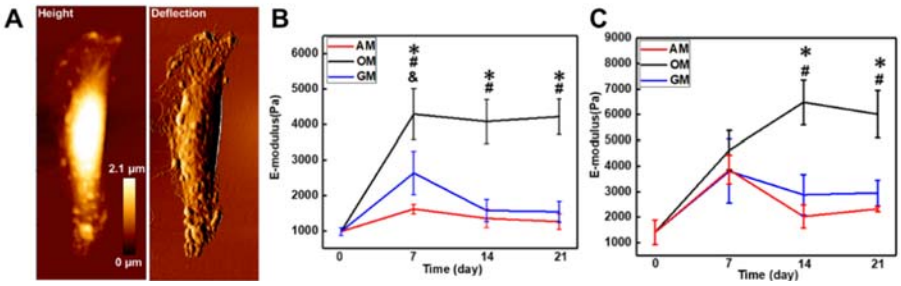

Fig. 1 Living cell morphology and cell mechanical properties determined by AFM. A Representative AFM scanning images of living cell morphology; B E-moduli of nuclei of cells were cultured under different conditions; C E-moduli of cytoplasm of cells were cultured under different conditions. (n = 5; * $p < 0.05$, OM vs GM; # $p < 0.05$, OM vs AM; & $p < 0.05$, AM vs GM).

Actin microfilaments (F-actin) are the major structure of the cytoskeleton, which regulates cell morphology, stiffness, and biological signal transmission as well as cell contractility [16]. Here, F-actin of cells cultured under different conditions was stained, and the images (Fig. 2) showed that the cells cultured in GM and OM had a stronger F-actin signal than that of cells cultured in AM. When increasing cell culture time, the F-actin signal of cells was enhanced in the OM group but decreased in the AM group. In the GM group, F-actin expression levels were the highest at day 7 when compared to those at day 14 or day 21. F-actin expression levels are known to regulate the E-moduli of the cytoplasm and nucleus, with higher F-actin expression levels resulting in higher E-moduli [17, 18]. F-actin staining results were consistent with the data on the E-modulus of cytoplasm.

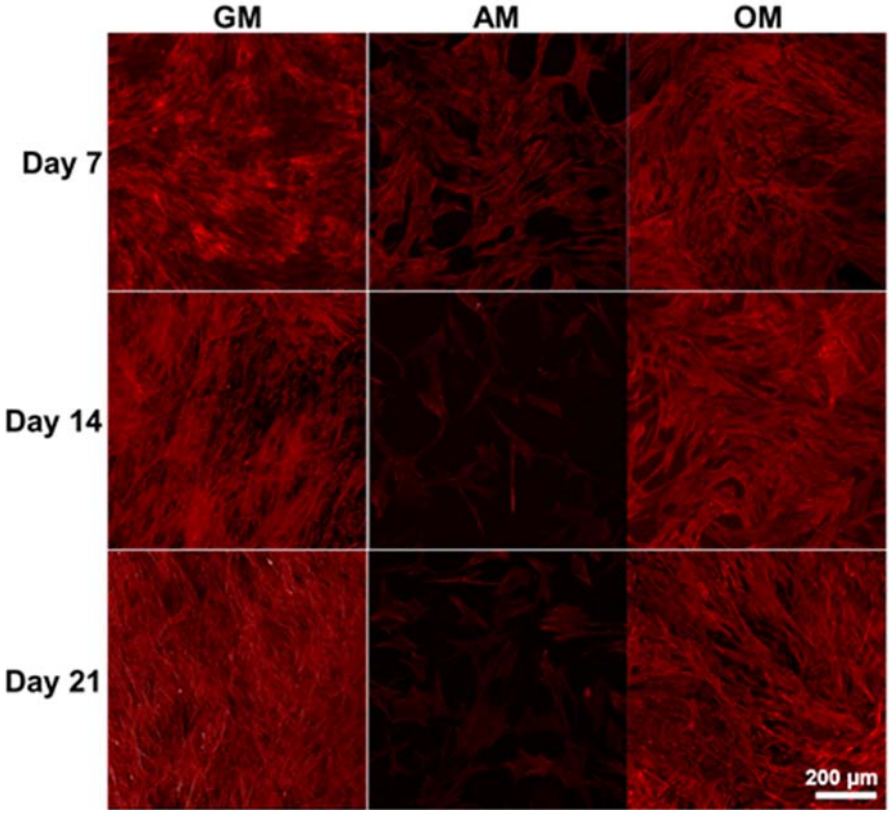

Fig. 2 Representative F-actin immunofluorescence staining images of hBMSCs cultured in different media at different time points (red: F-actin).

Cell contractility regulates stem cell differentiation. The contractile force was transmitted into the nucleus via the linker of nucleoskeleton and cytoskeleton (LINC) complex and regulated the compaction of chromatin [19]. The trimethylation of histone H3K27 (H3K27me3) is an epigenetic control point of MSC differentiation [20] and the expression level of H3K27me3 was directly related to the degree of chromatin compaction [21]. The H3K27me3 expression levels of cells cultured in different conditions were assessed. At day 14 and day 21, the cells cultured in OM or AM had significantly higher H3K27me3 expression levels than that of cells cultured in GM (Fig. 3). These results were consistent with a previous report [22], in which the H3K27me3 expression level was elevated during stem cell differentiation. H3K27me3 expression level regulates chromatin compaction degree [21], however, there was no significant difference in nuclei stiffness of cells cultured in AM and GM (Fig. 1B). This because the E-modulus of a nucleus is not only determined by the compaction of chromatin, but also the level of lamin A expression [23]. The cells cultured in AM had higher H3K27me3 expression levels, which resulted in increased compaction of chromatin, but the cells cultured in GM had higher cell contractility than that of cells cultured in AM, which led to higher lamin A/C expression of cells cultured in GM [18, 24].

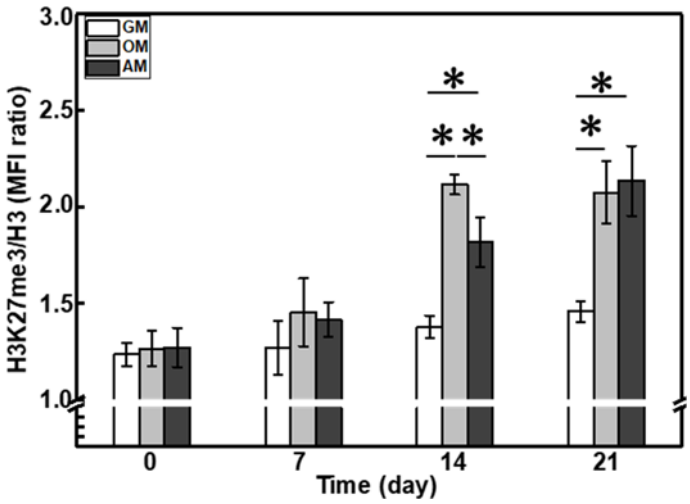

Fig. 3: H3K27me3/H3 MFI ratio of hBMSCs cultured under different conditions at different time points. The expression of H3K27me3 and H3 was quantified using flow cytometry and the MFI was calculated with Flowjo software (n = 5; * $p < 0.05$).

CONCLUSION

In our study, the mechanical properties of nuclei and cytoplasm of hBMSCs were measured by AFM during the process of adipogenic or osteogenic differentiation. F-actin and H3K27me3 expression levels were determined during the differentiation process over the same time period. The results indicated that osteogenic cells had nuclei and cytoplasm with strikingly higher E-moduli than those of cells cultured in AM or GM. F-actin expression level is directly related to the E-moduli of nuclei and cytoplasm, but H3k27me3 expression level did not remarkably influence nuclear stiffness. Our findings indicate that AFM is a facile and effective method to monitor stem cell differentiation, which could be a rapid and effective means for the evaluation of stem cells' differentiation potential and in distinguishing stem cells derived from different tissue sources.

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