Published in final edited form as:

J Biomech. 2015 April 13; 48(6): 1058–1066. doi:10.1016/j.jbiomech.2015.01.033.

Temporal heterogeneity in single-cell gene expression and mechanical properties during adipogenic differentiation

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Abstract

Adipose-derived stem/stromal cells (ASCs) respond heterogeneously when exposed to lineage-specific induction medium. Variable responses at the single-cell level can be observed in the production of lineage-specific metabolites, expression of mRNA transcripts, and adoption of mechanical phenotypes. Understanding the relationship between the biological and mechanical characteristics for individual ASCs is crucial for interpreting how cellular heterogeneity affects the differentiation process. The goal of the current study was to monitor the gene expression of peroxisome proliferator receptor gamma (*PPARG*) in adipogenically differentiating ASC populations over two weeks, while also characterizing the expression-associated mechanical properties of individual cells using atomic force microscopy (AFM). Results showed that ASC mechanical properties did not change significantly over time in either adipogenic or control medium; however, cells expressing *PPARG* exhibited significantly greater compliance and fluidity compared to those lacking expression in both adipogenic and control media environments. The percent of *PPARG*⁺ cells in adipogenic samples increased over time but stayed relatively constant in controls. Previous reports of a slow, gradual change in cellular mechanical properties are explained by the increase in the number of positively differentiating cells in a sample rather than being reflective of actual, single-cell mechanical property changes. Cytoskeletal remodeling was more prevalent in adipogenic samples than controls, likely driving the adoption of a more compliant mechanical phenotype and upregulation of *PPARG*. The combined results reinforce the

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Conflicts of interest

None of the authors have a conflict of interest associated with the work presented.

Authors' contributions

NRL and EMD designed the study. NRL performed mechanical testing, cytoskeletal characterizations, beacon-based gene expression experiments, and MATLAB and ImageJ programming. NRL and EMD analyzed the data and wrote the manuscript.

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importance of understanding single-cell characteristics, in the context of heterogeneity, to provide more accurate interpretations of biological phenomena such as stem cell differentiation.

Keywords

mechanical biomarkers; atomic force microscopy; molecular beacon; adipogenesis; adipose-derived stem cell

1. Introduction

Human adipose-derived stem cells (ASCs) show promise for regenerative therapies due in part to their multipotent differentiation capabilities (Zuk et al., 2001). The process of lineage commitment involves both biological and mechanical/morphological cues (Tee et al., 2011). Single-cell elastic and viscoelastic characterization can be used to assess cytoskeletal structure and mechanical properties, which influence cellular response (Ingber, 2006; Zhu et al., 2000). Of relevance to regenerative medicine, cellular “mechanical biomarkers” have been used to identify specific phenotypes (Darling et al., 2008) and proposed as potential biological indicators of lineage-specific differentiation potential (Gonzalez-Cruz et al., 2012).

During chemically induced differentiation, the actin cytoskeleton of stem cells undergoes reorganization concomitant with changes in mechanical properties and gene expression (Bongiorno et al., 2014; Keefer and Desai, 2011; Kwon et al., 2011; Titushkin et al., 2013; Vinckier and Semenza, 1998; Yourek et al., 2007; Yu et al., 2010). Specifically for adipogenesis, cells exhibit significant increases in their mechanical compliance and cytoskeletal disorganization (Kwon et al., 2011; Titushkin and Cho, 2007; Titushkin et al., 2013). Additionally, several lineage-specific genes are upregulated, including a master adipogenic regulator, peroxisome proliferator-receptor gamma (*PPARG*) (Rosen, 2005; Rosen and MacDougald, 2006; Spiegelman, 1998). Recent work from our lab demonstrated that molecular beacons, DNA-based probes which fluoresce in the presence of their target mRNA sequence, can be used to monitor the expression of lineage-specific genes during differentiation (Desai et al., 2014). By combining these techniques with mechanical testing, single-cell properties can be more accurately evaluated for positively differentiating cells.

The goals of this study were: (i) to characterize the heterogeneity and temporal changes in *PPARG* gene expression in human ASC populations undergoing adipogenesis and (ii) to test whether cells expressing *PPARG* mRNA (*PPARG*⁺) exhibit a unique mechanical phenotype compared to those that do not (*PPARG*[−]). The presence or absence of *PPARG* mRNA in individual ASCs was determined visually using a custom-designed molecular beacon. The elastic and viscoelastic mechanical properties of individual, *PPARG*^{+/−} ASCs were characterized via indentation and stress-relaxation tests using atomic force microscopy (AFM). Investigating the link between lineage-specific gene expression and mechanical properties will further our understanding of the dynamics of differentiation in heterogeneous stem cell populations and determine whether single-cell mechanical properties are reflective of the transcription factors responsible for lineage commitment.

2. Methods

2.1 Cell culture and differentiation

Human ASCs, pooled from the subcutaneous fat tissue of seven, healthy, non-diabetic, female donors between 18–60 years of age were purchased from Zen-Bio, Inc. (Research Triangle Park, NC; superlot #36). Cells were grown in expansion medium consisting of DMEM/F-12, 1% antibiotic/antimycotic (Hyclone, Logan, UT), 10% FBS (Zen-Bio), 1 ng/mL fibroblast growth factor, 5 ng/mL epidermal growth factor, and 0.25 ng/mL transforming growth factor- β 1 (R&D Systems, Minneapolis, MN) (Estes et al., 2008). ASCs were expanded three passages at 37°C in 5% CO₂ prior to experimentation.

For mechanical characterization, ASCs were seeded on glass coverslips at 20,000 cells/cm² in 50 mm, low-profile Petri dishes (BD Biosciences, San Jose, CA) containing polydimethylsiloxane well adaptors that exposed a 1.5 × 1.5 cm surface. For percent expression and cytoskeletal analysis experiments, ASCs were seeded at 15,000 cells/cm² in 24-well plates. All experiments included cells cultured in control or adipogenic induction medium. Control medium consisted of DMEM/F-12, 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic. Adipogenic induction medium consisted of control medium supplemented with 1 μ M dexamethasone, 10 μ M insulin, 0.5 μ M 3-isobutyl-1-methyl-xanthine, and 200 μ M indomethacin (Sigma-Aldrich, St. Louis, MO) (Zheng et al., 2006). Ninety percent of the medium was changed every two days.

2.2 Molecular beacon design

A molecular beacon was designed to target the master adipogenic regulatory gene, *PPARG*, specifically splice variant 2, which is the only isoform exclusive to cells with adipogenic commitment (Rosen, 2005; Rosen and MacDougald, 2006; Spiegelman, 1998). The process for designing molecular beacons has been described in detail previously (Bao et al., 2009). Briefly, a single-stranded region of the full, *PPARG* mRNA sequence was targeted using predictions of the secondary structure to allow for greater likelihood of binding. The reverse complement of this region comprised the loop portion of our molecular beacon. Sequence specificity to *PPARG* splice variant 2 was verified using NCBI Blast. Complementary stems were chosen such that the beacon would remain in a stem-loop structure under physiological conditions but allow binding of the loop region in the presence of the target mRNA. A fluorophore and quencher were attached to either end to complete the molecular beacon. DNA-based beacons were synthesized and HPLC purified by a commercial vendor (MWG Operon, Huntsville, AL). Table 1 includes all beacons used in this study.

2.3 Molecular beacon validation

The *PPARG* beacon was characterized by hybridization tests using target and off-target DNA sequences following previously described protocols (see Supplementary Material A) (Bratu et al., 2011; Tyagi and Kramer, 1996). Briefly, fluorescence was measured for buffer solution alone, then after addition of beacon, and again after addition of target/off-target DNA. The signal-to-noise ratio (SNR) was calculated by dividing the maximum fluorescence measured for the complementary target (CT) by the maximum fluorescence measured for a non-complementary, off-target (NCT; Eq. 1).

$$SNR = \left(\frac{maxRFU_{CT}}{maxRFU_{NCT}} \right) \quad (\text{Eq. 1})$$

SNR was determined to be 7 for the *PPARG* beacon used in this study (Fig. 1).

False-positive and -negative rates for beacon signal were assessed with validation experiments using random beacon sequences conjugated with either a fluorophore/quencher pair (negative control) or just a fluorophore (positive control). The random beacon sequence showed no matching targets using NCBI Blast. Quencherless, random beacon tests indicated the false-negative rate was $15 \pm 6\%$, while quenchered, random beacon tests indicated the false-positive rate was $40 \pm 11\%$ (Fig. 2B).

To ensure that mechanical changes did not result simply from the presence of molecular beacons, mechanical tests were also conducted on ASCs treated with a beacon targeting the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, Fig. 2C) (Nitin et al., 2004). No statistically significant differences were found between positively and negatively fluorescing cells ($p = 0.48$) (Fig. 2D).

2.4 Single-cell mechanical characterization

Alternating between control and adipogenic samples each day, the elastic and viscoelastic properties of individual *PPARG*⁺ ($n = 23\text{--}34$) and *PPARG*[−] ($n = 25\text{--}35$) ASCs were characterized over a 14-day induction period with an MFP-3D-BIO AFM (Asylum Research, Santa Barbara, CA). Twenty-four hours prior to mechanical tests of independent samples, $0.1 \mu\text{M}$ beacons were delivered to ASCs via lipofection with X-tremeGENE HP (1:4 ratio beacon:reagent, Roche Biotech, Pleasanton, CA). Positive or negative expression of the *PPARG* beacon was determined visually on a Nikon Eclipse Ti-U epifluorescent microscope (Nikon Instruments, Melville, NY) using a 3 second exposure on a QICAM 12-bit digital camera (QImaging, Surrey, BC, Canada). Individual cells were mechanically tested over the nucleus, stained with $0.1 \mu\text{g/mL}$ Hoechst 33342 dye (Sigma-Aldrich), using a spherically tipped cantilever ($5 \mu\text{m}$ diameter, average $k \sim 0.027 \text{ N/m}$, Novascan Technologies, Inc.) with an approach velocity of $10 \mu\text{m/s}$, force trigger of 1 nN , and 30 s relaxation phase (see Supplementary Material B). A modified, thin-layer Hertz model was used to determine the elastic modulus, E_{elastic} , from force (F) vs. indentation (δ) curves obtained from single-cell indentations (Eq. 2) (Dimitriadis et al., 2002). The viscoelastic properties, apparent viscosity (μ), instantaneous modulus (E_0), and relaxed modulus (E_R), were determined using a thin-layer, stress relaxation model of a viscoelastic solid (Eqs. 3–5) (Darling et al., 2007). In these equations, R is the effective radius of the spherical tip and ν is Poisson's ratio, assumed to be 0.5 for incompressible materials. The time constants, τ_σ and τ_ϵ , represent relaxation times under constant load and constant deformation, respectively. Lastly, a thin-layer correction factor, C , was included to account for indentation depth, radius of the spherical tip, and sample thickness (Dimitriadis et al., 2002).

$$F(\delta) = \frac{4R^{1/2}E_{elastic}}{3(1-\nu^2)}\delta^{3/2}C \quad (\text{Eq. 2})$$

$$F(t) = \frac{4R^{1/2}\delta_0^{3/2}E_R}{3(1-\nu)} \left(1 + \frac{\tau_\sigma - \tau_\varepsilon}{\tau_\varepsilon} e^{-t/\tau_\varepsilon} \right) C \quad (\text{Eq. 3})$$

$$E_0 = E_R \left(1 + \frac{\tau_\sigma - \tau_\varepsilon}{\tau_\varepsilon} \right) \quad (\text{Eq. 4})$$

$$\mu = E_R (\tau_\sigma - \tau_\varepsilon) \quad (\text{Eq. 5})$$

2.5 Quantification of PPARG mRNA expression

For percent expression experiments, ASCs were treated with 0.1 μM beacons 24 hours prior to nuclear, Hoechst staining and fixation with 3.7% paraformaldehyde (Thermo Fisher Scientific). Six images/well were taken at 10x magnification using a Cytation3 Cell Imaging Multi-Mode Reader (Biotek Instruments Inc., Winooski, VT) with an LED intensity of 10, gain of 15.5, and integration time of 320. ImageJ version 1.47 (National Institute of Health, Bethesda, MD) was used to post-process beacon signal images to remove bright debris, believed to be externally adhered, autofluorescent liposomes and degraded beacons within lysosomes (Nitin et al., 2004). Images were then analyzed with a custom MATLAB (MathWorks, Natick, MA) script to calculate the percentage of ASC nuclei associated with positive beacon signal in each image (see Supplementary Material C). In brief, the program assigned indices to each nucleus and each region of beacon signal, associating the two based on overlap to quantify the percentage of cells with and without localized expression (Fig. 3).

Simulations weighting the average mechanical properties for *PPARG*⁺ and *PPARG*[−] cells by their respective percent expression levels in each sample were used to provide an estimate of the elastic modulus of adipogenic and control cell populations over the induction period (Eq. 6).

$$\bar{E}_{elastic_{Population}} = \left(\bar{E}_{elastic_{PPARG^+}} * \frac{\%PPARG^+}{100} \right) + \left(\bar{E}_{elastic_{PPARG^-}} * \frac{(1-\%PPARG^+)}{100} \right) \quad (\text{Eq. 6})$$

2.6 Verification of adipogenesis

To verify *PPARG* upregulation in ASCs undergoing adipogenesis, total RNA was isolated from three, pooled samples of cells cultured in either adipogenic or control medium for 1, 7, or 14 days using a QuickRNA Miniprep Kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA was reverse-transcribed using a SuperScript III First Strand cDNA Synthesis Kit (Life Technologies, Grand Island, NY). TaqMan Gene Expression Assay human primers were chosen for *PPARG*, variant 2, (Hs01115510_m1) and a reference gene, *GAPDH* (Hs03929097_g1) (Life Technologies). Real-time

fluorescence signals were measured on an ABI 7900HT Fast Real-Time PCR Detection Instrument (Life Technologies). Using the comparative C_T method, relative expression of *PPARG* in each replicate was normalized to the mean cycle threshold of *GAPDH* per time point (Schmittgen and Livak, 2008).

Adipogenesis was also assessed by monitoring lipid aggregate formation. Samples used for *PPARG* expression experiments were stained with oil red O (ORO, Sigma-Aldrich), and bright-field images (6 per well) were taken at 10x magnification. Lipid aggregate sizes were quantified using a custom MATLAB program. Briefly, the script inverted image intensities so lipids appeared bright on a dark background and set area detection thresholds to identify blobs greater than a defined size. Resulting data included the number and average size of lipid aggregates (see Supplementary Material D).

2.7 Visualization and quantification of the actin cytoskeleton

To evaluate cytoskeletal structure, fixed samples corresponding to the *PPARG* expression experiments and lipid aggregate formation analyses were rinsed with PBS, and representative adipogenic and control wells from each plate were treated with 0.1% Triton X-100 (in PBS) for 5 minutes to permeabilize cell membranes. Samples were then blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes. For each well, 5 μ L of a 200 U/mL Alexa Fluor 594 phalloidin (Life Technologies) stock solution was diluted in 200 μ L of PBS with 1% BSA and applied to samples for 20 minutes at 25°C. Five images of each well were taken at 10x magnification using a 0.5 s exposure time.

2.8 Statistical analysis

Mechanical characterization, percent *PPARG* expression, and lipid quantification experiments were repeated in triplicate ($N = 3$), while cytoskeletal organization and qPCR validation data were acquired during a single iteration ($N = 1$). Mechanical moduli measuring negative values and moduli >2.5 standard deviations from the mean of each testing session were considered outliers and removed from the data set. Temporal trends of lipid areas, percent *PPARG* expression, and mechanical property data were assessed through linear regression analysis in SigmaPlot version 12.5 (Systat Software, San Jose, CA). A logarithmic transformation was performed to linearize data prior to regression analyses. To determine differences resulting from media type and *PPARG* expression, a Kruskal-Wallis non-parametric ANOVA was performed using R statistical analysis software version 3.1.1 (RCoreTeam, 2014). Violin plots were created using the R package vioplot (Adler, 2005). A Student's t-test was performed for qPCR comparisons.

3. Results

3.1 ASC mechanical properties

Over the 14-day induction period, the mechanical properties of ASCs did not change significantly in either adipogenic ($p > 0.261$) or control ($p > 0.056$) media (Fig. 4A, B). This lack of temporal response also existed for *PPARG*⁺ ($p > 0.061$) and *PPARG*[−] ($p > 0.167$) subpopulations. However, a clear difference was present between the overall, average mechanical properties for *PPARG*⁺ and *PPARG*[−] cells, independent of medium condition,

with *PPARG*⁺ cells exhibiting average elastic moduli 37% lower than *PPARG*[−] cells (0.72 vs. 1.13 kPa, respectively, $p < 0.001$, Fig. 4C). When comparing the mechanical properties of *PPARG*⁺ and *PPARG*[−] ASCs across media conditions, no statistical differences were observed ($p > 0.096$ and $p > 0.21$, respectively).

3.2 Heterogeneity of *PPARG* expression

Quantification of *PPARG* beacon signal revealed a significant increase in the percentage of *PPARG*⁺ ASCs in adipogenic samples compared to control samples after just three days of induction ($p < 0.016$). Most of the increase in adipogenic samples occurred by day 4 and reached a maximal level of 75% expressing cells by day 14 ($p < 0.001$). The percentage of *PPARG*⁺ cells in control samples showed a slight, yet statistically significant decrease from day 1 to day 14 ($p < 0.033$).

By interpreting *PPARG*^{+/−} mechanical property data in the context of *PPARG* expression percentages for each medium condition, estimated mechanical properties could be simulated for the overall cell population (Fig. 5B). The resulting values showed a decrease in the population-average, mechanical properties of ASCs cultured in adipogenic medium over time, which was driven by increasing numbers of cells exhibiting the more compliant phenotype associated with *PPARG*⁺ expression.

3.3 Cytoskeletal organization

Phalloidin staining of the actin cytoskeleton revealed distinct changes in the organization of these filaments in ASCs exposed to adipogenic medium compared to control medium (Fig. 6A). In cells exposed to adipogenic medium, actin filaments moved from the perinuclear region to the periphery of the cell, leaving less filaments crossing over the nucleus. Qualitatively, cells with positive beacon signal appeared to have fewer actin filaments, especially over the nucleus.

3.4 Verification of adipogenesis

Successful adipogenic differentiation of ASCs was demonstrated by extensive upregulation of *PPARG* gene expression and an increase in average, lipid aggregate area. Compared to day 1 samples, qPCR results revealed a 730-fold increase in the relative expression of *PPARG* in ASCs after 7 days of exposure to adipogenic induction medium ($p < 0.001$) and an 1100-fold increase after 14 days ($p < 0.001$). ASCs in control medium also experienced a significant increase in *PPARG* expression, though only 2-fold after 14 days in culture ($p < 0.02$). Based on these values, the relative expression of *PPARG* in adipogenic samples was 1800-fold higher than controls on day 14 ($p < 0.001$, Supplementary Material E).

Intracellular lipid analysis revealed that ASCs in adipogenic medium generated lipid aggregates with significantly larger areas than those cultured in control medium after just 5 days in culture ($p < 0.025$). After 14 days in culture, lipid aggregates in adipogenic samples were 75% larger than those in control samples ($p < 0.002$). Within adipogenic samples, average lipid aggregate area increased by approximately 120% from day 1 to day 14 of induction ($p < 0.001$, Fig. 7). In control samples, the average area of lipid aggregates also increased after 14 days in culture, though by only 40% ($p < 0.001$).

4. Discussion

The results of this study indicate that ASC populations exhibit heterogeneity at the single-cell level with respect to mechanical properties, lineage-specific gene expression, and cytoskeletal organization. As samples undergo adipogenesis, an increasing percentage of the cell population reorganizes their actin cytoskeleton to express a more compliant phenotype while also upregulating *PPARG* mRNA. Though both adipogenic and control ASC populations included *PPARG*⁺ cells, adipogenic samples experienced an increase in the percentage of those cells over time, while in controls this percentage remained largely unchanged. Cellular mechanical properties also did not change over time; however, *PPARG*⁺ cells were more compliant than *PPARG*[−] cells in both culture environments. By combining these data sets, a gradual decrease in cellular mechanical properties could be simulated for the adipogenic condition. In summary, adipogenic gene expression, cellular mechanical phenotype, and cytoskeletal organization were found to be interrelated during the process of stem cell differentiation.

Our simulation of population-average mechanical properties agrees with previous reports of a gradual increase in compliance for ASC samples after exposure to adipogenic induction medium (Kwon et al., 2011; Titushkin et al., 2013; Yu et al., 2010). However, *PPARG*-directed, AFM characterization revealed that these gradual, mechanical changes are not occurring at the single-cell level. In response to adipogenic induction medium, positively differentiating ASCs quickly reorganize their actin cytoskeletons, exhibiting a more compliant phenotype, and upregulate *PPARG* (Nobusue et al., 2014). As an increasing fraction of cells reorganize their cytoskeletal structures, the overall population appears to gradually increase in compliance; however, these changes are actually due to a shift in the ratio of two, mechanically distinct subpopulations identifiable by their *PPARG* expression.

Phalloidin staining revealed marked differences in the cytoskeletal organization of ASC populations exposed to the two medium types. Qualitative analysis of the images showed the development of a dense, organized actin cytoskeleton in control samples, while adipogenic samples exhibited disperse actin filaments with cortical localization, similar to results from previous studies (Swift et al., 2013; Titushkin et al., 2013). Though a distinction between *PPARG*⁺ and *PPARG*[−] ASCs was not feasible for quantification purposes, qualitatively, *PPARG*⁺ cells had fewer actin filaments crossing the nucleus. Mechanical measurements were made over the perinuclear region, providing an explanation for why *PPARG*⁺ cells were more compliant than *PPARG*[−] cells. This is consistent with the hypothesis that the actin cytoskeleton is largely responsible for whole-cell, mechanical phenotype (Docheva et al., 2008; Yim et al., 2010).

The increased compliance and altered actin cytoskeletal structure of ASCs exhibiting *PPARG* expression suggests an interaction between the cytoskeleton and *PPARG* gene transcription. Previous studies have reported that the disruption of the actin cytoskeleton with cytochalasin D results in the upregulation of *PPARG* and induces adipogenesis (Nobusue et al., 2014; Schiller et al., 2013). Mechanistically, adipogenic induction factors have been shown to downregulate RhoA-ROCK (Rho-kinase) signaling which disrupts actin stress fibers, increasing monomeric G-actin levels (Nobusue et al., 2014). These free G-actin

monomers interact with megakaryoblastic leukemia 1 (MKL1, also known as MAL or MRTF-A), a *PPARG* antagonist, preventing its translocation to the nucleus, which results in the upregulation of *PPARG* mRNA. Once upregulated, *PPARG* inhibits MKL1 resulting in positive feedback and ensures the cell continues to differentiate adipogenically (Nobusue et al., 2014). This mechanistic pathway suggests that the cytoskeletal remodeling of individual ASCs precedes the upregulation of *PPARG* and is supported by our empirical data which revealed consistent mechanical phenotypes of *PPARG*+ ASCs in both adipogenic and control samples. These findings also suggest that single-cell mechanical biomarkers may be better suited for the early detection of adipogenic differentiation than *PPARG* gene expression.

The interpretation of this study's results is necessarily constrained by the limitations inherent in our experimental setup. Due to the non-sterile environment of the AFM and the toxic effects of long-term exposure to Hoechst 33342 dye, repeated measurements on the same cells of a single sample could not be performed over the 14-day differentiation experiment. This prevented making a direct assessment of mechanical property changes in a single cell over time. Distinguishing true beacon signal from noise also complicated data interpretation. When analyzing *PPARG*+ signal in adipogenic cultures, we observe a change from ~50% expression to ~75% expression, which is appropriate based on the fidelity of the beacon-based approach used in the study. Validation experiments determined that a potential 40% false-positive baseline existed in samples, along with a potential 15% false-negative rate. ASCs were expected to express *PPARG* at nominal levels, which was observed in control samples. Emphasis was thus placed on the difference between adipogenic and control samples, independent of the background noise in the system. The same experimental trends resulted across multiple iterations, confirming that the observations were real and not due to random noise. Analysis of error between hand-counts and program-counts of *PPARG* + cells showed agreement within $\pm 10\%$ expression (see Supplementary Material F).

Individual ASCs can differ greatly in their mechanical properties and differentiation potential. This study showed that cellular mechanical properties relate to *PPARG* expression and cytoskeletal organization in adipogenically differentiating ASCs. However, we also showed that the mechanical changes observed for the overall population are dramatically influenced by the number of differentiating cells at a given time, rather than being truly reflective of the single-cell mechanical properties. These findings emphasize the importance of understanding how cellular heterogeneity may bias data interpretation in any experiment. Changes in the proportion of cellular subpopulations can be a driving factor in what is observed at the population level, including data such as mechanical properties, gene expression, or protein expression. Complete cellular homogeneity is rare, if not impossible, to find in biological systems since each cell may respond slightly differently to surrounding stimuli, and this is especially true of mesenchymal stem cell populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Jessica S. Sadick for performing qPCR tests and analyses. This work was supported by awards from the National Science Foundation (EMD, CAREER Award, CBET1253189), National Institute of Arthritis and Musculoskeletal and Skin Diseases (EMD, R01AR063642), and National Institute of General Medical Sciences (EMD, P20GM104937).

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Fig. 1. *PPARG* beacons show target specificity

A molecular beacon hybridization assay confirmed specificity of the custom-designed *PPARG* beacon for its complementary target. Fluorescence intensity measurements were taken for (i) only hybridization buffer solution, (ii) *PPARG* molecular beacon in hybridization buffer solution, and (iii) the hybridization reaction with complementary *PPARG* target (blue series) and non-complementary, off-target (red series). Error bars represent standard deviations.

Fig. 2. Validation of beacon detection approach

(A) Raw fluorescence and postprocessed signals were evaluated in a control population of ASCs. Validation experiments included treatment with a quencherless random beacon, fluorophore/quencher random beacon, and *PPARG* beacon. Positive signals (green) overlapping with cell nuclei (blue) were used to calculate the percent of positive cells in a population. (B) Results indicated baseline, non-specific signal was approximately 30 – 40%, whereas maximal signal was approximately 80 – 90%, representing our limits of detection. (C) ASCs were treated with a beacon for the housekeeping gene *GAPDH*, which should be present in all cells. A small fraction of cells did not exhibit positive signal (false-negatives). (D) The mechanical properties of single, *GAPDH*⁺ and *GAPDH*⁻ cells were characterized with AFM; E_{elastic} is illustrated here with violin plots. No significant differences were detected between positive and negative signal groups ($p = 0.48$), indicating the presence of beacon did not affect measured, cellular mechanical properties.

Fig. 3. Quantification of *PPARG* expressing cells

Post-processing of images was performed to remove false signal and allow for batch analysis of beacon signal. To begin (i)→(ii), bright debris noise was removed using a custom, ImageJ macro script. (ii)→(iii) MATLAB's image processing toolbox was used to detect positive beacon signals above a given threshold and remove background signal. (iv)→(v) Nuclei were detected and their perimeter expanded by 2 pixels to encompass cytoplasmic beacon signal in the perinuclear region. (vi) Beacon signal overlapping this region was used to designate positive cells. (vii) Finally the beacon signals were paired with their respective nuclei for calculating the percentage of *PPARG*⁺ cells.

Fig. 4. ASCs exhibit distinct mechanical phenotypes associated with $PPARG$ expression

No significant, temporal changes were observed in the elastic modulus of $PPARG^{+/-}$ ASCs exposed to (A) adipogenic medium or (B) control medium. This was true for other mechanical parameters as well. Dashed lines represent the logarithmic fits of 95% confidence intervals. (C) Violin plots of the compiled, 14-day data revealed that $PPARG^{+}$ cells in adipogenic/control medium were significantly more compliant and larger than $PPARG^{-}$ cells in adipogenic/control medium (* $p < 0.01$). Comparisons of $PPARG^{+}$ and $PPARG^{-}$ cells across medium conditions showed no differences in elastic or viscoelastic properties.

Fig. 5. Temporal changes in *PPARG* expression heterogeneity drive a perceived drop in elastic modulus for the overall population

(A) The percentage *PPARG*+ cells increases by approximately 25% in adipogenic induction medium (red curves) over the 14-day differentiation period, while ASCs grown in control medium (blue curves) exhibited no significant change in expression, remaining near the baseline level of 40–50% *PPARG*+. Solid lines represent the logarithmic fit of the average percentage of *PPARG*+ cells, and dotted lines represent the logarithmic fit of the 95% confidence intervals of expression (adipogenic vs. control, * $p < 0.001$; † $p < 0.025$). (B) Using the average values for the elastic modulus of *PPARG*+/- ASCs, weighted by their relative expression levels, an estimate of the mechanical properties of the entire population was simulated. Results showed that control samples were not expected to undergo changes in their mechanical properties over time while adipogenic samples should experience a gradual decrease, the observed mechanical change associated with adipogenesis reported in literature.

Fig 6. Cytoskeletal changes in adipogenically differentiating cells

Phalloidin staining revealed an increase in actin filament density in ASC populations cultured in control medium compared to adipogenic medium from day 1 to day 14 of culture. ASCs in adipogenic medium were less confluent, larger in size, and had more cortically distributed actin filaments, leaving fewer supporting structures in the perinuclear region of cells where mechanical testing occurred.

Fig. 7. Adipogenic induction produces larger lipid aggregates

(A) Oil red O staining confirmed the production of intracellular lipids in both control (top row) and adipogenic (bottom row) samples, although much larger lipids were formed in samples exposed to adipogenic induction factors. (B) Quantification of lipid aggregate area showed an increase in average size for adipogenic samples (red) but minimal changes for controls (blue). By day 5 of the induction period, differences in average lipid aggregate sizes in adipogenic and control samples were statistically significant (* $p < 0.002$; † $p < 0.025$). Dashed lines represent the exponential fit of 95% confidence intervals.

Table 1**Molecular Beacon and Target Sequences**

Sequence 5' $\Rightarrow$ 3'	
<u>Beacon</u>	
<i>PPARG</i>	6FAM- <i>ccggtc</i> GCATGGAATAGGGGTTT <i>Ggaccgg</i> -BHQ1
Random (Quenched)	6FAM- <i>gaggcc</i> AAAAAAGTGTGGAAATGTTGGAAAA <i>Gggcctc</i> -BHQ1
Random (Quencherless)	6FAM- <i>gaggcc</i> AAAAAAGTGTGGAAATGTTGGAAAA <i>Gggcctc</i>
<i>GAPDH</i>	6FAM- <i>cgacg</i> GAGTCCTTCCACGATACCA <i>cgtcg</i> -BHQ1
<u>Target</u>	
<i>PPARG</i> Target	CAAACCCCTATTCCATGC
Off-Target	CTTATGAAATGGGCTTTAACA

Loop regions (reverse complement of target mRNA sequence) are capitalized. Stem regions are italicized lowercase. Where specified, 6-FAM fluorophores and BHQ1 quenchers were conjugated to 5' and 3' ends of sequences. DNA sequences were custom-designed with the exception of *GAPDH* (Nitin et al., 2004).