Adipose-derived stem cell fate is predicted by cellular mechanical properties

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Keywords: mesenchymal stem cell, fat, cell mechanics, regenerative medicine, atomic force microscopy, differentiation, stem cell enrichment, passaging, cell sorting

Abbreviations: ASC, adipose derived stem cell; MSC, mesenchymal stem cell; AFM, atomic force microscopy; E_{elastic}, elastic modulus; E_{equil}, equilibrium modulus; E_0, instantaneous modulus; E_R, relaxed modulus; μ_{app}, apparent viscosity

Adipose-derived stem cells (ASCs) show great promise for tissue engineering applications and cell-based therapies because of their multipotency, relative abundance and immunosuppressive properties. However, ASCs must be isolated from heterogeneous cell populations present in adipose tissue. In this brief report, we provide a concise summary of the history and use of cellular mechanical properties as novel, label-free biomarkers to predict the differentiation potential of ASCs toward adipogenic, osteogenic and chondrogenic lineages. Additionally, we have found that passage number influences the mechanical properties of ASCs along with a discussion of potential environmental factors that could affect these properties. Altogether, this report provides evidence for the reliability of cellular mechanical properties as biomarkers for ASC differentiation potential and outlines how they can be used to sort ASCs with lineage-specific preferences for particular applications.

Introduction

Human, white adipose tissue harbors a population of mesenchymal stem cells (MSCs) known as adipose-derived stem cells (ASCs).1 These stem cells can differentiate into cells of mesodermal origin, including adipocytes, osteoblasts, chondrocytes, and endothelial cells, among others.1,2 However, ASCs are highly plastic and their differentiation potential is not limited only to mesodermal tissues (for a review on ASC plasticity, see ref. 3). ASCs have also been shown to differentiate into cells of ectodermal origin, such as neurons and epithelial cells, and cells of endodermal origin, such as hepatocytes, making them an extremely versatile cell source for regenerative therapies.3

ASCs are unique because they are more abundant and easier to isolate when compared with other MSC populations in the human body.4 Additionally, ASCs exhibit immunosuppressive properties, as shown by their ability to inhibit T cell activation responsible for graft vs. host disease in animals and humans.5,6 Because of the desirable features these cells possess, ASCs are an attractive source for cell-based regenerative therapies and tissue engineering of fat, bone, and cartilage tissues (for reviews of tissue engineering applications using ASCs, see refs. 7–9). ASC-based therapies have already been conducted in preclinical animal studies and clinical human trials.10

ASC Isolation: A Sorting Problem

ASCs must first be isolated from adipose tissue before being used for cell-based studies. However, lipoaspirate contains other cell types besides ASCs that are often undesirable for regenerative therapies.11 These cells include fibroblasts, endothelial cells, pericytes and smooth muscle cells. Since they are fully differentiated cell types, their presence can diminish the overall response of the population for tissue-specific applications.12 Stem cell biologists and engineers are addressing this barrier by exploring different methods to isolate ASCs from heterogeneous populations.

The gold standard for sorting and collecting cells is fluorescence activated cell sorting (FACS). This method consists of identifying cell types based on the expression of surface markers and has been used extensively to isolate many cell types, including ASCs.11,13–15 Unfortunately, ASCs share several surface receptors with other cells present in adipose tissue.16,17 Therefore, FACS-based strategies require the use of multiple surface marker labeling schemes to isolate ASCs. Increasing the number of antibody labels used for sorting enhances the specificity of the sorting process itself but also results in a very low cell yield of purified ASCs. As a result, such an approach drastically reduces the total number of cells available for research and clinical applications.
This sorting problem reaches a new level of complexity when considering the expression of these markers as a function of monolayer expansion, as designated by passage number. Specifically, ASCs sorted directly after their isolation from lipoaspirate have different surface receptor profiles than ASCs expanded in culture. As we will discuss later in this report, recent studies suggest that passage number is an important factor affecting ASC differentiation potential. This is problematic because cell-based therapies often require multiple passages to obtain sufficient cell numbers. Altogether, FACS-based sorting strategies are limited by their low cell yield, complex surface receptor labeling schemes required for sorting and passage-dependent surface marker composition.

**Cellular Mechanical Properties: A Reference Point**

Cellular mechanical properties have emerged as biomarkers for determining cellular phenotype in several contexts, including cancer cell characterization and stem cell differentiation. By mechanical properties, we refer to the cell’s intrinsically resistance to deformation (elasticity) and flow (viscosity). A material that exhibits elastic properties can fully recover its shape after being deformed by an applied stress. Parameters that help describe a material’s elasticity include the elastic modulus ($E_{\text{elastic}}$) and equilibrium modulus ($E_{\text{equil}}$). $E_{\text{elastic}}$ provides information about the compliance of a material immediately following the application of a stress while $E_{\text{equil}}$ provides information about the compliance of a material once the deformation response has reached equilibrium. Materials with high $E_{\text{elastic}}$ and $E_{\text{equil}}$ values are considered non-compliant or stiff whereas materials with low $E_{\text{elastic}}$ and $E_{\text{equil}}$ values are considered compliant or soft. On the other hand, materials that are viscous will flow when a stress is applied. Viscous materials can be described by a property termed apparent viscosity ($\mu_{app}$). Materials with high $\mu_{app}$ exhibit a greater resistance to flow than materials with low $\mu_{app}$ when a stress is applied.

Cells have both solid and liquid components that are responsible for their elastic and viscous behaviors. Because of this dual mechanical behavior, cells are considered viscoelastic materials. Viscoelasticity can be assessed using time- or frequency-dependent tests, and from these, additional parameters can be recorded: instantaneous modulus ($E_p$) and relaxed modulus ($E_g$). Materials with large $E_p$ and $E_g$ values are considered non-compliant whereas those with low $E_p$ and $E_g$ values are considered compliant. One key difference between elasticity and viscoelasticity is that viscoelastic materials do not fully recover their shape after a stress is applied. Therefore, the viscoelastic properties are time-dependent. $E_p$ represents the modulus at time $t = 0$ and depends on the rate of the applied stress while $E_g$ defines the relaxed modulus measured when the material reaches equilibrium. As would be expected, $E_p$ and $E_g$ often have similar values to $E_{\text{elastic}}$ and $E_{\text{equil}}$ even though they are extracted from a viscoelastic response curve. We have used these five mechanical properties, along with cell height, as a set of mechanical biomarkers to characterize a variety of cell types, including ASCs.

**Cellular Mechanical Properties Emerge as ASC Lineage-Specific Biomarkers**

Among the elastic and viscous components that comprise the cell, the actin cytoskeleton, which is responsible for the cell’s morphology, is also the primary contributor to cellular mechanical properties. In the context of ASC differentiation, previous studies indicate that undifferentiated ASCs have a different cytoskeletal organization than ASCs differentiated toward adipogenic or osteogenic lineages. If the organization of the cytoskeleton is changed, either by altering the geometry of the substrate or adding actin polymerization inhibitors to differentiation media, stem cell differentiation also changes. The role of the cytoskeleton in determining these mechanical properties has led researchers, including us, to investigate whether these mechanical properties could serve as biomarkers for lineage-specific phenotype. For purposes of this report, we will only mention studies that used atomic force microscopy (AFM)-based microindentation to explore this hypothesis (for a review of this technique, see ref. 28).

In two AFM studies, it was found that the mechanical properties of undifferentiated bone marrow MSCs changed during differentiation while undergoing cytoskeletal rearrangements. Specifically, undifferentiated MSCs had lower $E_{\text{elastic}}$ values than MSC-derived osteoblasts. The studies’ findings provided evidence that changes in cellular mechanical properties were tied to phenotypic changes, most likely as a direct consequence of the differentiation process. What was not investigated was whether the initial mechanical properties of individual MSCs could influence the differentiation response of the cell. In other words, do cellular mechanical biomarkers exist and can they be used to predict stem cell lineage preference?

Part of the answer to these questions came from another study that compared the mechanical properties of spherical and spread ASCs with those of differentiated adipocytes, osteoblasts and chondrocytes via AFM. Results showed clear differences in the mechanical properties among cell types. Specifically, adipocytes were the most compliant, followed by chondrocytes. Osteoblasts were the least compliant cell type. Interestingly, ASCs and MSCs had similar mechanical properties and their compliance was between that of chondrocytes and osteoblasts. Chondrocytes were the most viscous cell type while adipocytes had the largest cell height. The results of this study suggested that the mechanical properties of ASCs could be used as biomarkers to distinguish ASCs from differentiated cells.

Based on the collective findings from these reports, we hypothesized that ASCs in adipose tissue had mechanical properties that were indicative of their differentiation potential toward specific lineages. Because adipose tissue is heterogeneous, we established a set of 32 ASC clonal populations derived from single cells. After five passages, the mechanical properties of a subset of cells from each clonal population were tested using AFM. We acquired mechanical biomarkers for both spherical and spread cell morphologies. Interestingly, significant differences in properties were only found for cells in the rounded...
geometry, most likely because that effectively removed cell shape as a contributing factor. Another three clonal population subsets were subjected to adipogenic, osteogenic and chondrogenic differentiation. We assessed the differentiation of ASCs toward these lineages by quantifying lipid accumulation, calcium deposition, and secreted proteins such as collagen, respectively. By performing correlation analyses, we found that the mechanical properties of ASCs correlated with lineage-specific metabolite profiles. Specifically, we found that ASC clones that produced larger amounts of lipids (adipocytic) exhibited greater cell heights, clones that produced more calcified matrix (osteoblastic) exhibited greater $E_{\text{elastic}}$ and $E_{\text{app}}$ values and clones that produced more sulfated GAGs (chondrocytic) exhibited greater apparent viscosity values. The results of these experiments supported our hypothesis and showed that differences in the mechanical properties of undifferentiated ASCs could be used to predict their differentiation, and even matrix synthesis potential (Fig. 1).

The ASC clones included in the above study were not simply progenitor populations, showing multipotent differentiation capabilities along the adipogenic, osteogenic and chondrogenic lineages. However, the level of metabolite production varied widely among the clones, as did their mechanical properties, emphasizing the heterogeneity of the original ASC population. At this point, we asked whether sorting cells by mechanical biomarkers could produce populations with greater regenerative capacity. To investigate this possibility, we conducted simulations that inspected the mechanical properties of the top 25% of ASC clones for adipogenic, osteogenic and chondrogenic lineages. Indeed, we found that “high potential” ASC clones had similar mechanical properties within each lineage. For example, ASCs exhibiting robust osteogenesis had $E_{\text{app}}$ values above 200 Pa while ASCs exhibiting robust chondrogenesis had $\mu_{\text{app}}$ values above 1.6 kPa·s. Theoretically, sorting ASC populations for cells exhibiting these characteristics would result in a much more robust, lineage-specific response. The results of these experiments supported our hypothesis and showed that cellular mechanical properties can be used as biomarkers to identify ASCs exhibiting lineage-specific preferences. While certainly an exciting finding, it should be kept in mind that the correlations between mechanical biomarkers and differentiation response were not always strong. Many other factors influence stem cell behavior, with environmental conditions (e.g., biochemical, mechanical and topographical stimuli) being a primary contributor.

**Phenotypic Changes of ASCs as a Function of Passage Number: Does It Affect the Mechanical Properties Too?**

Despite the abundance of MSCs in adipose tissue when compared with other tissues of mesodermal origin, current isolation techniques such as FACS still yield very low cell numbers. As a result, purified ASC populations must be expanded in vitro until they reach a population size that is feasible for clinical applications. However, previous studies suggest that the time cells spend in culture could affect their phenotype and, subsequently, their differentiation potential. In one study that used FACS to sort ASCs, cell surface marker expression changed as a function of passage number. Immediately after being isolated, ASCs exhibited high expression levels of CD34, a stem cell-associated marker. However, CD34 expression decreased drastically after only one passage. Similarly, expression levels of hematopoietic marker CD45, initially low in freshly isolated cells, further decreased and became negligible soon after ASCs were expanded. On the other hand, the expression of surface markers such as CD49d, CD73 and CD90, which are expressed at moderate levels in freshly isolated cells, increased dramatically as ASCs were expanded in vitro. Altogether, these studies suggest that current ASC expansion conditions effectively enrich for multipotent stem cells of mesenchymal origin.

Additionally, other studies reported that the expression of other surface markers like CD44 and CD73, which are highly expressed in chondrocytes and osteoblasts, increased dramatically in expanded ASCs. These results suggest that ASC surface marker expression resembled that of chondrocytes and osteoblasts as their passage number increased. Separate studies, focusing on changes in ASC differentiation potential as a function of passage number, also reported striking differences between the adipogenic, chondrogenic and osteogenic potential of ASCs at different passages. Specifically, results indicated that ASC adipogenic potential was enhanced at early passages (P2–P5) but decreased at later passages. Conversely, ASC chondrogenic and osteogenic potential increased at later passages. Altogether, these results strongly indicate that the time ASCs spend in culture during their expansion strongly influences their fate.

Our previous work investigated the mechanical properties and differentiation potential of ASCs at a specific passage number (P5). Subsequent experiments investigated how ASC mechanical properties change over time in culture. We examined ASC elastic and viscoelastic properties at P3–P5 to determine the extent of any changes, as well as their potential impact on cell sorting.
3. Mizuno H, Tohita M, Uysal AC. Concise review: Substrate compliance could direct stem cell differentiation.33

Environments stimuli, substrate rigidity and bioactive molecules, are known to play a role in the differentiation potential of MSCs, including ASCs. Previous studies in which MSCs were cultured on substrates that mimicked tissue-specific elasticity showed that substrate compliance could direct stem cell differentiation.33

During their expansion, ASCs are adhered to a rigid plastic substrate and surrounded by a specialized media cocktail composed of nutrients and soluble growth factors. These two environmental stimuli, substrate rigidity and bioactive molecules, are known to play a role in the differentiation potential of MSCs, including ASCs. Previous studies in which MSCs were cultured on substrates that mimicked tissue-specific elasticity showed that substrate compliance could direct stem cell differentiation.33

Regarding the effects of passage number on ASC differentiation potential, previous studies show that ASCs expanded in monolayer cultures, but in the presence of chondrogenic-inducing soluble factors like bone morphogenetic protein 6, enhanced ASC chondrogenic potential.34 Interestingly, culturing and passaging mature chondrocytes in these two-dimensional conditions leads to their dedifferentiation, which occurs concomitantly with an increase in the cells’ viscoelastic moduli.35 However, the phenotype of these dedifferentiated chondrocytes can be partially rescued if they are re-seeded on a micropatterned, two-dimensional substrate that imparts the cells with a round morphology.36 In the end, passage number is an important factor in predicting stem cell fate because it is associated with how long ASCs are exposed to chemical and mechanical signals, which not only elicit biological changes, but also mechanical property changes.

### Implications and Future Directions

In this report, we provided an overview of the role cellular mechanical properties play in predicting ASC differentiation potential and showed that these mechanical properties can change as a function of time and passage. The collective findings of the aforementioned studies provide evidence that cellular mechanical properties can be used as label-free biomarkers to sort ASCs displaying lineage-specific preferences. By gaining an understanding of the factors that influence cellular mechanical properties, we can better understand how these properties might be used for sorting ASCs from heterogeneous populations. Once such knowledge is obtained, large-scale deformation-based sorting devices could be developed to sort large numbers of cells for tissue-specific applications.37 This approach is potentially revolutionary because sorting would not rely on any kind of labeling but instead on the intrinsic mechanical properties of cells.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

The authors thank Hetal V. Desai for providing insightful advice. This work was supported in part by National Institutes of Health grants AR054673 and GM104937.

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### Table 1. Mechanical properties of ASCs change as a function of passage number

| Passage | $E_{0\text{stem}}$ (Pa) | $E_{\text{equil}}$ (Pa) | $E_0$ (Pa) | $E_n$ (Pa) | $\mu_{\text{app}}$ (Pa·s) | Cell height (µm) |
|---------|-------------------|-------------------|-------------|-------------|-----------------|---------------|
| P3      | 345 ± 150         | 57 ± 34           | 206 ± 79    | 61 ± 33     | 321 ± 278       | 12.8 ± 4.8    |
| P4      | 884 ± 497         | 79 ± 78           | 523 ± 292   | 86 ± 69     | 734 ± 636       | 15.6 ± 3.7    |
| P5      | 800 ± 511         | 270 ± 226         | 583 ± 455   | 292 ± 254   | 1,690 ± 1,899   | 8.9 ± 3.7     |

The mechanical properties of P3, P4 and P5 ASCs were measured using AFM, as described previously.20 We tested a minimum of 18 cells per passage. Statistical significance between the mechanical properties from P3, P4 and P5 ASCs was determined by one-factor ANOVA with Fisher’s LSD post-hoc test ($p < 0.05$). Differences in $E_{0\text{stem}}$, $E_0$ and cell height were not significant between P3 and P4, but not P5, ASCs. However, differences in $E_{\text{equil}}$, $E_n$ and cell height were not significant between P3 and P4 ASCs but were significant between those passage groups and P5 ASCs. $\mu_{\text{app}}$ increased with passage number, and the observed differences across all passages were significant. Data are shown as arithmetic mean ± standard deviation.
