Published in final edited form as:

J Endod. 2015 January ; 41(1): 50–55. doi:10.1016/j.joen.2014.07.030.

Mechanical Changes in Human Dental Pulp Stem Cells during Early Odontogenic Differentiation

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Abstract

Cell adhesion and migration in bioactive scaffolds require actin cytoskeleton remodeling and focal adhesion formation. Additionally, human dental pulp stem cells (*hDPSCs*) undergo several changes in their mechanical properties during odontogenic differentiation. The effect of factors essential for odontogenesis on the actin stress fiber elasticity and focal adhesion formation is not known.

Methods—Live *hDPSCs* cultured in odontogenic media were imaged for cytoskeleton changes using an atomic force microscope. The Young's modulus (kPa) of the cytoskeleton was recorded as a function of culture medium for 10 days. Focal adhesion formation was assessed using immunofluorescence. Cultured *hDPSCs* were incubated with a monoclonal vinculin antibody and filamentous actins were visualized using 0.5 μ M phalloidin.

Results—Cytoskeletal elasticity significantly increased in response to odontogenic media. Both the number and physical size of focal adhesions in *hDPSCs* also increased. Up-regulation of vinculin expression was evident. The increase in the formation of focal adhesions was consistent with actin remodeling to stress fibers.

Conclusions—Our findings suggest that *hDPSCs* firmly attach to the glass substrate in response to odontogenic media. Successful regeneration of pulp-dentin tissue using biomimetic scaffolds will likely require cell-extracellular matrix (ECM) interactions influenced by biochemical induction factors.

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The authors deny any conflicts of interest related to this study.

Keywords

Adhesion; actin reorganization; regeneration; human dental pulp stem cells; attachment

Introduction

Promising options for the formation of reparative dentin include direct pulp capping (1) and human dental pulp stem cell (*hDPSC*) transplantation. Both alternatives to root canal therapy involve the interaction of a cell source with an extracellular bioactive material (2).

Extracellular matrix (ECM) scaffolds and inductive chemical factors in culture medium may affect cell adhesion, migration, proliferation and differentiation. Importantly, cellular actin cytoskeleton reorganization and focal adhesion formation have been shown to impact differentiation in human mesenchymal stem cells (2–3).

Adherent cells, including *hDPSCs*, must attach to a substrate before differentiating into terminal odontoblast-like cells (3), and inductive cues in culture medium could cause mechanical changes in *hDPSCs* affecting cell fate. Focal adhesion protein complexes are essential for cell attachment and migration (3–4). Vinculin, a structural adaptor protein found in focal adhesion complexes, anchors the actin cytoskeleton to signaling proteins that communicate with the ECM (5). The actin cytoskeleton is an important regulator of cell-body elasticity and plasma membrane signal transduction in response to external stimuli such as soluble factors and substrate elasticity (6–8). Stress fiber formation resulting in cytoskeleton reorganization and subsequent tissue remodeling affects stem cell viability, self-renewal and differentiation (9–10).

Recent studies have shown that pulp-capping materials, such as mineral trioxide aggregate (MTA) and BioAggregate™, enhance the association of focal adhesion and actin stress fibers *in vitro* (2). Actin remodeling has been shown to mediate decreased cell elasticity in human mesenchymal stem cells (*hMSCs*) subjected to osteodifferentiation factors (10–13). However, the role of odontogenic differentiation factors in altering the cytoskeletal organization in *hDPSC* remains unknown. In order to address this, we examined changes in vinculin expression and cell stiffness (Young's modulus) upon exposure to odontogenic biochemical factors *in vitro*.

Although there is no standardized induction cocktail for the odontoblast-like differentiation of *hDPSC*, the protocol reported by Gronthos et al has been widely used (15, 27). Using this induction protocol, we report key quantitative data for dental material and tissue engineering applications based on novel mechanical characterization. Our aim is to validate the hypothesis that soluble factors in odontogenic differentiation medium affect *hDPSC* cytoskeleton elasticity and focal adhesion formation. This research will inform future experiments designed to optimize the fabrication of biomimetic scaffolds for *hDPSC* differentiation requiring initial soluble factors *in vitro*.

Materials and Methods

Cell Culture

Human dental pulp stem cells (donor between the ages of 18–25) were kindly gifted from Dr. Songato Shi at the University of Southern California. Cells were cultured in minimum essential medium (α -MEM) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ M L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 μ g/mL streptomycin (15). Passage 4 cells were incubated at 37°C in a 5% CO₂ and the culture medium was exchanged every other day.

Odontogenic-like Induction

Cells between passages 4 and 5 were incubated with odontogenic induction media containing α -MEM supplemented with 20% FBS, 2 mM L-glutamine, 100 μ M L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 μ g/mL streptomycin, 1.8 mM of monopotassium phosphate, KH₂PO₄ and 10nM of dexamethasone (15). Samples were sub-cultured onto 22 $\times$ 22 mm glass coverslips at a density of 3×10^3 cells/cm². Samples for cell elasticity experiments were cultured at days 1,3,7 and 10. Samples for focal adhesion experiments were sampled over 5 days.

Cell Elasticity Measurements

The cell elasticity of live hDPSCs was measured with an atomic force microscope (Novascan Technologies, Ames, IA) using a microindentation technique. Briefly, a silicone nitride cantilever probe (Veeco, Santa Barbara, CA) 100 μ m in length was positioned over the cytoplasmic region of live hDPSCs in phosphate buffered saline (PBS) avoiding the perinuclear region. Each cell was indented with the AFM probe at three different locations over a 15 $\times$ 15 μ m area. Cells grown at days 1, 3, 7, and 10 were measured. A force curve was obtained by measuring the deflection of the cantilever corresponding to the applied force. Force-distance curves were analyzed using the Hertz model (10)

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

where F is the loading force, E is the Young's elastic modulus, ν is the cellular Poisson's ratio, δ is the cell indentation depth and R is the radius of the spherical indenter (5 μ m). The average Young's modulus for the hDPSCs cultured in odontogenic and control culture medium at four time points was calculated and analyzed using the two-way analysis of variance (ANOVA) test followed by the Friedman and Kruskal-Wallis test.

Immunofluorescence and fluorescent microscopy

To observe cytoskeletal actin microfilaments, hDPSCs were fixed with 4% formalin and permeabilized in cold (−20°C) acetone for 3 minutes. A 5% BSA solution was applied to the samples to block nonspecific binding sites for 30 minutes at room temperature. Actin microfilaments were stained with rhodamin-phalloidin (5 μ M) (Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Samples were imaged with a

fluorescent microscope at days 0, 1, 7 and 10. To determine the effect of actin filament disruption on cell elasticity, the cytoskeleton was disrupted with cytochalasin D (5 μ m), (Sigma-Aldrich, St.Louis, MO) following incubation for 30 min at 37°C (10). Samples for focal adhesion observations were incubated with mouse anti-human primary vinculin antibody overnight at 4°C, and further treated with FITC conjugated (green fluorescence) goat-anti-mouse secondary antibody for 1 hour at 37°C. (Millipore, Billerica, MD). F-actin were stained simultaneously with 0.5 μ M of TRITC- conjugated (red fluorescence) phalloidin (Millipore,Billerica, MD). Nuclei staining (blue fluorescence) was visualized using 0.5 μ M 4',6-diamidino-2-phenylindole (DAPI) for 3 minutes at room temperature. Staining was conducted at days 1, 3 and 5 in control and odontogenic induction medium in triplicate. Samples treated for immunocytochemistry were visualized with an E-800 Eclipse Nikon fluorescent microscope with a 60 $\times$ objective lens and a 16-bit charge-coupled device camera (Photometrics, Tucson, AZ). Images were pseudocolored with MetaMorph software (Molecular Devices, Downingtown, PA).

Statistical analysis

All values of statistical analysis are expressed as average values $\pm$ standard deviation. Statistical analysis of hDPSC cytoskeleton elasticity and focal adhesion formation was performed using the two -way analysis of variance (ANOVA) test followed by the Friedman and Kruskal-Wallis test. Actin disruption data was analyzed using the Student's t- test. P values ≤ 0.05 were considered significant.

Results

Effect of odontogenic induction medium on hDPSC cytoskeleton elasticity

To address whether odontogenic differentiation medium affects cytoskeletal changes, we examined hDPSC actin stress fiber formation using atomic force microscopy. Exposure time periods were selected to allow sufficient time for lineage commitment. As shown in Fig. 1A, cells exposed to odontogenic medium for days 7 and 10 showed a marked increase in the thickness when compared to days 0 and 1. Additionally, the Young's modulus, as measured using the AFM microindentation technique, exhibited an increase in kPa (4.11) in cells exposed to odontogenic medium on day 10. In contrast, cells exposed to odontogenic medium at days 1 and 3 showed a significantly less modulus kPa (2.25) (Fig. 1B). The increase in Young's modulus over time was significantly greater ($p \leq 0.05$) in hDPSCs cultured in odontogenic medium.

Influence of cytoskeleton disruption on the average Young's modulus

In continuation with our findings in Fig. 1B, we intended to test whether disruption of cytoskeleton hinders the cell stiffness in hDPSC. Interestingly, as observed in Fig. 2A, cells exposed to Cytochalasin D showed almost no stress fibers. Furthermore, cells treated with cytochalasin D showed a significant reduction ($p \leq 0.05$) in Young's Modulus, kPa when compared to cells treated with differentiation media alone (Fig. 2B).

Odontogenic differentiation increases focal adhesion proteins in *hDPSC*

In our next set of experiments, we tested whether the differentiation of *hDPSC* towards an odontogenic lineage affects focal adhesion presence and localization. We performed the experiment at 3 different time points; day 1, day 3, and day 5. In our experiments, we stained for Vinculin. Interestingly, our findings suggest an increase in the intensity of fluorescence, representing the levels of vinculin in *hDPSC* (Fig. 3A). An increase in the number of vinculin molecules are observed in cells treated with odontogenic medium. The average number of focal adhesions per cell in the microscopic field of view in experimental samples significantly increased ($p < 0.05$) over time compared to the control samples (Fig. 3B).

Discussion

Recent research reports pulp-capping and cell-based therapies as possible methods to regenerate compromised pulp tissue. Pulp-capping has been shown to maintain pulp vitality and enhance dentin repair mechanisms in damaged pulp tissue (1). Conversely, stem cells isolated from human dental pulp have been combined with cell delivery scaffolds for pulp regeneration applications (27). Odontoblast-like differentiation using *hDPSCs* and bioengineered ECM scaffolds may address shortcomings associated with pulp-capping materials such as limited reparative dentin formation (28), thus understanding the effect of odontogenic media on cell-ECM interactions in vitro is necessary for in vivo comparison.

Cell adhesion, migration and differentiation are three types of cell-ECM interactions influenced by actin cytoskeletal reorganization and focal adhesion formation (10,30). The contribution of soluble differentiation biochemical factors, such as dexamethasone, to these mechanical responses is unclear. Cell adhesion is required for cell attachment to a scaffold for adherent cell types (2,29), and our study reports the changes in the *hDPSC* cytoskeletal elasticity and vinculin protein expression of *hDPSCs* during early odontogenic differentiation.

In this study, we define early odontogenic differentiation as samples exposed to induction medium for less than 10 days. *HDPSCs* cultured on glass coverslips showed an increase in cytoskeletal elasticity. The Young's modulus increased in live *hDPSCs* induced with odontogenic medium from days 1 to 7, with an average increase between 3–4 kPa.

The actin stress fibers also thickened as time progressed, replacing initial thinner actin meshworks. This may be due to the dexamethasone in the induction medium penetrating the cell membrane and initiating an odontoblast-like lineage (3). The decrease in the modulus at day 10 may have been due to changes in gene expression as previously reported by Titushkin et al (10). Sample treatment with cytochalasin D significantly decreased the average elastic modulus of the *hDPSC*, most likely due to depolymerized stress fibers. This data demonstrates that actin filaments regulate cell stiffness.

Interestingly, *hMSCs* induced toward an osteogenic lineage with 10nM of dexamethasone between days 1 and 7 resulted in thick actin stress fibers replaced with thinner actin filaments with a decrease in Young's modulus at day 10 (10). Additionally, the

concentration of dexamethasone used to induce hDPSCs toward an odontoblast-like lineage was 10nM as previously reported by Gronthos et al (15). These results suggest that despite similarities in gene expression profiles between the two cell types (23) and the mechanical properties may be modulated differently. This hypothesis can be tested in future studies by comparing overlapping odontogenic and osteogenic gene markers at different time points with multiple hDPSC cell lines and donors.

Focal adhesion formation also required for cell adhesion and migration (30). Focal adhesions are flat, elongated structures ranging from 2 to 5 μ m in size (18–22). Our findings suggest that the hDPSCs become strongly attached to the substrate in response to the odontogenic factors. We used immunofluorescent staining to map the presence of vinculin, a 117-kDa protein at the hDPSC periphery. The mean number of focal adhesions per cell increased linearly with time for the samples exposed to odontogenic medium, but did not change in the control cells between days 3 and 5. This was potentially due to strong adhesions formed on the rigid glass restricting cell migration. Conversely, soluble signals promoting migration in the induction media or changes intercellular contact may have contributed to the increased average number of focal adhesions in the hDPSCs cultured in the odontogenic medium (25–26).

One method to better characterize this interaction would be to suppress focal adhesion kinase to determine if focal adhesion formation is inhibited at extended time points. Additionally, identifying the key integrins expressed by hDPSCs as a function of substrate stiffness would provide insight into the molecular interactions that regulate mechanical events. Integrin expression is necessary for the adherence of cells to synthetic scaffolds (19). A recent study examining stress fiber assembly and dental pulp cell adhesion formation to different pulp-capping agents showed preferential focal adhesion to BioAggregate, highlighting the clinical significance of this work (2).

In summary, the development of optimal scaffolds for dental pulp tissue engineering will likely require synergy between inductive factors in culture medium, scaffold material and cell sources. The current study shows that the elasticity of the hDPSC cytoskeleton increases over time and focal adhesion formation is enhanced by odontogenic medium. An important unknown factor is how hDPSC may respond to physical scaffold cues in the absence of induction medium in vivo. The next step in the use of data quantifying mechanical changes in differentiating hDPSCs would be to seed hDPSCs on scaffolds of varying matrix elasticity, as this has also been shown to direct stem cell lineage (3).

Acknowledgments

We acknowledge Dr. Amelia Zellander for her technical assistance with sample preparation. Support for this work was provided by a NIH/NIDCR grant DE019514-SBA.

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A

B

Figure 1.

(A) Actin filament reorganization of hDPSCs exposed to odontogenic medium at four time points as determined by phalloidin immunofluorescence staining. Scale bar = 30 μ m. Images represent samples from three independent experiments. (B) Effect of odontogenic medium on Young's modulus of hDPSCs as quantified using atomic force microscopy.

A

B.

Figure 2.

(A) Effect of cytochalasin D treatment on the cytoskeleton of hDPSCs. Scale bar = 30 μ m. Images represent samples from three independent experiments. (B) Effect of cytochalasin D treatment on the Young's modulus of hDPSCs.

A.

B.

Figure 3.

(A) Focal adhesion formation exposed to odontogenic induction medium as determined by immunofluorescence staining. hDPSC were treated with odontogenic medium for 5 days. Vinculin (green) was colocalized with the actin cytoskeleton and stained with phalloidin (red). Scale bar = 35 μ m. Images represent samples from three independent experiments. (B) The average number of focal adhesions per cell as a function of culture medium over time.