**Human Dental Pulp Stem Cell Adhesion And Viability Studies In Hybrid Hydrogels**

BY

TANEKA DENISE TAYLOR

B.S., Illinois State University, 2006
M.A., National-Louis University, 2009

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Master of Science in Bioengineering
in the Graduate College of the
University of Illinois at Chicago, 2014

Chicago, Illinois

Defense Committee:

Michael Cho, Advisor
Satish Alapati. Endodontics, Co-Advisor
Shan Sun, Bioengineering

This thesis is first and foremost dedicated to God followed by my husband, Markus L. Jones.  I would not have accomplished it without the support of both.  I would also like to dedicate this thesis to my father, William B. Taylor and mother, Yolanda M. Franklin.  Thank you for supporting me and encouraging me to realize my dreams.

# ACKNOWLEGDEMENTS

I would like to thank my thesis committee, Dr. Michael Cho, Dr. Satish Alapati and Dr. Shan Sun.  All three members provided me with support and assistance in finalizing my project goals.  I thank Dr. Michael Cho for welcoming me into his lab.  I thank Dr. Alapati for countless mentoring hours and Dr. Sun for her technical assistance and patience.  I especially acknowledge Dr. Aixa Alfonso for her encouragement, mentoring and contributions towards my professional career development.

There were a number of colleagues that assisted me with data collection.  Among them, I would like to thank Dr. Amelia Zellander for assisting me with specimen preparation and Amira Kefi for her support with meeting writing goals.

TDT

**TABLE OF CONTENTS**

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 2D | Two-dimensions |
| 3D | Three-dimensions |
| ECM | Extracellular Matrix |
| Da | Daltons |
| DAPI | 4',6-Diamidino-2-Phenylindole |
| DEX | Dexamethasone |
| DSPP | Dentin Sialophosphoprotein |
| GN | Gelatin |
| HA | Hyaluronic Acid |
| HDPSC | Human Dental Pulp Stem Cell |
| MSC | Mesenchymal Stem Cell |
| PBS | Phosphate Buffer Saline |
| PEGDA | Poly(ethylene glycol) |
| PEGSSDA | Poly(ethylene glycol) with a double-sulfide bond |
| RSS | Residual sum of squares |
| sECM | Semi-synthetic Extracellular Matrix |
| TCP | Tissue Culture Plastic |

# SUMMARY

Dental caries is the most prevalent disease that affects teeth and results in early tooth loss. According to data collected by the American Association of Endodontists, there are about forty-one thousand root canal treatment procedures per day in the United States to save natural dentition. Despite being a relatively common procedure to save teeth, these treatments are subject to failure when microorganisms are not fully eliminated or restorations are not sound (5).  Unsuccessful root canal therapy may result in tooth loss and increased restorative dental health care costs.  Current treatment protocols do not promote pulp-tissue regeneration, and the process of forming reparative tertiary dentin may require terminal stem cell differentiation (1).

Novel therapeutic intervention is needed to promote rapid healing of dental pulp tissue and the production of a new dentin barrier for regeneration of the pulp-dentin complex, so that conventional root canal therapy or permanent tooth extraction can be avoided.

Hystem-C™ is a commercially available semisynthetic extracellular matrix (sECM) product comprised of polyethylene glycol diacrylate (PEDGA), hyaluronuic acid (HA) and gelatin (Gn). These components can be manipulated to achieve favorable conditions for cell adhesion and viability in 3-dimensions (3D).  PEGDA can be substituted with a higher molecular weight disulfide bond product, (PEGSSDA).   The *objective* of this study was to investigate whether human dental pulp stem cells (hDPSCs) embedded in 3D PEGSSDA-HA-Gn hydrogel scaffolds enhance cell adhesion and viability.

Human dental pulp stem cells  embedded in 3D PEGSSDA HA-Gn scaffolds enhanced cell viability, while cell proliferation increased after 14 days in culture.  Our investigations to determine the optimal ratio of HA: Gn suggests that the concentration of a 1:3 ratio of HA: Gn significantly increased hDPSC viability embedded in PEGDA-HA-Gn 3D membranes.

# I. INTRODUCTION

## 1.1 Developmental Biology of the Dentin-Pulp Complex

### 1.1.1 <u>Developmental Oral Biology</u>

During embryonic tooth development, the cranial neural crest gives rise to developing dental epithelium. Ectomesenchymal cells originate from the dental epithelium and form the dental papilla. The dental papilla is an aggregate of differentiated ectomesenchymal cells called odontoblasts. During embryogenesis these highly specialized cells secrete primary dentin, a mineralized tissue surrounding the soft pulp tissue (2). Mineralization starts at the junction of the enamel organ and dental papilla, which gives rise to the enamel-dentin junction.

Odontoblasts also secrete post-developmental secondary dentin, with characteristic tubules that remain continuous with primary dentin. Tertiary dentin is formed in response to stimuli such as dental caries, trauma and excessive tooth wear (3) and is further classified as either reparative or reactionary. Mature dentin is composed of 70% hydroxyapatite, 20% organic matrix including type I and type V collagen, noncollagenous proteins such as dentin sialophosphoprotein (DSPP) and 10% water. Despite being the most mineralized tissue in the tooth, the elasticity of dentin provides flexibility for enamel (4).

Dental pulp is the soft connective tissue comprised of nerve endings and vasculature occupying the internal cavities of the tooth. The pulp space is divided into the pulp chamber and pulp root canals. Tubules of dentin surround the pulp cavity forming the pulp-dentin complex. The two tissues are considered a complex due to the structural and functional relationship. Odontoblasts are thought to be maintained by human dental pulp stem cells (hDPSC), a clonogenic precursor population of cells affiliated with perivascular cells (2).

1

### 1.1.2 Molecular characteristics of human dental pulp stem cells

The ability of odontoblasts to secrete reactionary and reparative dentin has led to the speculation that resident stem cells originating from the neural crest exist in dental pulp tissue (7). Stem cell populations in the dental pulp of human first and third molars have been identified that are clonogenic and can form dentin, adipose and neural cells when exposed to biological inductive factors (8-9). Interestingly, stem cell sources isolated from dental pulp tissue appear to be preferentially located in a specific perivascular microenvironment (10).

The immunophenotypes of isolated DPSCs can be compared with mesenchymal stem cells using flow cytometry. Subsequent flow cytometry data from recent groups show that human DPSC are negative for CD14 and CD34, and positive for CD146 and STRO-4 markers (9).

## 1.2 In vitro Culture of Human Dental Pulp Stem Cells

### 1.2.1. Growth factors in human dental pulp stem cell culture medium

In vitro experiments are conducted using single cell suspensions of hDPSCs. There are well documented techniques to isolate and expand hDPSC from adult third molars (2). Regular hDPSC growth medium contains a cocktail of amino acids, ribonulceosides and deoxyribonuleosides supplemented with antibiotics and ascorbic-acid 2-phosphate. There are specific growth factors that have been shown to regulate odontoblast differentiation and maturation from undifferentiated hDPSC.

The most commonly reported odontogenic-like differentiation growth factors reported in the literature are dexamethasone (DEX) , a glucocorticosteroid, and 1-alpha-25-dihydroxyvitamin $D_3$ (13). Both inducers influence differentiation pathways in a dosage

dependent manner. A phosphate compound such as β-glycerophosphate is usually added to the cocktail to encourage mineralization.

Wei et al, showed a mineralized matrix and upregulation of an odontogenic marker dentin sialoprotein (DSP) using an induction cocktail of dexamethasone, β-glycerophosphate and L-ascorbic acid 2-phosphate (12). When these cells were subjected to additional growth factors such as indomethacin, insulin and TGF-β₁, the hDPCSs progressed along adipogenic and chrondrogenic pathways respectively. Similarly, Jain et al demonstrated maintenance of hDPSC induced with vitamin D₃ for 14 days with upregulation of secreted osteocalcin, a late marker of osteogenesis (13). These results strongly suggest that a desired odontogenic differentiation program include the cocktail trio.

## 1.3 Regenerative Endodontics

### 1.3.1 Regenerative Endodontics

Endodontics is a specialized dental field concerned with treating infected and traumatized dental tissues. Dental pulp is the soft tissue inside of the tooth core that contains nerve and blood vessels subject to thermal, chemical, mechanical and bacterial insults. The American Association of Endodontists approximates that 16 million endodontic procedures are performed annually in the United States alone. Conventional treatment for patients with diseased dental pulp tissue results in the removal, or extirpation, of tissue from the tooth core. This "root canal therapy" is followed by disinfection and filling the hollowed tooth canal with a biologically inert material.

Restoration of tooth function following a root canal procedure is not likely due to removal of the nerve and vascular tissue. Complications associated with root canal

procedural failures such as reinfection and tooth fractures have encouraged merging research in the field of regenerative endodontics.

The most heavily researched aims of the field appear to be targeted towards pulp revascularization and pulp regeneration (1). Pulp revascularization is the promotion of angiogenesis in a root canal that has been treated by an endondontist. Pulp regeneration is a combination of pulp revascularization and the restored function of dental pulp tissue. Regeneration of the pulp-dentin complex requires a stem cell source, growth factor morphogens and an extracellular matrix (ECM) scaffold.

The successful design of a biological substitute for dental-pulp tissue will likely require a tissue engineering approach designed to restore, maintain or improve dental-pulp tissue function applying both regenerative endodontics and engineering principles (14).

1.3.2. <u>Bioengineering of dental pulp tissue</u>

There are two approaches in the literature using hDPSC for regenerative endodontics applications. Cell homing of autologous cells from the apical region were induced to migrate towards the root canal and differentiate into pulp tissue showing vascularization. A second approach is a cell delivery based strategy, where stem cells are transplanted directly into the root canal and induced to odontoblast-like lineages (11). The most favorable stem cell source for clinical application might be autologous hDPSC from individual patients, but is dependent on the viability and health of a patient's tooth.

A traditional tissue engineering approach would combine growth factors and the use of natural, synthetic or hybrid scaffolds with the stem cell source to maximize pulp regeneration efforts. The optimal scaffold for dental pulp tissue engineering has not been determined. Table I outlines the advantages and limitations of natural and synthetic scaffolds (15).

TABLE I
ADVANTAGES AND LIMITATIONS OF TISSUE ENGINEERING SCAFFOLDS

| Type of Scaffold | Advantages | Limitations |
| --- | --- | --- |
| Natural | Biodegradable<br>Biocompatible<br>Recognition domains for<br>cell attachment | Mechanically weak<br>Unfavorable immune<br>response<br>Structurally complex |
| Synthetic | Mechanically Tunable<br>Functional groups can<br>support<br>cell attachment/bind growth<br>factors | Inert microenvironment<br>Degradation cytotoxicity |

## 1.4 Scaffolds used in Dental Pulp Tissue Engineering

### 1.4.1 <u>The Extracellular matrix as a model scaffold</u>

The extracellular matrix (ECM) is the cells' physiological environment that modulates cellular behavior via chemical signals. Optimal scaffolds for tissue engineering should mimic the microenvironment of the cell as closely as possible. Effectively, this favorable cellular environment is the cells' niche. The ECM is a dynamic environment degraded and remodeled by cells (15).

A scaffold designed for dental pulp regeneration should closely mimic the native dental pulp tissue. Design considerations include the incorporation of growth factors for odontogenesis, mineralization potential and tunable cell adhesion potential for cell attachment and cell-matrix interactions. To date, there are no reported commercially available scaffolds specifically designed for dental pulp tissue engineering (26). Scaffolds designed as natural ECM analogs may be natural, synthetic or a hybrid of both.

### 1.4.2 <u>Natural scaffolds</u>

The native in vivo cellular environment provides the most benefit for tissue remodeling. Biomaterials derived from natural sources are inherently biodegradable and biocompatible which favors desirable host integration responses. Natural scaffolds include polysaccharides, collagen and hyaluronic acid (HA).

Collagen is the most abundant protein in the human body with excellent tensile strength and viscoelastic properties. ECM proteins such as collagen and fibronectin contain binding motifs necessary for cell-matrix interactions such as attachment. Kim et al, compared

hDPSC seeded on type I and III collagen, chitosan and gelatin and found that cellular adhesion and proliferation were optimal on collagen scaffolds (19.)

HA is a natural non-sulfated glycosaminoglycan found extensively in the ECM of connective tissues.   It is a hydrophilic molecule that is able to bear compressive loads in tissues (16).  HA is an attractive scaffold dental tissue engineering option because it is biodegradable, biocompatible, non-immunogenic and angiogenic (17).   In situ, ECM proteins such as collagen can be mixed with HA to aid in cell attachment (18).

1.4.3 Synthetic scaffolds

A major limitation of natural polymers such as HA is low mechanical stiffness and rapid biodegradation.  This drawback is addressed by crosslinking with synthetic polymers such as polyethylene glycol diacrylate (PEGDA).   Polyethylene glycol based scaffolds are generally well accepted for tissue engineering applications due to their solubility and porosity (20). Porous scaffolds allow cells to attach and migrate in material via interconnected pores, and studies have shown that different cell types require certain pore sizes for optimal attachment (23-24.

The mechanical strength and biological inertness of PEG-based synthetic polymers enables bulk and surface modification.  PEG hydrogels can be fabricated with growth factors that increase cell adhesion, migration and proliferation rates.  .  Cell adhesion can be mediated by the addition of (ECM) proteins to synthetic scaffolds that lack the physio-chemical information found in the ECM (15).  Gelatin, as denatured collagen, is an ECM mimic that has also been used as a porogen when added to synthetic ECM PEG based hydrogels (21).

1.4.4 <u>Three dimensional (3D) synthetic hybrid ECM scaffolds</u>

Tissue engineering scaffolds modeled after the in vivo physiological environment have been challenging but necessary.  Traditional two-dimension (2D) scaffolds are limited by exaggerated cell adhesion and minimized cell-cell interactions.  Fabrication of 3D synthetic scaffolds allow cell-scaffold interactions to be studied and may help to model and predict in vivo functional outcomes (27).

Gel-based culture models are one type of 3D mimic where cells are embedded. Hydrogels are a class of synthetic biomaterials with viscoelastic properties similar to in vivo connective tissue.  The hydrophilic nature of hydrogels enables absorption of up to a thousand times of their dry weight in water (15).  Similar to PEG based constructs, hydrogel surfaces may be modified with cell adhesive ECM proteins such as collagen.

A second advantage of hydrogels is the potential for clinical use.  Clinicians often use syringe applications for injectable materials. Injectable hydrogels can be molded into complex shapes and crosslinked in situ (21). Cavalcanti et al used an injectable self-assembling peptide hydrogel to examine viability and differentiation of human dental pulp stem cells (22).

Several groups have designed ECM analogues to facilitate cell attachment, proliferation, migration and differentiation.  Serban et al, designed a semisynthetic ECM (sECM) 3D culture hydrogel model using cross-linked derivatives of hyaluronan, gelatin and PEGDA. Interestingly, the compliance of the hydrogels can be altered by changing the concentrations of components (28).

1.4.4.1 <u>Clinical applications of semisynthetic ECM hyaluronan-derived hydrogels</u>

Cheng et al tested the regenerative potential of cardiac cells delivered in an injectable

hyaluronan-gelatin-polyethylene glycol (HA-GN-PEGDA) hydrogel (29). Embedded human

cardiocytes demonstrated improved cell spreading and viability in the constructs compared

with HA-PEGDA only scaffolds. Serban et al assessed the morphology and cell proliferation

rates of murine fibroblasts embedded in 3D HA- GN-PEGDA constructs. Results from

immunocompromised mice injected subcutaneously showed biodegradation of the scaffold

with simultaneous secretion of new ECM (28).

There have also been reported uses of sECM for stem cell delivery applications.

Autologous bone marrow derived mesenchymal stem cells were delivered in an injectable

hyaluronan sECM and mediated cell retention for endogenous cell biological repair (30).

Similarly, encapsulated chondrogenic derived from human embryonic stem cells mediated

spatiotemporal controlled remodeling of an osteochrondral defect (31).


**1.5 Cell-matrix Interactions**

1.5.1 <u>Cell-matrix adhesions</u>

Cell adhesion is a first step of cell-matrix interactions as cells must attach to the scaffold

to be viable and functional. The adhesion of cells to the ECM causes a signaling cascade that

relays information for anchorage dependent cells. When a cell attaches to a substrate with

integrins expressed on the cell membrane, the cytoskeleton transmits traction forces between

multiple attachment sites which allow cells to respond to the substrate stiffness (32).

Lucchini et al determined that the αVβ3 integrin expressed by hDPSC could play a role in adhesion and odontoblast binding to the dentin-pulp matrix (33). Clusters of integrins are called focal adhesions, structural complexes that link the ECM to the actin cytoskeleton (34).

Focal adhesion complexes involve highly regulated processes where transmembrane integrin proteins cluster with adhesion signaling molecules. Cell adhesions are generally classified based on morphology, size and subcellular distribution (40).

There are four known adhesion types; focal complexes, podosomes, fibrillar adhesions and focal adhesions (41). Focal adhesions are flat, elongated structures ranging from 2 to 5 um in size. These adhesions are located near the cell periphery at the ends of actin stress fibers (41-45.) Pasquinelli et al showed the adhesion of rat MSCs to a HA-based microfibrous scaffold in the absence of chemical induction (35).

This clustering between integrins and ECM bonds increases the adhesive force by increasing the number of force bearing members within an area (36). Additionally, signaling cascades initiated by focal adhesion assembly may enhance adhesive force by distributing mechanical loads among integrins.

Integrins associate intracellularly with structural adaptor proteins. Vinculin is a universal focal adhesion adaptor protein responsible for linking actin filaments to other protein complexes. These interactions result in the propagation of cellular responses including actin reorganization (37-39). In addition to structural function, focal adhesions initiate signaling cascades following preliminary adhesion. Cell adhesion and migration are critical for cell colonization in biomimetic scaffolds, thus fabrication of substrates modeled after in vivo dentin-pulp tissue moduli is timely.

### 1.5.2 Cell-Matrix Elasticity

Matrix elasticity has been shown to direct naïve mesenchymal stem cell lineage to committed phenotypes (25).  Cell morphology, spreading and function are also influenced by substrate stiffness (54.)  Mechanical properties such as substrate stiffness and biodegradation rates can be adjusted by several parameters including varying the molecular weight of the HA and/or varying the molecular weight of synthetic crosslinking agents.

Skardal et al encapsulated NIH 3T3 cells in thiolated hyaluronic acid crosslinked with thiolated gelatin and polyethylene tetra-acrylate (PEDTA) to prepare printed tissue constructs with better mechanical properties than PEGDA 3400 (55).

PEGDA 3400 is frequently for tissue engineering purposes, but requires digestion with enzymes such as hyaluronidase to recover embedded cells.  Such treatment will disrupt cell-matrix interactions and possibly result in low cell recovery or altered cell phenotype (53). The higher molecular weight of PEGSSDA 8400 was clinically attractive, as dentin is a mineralized tissue.

PEGSSDA overcomes the cell recovery limitation by the inclusion of a single disulfide block.  Non-enzymatic treatment via a thiol-disulfide exchange reaction encourages the dissolution of gels.  PEGSSDA 8400 is also higher in molecular weight, resulting in a higher elastic modulus compared to PEGDA 3400.

## II. HYPOTHESES

### 2.1 Investigation of Focal Adhesion Formation

2.1.1 Hypothesis

To investigate focal adhesion formation of hDPSCs on 2D glass as a function of culture medium, we hypothesized that focal adhesion formation is modulated by osteogenic growth medium. Observation s of cell attachment was used to assess cell viability and attachment for subsequent 3D hydrogel experiments.

### 2.2 Determination of Effect of Crosslinker Variation on Cell Viability

2.2.1 Hypothesis

To determine the optimal crosslinker for hDPSC viability in 3D, we compared live/dead staining of hDPSC as a function of PEGDA 3400 or PEGSSDA8400. We hypothesized that the PEGSSDA 8400 will be optimal for hDPSC viability.

### 2.3 Quantification of Effect of PEGSSDA Concentration on Gelation Time

2.3.1 Hypothesis

To quantify the gelation time of the 3D hydrogels as a function of PEGSSDA concentration, we prepared five concentrations of PEGSSDA-HA-Gn hydrogels. We hypothesized that higher concentrations of PEGSSDA would result in decreased gelation times of the PEGSSGA-HA-Gn injectable hydrogels.

### 2.4 Optimization of Hyaluronic Acid to Gelatin Volume Ratios for hDPSC Attachment

2.4.1 Hypothesis

We hypothesized that a commercial PEGSSDA-HA-Gn product will encourage cell spreading if the ratio of HA: GN is optimized. Cell attachment and spreading should be enhanced by GN addition.

# III.    STATISTICAL ANALYSIS

## 3.1 <u>Statistical Methods</u>

### 3.1.1 <u>Calculations of statistical significance</u>

Statistical analysis was calculated using SPSS software (Chicago, IL, USA).  Mean values and standard deviations were computed (n=3).  The Student's t-test and analysis of variance (ANOVA) used to assess the statistical significance for focal adhesion formation and experiments designed to determine the effect of crosslinker and hyaluronic acid: gelatin on hydrogel stiffness.  A p-value $< 0.05$ was considered significant.

### 3.1.2 <u>Calculations and modelling of non-linear regression</u>

Data obtained from plotting gelation time as a function of crosslinker concentration was fit to a curve using SPSS software (Chicago, IL, USA).  Non-regression logarithmic and exponential models were used to fit the curved data.  The residual sum of squares was used to measure the discrepancy between the experimental and theoretical models.

# IV. MATERIALS AND METHODS

## 4.1 Investigation of Focal Adhesion Formation

### 4.1.1 Cell Culture

Human dental pulp stem cells were kindly gifted by Dr. Songato Shi at the University of Southern California and expanded in vitro as previously reported by Gronthos et al, (2). Single cell suspensions were seeded in a 25cm2 culture flask containing α-MEM (Gibco, Grand Island, NY,USA) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA,USA) 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37°C in a 5% $CO_2$ atmosphere until they reached ~80% confluence. The medium was changed every other day.

### 4.1.2 Odontogenic Induction:

Passage 4 hDPSCs were incubated with odontogenic induction media containing α-MEM (Gibco, Grand Island, NY,USA) supplemented with 20% fetal bovine serum ( FBS; Atlanta Biologicals, Flowery Branch, GA,USA), 2 mM L-glutamine, 100 uM L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 μg/mL streptomycin ,1.8 mM of monopotassium phosphate, KH2PO4 and 10nM of dexamethasone. Samples were subcultured onto 22x22 mm coverslips at a density of 3000 cells/4.84 $cm^2$

### 4.1.3 Immunofluorescence

Cultured human dental pulp stem cells 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. Samples 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, USA).  F-actins were stained simultaneously with 0.5 uM of TRITC- conjugated (red fluorescence) phalloidin (Millipore, Billerica, MD, USA).  Nuclei staining (blue fluorescence) was visualized using 0.5 uM 4',6-diamidino-2-phenylindole for 3 minutes at room temperature. Samples were imaged at Days 1, 3 and 5 for control (DPSC regular growth medium) and osteogenic media.  Each time point included 4 samples per media type.

### 4.1.4  Fluorescent Microscopy

Samples treated for immunocytochemistry were visualized with an E-800 Eclipse Nikon (Melville,NY,USA)fluorescent microscope with a 60x objective lens and a 16-bit charge-coupled device camera (Photometrics,Tuscan,AZ,USA).  Images were pseudocolored with MetaMorph software (Molecular Devices, Sunnyvale, CA,USA).

## 4.2 Determination of Effect of Crosslinker Variation  on Cell Viability

### 4.2.1 Cell Culture

Human DPSC were kindly gifted by Dr. Songato Shi at the University of Southern California and expanded in vitro as previously reported by Gronthos et al, (2). Single cell suspensions were seeded in a $25cm^2$ culture flask containing α-MEM supplemented with

20% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 µg/mL streptomycin.

4.2.2 <u>Variation of PEGDA Crosslinker Concentration</u>

Extracel™ hydrogels (Glycosan Biosystems, Salt Lake City, UT,USA) were prepared by mixing 1% w/v thiolated hyaluronic acid (Glycosil) with 1% w/v thiolated denatured porcine collagen (Gelin-S). The solutions were cross-linked with a 2.0% w/v concentration of polyethylene glycol derivatives (PEGDA ~3400 Da and PEGSSDA ~8400 Da) in a 1:4 ratio of PEG-HA: GN to vary the hydrogel stiffness. All components were dissolved in sterile phosphate buffer saline (PBS) pH 7.4 and allowed to gel at room temperature for one hour.

4.2.3 <u>Human Dental Pulp Stem Cell 3D Embedding</u>

Passage 4 human dental pulp stem cells were embedded in 100 ul of HA-GN-PEGDA or HA-GN-PEGSSDA hydrogels at a density of $3.0 \times 10^4$ cells per insert (Millipore, Billerica, MD, USA). Cell culture inserts dimensions were 13mm in diameter, 10.5mm in height and a pore size of 8.0um. Samples were prepared in a 24 well plate in triplicate. Control samples were prepared in 24 well plates made of tissue culture plastic (TCP) at the same cell density in triplicate.

4.2.4 <u>Live/dead cell viability assay</u>

Cell viability was measured with a commercially available live/dead staining kit (Molecular Probes, Grand Island, NY, USA) after twenty-four hours. Cell viability was evaluated using calcein AM.

Intracellular cleavage emits green fluorescence in live cells when excited at 495 nm. A solution of 2μM calcein AM was prepared and applied directly to cells.

### 4.2.5 Fluorescent Microscopy

Samples treated for immunocytochemistry were visualized with an E-800 Eclipse Nikon fluorescent microscope with a 10x objective lens and a 16-bit charge-coupled device camera (Photometrics, Tuscan,AZ,USA). Images were pseudocolored with MetaMorph software (Molecular Devices, Sunnyvale, CA,USA).

## 4.3 Quantification of Effect of PEGSSDA Concentration on Gelation Time

### 4.3.1 Variation of PEGSSDA (w/v) concentrations

To determine the optimal gelation time of PEGSSDA (Glycosan Biosystems) as a function of concentration, the (w/v) of PEGSSDA was varied from 0.5% to 8%. The following concentrations were prepared in triplicate: 0.5%, 1.0%, 2.0%, 4.0% and 8.0%. Each concentration was diluted in a 1:1 (v/v) ratio of hyaluronic acid: gelatin (Glycosan Biosystems). The gelation time was plotted as a function of concentration and analyzed using non-linear regression modeling SPSS software (Chicago,IL,USA).

## 4.4 Optimization of Hyaluronic Acid to Gelatin Volume Ratios for hDPSC Attachment

### 4.4.1 Cell Culture

Human DPSC were kindly gifted by Dr. Songato Shi at the University of Southern California and expanded in vitro as previously reported by Gronthos et al, (2). and expanded in vitro as previously reported by Gronthos et al, (2). Single cell suspensions were seeded in

a 25cm$^2$ culture flask containing α-MEM supplemented with 20% FBS, 2 mM L-glutamine, 100 mM L-ascorbic acid 2-phosphate, 100 U/mL penicillin and 100 µg/mL streptomycin.

### 4.4.2 <u>Variation of the ratio of Hyaluronic Acid to Gelatin Concentration</u>

Under aseptic conditions, 1.0 mL of sterile PBS pH 7.4 was added to one vial each of hyaluronic acid and gelatin. To adjust hydrogel stiffness, the volume ratio of HA: GN was varied from 0-75% HA: GN concentration.  The PEGSSDA concentration remained constant at 2.0% w/v.  All components were dissolved in sterile phosphate buffer saline (PBS) and allowed to gel at room temperature for one hour.  Table 2 shows the volume ratio of HA: GN used for the experiment.

TABLE II

VOLUME RATIO OF THIOLATED HYALURONIC ACID TO THIOLATED GELATIN

| Condition | Thiolated HA, mL | Thiolated GN, mL | PEGSSDA, mL | Ratio of HN:GN |
|-----------|------------------|------------------|-------------|----------------|
| A | 0.500 | 0 | 0.125 | 100:0 |
| B | 0.375 | 0.125 | 0.125 | 75:25 |
| C | 0.250 | 0.250 | 0.125 | 50:50 |
| D | 0.125 | 0.375 | 0.125 | 25:75 |

### 4.4.3 Human Dental Pulp Stem Cell 3D Embedding

Human dental pulp stem cells were embedded in 100 ul of HA-GN-PEGSSDA hydrogels at a density of 3.0 x $10^4$ cells per cell culture insert (Millipore) at passage 4. Each volume condition from table II was prepared in triplicate. Cell culture inserts dimensions were 13mm in diameter, 10.5mm in height and a pore size of 8.0um. Control samples were prepared on tissue culture plastic (TCP) at the same cell density in triplicate.

### 4.4.4 Live/dead cell viability assay

Cell viability was measured with a commercially available live/dead staining kit (Molecular Probes) at days 0, 7 and 14. Cell viability was evaluated using calcein AM. Intracellular cleavage emits green fluorescence in live cells when excited at 495 nanometers (nm). The ethidium homodimer (EthD-1) enters cells with damaged membranes and binds to nucleic acids when excited at 528 nm. A solution of 2µM calcein AM (2.5 uL) and 4 µM EthD-1 (10uL) was diluted with 5 ml of phosphate buffer saline (PBS). One mL of the solution was added to the samples and incubated in the dark for one hour at 37°C and 5% $CO_2$.

### 4.4.5 Fluorescent Microscopy

Samples treated for immunocytochemistry were visualized with an E-800 Eclipse Nikon fluorescent microscope with a 20x objective lens and a 16-bit charge-coupled device camera (Photometrics). Images were pseudocolored with MetaMorph software (Molecular Devices).

<h1 style="text-align: center;">V. RESULTS</h1>

## 5.1 Investigation of Focal Adhesion Formation

### 5.1.1 Focal adhesion formation on 2D glass

Focal adhesion studies provide key information for researchers interested in cell-matrix interactions, and the aim of this objective was to determine if human dental pulp stem cells physically modulate focal adhesion formation in response to osteogenic induction medium. Dexamethasone was used to chemically induce hDPSC differentiation towards an osteogenic lineage over a five day period.  Immunocytochemistry was used to examine the intracellular structural link between actin filaments and the hDPSC extracellular matrix on 2D glass. FITC-labeled goat-anti-mouse secondary antibody for Vinculin staining was used to label the local orientations of focal adhesion formation and DAPI staining fluorescently -labeled hDPSC nuclei.  TRITC-labeled Phalloidian stained filamentous actin.  The merged focal adhesion, actin and nuclei images are shown in figure 1.

The average number of focal adhesions per cell increased linearly with time for the samples exposed to osteogenic medium, but did not change in the control cells between days 3 and 5 (Fig. 2).

## 5.2  Determination of Effect of Crosslinker Variation of Cell Viability

### 5.2.1 Cell viability of hDPSC embedded in PEG scaffolds

To determine the optimal crosslinker for hDPSCs in the HA-GN-PEG system, we subjected cells to PEGDA 3400 and PEGSSDA 8400 and examined hDPSCs for live/dead viability.   The concentration of the both PEG crosslinkers was maintained at 2.0% w/v.  The ratio of HA: GN was held constant at 1% w/v. We hypothesized that PEGSSDA can support hDPSC viability.  The immunofluorescent results are shown in Fig 4 a-f.

<p style="text-align: center;">21</p>
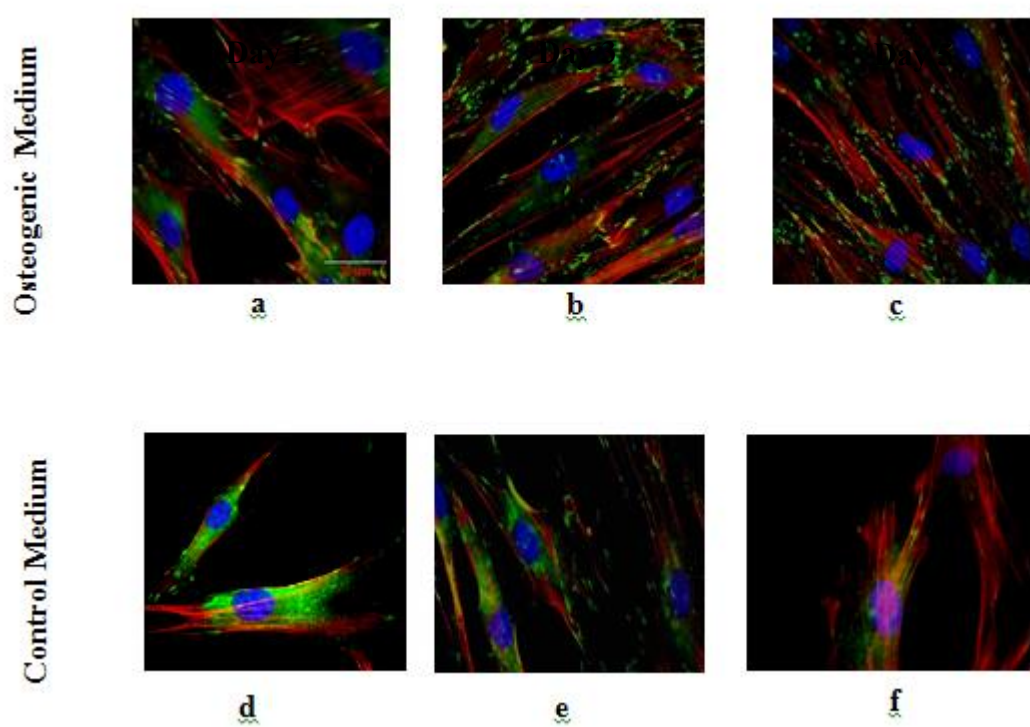
**Figure 1**: Merged images of immunofluorescence results for focal adhesion staining. Images represent randomly selected cells cultured in osteogenic medium (a-c) or regular growth medium (d-f) at days 1,3 and 5. Green= FITC vinculin staining. Red= TRITC F-Actin staining. Blue= DAPI nucleus staining. Magnification =60x. Scale bar=35 um.
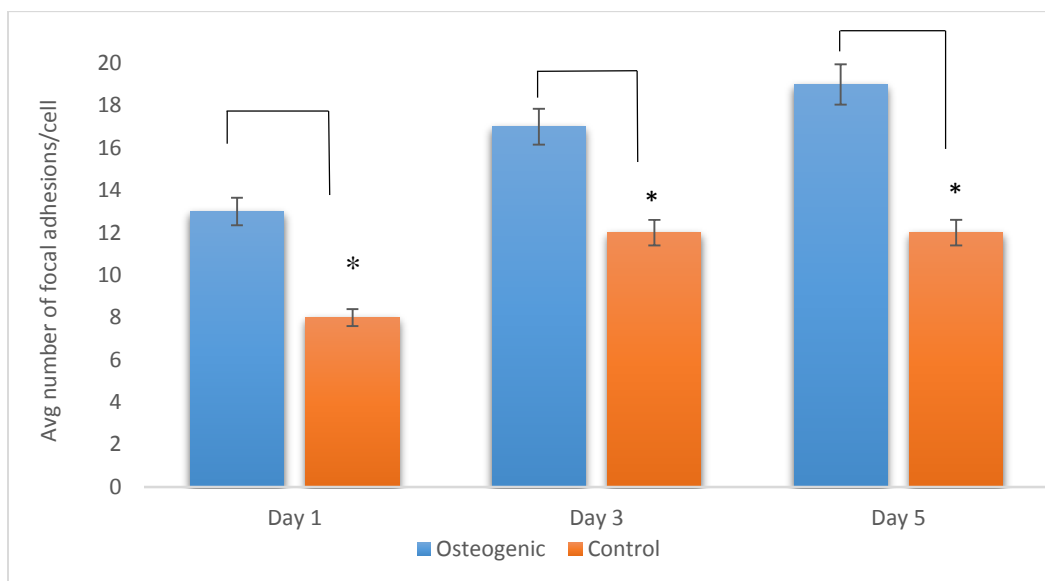
**Figure 2**: The average number of focal adhesions for DPSCs exposed to osteogenic medium increases linearly with time. *p<0.05
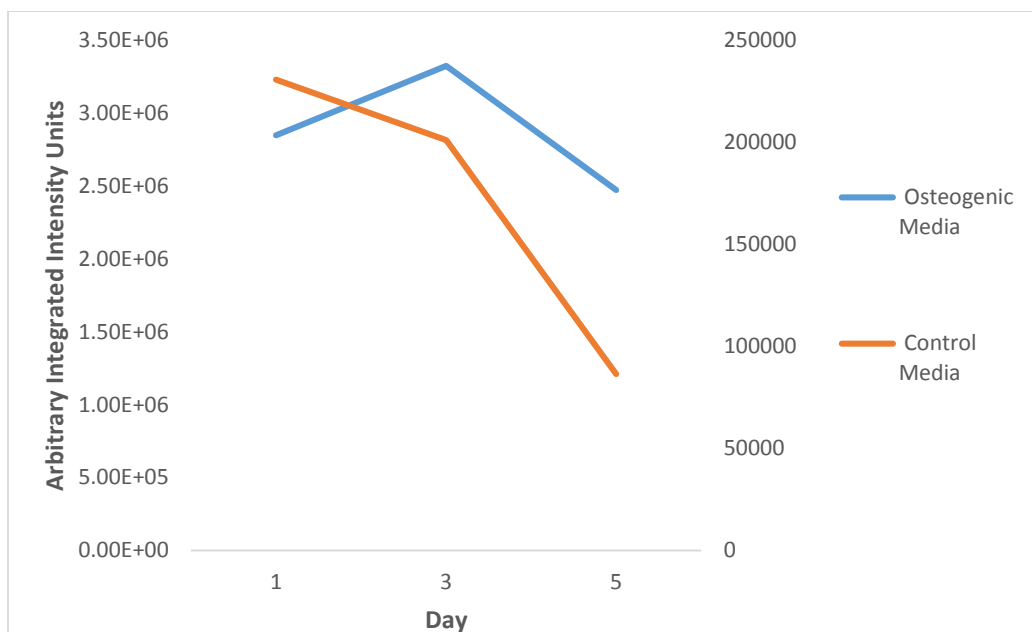
**Figure 3:** The integrated intensity of vinculin staining is substantially higher in samples cultured in osteogenic media over time.

**Day 0**     **Day 7**
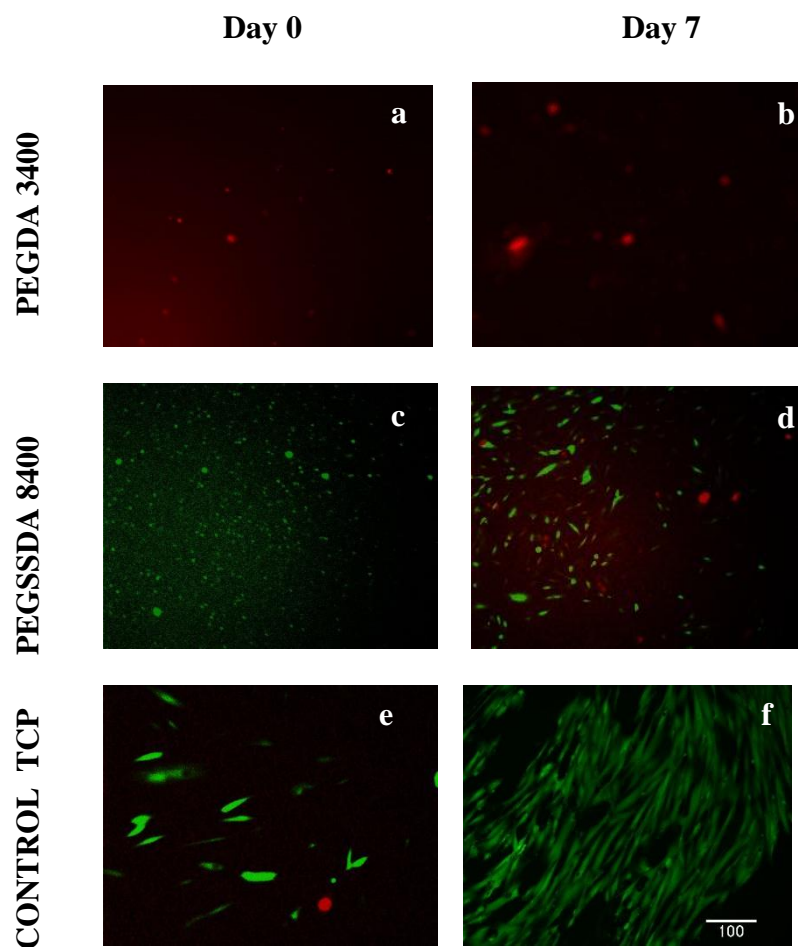


**Figure 4**: a-f  Immunofluorescence results for live/dead staining of human dental pulp stem cells. Green= live cells.  Red= dead cells. Magnification =20x.  Scale bar=100 um.  TCP= Tissue Culture Plastic.

**5.3 Quantification of Effect of PEGSSDA Concentration on Gelation Time**

5.3.1 <u>PESSDA gelation time as a function of crosslinker concentration</u>

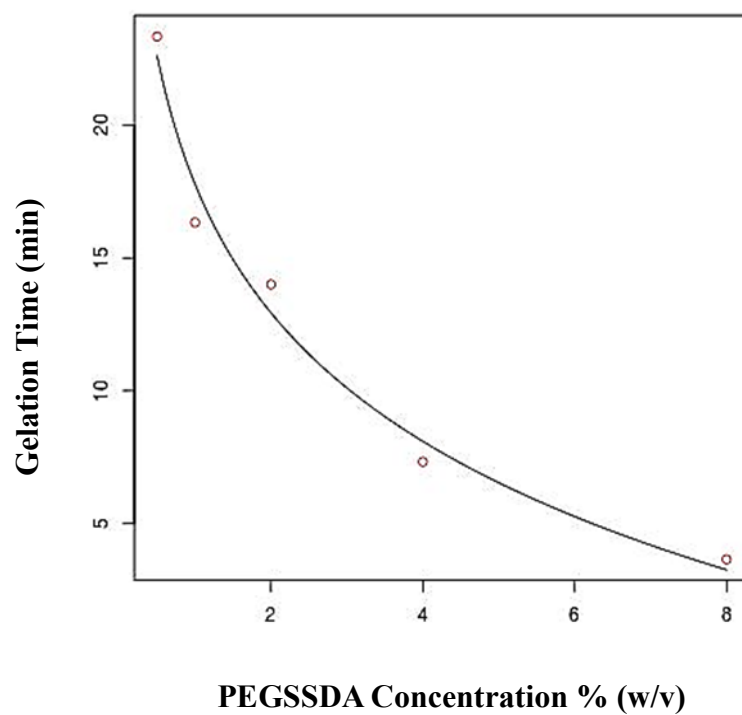Varying concentrations of PEGSSDA hydrogels were prepared to determine the gelation time of PEGSSDA as a function of gel concentration (w/v).   Five PEGSSDA concentrations were prepared in PBS ranging from 0.5% to 8.0% in concentration. The (v/v) ratio of hyaluronic acid to gelatin was maintained at a 1:1 ratio.  The results of non-linear regression modelling to predict the average gelation time of PEGSSDA are shown in figure 5 on a logarithmic scale and figure 6 on an exponential scale.  The data represents the average gelation times of experiments conducted in triplicate.

**5.4 Optimization of Hyaluronic Acid to Gelatin Volume Ratios for hDPSC Attachment**

5.4.1 <u>Variation of the ratio of Hyaluronic Acid to Gelatin Concentration</u>

To test the hypothesis that increased gelatin addition would encourage cell spreading in 3D HN-GN-PEGSSDA scaffolds, we varied hyaluronic acid and gelatin volume ratios.  A 1% (w/v) solution of hyaluronic acid and gelatin was used to prepare four experimental (v/v) conditions.  A 2.0% (w/v) solution of PEGSSDA was maintained as the hydrogel crosslinker. The experimental results are shown in figures 7 a-h.

Quantitative data showing the average number of live cells/field of view is shown in figure 8.  Based on the limited number of cells in the field of view for the 100:0 and 75:25 HA:GN ratios, we normalized the data by counting the total number of cells per field of view to determine if cell proliferation increased from day 7 to day 14 (Fig.9).  The percentage of non-viable cells based on hyaluronic acid: gelatin ratios for the 50:50 and 25:75 mixtures are provided in figure 10.

**PEGSSDA Concentration % (w/v)**

**Figure 5**: Gelation time as a function of PEGSSDA concentation
fit to a non-linear logarithmic regression model. Open circles represent
experimental data.  The curved line is theoretical data. RSS= 4.482
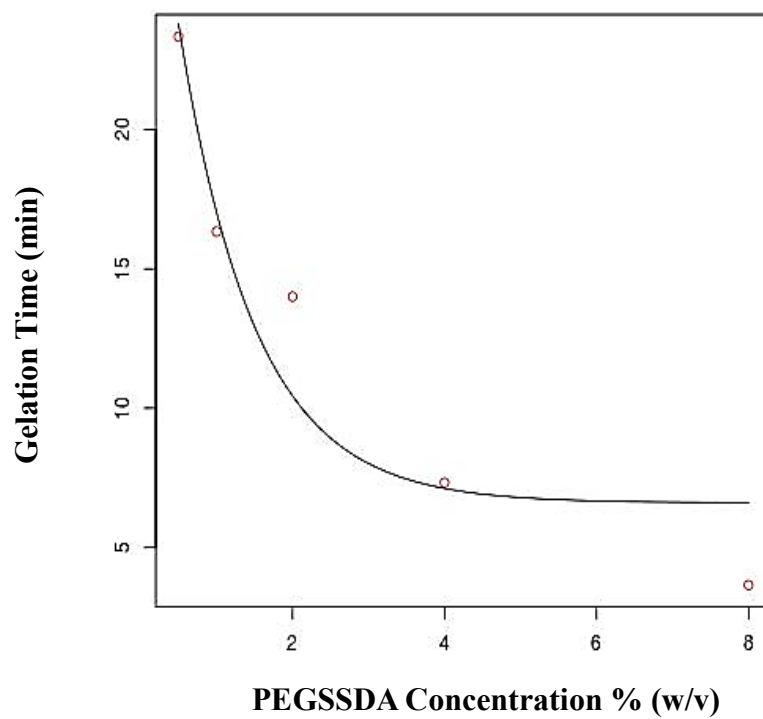
Figure 5: Gelation time as a function of PEGSSDA concentration fit to a non-linear exponential regression model. Open circles represent experimental data. The curved line is theoretical data RSS= 22.12

**Volume Ratio of Hyaluronic acid: Gelatin**

|  | 100:0 | 75:25 | 50:50 | 25:75 |
|---|---|---|---|---|
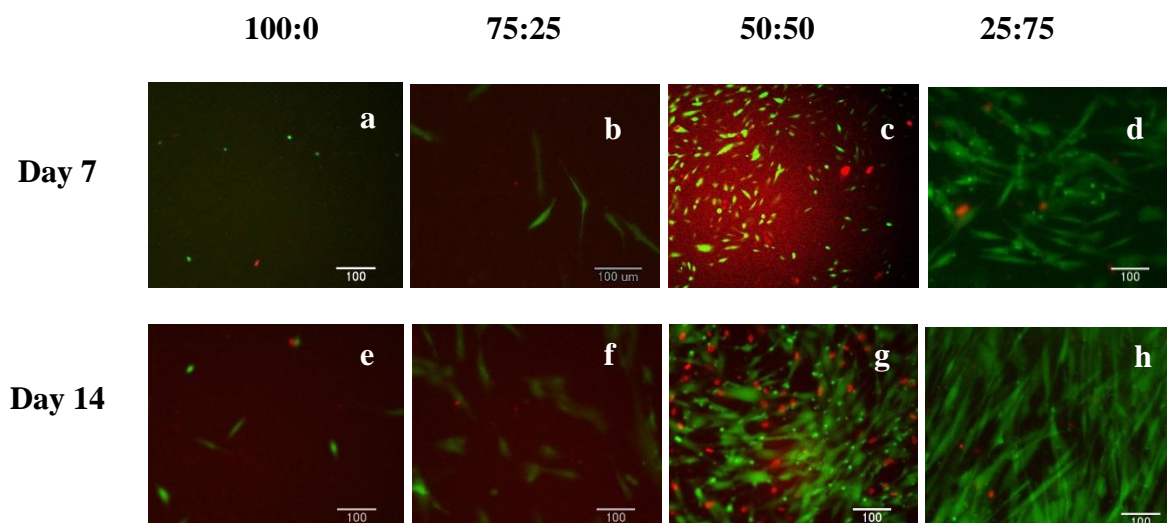


**Figure 7**: Immunofluorescence results for live/dead staining of human dental pulp stem cells embedded in 2.0% (w/v) PEGSSDA and different ratios of HA:GN. Green= live cells.  Red= dead cells.  Magnification =20x.
Scale bar=100 um.
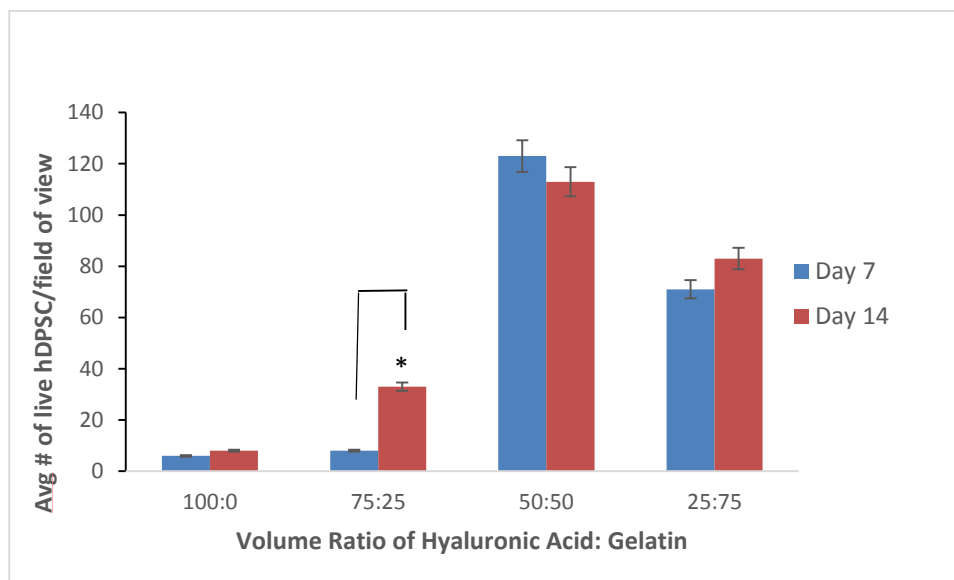
**Figure 8**: Average number of live human dental pulp stem cells/field of view from a 20x objective. *p<0.05
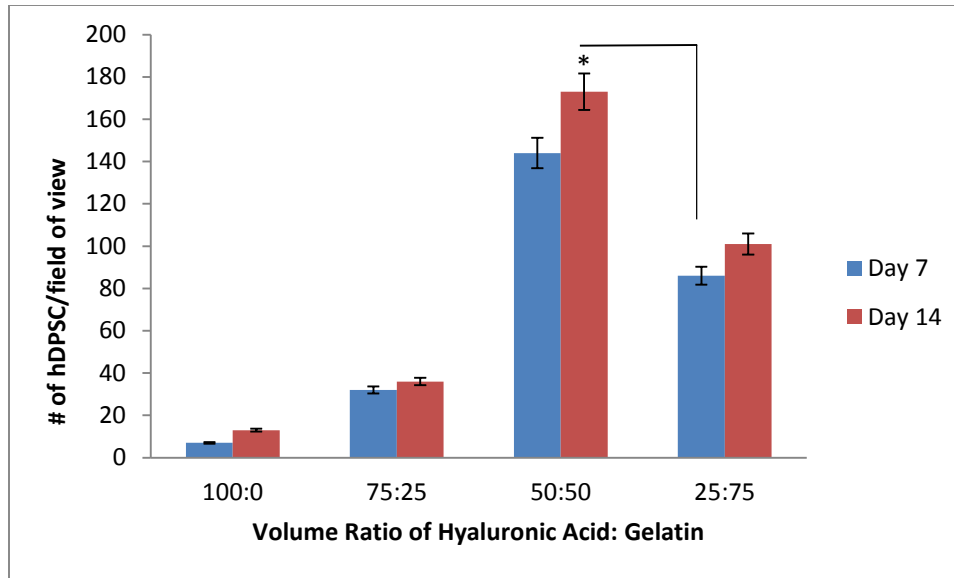
**Figure 9**: The average total number of human dental pulp stem cells/field of view from a 20x objective. *p<0.05
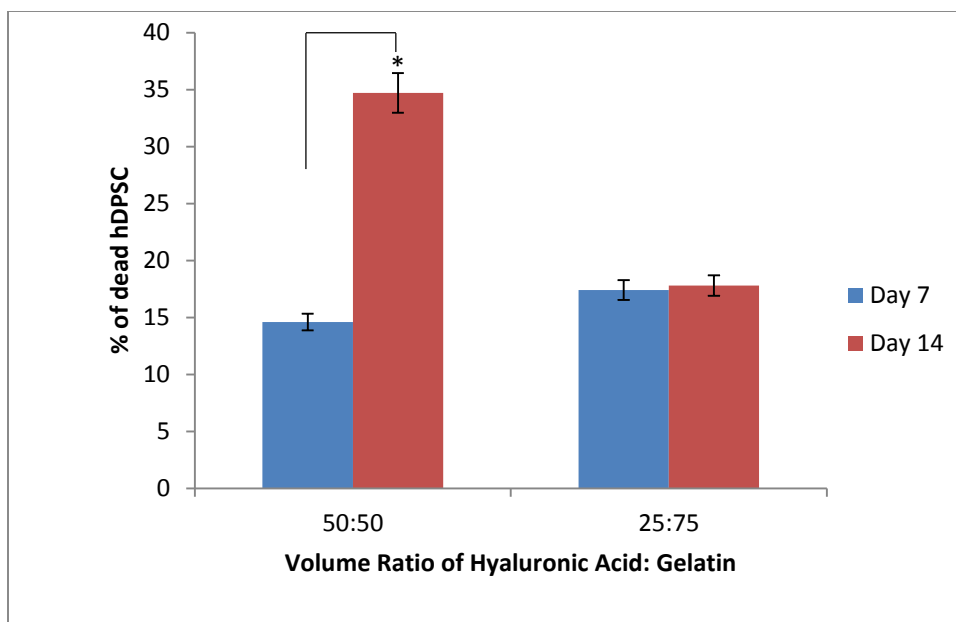
**Figure 10**: The percentage of non-viable human dental pulp stem cells embedded in 3D HA-PEGSSDA-GN scaffolds between two ratios of HA:GN. *p<0.05

# VI. DISCUSSION

## 6.1 Investigation of Focal Adhesion Formation

### 6.1.1 Focal adhesion formation on 2D glass

Between days 1 and 5, the cytoskeletal actin stress fibers were more prominent in the hDPSC cultured in osteogenic medium compared to the control (Fig.1).  This may be due to the dexamethasone penetrating the cell membrane and initiating an odontoblast-like lineage (25).  Interestingly, hMSC induced with 10nM of dexamethasone between days 1 and 3 resulted in thick actin stress fibers replaced with thinner actin filaments (46).  These results suggest that despite the similarities in gene expression profiles between the two cell types (47), the mechanical properties may be modulated differently.

A lack of focal adhesion increase in hDPSC cultured in control medium between days 3 and 5 may be due to strong adhesions unable to detach and migrate because of the rigid glass.  Soluble signals promoting migration in the induction media or changes in the cell-cell contact may have contributed to the increased average number of focal adhesions in the hDPSCs cultured in the osteogenic medium (48-49).  The results in figure 3 validate these findings.   The average integrated intensity arbitrary units of the FITC-labeled vinculin focal adhesions are higher in samples treated with osteogenic media over time.  Importantly, the number of cells/view are different in the osteogenic vs. control samples which may have skewed the data.

While it is accepted that focal adhesion formation on 2D substrates such as glass may be exaggerated (50), such studies provide excellent controls for 3D experiments where cell fate processes such as migration are complicated.  To our knowledge, focal adhesion modulation has not been reported for hDPSC- synthetic substrate interactions.

33

One method to better characterize this interaction would be to suppress focal adhesion kinase to determine if focal adhesion formation is inhibited. Additionally, identifying the key integrins expressed by hDPSCs would provide insight into the molecular interactions that regulate mechanical events. Integrin expression is necessary for the adherence of cells to synthetic scaffolds (51).

**6.2 Determination of Effect of Crosslinker Variation of Cell Viability**

6.2.1 Variation of PEGDA Crosslinker Concentration

The elastic moduli of hydrogel systems can be optimized for specific cell types. The molecular weight of polymer chains has been shown to effect the shear modulus, an important parameter for injectable hydrogels (52). At day 0, there were very little live hDPSC in the hydrogels embedded within the HA-GN-PEGDA hydrogel (Fig.4a).

We expected to see more cells in the images as the cell density for the experiments were $3 \times 10^4$ cells/insert, with 100 ul of gel in each insert. Based on this density, the cells may have been washed away during staining if they were not attached in the gel. At day 7, there were no observable viable cells in the PEGDA based hydrogel (Fig. 4b). This may have been because the PEGDA was not stiff enough to support hDPSC viability. The reported shear modulus of 2% PEGDA after mixing with a 1% (w/v) HA-GN solution in PBS is ~ 550 Pa, whereas a 4.5% (w/v) solution is reported as 4270 Pa (56).

The hDPSCs embedded within the HA-GN-PEGSSDA sECM were viable with a round morphology as evidenced by live/dead staining at day 0 (Fig.4c). We presumed that the gel was not toxic to the hDPSC, but attributed the rounded morphology to two possibilities. The gel may have lacked the required stiffness to promote immediate cell spreading, or the PEGSSDA did not have sufficient gelatin concentration to encourage immediate cell

spreading.  The cell morphology at day 7 was more characteristic of hDPSC compared to the control on TCP (Fig.4d-f), but we observed dead, rounded cells at day 7.  Based on these results, we decided to vary the ratio of HA: GN and maintain a constant PEGSSDA concentration at 2% (w/v).

## 6.3 Quantification of Effect of PEGSSDA Concentration on Gelation Time

6.3.1 <u>PESSDA gelation time as a function of crosslinker concentration</u>

Non-linear regression modelling was used to determine the relationship between gelation time and PEGSSDA concentration.  The residual sum of squares (RSS) data showed that the logarithmic curve (fig. 5) was a better fit for the experimental data compared with the exponential curve (fig. 6), with values of 4.482 and 22.12 respectively.

There was a clear relationship between the gelation time and the PEGSSDA concentration.  The higher the concentration, the lower the gelation time.  The 2.0 % w/v PEGSDDA concentration used in the experiments had an average gelation time of 13 minutes.   Although a 4% concentration may be optimal for clinicians with an average gelation time of 7 minutes, we maintained 2.0% PEGSSDA concentration to maximize product efficiency.  Based on these experimental results, we can potentially develop a kinetic model to predict the elastic modulus of the hydrogel during the crosslinking process.

## 6.4 Optimization of Hyaluronic Acid to Gelatin Volume Ratios for hDPSC Attachment

6.4.1 <u>Variation of the ratio of Hyaluronic Acid to Gelatin Concentration</u>

At day 7, hDPSCs embedded in the gel inserts were viable with a round morphology due to the lack of gelatin in the hydrogel (Fig. 7a). Interestingly, at day 7 the cells subject to a 75:25 HA: GN ratio were spread apart, but did not present a large number of cells in the microscopic field of view.  Similarly, there were only a few viable cells at day 14 embedded

with the 100:0 ratio of HA: GN.  We did note apparent cell spreading, which was surprising

due to the lack of gelatin (Fig. 7d).

Data shown in figure 9 suggests that the greatest hDPSC cell viability, spreading and

adhesion resulted from cells embedded in 50:50 and 25:75 ratios of HA: GN between days 7

and 14.  Although the data shows that the average total number of hDPSC over time in the

50:50 HA: GN ratio is greater than the 25:75 HA: GN, the latter appears more optimal for

cell adhesion, spreading and proliferation.  This is based on the calculated percentage of non-

viable cells in the field of view (fig. 10.)  Roughly 35% of cells in the 50:50 HA: GN ratio

were non-viable compared to 18% of cells in the 25:75 ratio after 14 days.  This may have

been due to an initial higher proliferation rate in the 50:50 HA: GN ratio that was not optimal

for the cell culture inserts diameter.

The orientation of the cells at day 14 (Fig. 7g) was also interesting.  There appeared to be

a preferential direction of cell spreading in cells subject to the 25:75 HA: GN ratio. We did

not examine this relationship further, but speculate that the cells may have been spreading on

the pore insert membrane or that the hydrogel solution volume (100 ul) may have influenced

cell orientation.

.

## VII. CONCLUSION

The use of an injectable synthetic polyethylene glycol-hyaluronic acid-gelatin scaffold may be a viable option for dental tissue engineering scaffolds. The mechanical properties may be altered for cell-specific needs. Two methods to manipulate hydrogel compliance include varying the crosslinker density and/or concentrations of thiolated gelatin to thiolated hyaluronic acid volume ratios. The ability to adjust these parameters is interesting to our work as recent literature has shown that fate, function and morphology are heavily dependent on substrate stiffness (54). To accurately predict when an injected hydrogel will transition from a liquid to a gel, a quantitative understanding of the gelation kinetics is required

In this work we report four conclusions. First, the number and physical size of focal adhesions increase in response to odontogenic induction culture medium. This observation is consistent with actin cytoskeletal changes in 2D. Next, PEGDDSA 8400 supports hDPSC adhesion and viability compared to PEGDA 3400 when used in PEG-HA-GN synthetic extracellular matrices. Third, higher concentrations of PEGSSDA (w/v) result in reduced gelation times. . Finally, embedded human dental pulp stems maintained viability for at least 14 days in a semi-synthetic ECM mimic. The PEGSSDA-HA-GN scaffold with a PEGSSDA concentration of 2.0% (w/v) and 25:75% ratio of HA: GN were the optimal conditions for hDPSC viability and spreading in 3D.

Future directions for this work include substituting fibronectin with gelatin to determine if cell adhesion is enhanced. We are also human dental pulp stem differentiation as a function of the mechanical stiffness of the human tooth in the absence of biological factors.

# CITED LITERATURE

1.  Mao, J., Kim,S.,Zhou,J.,Ye,L.,Cho,S.,Suzuki,T.,Fu,S.,Yang,R.,Zhou,X. (2012). Regenerative Endodontics: Barriers and strategies for clinical translation. *Dental Clinics of North America*, 639-649.

2.  Gronthos, S., Agnieszka, A., Bartold, P., & Songtao, S. (2011). A method to isolate and culture expand human dental pulp stem cells. *Methods in Molecular Biology*, 107-121.

3.  Cox, C., White, K., Ramus, D., Farmer, J., & Snuggs, H. (1992). Reparative dentin: factors affecting its deposition. *Quintessence Int*, 23, 257.

4.  Kinney, J., Marshall, S., & Marshall, G. (2003). The mechanical properties of human dentin: a critical review and reevaluation of the dental literature. *Crit Rev Oral Biol Med, 14*, 13.

5.  Sundquivst, G., Figdor, D., Persson, S., & Sjogren, U. (1998). Microbiological analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surgey, Oral Medicine, *Oral Pathology, Oral Radiology and Endodontology*., 85, 86.

6.  Peterson, P., Bourgeois, D., Ogawa, H., Estupinan-Day, S., & Ndiaye, C. (2005). The global burden of oral diseases and risks to oral health. *WHO Bulletin, 83*, pp. 661-9.

7.  Tecles, O.,Laurent,P., Zygouritsas,S., Burger, A.S., Camps, J. (2005). Activation of human dental pulp progenitor/stem cells in response to odontoblast injury. *Arch Oral Biol 50*, 103-108.

8.  Gronthos, S.,Brahim,J., Li,W., Fisher,L.W., ChermanmN., Boyde,A., DenBesten,P., Robey,P., & Shi,S. (2002). Stem cell properties of human dental pulp stem cells. *Journal of Dental Research 81,* 531-535.

9.  Gronthos, S.,Mankani,M.,Brahim,J.,Robey,P.G., & Shi,S. (2000). Postnatal human dental pulp stem cells (DPSC) in vitro and vivo. *Proc Natl Acad Sci USA 97*, 13625-13630.

10. Shi,S., and Gronthos,S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *Journal of Bone and Mineral Research 18*, 696-704.

11. Sakai,V.,Cordeiro,M.,Dong,Z.,Zeitlin,B., & Nor,J. (2011). Tooth slice/Scaffold Model of Dental Pulp Tissue Engineering. *Adv Dent Res 23*, 325-332.

12. Wei, X., Ling, J., Wu, L., Liu, L., & Xiao, Y. (2007). Expression of mineralization markers in dental pulp stem cells. *Journal of Endodontics 33*, 703-708.

13. Jain,R.,Mannerstrom,B.,Vuorinen,A.,Sandor,G.,Suuromem,R., &Miettinen,S. (2012). Osteogenic differentiation of human dental pulp stem cells on β-tricalcium phosphate/poly (L-Lactic/caprolactone) three dimensional scaffolds. *Journal of Tissue Engineering 3*, 1-11.

14. Langer,R., and Vancanti,J. (1993). Tissue Engineering. *Science 260*, 920-926.

15. Galler, K., D'Souza R, Hartgerink, J., & and Schmalz, G. (2011). Scaffolds for dental pulp tissue engineering. *Advanced Dental Research 23*, 333-339.

16. Allison, D., & Grande-Allen, K. (2006). Review. Hyaluronan: A Powerful Tissue Engineering tool. *Tissue Engineering 12*, 2131-2140.

17. Li, Y., Rodrigues, J., &Tomas, H. (2012). Injectable and biodegradable hydrogels: gelation,biodegradation and biomedical applications. *Chem. Soc. Rev 41*, 2193-2221.

18. Cheng, K., Blusztajn, A., Shen, D., Sun, B., & Galang, G. (2012). Functional performance of . human cardiosphere-derived cells delivered in situ polymerizable hyaluronan-gelatin hydrogel. *Biomaterials 33*, 5317-5324.

19. Kim N., Lee,D., Chung,P., &Yang,H. (2009). Distinct differentiation properties of human dental pulp stem cells on collagen, gelatin, and chitosan scaffolds. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 108, 94-100.

20. Vanderhooft,J.,Mann,B., & Prestwich,G. (2007). Synthesis and characterization of novel thiol-reactive poly(ethylene glycol) cross-linkers for extracellular matrix mimetic biomaterials. *Biomacromolecules 8*, 2883-2889.

21. Shu,X.,Liu,Y.,Palumbo,F.,Luo,Y.,& Prestwich,G. (2004). In situ crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials 25*, 1339-1348.

22. Cavalcanti,B.,Zeitlin,B., & Nor, J. (2013). A hydrogel scaffold that maintains viability and supports differentiation of dental pulp stem cells. *Dental Materials 29*, 97-102.

23. Kelly, A. (2007). Why engineer porous materials? *Phil Trans A Math Phys Eng Sci 364*, 5-14.

24. Whang, K., Healy, E., & Elenz, D. (1999). Engineering bone regeneration with bioabsorbable scaffolds with novel microarchitecture. *Tissue Eng 5*, 35-51.

25. Engler, A., Sen, S., Sweeney, H., & Discher, D. (2006). Matrix elasticity directs stem cell lineage specification. *Cell 126*, 677-689.

26. Galler,K.,Hartgerink,J.,Cavendar,A.,Schmalz,G.,& D'Souza,R. (2012). A customized self-assembling peptide hydrogel for dental pulp tissue engineering. *Tissue Engineering Part A 18*, 176-184.

27. Petersen, M. C., Lazar, J., Jacob, H. J., & Wakatsuki, T. (2007). Tissue engineering: a new frontier in physiological genomics. *Physiological genomics 32*, 28-32.

28. Serban,M.,Yanchun,L., & Prestwich,G. (2008). Effects of extracellular matrix analogues on primary human fibroblast behavior. *Acta Biomaterialia 4*, 67-75.

29. Cheng,K.,Blusztajn,A.,Shen,D.,Sheng,L.,Sun,B., et al. (2012). Functional performance of human cardiosphere-derived cells delivered in an in situ polymerizable hyaluronan-gelatin hydrogel. *Biomaterials 33*, 5317-5324.

30.  Liu,Y.,Shu,X.,& Prestwich,G.  (2006). Osteochondral defect repair with autologous bone-marrow derived mesenchymal stem cells in an injectable, in situ, cross-linked synthetic extracellular matrix.  *Tissue Engineering 12*, 3405-3416.

31.  Toh,W.,Lee,E.,Guo,X.,Chan,J.,Yeow,A., et al. (2010).  Cartilage repair using hyaluronan hydrogel-encapsulated human embryonic stem cell-derived chrondrogenic cells.  *Biomaterials 31*, 6968-6980.

32. Vanderhooft, J., Alcoutlabi, M., Magda, J., & Prestwich, G. (2009). Rheological Properties of Cross-linked Hyaluronan-Gelatin Hydrogels for Tissue Engineering. *Macromolecular Bioscience, 9*, 20-28.

33. Lucchini.,Couble,M.,Romeas,M.,Staquet,M.,Bleicher,F.,et al. (2004).  $\alpha V\beta 3$ Integrin expression in human odontoblasts and co-localization with osteoadherin.  *Journal of Dental Research 83*, 552-556.

34. Yamada, K.,& Geiger, B. (1997).  Molecular interactions in cell adhesion complexes.  *Curr Opin Cell Bio 9*, 76-85.

35. Pasquinelli, G., Orrico, C., Foroni, L., Bonafe, F., Carboni, M., et al.  (2008).  Mesenchymal stem cell interaction with a non-woven hyaluronan-based scaffold suitable for tissue repair. *J.Anat 213*, 520-530.

36. Michael, K., Dumbauld, D., Burns, K., Hanks, S., & Garcia, A. (2009). Focal Adhesion Kinase Modulates Cell Adhesion Strengthening Via Integrin Activation. *Molecular Biology of the Cell 20*, 2508-2519.

37.  Burridge,K. Nuckolls, G., Otey,C.,Pavalko,F.,Simon,K., & Turner, C. (1990). Actin-membrane interaction in focal adhesions.  *Cell Diff Dev 32*, 337-342.

38. Turner, C., & Burridge,K. (1991). Transmembrane molecular assemblies in extra-cellular interactions.  *Curr Opin Cell Biol 3*, 849-853.

39. Burridge,K, &  Chrzanowska-Wodnicka,M.  (1996). Focal adhesions, contractility, and signaling. *Ann Rev Cell Div Biol 12,* 463-518.

40. Dubash,A.,Menold,M., Samson,T., Boutler,E.,Garcia,R.,Doughman,R., & Burridge,K. (2009).  Focal adhesions: New angles on an old structure.   Int Rev Cell Mol Biol 277, 1-65.

41.  Geiger,B, Bershadsky,A, Pankov,R,& Yamada,KM. (2001). Transmembrane crosstalk betweenthe extracellular matrix and the cytoskeleton. *Nat Rev Mol Cell Biol 2*,793-805.

42. Geiger,B, Spatz,J, Bershadsky,A.  (2009). Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol. 10*, 21-33.

43. Kaverina I, Krylyshkina ,O Small,J. Regulation of substrate adhesion dynamics during cell motility. TheInternational Journal of Biochemistry & Cell Biology. 2002; 34:746–61.

44. Pollard ,T, Borisy ,G. Cellular motility driven by assembly and disassembly of actin filaments. Cell 2003; 112: 453–465.

45. Zaidel-Bar R., Ballestrem C., Kam Z., & Geiger, B. (2003). Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *Journal of Cell Science 116*, 4605–4613.

46. Titushkin, I & Cho, M (2007). Modulation of cellular mechanics during osteogenic differentiation of human mesenchymal stem cells. *Biophys J 10*, 3693-3702.

47. Shi,S., Robey,P., & Gronthos,S. (2001).Comparison of human dental pulp and bone marrow stromal cells by cDNA microarray analysis. *Bone 29*, 532-539.

48. Lutolf,M & Hubbell, J. (2005)Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nat Biotechnol.23*, 47-55.

49. Ridley,A.,Schwartz,M.,Burridge,K.,Firtel,R.,Ginsberg,M.,Borisy,G.,Parsons,J & Horwitz,A. (2003). Cell migration: Integrating signals from front to back. *Science 302*, 1704- 1709.

50. Nemir,S & West,J. (2010). Synthetic materials in the study of cell response to substrate rigidity. *Ann  Biomed Eng. 38*, 2-20.

51. Geiger,B & Yamada,K. (2011).  Molecular architecture and function of matrix adhesions. *Cold Spring Harb Perspect Biol. 3*, 1-22.

52.  Vanderhooft, J., Alcoutlabi, M., Magda, J., & Prestwich, G. (2009). Rheological  properties of cross-linked hyaluronan-gelatin hydrogels for tissue engineering. *Macromolecular Bioscience, 9*, 20-28.

53.  Zhang,J.,Skardal,A.,& Prestwich,G. (2008).  Engineered extracellular matrices with cleavable crosslinkers for cell expansion and easy cell recovery.  *Biomaterials 29*, 4521-4531.

54.  Kshitz., Park,J.,Kim,P.,Helen,W.,Engler,A.,Levchenko,A.,& Kim,D. (2012).  Control of stem cell fate and function by engineering physical microenvironments. *Integr Biol 4*, 1008-1018.

55. Skardal, A. Z., & Prestwich, G. (2010). The generation of 3-D tissue models based on hyaluronan hydrogel-coated microcarriers. *Biomaterials 31*, 6173-6181.

56.  Ghosh,K.,Pan,Z.,Guan,E.,Shouren,G.,Liu,Y.,Nakamura,T.,Ren,X.,Rafailovich,M.,& Clark,A. (2007).   Cell adaptation to a physiologically relevant ECM mimic with different viscoelastic properties.  *Biomaterials 28*, 671-679.

57.  Chai,Y.,Jiang,X.,Ito,Y.,Bringas,P.,Han,J.,Rowitch,D., Soriano,P., McMahon,A.,& Sucov,H. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development, 127*, 1671-1679.

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

**Determination Notice**
**Research Activity Does Not Involve "Human Subjects"**

March 2, 2011

Satish Alapati, BDS, MS, PhD
Endodontics
801 S Paulina, Rm#304 D
Chicago, IL 60612
Phone: (312) 413-1334 / Fax: (312) 996-3375

RE:    Research Protocol # 2011-0129
          "A Novel Approach for Biomaterials Assisted Regeneration of Pulp-Dentin
Complex"

| | |
|---|---|
| **Sponsor:** | NIH-NIDCR |
| **PAF#:** | 2010-07538 |
| **Grant/Contract No:** | Not available |
| **Grant/Contract Title:** | A Novel Approach for Biomaterials Assisted Regeneration of Pulp-Dentin Complex |

Dear Dr. Alapati:

---

Please note that this determination is limited to the proposed testing on discarded teeth.
There will be no information attached/linked to the teeth. Consequently, the proposed
research will not involve: a) any interactions or interventions with living individuals for
research purposes; or b) the use or disclosure of any private identifiable information.

It is understood that this initial phase may subsequently lead to human subject
research, but if it does, prospective written IRB approval or an exemption
determination will be obtained.

---

The above proposal was reviewed on March 1, 2011 by OPRS staff/members of IRB #2.
From the information you have provided, the proposal does not appear to involve "human
subjects" as defined in 45 CFR 46. 102(f).

The specific definition of human subject under 45 CFR 46.102(f) is:

> *Human subject* means a living individual about whom an investigator (whether professional or student)
> conducting research obtains
>
> (1)  data through intervention or interaction with the individual, or
> (2)  identifiable private information.

**NAME:**              Taneka Denise Taylor


**EDUCATION:**         B.S., Biology, Illinois State University, Bloomington, Illinois, 2006

M.A.T., Teaching, Secondary Education Science, National-Louis University, Chicago, Illinois, 2009

M.S., Bioengineering, University of Illinois at Chicago, Chicago, Illinois 2014

**TEACHING:**          Department of Science, South Shore International College Prep High School, Chicago, Illinois 2011-2012

Department of Science, Morgan Park High School, Chicago, Illinois, 2007-2010

**HONORS:**            Diversifying Faculty in Illinois Fellowship Recipient, University of Illinois at Chicago, Chicago, Illinois, 2011-2014
Chancellor's Fellowship Recipient, University of Illinois at Chicago, Chicago, Illinois, 2014-2016

**PROFESSIONAL**      Society of Women Engineers
**MEMBERSHIP:**       American Association of Dental Research

**ABSTRACTS**:        Taylor, T.D. (2013, April). Functional Performance of Human Dental Pulp Stem Cells Delivered in an In Situ Polymerizable Hyaluronan/Polyethylene Glycol/Gelatin Hydrogel.  Poster presented at the Illinois African American and Latino Higher Education Alliance Diversity Dialogue and Research forum.

Taylor, T.D. (2012, March).  Investigation of Focal Adhesions during Dental Pulp Stem Cell Differentiation.  Poster presented at the International Association of Dental Research Conference, Tampa, Fl.

Taylor, T.D. (2010, March). Human Mesenchymal Stem Cell Behavior on 3D PEGDA Superporous Hydrogels. Poster presented at the National Science Teachers Association Conference, Philadelphia, PA.

Taylor, T.D. (2010, March). Biochemical Manipulation of Neuronal Cells May Influence Characterization.
Poster presented at the National Science Teachers Association Conference, Philadelphia, PA.