CHARACTERIZATION OF FIBRONECTIN ADHESION ISOLATED DENTAL PULP PROGENITOR CELL POPULATIONS

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Abstract

CHARACTERIZATION OF FIBRONECTIN ADHESION ISOLATED DENTAL PULP PROGENITOR CELL POPULATIONS

By

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Background: The DPSC constitutes a significantly small population, comprising approximately 1% of the total cells in the pulp tissue. Therefore, it is imperative to successfully culture and populate DPSCs in vitro before utilizing them for therapeutic purposes. Understanding DPSC heterogeneity is crucial for innovative regenerative therapies, prompting the investigation of mixed populations using fibronectin as a biological marker. It is reported that cellular phenotype and biological function may undergo alterations as cells replicate in a cultured environment or the substrate to which the cells adhere.

Objectives: In this study, we compared and analyzed the impact of various isolation methods on the expansion, diversity, and variability in the expression of mesenchymal cell surface markers of DPSCs as well as the cellular expression during osteodifferentiation. The study aims to examine the relationship between fibronectin adherent, fibronectin non-adherent, and explant DPSC populations in terms of mesenchymal cell surface markers and mineralizing lineage differentiation potential. Identifying markers that consistently and specifically represent distinct subtypes of DPSCs within mixed populations will streamline direct purification and will help us to give more insight into DPSC heterogeneity.

Methods: Two distinct sets of dental pulp stem cells (DPSC) were derived from separate donors using enzymatic digestion or explant outgrowth methods. The experiments were replicated for each set. DPSC underwent a selection process with fibronectin coating on plates, resulting in two variables: fibronectin-adherent cells and non-adherent cells. DPSCs went through passages, population doublings were calculated, and the proliferation rate was evaluated. Flow cytometric analysis was conducted to assess mesenchymal cell surface marker expression for each group. After treatment with osteogenic differentiation media, DPSCs' total RNA was extracted. A cDNA library was generated using RT-PCR, and qPCR assessed gene expression related to the mineralization lineage pathway.

Results: The DPSCs isolated through fibronectin adhesion had greater cell growth than those without selection (P-value <0.05). Flow cytometry analysis of DPSCs from various donors, including HADAN II and HADAN III, demonstrated comparable percentages of quadruplicate positive cells cell surface markers and negative markers in both fibronectin-adherent and non-fibronectin cells. HADAN II exhibited reduced osteopontin (OPN) expression levels and elevated osteocalcin (OCN) expression levels (P-value <0.05). Conversely, both the HADAN III

fibronectin-adherent isolate and non-adherent isolates displayed increased OPN expression, with no significant difference in BGLAP mRNA expression levels. OPN is recognized as an early marker of mineralization pathways, while OCN is acknowledged as a later marker in the osteogenic process. The data suggests that DPSCs in HADAN III may be less differentiated than those in HADAN II after a 7-day of incubation in the osteogenic medium. While both types of isolates demonstrated osteodifferentiation capacity, the fibronectin-adherent isolate exhibited a higher level of osteogenic differentiation markers compared to non-adherent dental pulp progenitor cells.

Conclusion: While all isolates of dental pulp progenitor cells displayed similar proportions of mesenchymal stem cell markers, those selected through fibronectin exhibited an accelerated division rate and demonstrated potentially enhanced osteogenic potential across two donors. Our findings support fibronectin as a valuable selection tool capable of promoting MSC expansion and increased differentiation potential without compromising their stem cell characteristic. Nevertheless, it is essential to recognize the substantial individual variations in mesenchymal progenitor proportions, phenotype, and behavior. In addition, the inherent heterogeneity of human dental pulp stem cells (hDPSCs) presents a barrier to discerning the quality of stem cells solely through the examination of a single cellular marker. This challenge complicates the effective characterization and study of DPSCs.

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Chapter 1: Introduction

Background and Significance

Stem cells are undifferentiated cells that exhibit self-renewal and multi-lineage differentiation capacity and have long-term proliferative potential (Amit et al., 2000). They can be induced to differentiate into various cell types with distinct functions when exposed to specific stimuli, such as growth factors in specific media (Chen, Reuveny, & Oh, 2013). Stem cells can be categorized into embryonic pluripotent stem cells and adult stem cells, which are known as tissue-specific stem cells. Adult stem cells are undifferentiated cells located within the specialized tissues of our bodies after development. These cells enable the healing, growth, and replacement of injured or dead tissue at the parental site (Chen, Reuveny, & Oh, 2013; Zakrzewski, Dobrzyński, Szymonowicz, & Rybak, 2019). Adult stem cells are frequently investigated for regenerative medicine and tissue repair, and they are less controversial than embryonic stem cells, primarily because they raise fewer ethical concerns.

Adult stem cells can be broadly categorized into mesenchymal stem cells (MSCs) and hematopoietic stem cells. While hematopoietic stem cells differentiate into white blood cells, red blood cells, and platelets, mesenchymal stem cells can generate various cell types generally associated with connective tissue, including epithelial, skeletal, glial, adipocytes, fibroblasts, osteoblasts, and chondrocytes (Oh & Choo, 2011). Bone marrow serves as the main source of mesenchymal stem cells, but a more accessible alternative involves extracting them from teeth, commonly referred to as dental pulp stem cells. Dental pulp stem cells (DPSCs) are a type of mesenchymal stem cell that can be isolated from the dental pulp cavity of permanent or primary dentition. These cells are noteworthy due to their multipotential to differentiate into various cell

types, such as odontoblasts, adipocytes, myocyte, and neural-like cells; and have similar regenerative properties to bone marrow-derived MSCs (Huang, G. T. -J, Gronthos, & Shi, 2009) (Kok et al., 2022). In fact, proliferation rates of DPSCs have been demonstrated to exceed that of bone-marrow derived MSCs (Shi, Robey, & Gronthos, 2001). Dental pulp stem cells (DPSCs), usually extracted from wisdom teeth, generally exhibit a less mature phenotype when compared to mesenchymal stem cells derived from bone marrow. This disparity in maturation is attributed to variations in developmental stages, resulting in distinct proliferation rates across different sources (Rola Mortada, Mortada, Mortada Beirut, & Lebanon, 2018a; Shi, Robey, & Gronthos, 2001). For all these reasons, DPSCs emerge as an excellent resource for studying MSCs.

Dental Pulp Stem Cells

Cellular and molecular markers are utilized to characterize MSCs and gain insights into their distinctive phenotypes. DPSCs meet the basic criteria for being classified as MSCs according to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cell and Gene Therapy (ISCT): they positively express markers associated with MSCs such as CD73 (ecto-5'-nucleotidase), CD90 (Thy-1), CD 44 (HCAM) and CD105 (endoglin) and do not express CD34 (hematopoietic marker), CD45 ((leukocyte common antigen), or CD 14 (monocyte marker) (Yamada et al., 2010)(Al Madhoun et al., 2021). The factors that contribute to the appeal of DPSCs include their ease of extraction from discarded teeth, marginal ethical concerns regarding procurement, and the ability of cryopreserved DPSCs to retain their potential for differentiation into multiple lineages (Xuan et al., 2018). DPSCs can serve as valuable resources for studying their embryonic origin, and biological characteristics during pre-clinical and clinical applications. Their accessibility, along with their ability to differentiate into different cell lineages, makes them

valuable for potential therapeutic use. The tooth's capacity to produce reparative dentin after injury stands as evidence that resident stem cell populations within the dental pulp fulfill a natural therapeutic function within the tooth complex(Sui et al., 2020). Due to their versatile nature, DPSCs are considered a promising source of adult stem cells for regenerative medicine and tissue engineering applications.

Isolation

In 2000, Gronthos et al. were the pioneers in identifying and describing dental pulp stem cells (DPSCs) (Gronthos, Mankani, Brahim, Robey, & Shi, 2000). Subsequent studies by various researchers have delved into the isolation, characterization, differentiation, and preservation of DPSCs. MSCs in the bone marrow constitute an extremely rare population, ranging from 0.01% to 0.001% (Friedenstein, Latzinik, Grosheva, & Gorskaya, 1982). The DPSC population is very small as well, constituting around 1% of the total cells in the pulp tissue (Raoof et al., 2014a). Therefore, it is imperative to successfully culture and populate DPSC in vitro before utilizing them for therapeutic purposes. Though there remains much to be understood regarding DPSCs, it is observed that DPSCs consist of progenitor cells distinguished by various features, including clonal heterogeneity, the ability to differentiate into multiple lineages, and phenotypic complexity (Kok et al., 2022). Research has demonstrated that the conditions and components of the media employed can induce potential phenotypic and functional alterations in freshly extracted DPSCs (Al Madhoun et al., 2021). Additionally, the procedures for isolating these cells may impact their heterogeneity (Rodas-Junco & Villicaña, 2017). Two commonly utilized approaches for cultivating dental pulp stem cells include the enzyme digestion method and the explant outgrowth method. Raoof *et al.* and Bronckaers *et al.* has shown that, while isolating DPSCs through

enzymatic digestion yields a substantial number of cells at a low passage rate, using tissue explants allows for the isolation of a more homogeneous cell population (Bronckaers et al., 2013) (Raoof et al., 2014b). Moreover, Raoof *et al.* and Huang *et al*. revealed that cells isolated through enzyme digestion exhibited a higher rate of proliferation compared to those isolated using the explant outgrowth method (Raoof et al., 2014b) (Huang, George T. -J, Sonoyama, Chen, & Park, 2006). In this study, we compare and analyze the impact of various isolation methods on the expansion, diversity, and variability in the expression of mesenchymal cell surface markers of DPSCs and well as their capacity for osteodifferentiation.

Selection

Understanding the diverse biological traits of DPSCs is essential for creating strategies to utilize them in innovative therapies for tissue regeneration in clinical applications, considering their well-known heterogeneity. An effective approach to categorizing cellular diversity is by selecting them based on their cell adhesion molecules. The expression of mesenchymal cell surface markers is related to various factors, such as the stem cell niche and the substrate to which cells adhere. An established method for isolating progenitor populations entails employing differential adhesion to fibronectin. Fibronectin is a key extracellular matrix protein that can impact cell behavior and phenotype (Zhu, Safavi, & Spangberg, 1998). Attachment of cells to the fibronectin matrix takes place through the interaction between the cell-binding domain of fibronectin and the integrin receptor on the cell surface, specifically the alpha-5/beta-integrin type in human DPSCs, triggering intracellular signaling pathways that can influence cell phenotype. (Zhu, Safavi, & Spangberg, 1998). This interaction between fibronectin and integrin receptors can activate intracellular mechanisms that facilitate odontoblast differentiation and the initiation of reparative

dentinogenesis when pulp cells respond to calcium hydroxide (Yoshiba, Yoshiba, Nakamura, Iwaku, & Ozawa, 1996) (Tziafas, Panagiotakopoulos, & Komnenou, 1995). Earlier studies have employed this technique to isolate multipotent progenitors from both skin and bone marrow (D'Ippolito et al., 2004) (Jones & Watt, 1993). However, isolation of DPSCs populations based on differential adhesion to fibronectin is much less well studied. In a recent study, progenitor cells derived from articular cartilage and isolated through fibronectin adhesion were observed to express MSC cell surface markers (such as CD90, CD105, and Notch), exhibit increased telomerase activity, possess a high proliferation capacity, and demonstrate multilineage differentiation potential (Korpershoek et al., 2021; Williams et al., 2010).

Differentiation

DPSCs can undergo transcriptional changes and transition to committed precursor cells during a more limited developmental stage presenting distinct phenotypes (Liu, Zhou, & Liu, 2016). The transformation of DPSCs into specific mature cell types, like osteocytes, is a regulated process by special chemical signals, growth factors, cytokines, and elements of the extracellular matrix (ECM) (Jaiswal, Haynesworth, Caplan, & Bruder, 1997). While there is currently no singular marker for identifying mineralizing lineage pathways in DPSCs, various expression markers have been explored to examine the osteodifferentiation process. The osteodifferentiation specific markers include Runx2 and osteopontin (OPN), osteocalcin (OCN), Runx2. These markers are related to differentiation into osteoblast-like cells that generate a mineralized matrix (Nakamura et al., 2009) (Saygin, Giannobile, & Somerman, 2000).

Runx2 (Runt-related transcription factor 2) is a master transcription factor that encodes a nuclear protein with a Runt DNA-binding domain, and it is involved in the formation of bone and

teeth (Kim, Shin, Kim, Kim, & Ryoo, 2020). As a transcription factor, Runx2 regulates the expression of various genes involved in osteoblast differentiation, extracellular matrix formation, and mineralization. It is essential for the development of skeletal tissues and is considered a central player in the process of osteogenesis. The high expression of Runx2 mRNA takes place in preosteoblasts, committed to osteogenesis at the osteogenic fronts and developing membranous parietal bones during the initial stages of the embryonic period (Kim, Shin, Kim, Kim, & Ryoo, 2020). Osteocalcin (OCN) is a non-collagenous protein that is most abundant in mineralized extracellular matrix and is required during osteogenic maturation (Tsao et al., 2017). It is typically present in both bone and dentin and plays a role in the mineralization of hard tissues (Rola Mortada, Mortada, Mortada Beirut, & Lebanon, 2018b). Its synthesis is limited to cells involved in mineralization, such as osteoblasts, odontoblasts, and cementoblasts (Sun, Wu, Dai, Chang, & Tang, 2006). Bakopoulou *et al* showed notable elevation in the expression of OCN in DPSCs subjected to osteoinductive stimuli (Bakopoulou et al., 2011). Osteopontin (OPN) is another bone matrix protein that is upregulated during osteogenic differentiation. The detailed function of OPN is still unclear; however, it is believed to be essential for the process of mineralization and the repair of mineralized tissue. (Rola Mortada, Mortada, Mortada Beirut, & Lebanon, 2018b). Additionally, it plays a role in cellular division, chemotaxis, migration, adhesion (Faccio et al., 1998), cytodifferentiation of osteoclasts, and intracellular signaling (Miyauchi et al., 1991)all of which are crucial for the generation of a new set of odontoblasts. *BGLAP* (Bone Gamma-Carboxyglutamate Protein) is a gene responsible for producing OCN protein. *SPP1* (Secreted Phosphoprotein 1) is a gene responsible for producing OPN protein.

These three molecular markers provide insight into the differentiation potential and functional characteristics of MSCs. The expression levels of these markers may vary depending

on the selected culture conditions of MSCs. Selecting cells based on specific cell adhesion molecules, particularly fibronectin receptors, may activate intracellular signaling pathways, influencing the cell phenotype and resulting in distinct expression patterns of certain molecular markers (Korpershoek et al., 2021) (Williams et al., 2010). In this study, we explore the relationship between fibronectin adherent and non-adherent isolates in terms of mesenchymal cell surface markers and their potential for differentiation along a mineralizing lineage pathway. After placing DPSCs into osteogenic medium, we analyzed the mRNA expression of *BGLAP, SPP1*, and *RUNX2* using reverse transcriptase-PCR and quantification PCR.

Rationale and Objective:

This study aims to isolate and analyze mixed populations of DPSCs originating from specific subtypes. By employing the specific biological marker fibronectin, the goal is to study different expression patterns of certain cell surface markers and molecular markers and selectively acquire more precise subgroups tailored for applications in regenerative medicine.

DPSCs constitute a relatively small population, comprising approximately 1% of the total cells in the pulp tissue. Therefore, it is imperative to successfully isolate, and culture refined populations of DPSCs in vitro before utilizing them for therapeutic purposes. Understanding DPSC heterogeneity is crucial for innovative regenerative therapies, prompting the investigation of mixed populations using fibronectin as a selection tool. It is reported that cellular phenotype and biological function may undergo alterations as cells replicate in a cultured environment or the substrate to which the cells adhere.

Here, we compared and analyzed the impact of various isolation methods on the expansion, diversity, and variability in the expression of mesenchymal cell surface markers of DPSCs as well

as the cellular expression during osteodifferentiation. The study aims to examine the relationship between fibronectin adherent, nonadherent, and explant DPSC populations in terms of mesenchymal cell surface markers and mineralizing lineage differentiation potential. Identifying markers that consistently and specifically represent distinct subtypes of DPSCs within mixed populations will streamline direct purification and will help us to give more insight into DPSC heterogeneity.

Research Questions

1. Does the fibronectin adherent cell population exhibit a faster growth rate compared to the non-adherent cell population?

o Null hypothesis (H0): Fibronectin adherent cell population grow at the same rate as the non-adherent cell population

o Alternative hypothesis (HA): Fibronectin adherent cell population grow faster than the non-adherent cell population

2. Do cells adherent to fibronectin display distinct expression patterns of mesenchymal cell surface markers compared to those in non-adherent cell populations?

o Null hypothesis (H0): Cells adherent to fibronectin display patterns of mesenchymal cell surface markers similar to those in non-adherent cell populations

o Alternative hypothesis (HA): Cells adherent to fibronectin display distinct expression patterns of mesenchymal cell surface markers compared to those in non-adherent cell populations

3. Do cells adherent to fibronectin exhibit greater osteogenic potential compared to those in non-adherent cell populations?

o Null hypothesis (H0): Cells adherent to fibronectin exhibit similar osteogenic potential compared to those in non-adherent cell populations

o Alternative hypothesis (HA): Cells adherent to fibronectin exhibit greater osteogenic potential compared to those in non-adherent cell populations

Chapter 2: Materials and Methodology

2.1 Isolation of human Dental Pulp Cells

Dental pulp tissues were harvested from normal, mature, and noncarious permanent teeth at the Dental clinic of the University of Nevada Las Vegas School of Dental Medicine. The teeth were extracted as part of regular operative procedures, and the information of the donor such as the age and health status were kept undisclosed to both the sample collectors and the investigators. The extracted teeth were considered biowaste; therefore, informed consent was not specifically required, however patients signed a consent to treatment form which makes them aware that their waste tissue may be used for research or teaching purposes. The study proposal was approved by UNLV Institutional Review Board before data collection for a study involving human subjects. The approval was based on an appropriate risk/benefit ratio and a protocol design in which the risks have been minimized.

Teeth (n=8) from two donors were used for the experiments, with the DPSC derived from the first donor designated as HADAN II, and those from the second donor referred to as HADAN III. The teeth consisted of a mix of premolars and third molars. Notably, one tooth from the second donor featured entirely intact pulp tissues, permitting an explant to be used as an additional aspect of the experiment.

The extracted teeth were placed into the α-MEM media (Gibco™ Modified Essential Medium α (1x) + GlutaMAX ™ with 10% v/v FBS, 1% L-ascorbic acid, 1% v/v penicillin/streptomycin) and stored no more than 1 hour prior to pulp extraction. The majority of attached soft tissues were removed and discarded. Teeth were soaked in 70% ethanol for approximately 10 seconds. To expose the pulp chamber, 2mm grooves were made along the

cement-enamel junction using a rotary tool (*Dremel*®) while ensuring there was abundant irrigation with a saline solution, facilitating the subsequent sectioning process. Using this groove, the teeth were cracked open using a wafer tweezer, exposing the pulp chambers. The entire pulp tissues were collected using forceps or NiTi Endo Hand K-Files in the pulp canals and combined into the media. The pulps were finely shredded using a scalpel and treated with pre-warmed 4mg/ml collagenase/dispase and incubated at 37° C, 5% CO_2 for 1hr with intermittent shaking. The digested tissues were filtered through a 70mm cell strainer and washed with an additional 5-10mL serum containing α-MEM media. The filtered cells were centrifuged at 400 rpm for 5 min. Afterwards, the supernatant was discarded, and the cell pellet was suspended in a fresh culture medium. Centrifugation was repeated to ensure complete removal of collagenase/dispase. The cells were then resuspended in a serum-free medium for a cell isolation procedure. For the explant taken from the second donor (HADAN III), the intact pulp explant was placed directly in a plate well with the α-MEM media for 5 days and then removed. The remaining cells were retained in the well and were collected for subsequent experiments.

2.2 Fibronectin selection

For both donors, fibronectin at a concentration of 10 μg/mL, derived from human plasma, was reconstituted in 0.1M PBS+ (PBS containing 1mM Ca^{2+} and 1mM Mg^{2+}, pH 7.4). This suspension was used to precoat the base of 10cm^2 6-well plates (Greiner Bio-One, UK) with 1 mL/well, kept at 4°C overnight. The plates were aspirated before adding cells. A single-cell suspension, resuspended in 1 mL serum-free medium, was seeded into the pre-coated wells at a density of 4x10^3 cells/cm^2 for 20 minutes at 37°C 5% CO_2 incubator. Adherent cells were retained in the wells until confluent, constituting the fibronectin adherent (FA) populations. Any non-

adherent cells were then collected and seeded into separate wells and cultured. These cells served as the Non-Adherent (NA) cell population used in subsequent experiments.

2.3. Sub-culturing of DPSC and calculating cumulative population doublings

Cell population doubling rates were recorded for Fibronectin Adherent (FA) and Non-fibronectin Adherent (NA) populations from both donors as well as cells isolated from the explant of the HADAN III donor. Daily observation of cell growth was conducted, with the medium refreshed every three days. When DPSCs reached 80% confluence sub-culturing was initiated. The cells were observed under a light microscope to monitor proliferation and estimate the confluency at the bottom of the flask. Upon confirming the desired confluency, cell media were removed and a thorough wash with 10 mL of sterile PBS followed, aimed at removing cell debris and any remnants of old media. Subsequently, 3 mL of Accutase (StemPro® Accutase®) was added and allowed to incubate for 3-5 minutes in a 37°C, 5% CO2 incubator. Confirmation of cell detachment was achieved through observation under a light microscope, after which 2 mL of α-MEM media was added. The cells were then collected and centrifuged at 900 rpm for 5 minutes. The supernatant was carefully removed, and the pellets were resuspended in 1000 µL of α-MEM media. Cell count was done using a hemocytometer. The volume of the cell suspension required for reseeding into each flask was calculated using the following formula:

$$Vf = \frac{Ci}{Cf} \; x \; Vi$$

V_f *= volume of cell suspension for reseeding for passage n*
V_i *= volume of cell suspension (1000 µL)*
C_i *= initial cell number seeded at passage n-1*
C_f *= final cell yield cells counted at passage n*

A total of 8 to 14 passages were conducted for both HADAN II and HADAN III. Subculturing was concluded upon reaching senescence in the cells. For every passage, the date, the total number of cells counted, and the number of cells reseeded were recorded. The Total Population Doublings (PD_t) was calculated based on the specified formula.

$$PDt = \text{PDi} + \frac{log\ Cf - log\ Ci}{log\ 2}$$

***PD**$_t$= Total Population doubling level*
***PD**$_i$= initial population doubling level*
***C**$_i$= initial cell number seeded at passage n-1*
***C**$_f$= final cell yield cells counted at passage n*

The total population doublings mean the total number of times a population of cells doubled during cell culture. The correlation between cumulative PDL and the duration in days has been plotted in a line graph.

2.4 Flow cytometric surface marker expression analysis

Cells from the 4th passage for HADAN II and the 10th passage for HADAN III were utilized in the experiments. The antigen profiles of fibronectin adherent and non-adherent isolates from HADAN II and HADAN III and explant from HADAN III were analyzed by detecting the expression of the stem cell surface markers CD73, CD90, CD105, CD 44 using flow cytometry. This analysis adhered to the (ISCT) guidelines for identifying human MSCs. The kit used for this analysis (BD Biosciences™) included a panel recommended by the International Society for Cellular Therapy ISCT and was designed to identify MSCs expression of CD73, CD90, CD105, CD 44 and absence of CD34, CD45, CD11b, CD19, and HLA-DR.

Cells were harvested by treatment with BD™ Accutase™ Cell Detachment Solution for 3 min, washed, and resuspended at 1x10^7 cells/ml in BD Pharmingen™ Stain Buffer. The antibody tubes from the kit were prepared following the manufacturer's guidelines. Then,100 µl of the cell suspension was carefully added to each antibody or cocktail tube. Tubes were incubated in the dark for 30 minutes at room temperature. Cells were washed twice with BD Pharmingen™ Stain Buffer (FBS) and resuspended in 500 µls of this same buffer. Cells were then analyzed using an Attune flow cytometer (ThermoFisher™), with 20,000 events collected for each sample. Compensation settings were set using UltraComp™ compensation beads (Thermofisher) and antibodies conjugated with each fluorophore examined during experiments. Gating was performed on a forward scatter (FSC) and side scatter (SSC) to exclude debris and other outliers and subsequently gated on a FSC-H vs FSC-W plot to remove potential doublets.

2.5 Incubation in inductive media

Cells from the 4th passage for HADAN II and the 10th passage for HADAN III were utilized in the experiments. The passage to initiate experiments was chosen based on the relative stability and growth of cells from each donor as well as their number, as subsequent experiments required varying numbers of cells. The fibronectin-adherent and non-adherent isolates from both HADAN II and HADAN III, along with the explant variable from HADAN III, were incubated in MSC Osteogenic Differentiation Medium (PromoCell GmbH, Heidelberg, Germany) for 7 days. The same variables were also subjected to a basic medium, serving as the negative control.

2.6 RNA isolation

Total RNA was extracted from cells cultured in osteogenic differentiation media and the negative control, employing the RNeasy® mini-prep kit from Qiagen following the manufacturer’s

guidelines. The quality and quantity (ng/µl) of the RNA were assessed using Thermo Scientific™ NanoDrop™ Spectrophotometers, evaluating absorbance ratio measurements at 260 and 280 nm (A260/A280 ratio). All samples exhibited ratios within the range of 1.92-2.06, indicating minimal protein contamination. Each experiment was conducted in triplicate and replicated for HADAN II and HADAN III.

2.7 RT-PCR amplification for cDNA synthesis

The cDNA synthesis process utilized the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Total RNA concentration was adjusted to 0.5 µg in nuclease-free water per reaction. To create the cDNA master mix, precise quantities of 10x RT buffer, 25x dNTP Mix, 10X RT Random Primers, MultiScribe™ Reverse Transcriptase, and nuclease-free water were mixed. The 30µl of the resulting master mix was then added to 30µl of RNA template into each PCR tube. The reverse transcriptase PCR amplification was performed by a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems) under the following conditions: primers annealed at 25°C for 10 minutes, RNA reverse transcribed at 37°C for 120 minutes, enzyme inactivated at 85°C for 5 minutes, followed by a cooling step at 4°C. The cDNA library was stored in a -20°C freezer until it was ready for use.

2.8 Gene expression analysis via qPCR

For the quantification PCR experiment, TaqMan® Fast Advanced Master Mix was used (Applied Biosystems, USA). The TaqMan master mix was prepared for each gene of interest (RUNX2, BGLAP, SPP1) adding 2 x PCR Buffer, primers for housekeeping gene with probe (RPLPO_Vic[1]), primers for a gene of interest with probe (GOI_Fam), and nuclease-free water. [1]The Applied Biosystems™ Human RPLPO (large ribosomal protein with VIC probe) is intended

as an endogenous control. When combined with other gene expression assays, it enables the quantification of relative gene expression in cDNA samples. The 14 µl of each gene specific master mix were transferred to the corresponding well of an optical 96-well plate. Then, 6 µl of cDNA samples were transferred to each well. The negative control was generated by consolidating the remaining gene-specific master mix and dispensing 20µl into the designated wells. Additionally, 20µl of an internal positive control (IPC) was dispensed into the assigned well. Each experiment was conducted in triplicate and replicated for HADAN II and HADAN III.

The amplification was performed by the QuantStudio™ 3 Real-Time PCR instrument (Applied Biosystems, Thermo Scientific™, USA) following the thermal protocol and fast cycling mode with the following steps: enzyme activation at 95°C for 20 seconds followed by 40 cycles of denaturation at 95°C for 1 second and annealing-extension at 60 °C for 20 seconds. The designated assay ID from Thermo Fisher Scientific™ for each gene primer is as follows:

Table 1. Assay ID for each gene primer used from Thermo Fisher Scientific™

Genes	Assay ID
BGLAP	Hs01587814_g1
SPP1	Hs00959010_m1
RUNX2	Hs01047973_m1
RPLPO_VIC	Hs00420895_gH

The Cycle Threshold (CT) mean for each untreated control and treatment group was derived using the QuantStudio™ 3. ΔCt was computed by subtracting the CT of the gene of interest

from the CT of RPLPO (housekeeping gene). Then ΔΔCt was calculated as ΔCt minus the average ΔCt of the control group. The relative quantification (RQ) or mean fold change in expression, comparing control and treatment groups, was determined using the following formula:

$$RQ = 2^{-(\Delta\Delta\, Ct)}$$

2.9 Statistical Analysis

The cumulative population doublings data for Dental Pulp Stem Cells (DPSCs) for HADAN II isolates (fibronectin adherent vs non-fibronectin adherent populations) were compared using a paired T-test, with statistical significance set at $p < 0.05$. For HADAN III isolates, the cumulative population doublings for fibronectin adherent, non-fibronectin adherent, and explant-derived populations were compared using a repeated measures ANOVA analysis, with statistical significance set at $p < 0.05$. Repeated Measures ANOVA and Paired T-Tests were performed using GraphPad Prism Software.

For the q-PCR analyses, all assays were conducted in two independent experiments ($n = 2$), each comprising three replicates. The standard error of the mean (SEM) of the RQ was calculated. The SEM approaching zero suggests that the estimated value is nearly identical to the true value. The Mann-Whitney U Test (two-tail T-test) analysis was employed to assess the significance of relative expression between the untreated control group and the treated group, with significance assumed for $p < 0.05$.

Chapter 3: Results

3.1 Cumulative Population Doublings Level

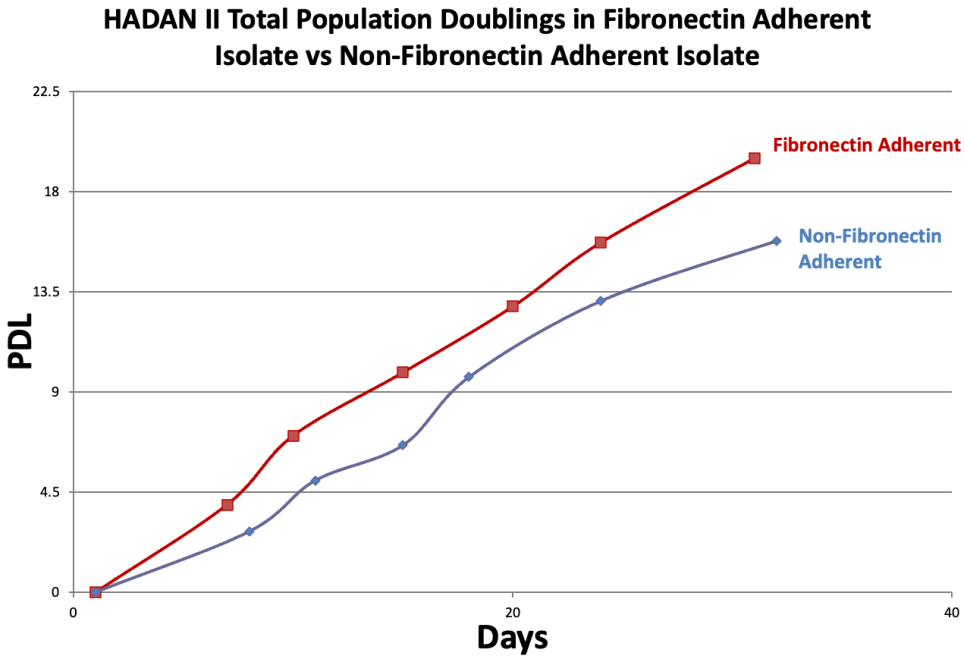

Figure 1. The graph illustrates the correlation between the cumulative Population Doublings (PDs) over time for HADAN II (fibronectin adherent isolate and non-fibronectin adherent isolate). Each data point represents a passage. Cell counts were performed in quadruplicate. Paired T Test: Between Groups P=.0036, demonstrates significantly different cumulative population doublings between the two groups over time**.**

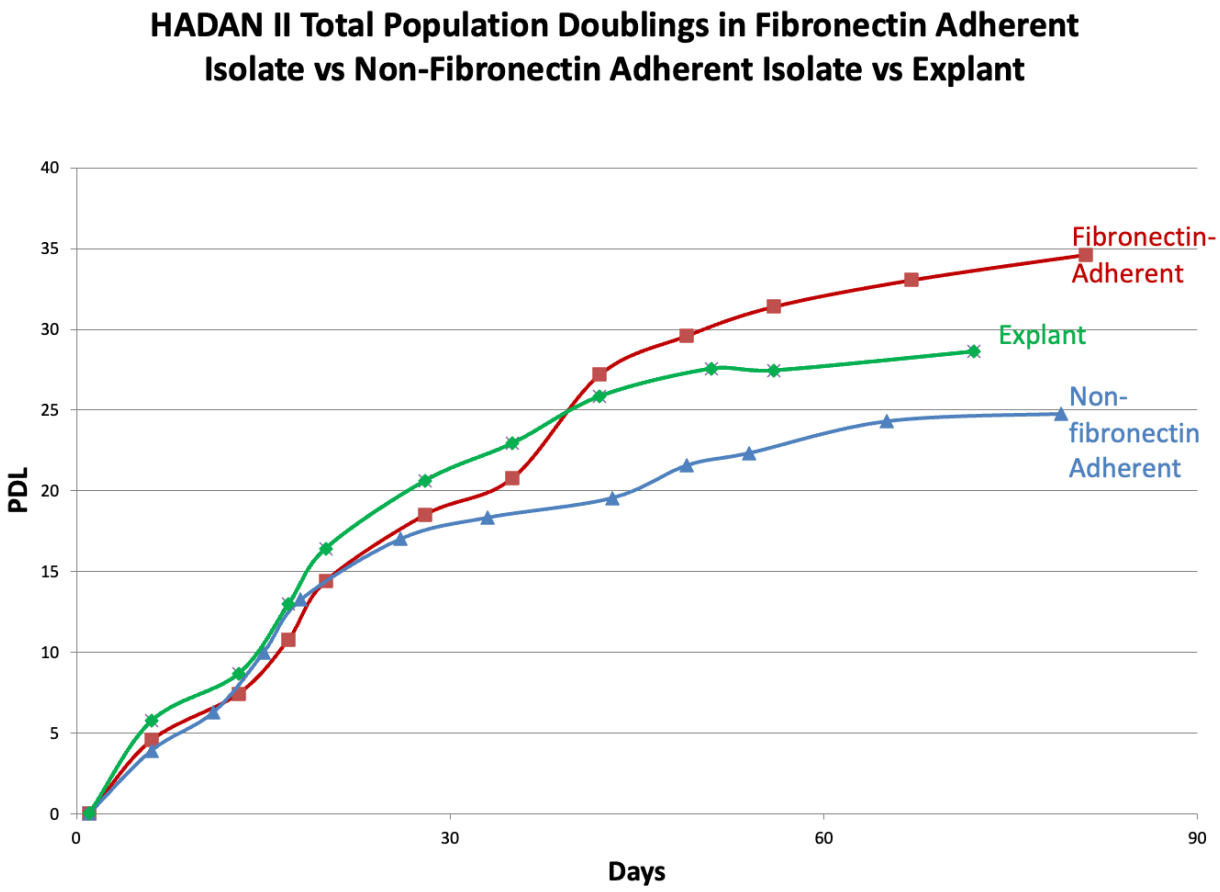

Figure 2. The graph illustrates the correlation between the cumulative Population Doublings(PDs) and the duration in days for HADAN III (fibronectin adherent isolate, non-fibronectin adherent isolate, and explant population). Each data point represents a passage. Paired T Test: Between Groups P=.0001, demonstrates significantly different cumulative population doublings over time.

The population doubling (PDs) is the cumulative number of times a cell population doubles during cell culture. The two experimental groups of HADAN II completed a total of 8 passages within 31-32 days. The three experimental groups of HADAN III completed a total of 12–13 passages within 72–81 days. **(Figure 1)** shows the two HADAN II groups demonstrating an increase in population in a comparable pattern. The non-fibronectin adherent group displayed the

steepest increase in population doublings (PDs) between passages 3-4 and 5-6, while the fibronectin adherent group showed the highest increase in PDs at passages 3-4. It also shows fibronectin adherent isolate consistently maintains a higher PD rate than the non-fibronectin adherent isolate across all passages. The comparison of cumulative HANDAN II PDs between the two isolates using a paired T-test reveals statistically significant differences in population doublings between groups, evidenced by a P-value of.0036.

In **(Figure 2),** the three HADAN III groups illustrate a similar, sigmoidal growth pattern, characterized by an increase in cell culture at the initial stages, followed by a steady population growth over time. This indicates that the rate of population doublings is most rapid at the outset, gradually slowing down after reaching its peak. This growth curve is observed consistently across all three isolates—fibronectin adherent, non-fibronectin adherent, and explant—displaying a similar growth trajectory. Explant showed the highest population doublings from passage 0-6. After passage 6 (beyond 40 days), the fibronectin adherent isolate surpassed the growth of the explant cells, consistently maintaining the highest population throughout the subsequent days. Throughout the experiment, the fibronectin isolated DPSC always had higher population doublings than the NA isolate. All isolates exhibited the most significant increase in Population Doublings between passages 5-6. Additionally, the explant-derived cell doubling rate began to plateau slightly earlier than the other two groups. The termination of passaging occurred when there were no discernible signs of cell growth. The examination of cumulative HANDAN III PD numbers through repeated measures ANOVA demonstrates significant differences in the cumulative population doublings between groups, demonstrated by a P-value of .0001.

3.2 Immunophenotypic Characterization of Dental Pulp Stem Cells

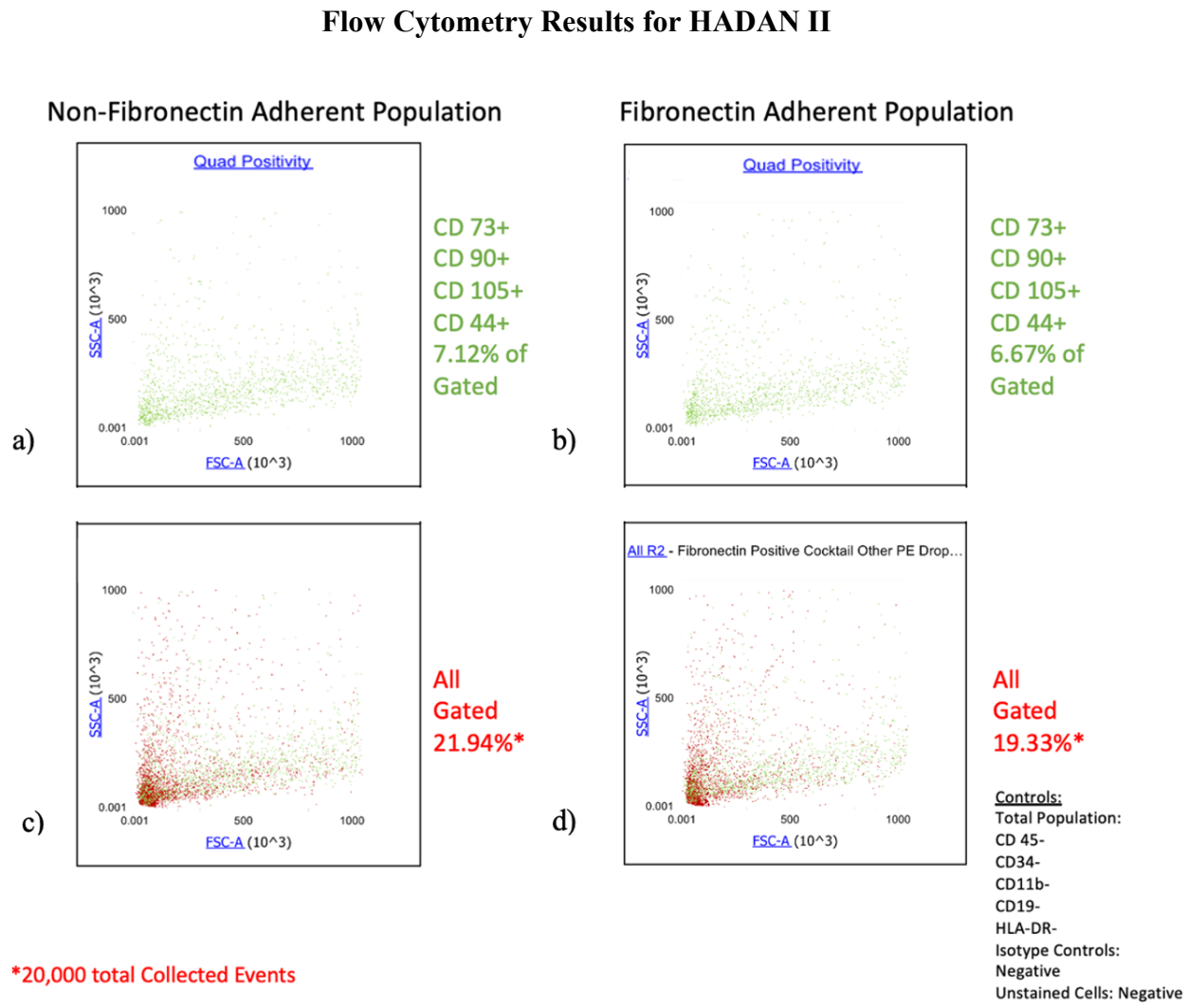

Fig. 3 – (a–d). Flow cytometric analysis of mesenchymal cell surface marker expression on populations isolated from HADAN II. Cells are displayed on a forward scatter vs side scatter plot. **a)** shows the % and distribution of the quadruplicate positive cells labeled in green for the NA population **b)** shows the distribution of the total population of gated cells (red) with the quadruplicate labeled cells superimposed (green) for the NA population **c)** shows the % and distribution of the quadruplicate positive cells labeled in green for the FA population **d)** shows the distribution of the total population of gated cells (red) with the quadruplicate labeled cells superimposed (green) for the FA population

Flow Cytometry Results for HADAN III

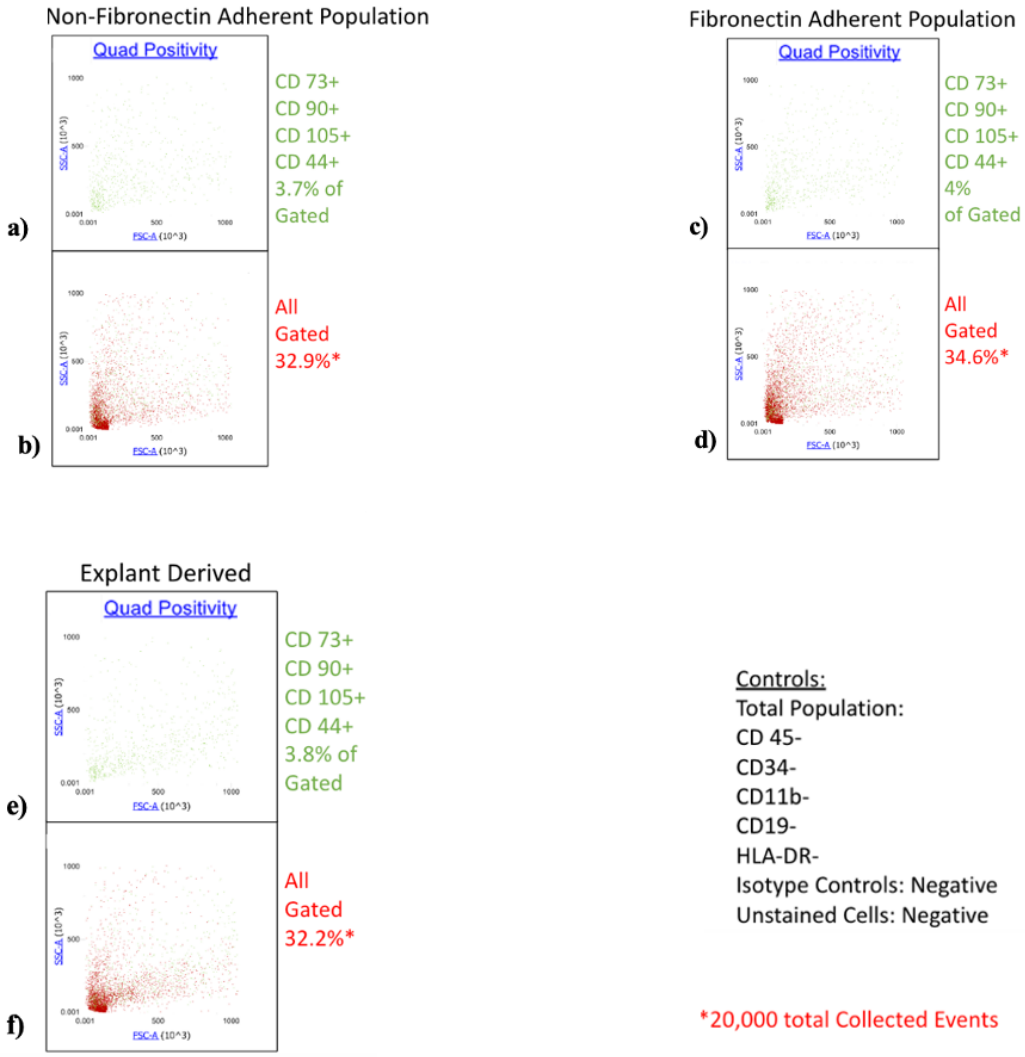

Fig. 4 – (a–f). Flow cytometric analysis of mesenchymal cell surface marker expression on populations isolated from HADAN III. Cells are displayed on a forward scatter vs side scatter plot. **a)** shows the % and distribution of the quadruplicate positive cells labeled in green for the NA population **b)** shows the distribution of the total population of gated cells (red) with the quadruplicate labeled cells superimposed (green) for the NA population **c)** shows the % and distribution of the quadruplicate positive cells labeled in green for the FA population **d)** shows the distribution of the total population of gated cells (red) with the quadruplicate labeled cells superimposed (green) for the FA population. **e)** shows the % and distribution of the quadruplicate positive cells labeled in green for the explant population. **f)** shows the distribution of the total population of gated cells (red) with the quadruplicate labeled cells superimposed (green) for the explant population.

As illustrated in (**Figure 3)**, the flow cytometry analysis of HADAN II revealed that the non-fibronectin adherent (NA) population had 21.94% total gated cells and of those gated cells, 7.12% were quadruplicate positive cells (having a simultaneous expression of CD90, CD44, CD105, CD73). The fibronectin adherent (FA) population of HADAN II displayed 19.33% total gated cells and of those gated, 6.67% quadruplicate positive cells. These cells were also negatively controlled for CD45, CD34, CD11b, CD19, and HLA-DR expression.

In (**Figure 4)**, the flow cytometry analysis of HADAN III indicated that the NA population exhibited 32.9% total gated cells, and of the gated cells, 3.7% were quadruplicate positive cells. The FA population of HADAN III showed 34.6% total gated cells with 4% quadruplicate positive cells from the gated cells. Fibronectin-isolated populations displayed slightly higher quadruplicate positive expression than the NA population in HADAN III. Explant-derived cells demonstrated comparable proportions of all gated cells (32.2%) and quadruplicate positive cells (3.8%) and to both NA and FA isolate groups.

(Table 2) presents the proportions of individual mesenchymal cell surface markers from DPSCs isolated from HADAN II and HADAN III (CD90, CD44, CD105, CD73). In HADAN II, the NA isolate exhibited slightly higher percentages of cells than the FA group in all cell surface markers except CD105, with the difference being relatively minor. HADAN III populations displayed comparable expression levels in both fibronectin-adherent and non-fibronectin-adherent groups. Cells derived from explants exhibited marginally elevated percentages of CD90, CD44, CD105, and CD73 expression, with a difference of less than 5%.

Table 2. % Gated for individual MSC cell surface markers for each isolate

MSC Cell Surface Markers	HADAN II		HADAN III		
	Fibronectin Adherent	**Nonfibronectin Adherent**	**Fibronectin Adherent**	**Nonfibronectin Adherent**	**Explant**
CD90	60.3%	66.8%	28.4%	29.8%	36.5%
CD44	59.2%	61.2%	31.3%	33.3%	33.9%
CD105	36.7%	35.5%	12.6%	11.8%	13.6%
CD73	60%	64.9%	29.9%	31.6%	37.7%

3.3 mRNA Expression of Osteodifferentiation Genes

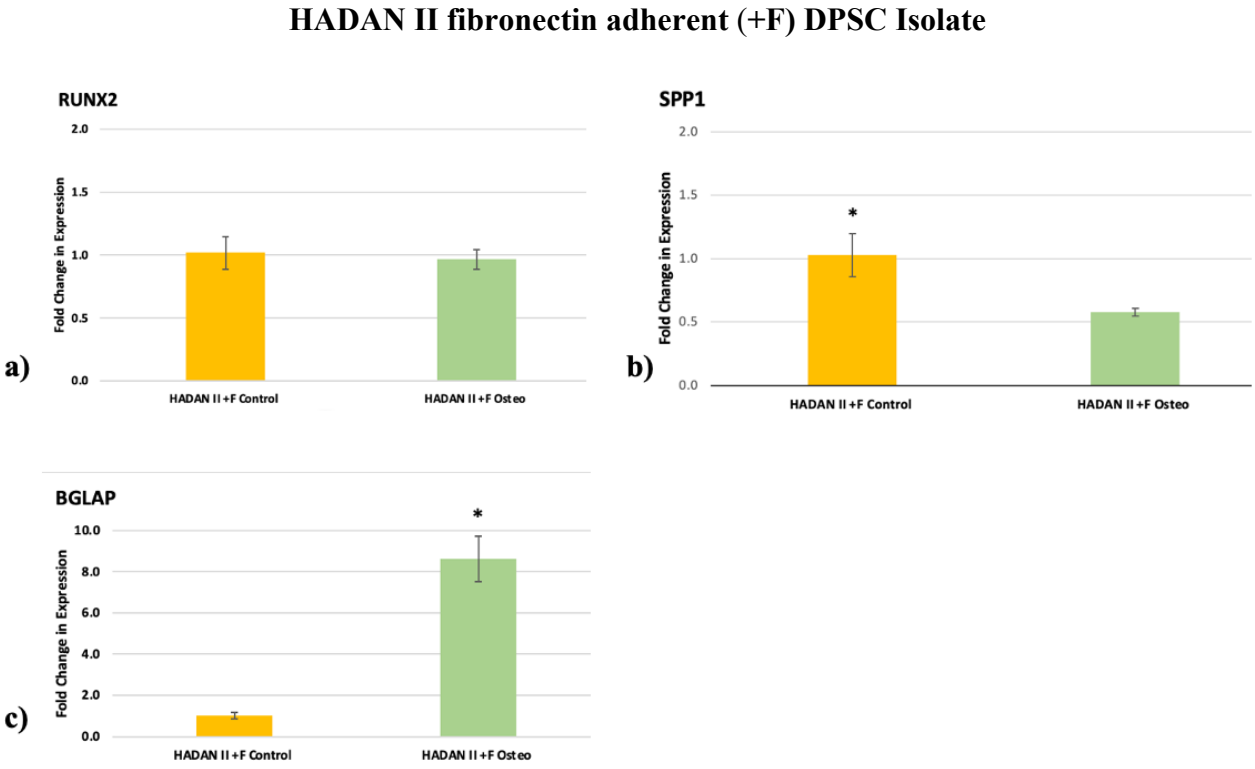

Fig. 5 – (a–c) Mean fold changes, or relative quantification (RQ), for the expression of the gene of interest following a 7-day exposure to osteogenic differentiation media. The **HADAN II fibronectin adherent** (**+F**) DPSC isolate was compared between the control and the osteogenic treatment groups (Osteo). The asterisk indicates statistically significant differences in gene expression between the untreated control and the Osteo-treated group ($p < 0.05$).

a) For *RUNX2* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.13, while the treatment group exhibits SEM of 0.08. The P-value for this comparison is 0.85408.
b) For *SPP1* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.168, while the treatment group exhibits SEM of 0.035. The P-value for this comparison is 0.0213, indicating a statistically significant difference.
c) For *BGLAP* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.256, while the treatment group exhibits SEM of 1.109. The P-value for this comparison is 0.0213, indicating a statistically significant difference.

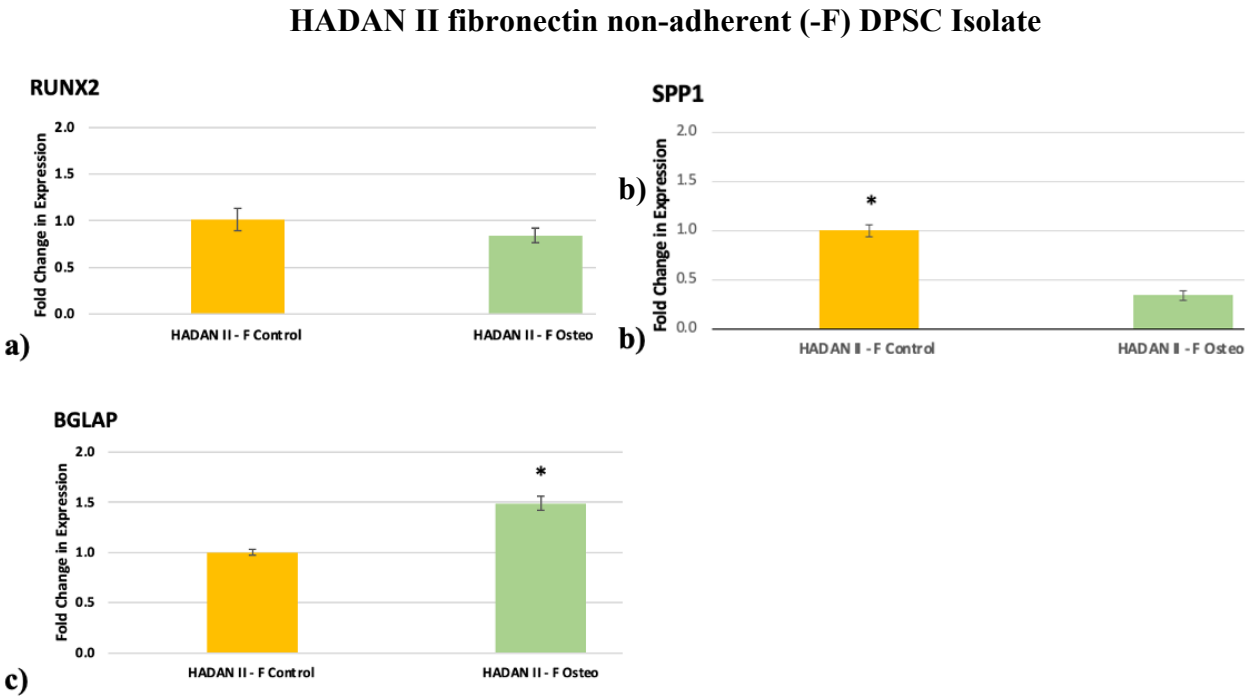

Fig. 6 – (a–c) Mean fold changes, or relative quantification (RQ), for the expression of the gene of interest following a 7-day exposure to osteogenic differentiation media. The **HADAN II Non-fibronectin adherent (- F) DPSC** isolate was compared between the control and the osteogenic treatment groups (Osteo). The asterisk indicates statistically significant differences in gene expression between the untreated control and the Osteo-treated group ($p < 0.05$).

a) For *RUNX2* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.12, while the treatment group exhibits SEM of 0.08. The P-value for this comparison is 0.326.

b) For *SPP1* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.0556, while the treatment group exhibits SEM of 0.0475. The P-value for this comparison is 0.0213, indicating a statistically significant difference.

c) For *BGLAP* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.0258, while the treatment group exhibits SEM of 0.0658. The P-value for this comparison is 0.0213, indicating a statistically significant difference.

HADAN III fibronectin adherent (+F) DPSC Isolate

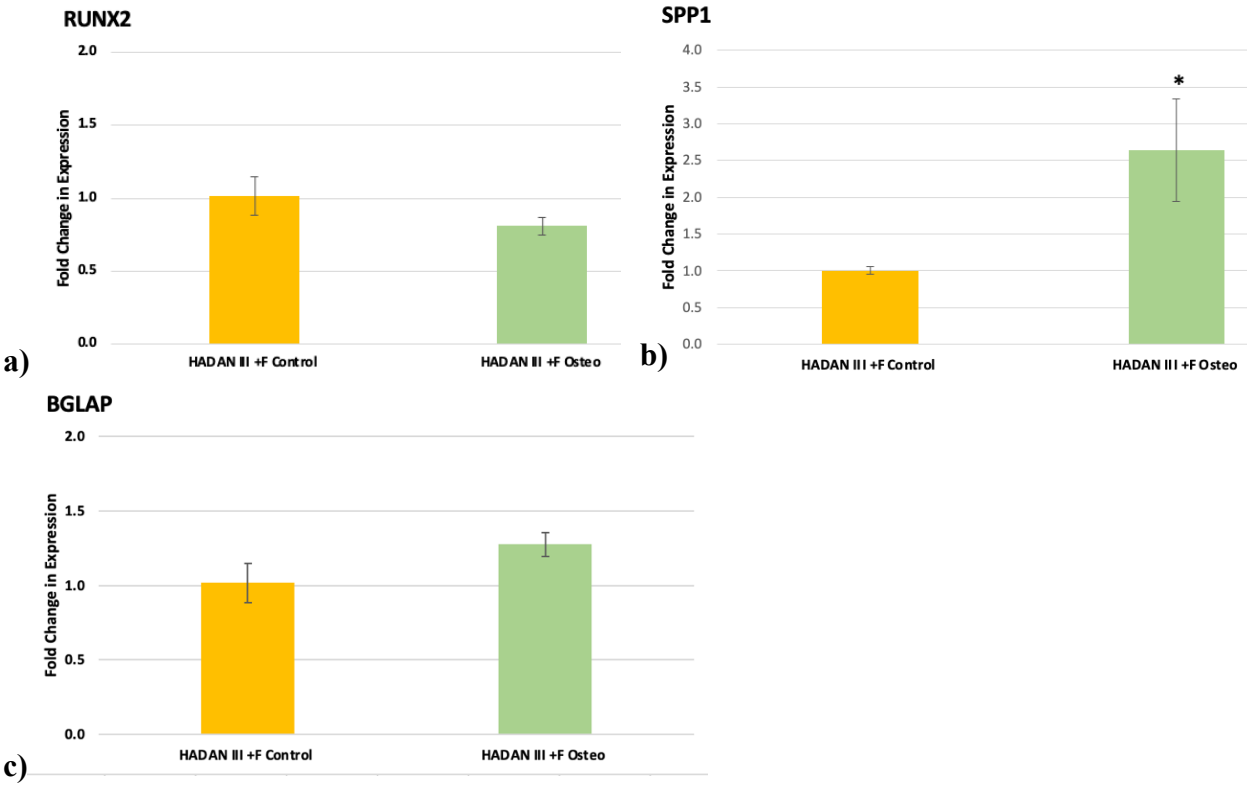

Fig. 7 – (a–c) Mean fold changes, or relative quantification (RQ), for the expression of the gene of interest following a 7-day exposure to osteogenic differentiation media. The **HADAN III Fibronectin adherent** (**+F**) DPSC isolate was compared between the control and the osteogenic treatment groups (Osteo). The asterisk indicates statistically significant differences in gene expression between the untreated control and the Osteo-treated group ($p < 0.05$).

a) For *RUNX2* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.13, while the treatment group exhibits SEM of 0.06. The P-value for this comparison is 0.326.
b) For *SPP1* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.0515, while the treatment group exhibits SEM of 0.7062. The P-value for this comparison is 0.0213, indicating a statistically significant difference.
c) For *BGLAP* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.13, while the treatment group exhibits SEM of 0.08. The P-value for this comparison is 0.1347, indicating a statistically significant difference.

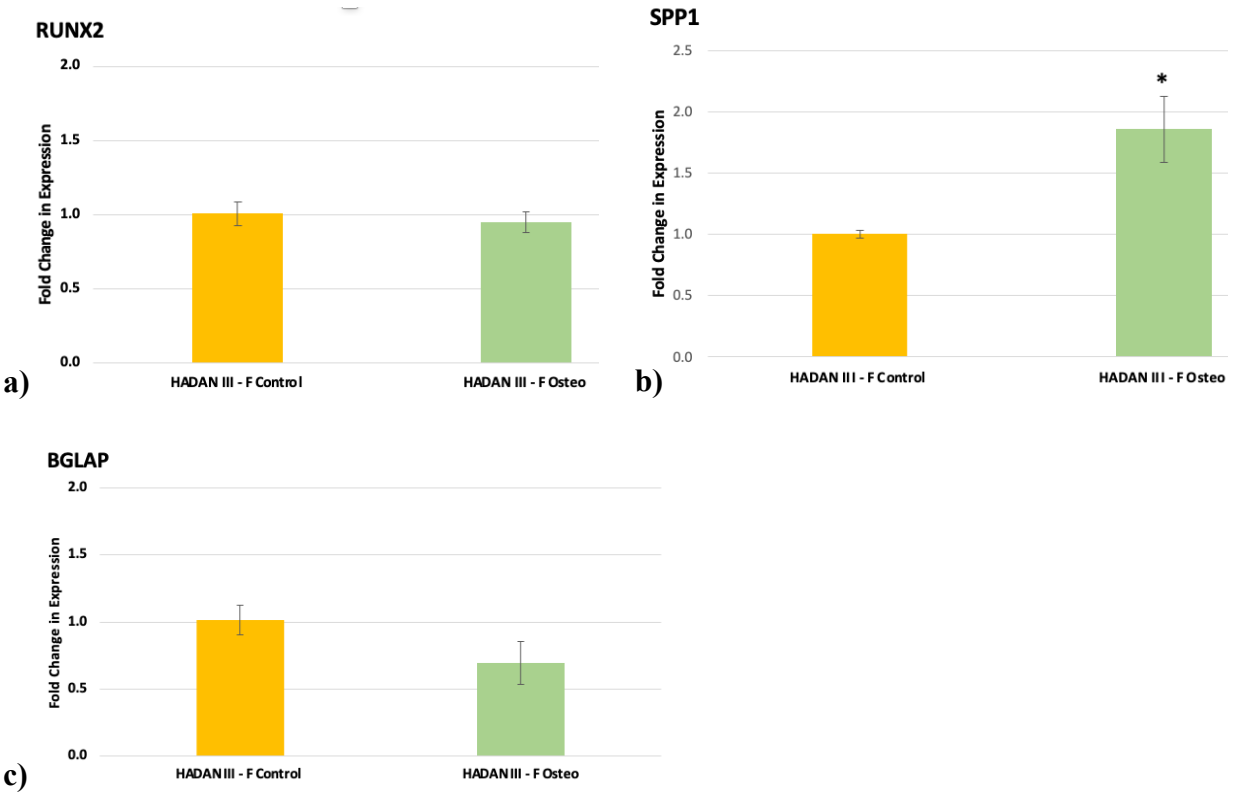

Fig. 8 – (a–c) Mean fold changes, or relative quantification (RQ), for the expression of the gene of interest following a 7-day exposure to osteogenic differentiation media. The **HADAN III Non-fibronectin adherent** (**-F**) DPSC isolate was compared between the control and the osteogenic treatment groups (Osteo). The asterisk indicates statistically significant differences in gene expression between the untreated control and the Osteo-treated group ($p < 0.05$).

a) For *RUNX2* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.13, while the treatment group exhibits SEM of 0.06. The P-value for this comparison is 0.57339.
b) For *SPP1* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.0266, while the treatment group exhibits SEM of 0.2698. The P-value for this comparison is 0.0213, indicating a statistically significant difference.
c) For *BGLAP* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.11, while the treatment group exhibits SEM of 0.16. The P-value for this comparison is 0.1347.

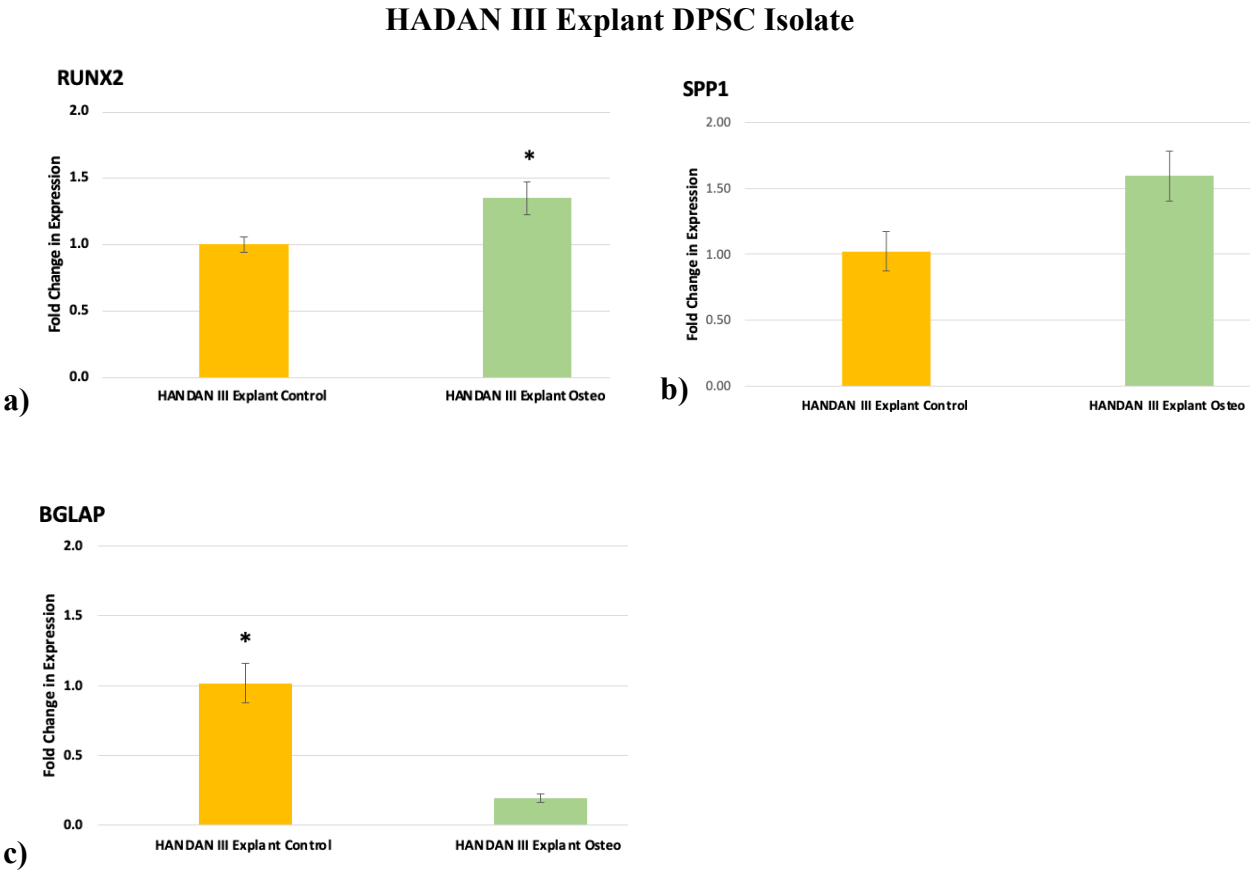

Fig. 9 – (a–c) Mean fold changes, or relative quantification (RQ), for the expression of the gene of interest following a 7-day exposure to osteogenic differentiation media. The **HADAN III Explant** DPSC was compared between the control and the osteogenic treatment groups (Osteo). The asterisk indicates statistically significant differences in gene expression between the untreated control and the Osteo-treated group ($p < 0.05$).

a) For *RUNX2* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.06, while the treatment group exhibits SEM of 0.12. The P-value for this comparison is 0.0213, indicating a statistically significant difference.
b) For *SPP1* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.152, while the treatment group exhibits SEM of 0.1935. The P-value for this comparison is 0.1347.
c) For *BGLAP* gene in the fibronectin adherent control, the standard error of the mean (SEM) is 0.14, while the treatment group exhibits SEM of 0.03. The P-value for this comparison is 0.0213, indicating a statistically significant difference.

The HADAN II fibronectin-adherent (+F) DPSC isolate displayed no significant difference in *RUNX2* expression between the untreated control and treatment groups. The HADAN II fibronectin-adherent (+F) DPSC isolate showed a 42% reduction in *SPP1* expression in the osteogenic media treatment group compared to the untreated control group (P-value of 0.0213). There was an approximately 800% increase in *BGLAP* mRNA expression following osteogenic media treatment (P-value of 0.0213). The standard error of the mean for all groups in three genes ranged from 0.035 to 1.109, indicating that the calculated RQs closely align with the true values **(Figure 5 (a-c)).**

The HADAN II fibronectin non-adherent (-F) DPSC isolate displayed no significant difference in *RUNX2* expression between the untreated control and treatment groups. The HADAN II fibronectin non-adherent (-F) DPSC isolate showed a 66% reduction in *SPP1* mRNA expression in the osteogenic media treatment group compared to the untreated control group (P-value of 0.0213). There was an approximately 50% increase in *BGLAP* mRNA expression following osteogenic media treatment (P-value of 0.0213). The standard error of the mean for all groups in three genes ranged from 0.026 to 0.12, indicating that the calculated RQs closely align with the true values **(Figure 6 (a-c)).**

The HADAN III fibronectin adherent (+F) DPSC isolate displayed no significant difference in *RUNX2* and *BGLAP* expression between the untreated control and treatment groups. The HADAN III fibronectin adherent (+F) DPSC isolate displayed a 160% increase in *SPP1* mRNA expression in the osteogenic media treatment group compared to the untreated control group (P-value of 0.0213). The standard error of the mean for all groups in three genes ranged

from 0.06 to 0.706, indicating that the calculated RQs closely align with the true values **(Figure 7 (a-c)).**

The HADAN III fibronectin non-adherent (-F) DPSC displayed no significant difference in *RUNX2* and *BGLAP* expression between the untreated control and treatment groups. The HADAN III fibronectin non-adherent (-F) DPSC isolate displayed an 86% increase in *SPP1* mRNA expression in the osteogenic media treatment group compared to the untreated control group (P-value of 0.0213). The standard error of the mean for all groups in three genes ranged from 0.027 to 0.27, indicating that the calculated RQs closely align with the true values **(Figure 8 (a-c)).**

The HADAN III explant DPSC isolate demonstrated *RUNX2* mRNA expression increased by 35% in the osteogenic media treatment group with a P-value of 0.0213. Although there was an increase in *SPP1* mRNA expression in the treatment group, it did not reach statistical significance (P-value 0.1347). There was an 82% reduction in *BGLAP* mRNA expression in the osteogenic media treatment group compared to the untreated control group (P-value of 0.0213). The standard error of the mean for all groups in three genes ranged from 0.03 to 0.19, indicating that the calculated (RQs) closely align with the true values **(Figure 9 (a-c)).**

Chapter 4: Discussion

The measurement most employed to assess cell culture growth rate is the population doubling time, representing the duration required for a cell population to double in size (Greenwood et al., 2004). In our study, we observed a notable difference in population doublings between dental pulp stem cells isolated through fibronectin selection and those not subjected to this process. The significant increase in population doublings was consistently observed in both HADAN II and HADAN III, despite different donor origins. This suggests that fibronectin isolation contributes to improved population growth, as evidenced by the consistent increase in population doubling over time—a direct reflection of population growth. These findings align with previous studies indicating that human MSCs isolated through fibronectin adhesion exhibit enhanced telomerase activity and possess a robust proliferation capacity (Korpershoek et al., 2021; Williams et al., 2010). This becomes a crucial consideration for harvesting ample quantities of human DPSC subpopulations, assessing, and developing them for clinical use. Moreover, the enhanced proliferative potential of fibronectin-adherent MSCs may be valuable for sustaining long-term tissue regeneration upon reinfusion (Kok et al., 2022).

In contrast to the findings of previous studies by Raoof et al. and Huang et al., which suggested that cells obtained through enzymatic digestion exhibited a higher proliferation rate compared to those isolated via the explant outgrowth method, our experiment yielded opposing results, at least initially. DPSCs from the explant outgrowth method demonstrated an increased population doubling, indicating a higher proliferation rate, but only during the earlier passages. Moreover, explant-derived DPSCs appeared to reach senescence earlier than those obtained through enzymatic digestion. However, it is important to acknowledge the limitation of the low

sample size. Given the high level of individual variation between donors found here and by others, there remains much to be determined regarding enzymatic digestion vs explant derivation of DPSCs, even though both techniques yielded viable DPSC populations.

Regarding the phenotypic characterization of dental pulp progenitor cells, the expression of specific markers, such as CD90, CD73, CD105, and CD44, served as an indication of their mesenchymal stem cell phenotype under ISCT guidelines. All isolates displayed relatively low proportions of quadruplicate positive cells, aligning with the understanding that DPSCs constitute a minority cell type within the pulp, and no enrichment measures were employed beyond fibronectin adhesion. Although HADAN II and III exhibited different proportions of total gated cells, the quantity of quadruplicate positive cells remained comparable across the groups. The HADAN II's fibronectin-isolated group (FA) and non-fibronectin adherent (NA) counterparts demonstrated a negligible difference in the expression of MSC cell markers. Similarly, HADAN III displayed minimal disparities in MSC cell marker expressions between the FA group and the NA group. Hence, it does not seem that fibronectin significantly enhanced the proportion of cells positive for these four MSC cell surface markers. Similarly, the proportion of cells positive for any single MSC cell surface marker, despite variation between donors, was comparable across all populations.

This finding suggests that, while fibronectin influences the proliferation rate of MSCs, it does not significantly alter the expression of key MSC cell surface markers. It underscores the utility of fibronectin in enhancing the growth rate of MSCs without altering their stem cell characteristics, which is crucial for their identification and subsequent applications in regenerative medicine and tissue engineering. Despite the consistency in MSC cell surface markers across our selection and isolation processes, significant individual variations can still play a role in the

proportions of mesenchymal progenitors. Various factors contribute to the individual variability of stem cells, with aging being well-established as a factor influencing both the quantity and quality of stem cells (Sloan & Smith, 2007; Raoof et al., 2014b). It is important to note that our study did not include controls for the age or health status of individual donors, due to the irretrievable anonymization of the donors, and these unaccounted-for variables have the potential to impact the biological and regenerative characteristics of DPSCs in any isolate.

It is important to note that HADAN II cells from the 4th passage and HADAN III cells from the 10th passage underwent immunophenotyping for MSC markers. It has been reported that the cells from different passages may influence the expression of MSC markers. A study by Ghaneialvar *et al*. on murine MSCs revealed that MSCs at passage 3 exhibited the highest expression of MSC markers (CD29+, CD44+, CD105+, CD106+, and Sca-1+) (Ng, Mak, Popp, & Ng, 2020). However, by passage 7, there was a reduction in MSC markers, indicating a potential loss of the stem cell population after prolonged culturing. While cells from different donors require different numbers of passages to reach the stage where they can be utilized in experiments, future studies should consider a consistent number of passages when immunotyping MSC markers. The potential volatility of MSC marker expression in culture presents a possible challenge in considering the use of these cells in regenerative medicine/dentistry, where multiple passages will be required to bring cells to usable numbers.

The quantification results of osteodifferentiation markers indicate that both the HADAN II fibronectin-adherent isolate and non-adherent isolates exhibited decreased SPP1 mRNA expression and increased BGLAP mRNA expression after 7 days of osteogenic differentiation media treatment. This implies reduced osteopontin (OPN) expression levels and heightened osteocalcin (OCN) expression levels in HADAN II. OPN is recognized as an early marker of

mineralization pathways, while OCN is acknowledged as a later marker in the osteogenic process (Zohar, Cheifetz, McCulloch, & Sodek, 1998) (Tsao et al., 2017). OPN is a multifunctional ECM protein reported to stimulate cell-cell adhesion, facilitate cell-ECM communication, and promote cell migration (Faccio et al., 1998) (Staniowski, Zawadzka-Knefel, & Skośkiewicz-Malinowska, 2021). The study by Zohar et al. demonstrated that OPN is associated with cell migration and is expressed early in mesenchymal cell differentiation (Zohar, Cheifetz, McCulloch, & Sodek, 1998). This can be attributed to its role in fulfilling the requirements during the early differentiation phase. It is also noted that that OPN can inhibit both growth and differentiation indicating that OPN is a potent negative regulator for the development of osteoblasts. Moreover, findings from the study by Huang *et al*. revealed that OPN negatively regulates preosteoblast development by impeding both proliferation and osteogenic differentiation. Elevated OPN expression led to decreases in the expression of osteocalcin and bone sialoproteins, while a decrease in OPN levels resulted in the opposite effect. (Huang, Weibiao et al., 2004). *In vivo*, during mandibular bone healing in rats, it has also been demonstrated that OPN is expressed early in healing, subsequently diminishing as bone healing progresses and OC expression increases (Colombo et al., 2011). Based on this information, the observed results in the HADAN II control/treatment group, indicating decreased expression of OPN and increased expression of OCN, suggest that HADAN II is in the later stages of osteoblast differentiation after 7 days in the osteogenic medium.

Both isolates yielded consistent results, given that they are dental pulp progenitor cells from the same donors. However, the osteocalcin markers in fibronectin-adherent isolates exhibited an 800% increase, whereas there was only a 50% increase in non-fibronectin-adherent cells when exposed to osteoinductive differentiating media. This suggests that the fibronectin isolated dental

pulp progenitor cells in HADAN II underwent a more substantial degree of differentiation compared to their NA counterparts.

Both the HADAN III fibronectin isolated populations and NA isolates displayed elevated SPP1 mRNA expression and no significant difference in BGLAP mRNA expression after 7 days of osteogenic differentiation media treatment. This suggests that HADAN III isolates were in the early stages of differentiation at the 7-day treatment mark. While both isolates produced consistent results, the fibronectin-adherent isolate exhibited a 164% increase compared to the untreated control, whereas the non-fibronectin isolate showed an 86% increase. Taken together these findings suggest that fibronectin selection yielded a population of cells with a somewhat greater capacity for osteodifferentiation, despite potential donor variation in the rate at which differentiation occurs.

In the process of osteogenic differentiation in BM-MSCs, Runx2 is considered a crucial osteogenic transcription factor and serves as an early osteogenic differentiation marker (Xu, Li, Hou, & Fang, 2015). Nevertheless, our findings revealed no discernible differences in the expression of RUNX2, across both types of isolates and donors, except in the case of HADAN III explant, which showed 35% increased expression in Osteo treatment group.

The data suggests that DPSCs in HADAN III may be less differentiated than those in HADAN II after 7 days of incubation in the osteogenic medium. Notably, HADAN II cells from the 4th passage and HADAN III cells from the 10th passage underwent differentiation. This observation aligns with previous studies indicating that pre-osteoblastic cells at higher passage numbers exhibit weakened osteogenic capacity and elevated OPN mRNA expression (Huang, Weibiao et al., 2004). Huang *et al*. also proposed that the capability of progenitor cells to

proliferate and differentiate significantly diminishes after a finite period. It is plausible that HADAN III cells, possibly owing to their later passages, exhibited not only diminished osteogenic capacity but also a reduction in MSC markers. According to the literature, the optimal time to study osteogenic capacity and express the most MSC cell surface markers, or exhibit a more stem cell-like state, is during passages 4-5. A study by Bilic *et al*. revealed that in passages 0 and 1, MSCs exhibited weak and insignificant osteogenic differentiation, while by passage 5, MSCs displayed notable osteogenic capacity *in vivo* (Bilic, Zeisberger, Mallik, Zimmermann, & Zisch, 2008; Diao, Ma, Cui, & Zhong, 2009).

Distinct variations in osteogenic potentials were apparent between fibronectin-isolated and non-adherent counterparts, even among cells exhibiting a similar MSC phenotype. The fibronectin selection demonstrated differences in the expression of molecular markers without corresponding changes in cellular markers. This highlights the intrinsic heterogeneity within subpopulations of DPSCs, which are remarkably diverse. The reliance solely on phenotypic markers may prove insufficient for effectively distinguishing subpopulations with varying osteogenic capacities.

To facilitate more direct comparisons between donors, it would be critical to replicate this experiment using identical passages for diverse donors. Alternatively, repeating the experiment with cells from a single donor at various passages would serve to validate the findings to some extent, although the individual variations seen here call into question our ability to assume that isolates from one donor would behave like isolates from another. We have observed potential donor variations in proliferation capacity, the rate at which differentiation occurs, and the osteogenic potential. Therefore, a significant challenge in this study is the inherent individual

variations present in DPSCs from different donors. These traits pose a significant obstacle to the translational development of human DPSC-based therapies for clinical applications.

Chapter 5: Conclusion

While all isolates of dental pulp progenitor cells displayed similar proportions of mesenchymal stem cell markers, those selected through fibronectin exhibited an accelerated division rate and demonstrated potentially enhanced osteogenic potential across different donors. Our findings support fibronectin as a valuable selection tool capable of promoting MSC expansion and increased differentiation potential without compromising their stem cell characteristic. The use of fibronectin selection revealed changes in the expression of molecular markers without concurrent alterations in cellular markers. This emphasizes the inherent diversity within subpopulations of DPSCs. Relying solely on phenotypic markers may fall short of accurately distinguishing subpopulations with different osteogenic capacities. Nevertheless, it is essential to recognize the substantial individual variations in mesenchymal progenitor proportions, phenotype, and behavior. In addition, the inherent heterogeneity of human dental pulp stem cells (hDPSCs) presents a barrier to discerning the quality of stem cells solely through the examination of a single cellular marker. This challenge complicates the effective characterization and study of DPSCs. As a result, understanding these complex biological genotypes and phenotypes becomes imperative for future research endeavors. Emphasizing the need for control over variations is crucial when identifying markers that consistently and specifically delineate superior subtypes of DPSCs within mixed populations.

Appendix

Institutional Review Board Approval Notice

Biomedical - Full Committee Review

Approval Notice

DATE: June 30, 2022

TO: John Colombo
FROM: Biomedical

PROTOCOL TITLE: UNLV-2022-28 Isolation of Dental Pulp Progenitor Cells From Extracted Human Teeth
SUBMISSION TYPE: Initial

ACTION: Approved
APPROVAL DATE: June 30, 2022
EXPIRATION DATE: June 29, 2023
REVIEW TYPE: FULL COMMITTEE REVIEW

Thank you for submission of materials for this proposal. The Biomedical IRB has approved your study. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission. Only copies of the most recently submitted materials may be used.

Waiver of Informed Consent has been approved for this study.

PLEASE NOTE:

1. **An online Initial Submission form will need to be submitted for any future research using the stored data that is different from the current proposal.**

Should there be any change to the study, it will be necessary to submit a **Modification** for review. No changes may be made to the existing study until modifications have been approved/acknowledged.

All unanticipated problems involving risk to subjects or others, and/or serious and unexpected adverse events must be reported promptly to this office. All FDA and sponsor reporting requirements must also be followed where applicable.

Any non-compliance issues or complaints regarding this protocol must be reported promptly to this office.

All approvals from appropriate UNLV offices regarding this research must be obtained prior to initiation of this study (e.g., IBC, COI, Export Control, OSP, Radiation Safety, Clinical Trials Office, etc.).

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your study title and study ID in all correspondence.

Biomedical - Expedited Review

Continuing Review Approved

DATE: June 21, 2023

TO: John Colombo
FROM: Biomedical

PROTOCOL TITLE: UNLV-2022-28 Isolation of Dental Pulp Progenitor Cells From Extracted Human Teeth
SUBMISSION TYPE: Renewal

ACTION: Approved
APPROVAL DATE: June 20, 2023
NEXT REPORT DUE: December 31, 2999
REVIEW TYPE: EXPEDITED REVIEW

Thank you for submission of renewal/continuing review/progress report materials for this protocol. The Biomedical IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a protocol design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

PLEASE NOTE:

1. **Approved under Expedited Category 9, "*Continuing review of research, not conducted under an investigational new drug application or investigational device exemption where categories two (2) through eight (8) do not apply but the IRB has determined and documented at a convened meeting that the research involves no greater than minimal risk and no additional risks have been identified.*"**
2. **Affirmed Subpart D 45CFR46.404, "*Research not involving greater than minimal risk*" is applicable to this study. Also, confirmed that waiver of informed consent meets criteria 45CFR46.116(f)(3).**

Only copies of the most recently submitted and approved/acknowledged Informed Consent materials may be used when obtaining consent.

Should there be any change to the protocol, it will be necessary to submit a **Modification** for review. No changes may be made to the existing protocol until modifications have been approved/acknowledged.

All **unanticipated problems** involving risk to subjects or others, and/or **serious and unexpected adverse events** must be reported promptly to this office. Please use the appropriate reporting forms for this procedure. All FDA and sponsor reporting requirements must also be followed where applicable.

Any **non-compliance** issues or **complaints** regarding this protocol must be reported promptly to this office.

This study is still determined to be minimal risk.

Please remember that all approvals regarding this research must be sought prior to initiation of this study (e.g., IBC, COI, Export Control, OSP, Radiation Safety, Clinical Trials Office, etc.).

If you have questions, please contact the Office of Research Integrity - Human Subjects at IRB@unlv.edu or call 702-895-2794. Please include your protocol title and protocol ID in all correspondence.

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Effects of relative centrifugation force on L-PRF: An in vivo submandibular boney defect regeneration study. Tovar N, Benalcázar Jalkh EB, Ramalho IS, Rodriguez Colon R, Kim H, Bonfante EA, Torroni A, Coelho PG, Witek J Biomed Mater Res B Appl Biomater. 2021 Dec;109(12):2237-2245. doi: 10.1002/jbm.b.34885. Epub 2021 Jun 3. PMID: 34080775.

Bone regeneration at extraction sockets filled with leukocyte-platelet-rich fibrin: An experimental pre-clinical study. Park, G, Jalkh, EBB, Boczar, D, Bergamo, ETP, Kim, H, Kurgansky, G, Torroni, A, Gil, LF, Bonfante, EA, Coelho, PG & Witek, L. Medicina Oral, Patologia Oral y Cirugia Bucal. 2022 Sep;27(5):e468-e475. DOI: 10.4317/medoral.25462. PMID: 35975804; PMCID: PMC9445612.

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