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Biological responses of dental pulp cells to surfaces modified by collagen 1 and fibronectin

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Abstract

Collagen 1 (COL1) and fibronectin (FN) are extracellular matrix proteins that contribute in cell activity and involve in regulating dental pulp cells (DPCs). The purpose of this study was to investigate the effect of COL1 and FN on the behavior of DPCs. Here, DPCs were grown under three different conditions: COL1 coating, FN coating, and control group without coating. The proliferation and differentiation of DPCs were investigated. DPCs in osteogenic media were able to differentiate into osteoblastic phenotype. The morphological analysis revealed no obvious difference on the shape of cells. Cells had spread well on both coated and noncoated culture plates with slightly more spreading in the coated plates after 24 hr. The MTT analysis did not demonstrate a significant difference at 1 and 3 hr among the groups, but interestingly, the analysis disclosed more cells on the coated plates after longer cultures, which indicated a higher proliferative capacity in response to COL1 and FN. RT-PCR, Western Blotting and mineralization assays did not reveal significant differences between the coated and noncoated surfaces in relation to osteogenic differential potential. Our data suggested that the surface coating of COL1 and FN were able to promote cellular proliferation and the osteogenic differentiation tendency of DPCs was also observed in vitro.

KEYWORDS

biological responses, collagen 1, dental pulp cells, fibronectin

1 | INTRODUCTION

Bone defects have been reported as one of the most common challenges in clinical dentistry (Kneser, Schaefer, Polykandriotis, & Horch, 2006). The periodontal diseases, tumors, trauma, and congenital anomalies always result in bone defects. The needs for engineered functional tissue have been increasing to overcome the obstacles faced by using different types of bone transplantations. Bone tissue engineering (BTE) relays on three main components: cells, scaffold as cell carrier and growth factors (Langer & Vacanti, 1993). Successful BTE requires osteogenic cells stimulating bone regeneration. Stem cells were reported to be appropriate for BTE (Derubeis & Cancedda, 2004; Dubey & Mequanint, 2011). Dental pulp stem cells (DPSCs) have the capacity to self-renew, and the ability to differentiate into multiple lineages: neurons, adipocyte, osteoblasts, and dentin-like structure with mineralized complex structure with dentinal tubules. DPSCs can also differentiate into fibrous-like structure containing blood vessels (Graziano, d'Aquino, Laino, & Papaccio, 2008). Therefore, DPSCs are suitable candidates for BTE.

The extracellular matrix (ECM) is a dynamic microenvironment whose components provide the structural support to the cells, in addition to the regulatory effects in term of controlling cellular signaling,

shape, migration, proliferation, and differentiation (Dominici et al., 2006; Hibi, Yamada, Ueda, & Endo, 2006; Zheng, Tian, Fan, & Xu, 2019). The main task for the scaffold in BTE is to create an environment that provides the cells with mechanical support required for 3D proliferation and growth. Surface properties of scaffolds as topography and bioactivity are crucial for determining cellular attachment, proliferation and differentiation (Garcia & Reyes, 2005). Instead of sensing the surface of the material directly, the cell senses it through a layer of adsorbed proteins whose conformation and distribution will determine integrin binding and the organization of focal adhesions. And this will further affects cell signaling and fate (Rico, Mnatsakanyan, Dalby, & Salmerón-Sánchez, 2016). It has been reported that the ECM proteins such as collagen 1 (COL1) and fibronectin (FN) molecules contribute to cellular activity (Garcia, Ducheyne, & Boettiger, 1998).

COL1 and FN are ECM proteins that contribute to cell activity and involve in regulating DPSCs. They play crucial roles in adhesion, migration, and osteogenic differentiation of stem cells (Alanazi et al., 2019; Kang et al., 2017; Mittag et al., 2012; Zheng et al., 2019). COL1 as collagen sponges is a natural material has the advantages of its biocompatibility, abundance, and its highly porous. In some cases, collagen sponges used in combination with a chondro/osteoinductive material to enhance more functional engineered tissue (Glowacki & Mizuno, 2008). FN plays an important role in attaching the cells to COL1 or proteoglycan substrates. It mediates the cell–cell interaction and cell-ECM by binding to different components of ECM. Its glycoprotein contains repeating units of amino acids which can enhance cell attachment to biomaterial surface with its central-binding domain RGD sequence(Anselme, 2000; Bhuvanesh et al., 2017; Singh, Carraher, & Schwarzbauer, 2010).

The COL1 and FN coating in the 2D might induce cellular changes and osteogenic differentiation even without the additional osteoinductive supplements. This might be used as a substrate to modify scaffold materials used in BTE. It has been reported that FN stimulates the expansion and osteogenic differentiation of stem cells (Andreeva, Leonova, Popenko, & Belyavsky, 2016; Kang et al., 2017; Zheng et al., 2019), but no previous experiments have investigated the effect of COL1 and FN coating on osteogenic differentiation of dental pulp cells (DPCs). This in vitro study was aimed to investigate the influence of COL1 and FN coating on attachment and proliferation of hDPCs and the effect of COL1 and FN on osteogenic differentiation of DPCs at gene and protein levels.

2 | MATERIALS AND METHODS

2.1 | Isolation of human DPCs

DPCs were harvested from normal mature third molars of healthy adult patients aged 16–20 at the Dental Clinic at the Faculty of Medicine and Dentistry, University of Bergen, Norway. Teeth were removed during routine operative procedures and collected under informed consent approved according to guidelines by the Ethics Committee at the Faculty of Medicine and Dentistry, University of Bergen, Norway. DPCs were isolated by enzyme dissociation method according to earlier reported protocol (Gronthos, Mankani, Brahim, Robey, & Shi, 2000). Dental pulp tissue was isolated by creating a groove (0.5–1 mm) with a sterile high-speed drill under copious irrigation with sterile saline solution along the cement–enamel junction to reveal the pulp chamber. Harvested tissue was sectioned into small pieces and then immersed in an enzymatic digestive solution of collagenase type 1 (4 mg/ml) (Sigma Aldrich) and dispase (2 mg/ml) for 1 hr at 37°C. The digested tissue was centrifuged at 1400 rpm for 10 min, and thereafter filtered through a 70 μm strainer. Cell suspension was cultured and expanded with Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, before incubation at 37°C in 5% CO_2 atmosphere. The culture medium was changed two times a week. When the cells reached ~75% confluence, they were either subcultured or stored in liquid nitrogen. Primary cells of DPCs from 2 to 4 passages were used in the experiments.

2.2 | Phenotypic characterization of DPCs

For phenotypic characterization of cells, flow-cytometric analysis was carried out using the following conjugated antibodies against cell surface molecules and their respective isotype controls were used: Stro-1 PerCP-Cy5.5 (Santa Cruz biotechnology, Inc, Texas), Fluorescein isothiocyanate (FITC) Mouse Anti-Human CD90 (BD Biosciences, New Jersey) and Allophycocyanin (APC) Conjugated Mouse Anti-human CD105 (Southern Biotech, Alabama). Concentrations of antibodies were titrated and optimized from a pilot study (data not shown). Cells derived dental pulp tissue at passage 1 was applied for this analysis. Briefly, 5×10 (Graziano et al., 2008) cells per tube were blocked by 20 μl blocking reagent (0.5% BSA in PBS) for 10 min at room temperature (RT). The cells were stained by incubation with conjugated antibodies in the dark for 1 hr, washed with 200 μl of PBS and then centrifuged at 250–300*g* for 5 min at 4°C. Supernatant was removed, and cells were resuspended in 300 μl of 4% paraformaldehyde and stored at 4°C to the time of analysis. Flow-cytometric analysis was performed using a BD Accuri C6 flow cytometer (BD Accuri Cytometers Inc, New Jersey). A total of 100,000 events were used for each sample. To confirm the osteogenic potential capacity, 1×10 (Dubey & Mequanint, 2011) cells/cm^2 in 6-well plate were cultured in either the growth media (control) or in the osteogenic induction media (OM) containing ascorbate, dexamethasone, and β-glycerophosphate (StemCell Technologies) for 21 days (Table 1).

TABLE 1 Osteoinductive medium supplements

50 μg/ml ascorbic acid
10^{-8} M dexamethasone
3.5 mM β-glycerophosphate

2.3 | Cell culture under different conditions

DPCs were cultured as mentioned above till they reached 70% confluence. Cells were then harvested using trypsin and prepared for experiments. The cells were seeded into three different conditions: (a) DPCs in noncoated well-plates (Nunc A/S, Denmark) with DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin. (b) DPCs with DMEM, 10 %FBS, 1% penicillin and streptomycin, in well-plates coated with COL1 (5 μg/cm^2) according to manufacture (Sigma Aldrich), COL1 was diluted in PBS and applied on the culture plates and kept inside laminar hood for overnight. (c) DPCs with DMEM, 10% FBS, 1% penicillin and streptomycin, in well plates coated with FN (5 μg/cm^2) according to manufacture (Sigma Aldrich). FN was diluted in PBS and applied on the culture plates and kept it for 45 min at room temperature. The remaining solution was removed.

2.4 | Morphological assay

DPCs which were cultured under the three different conditions (uncoated, COL1 coated and FN coated) were seeded in 24 well-plates (Nunc A/S, Denmark) at 20,000 cells/well with three replicates. At 1, 3, and 24 hr in culture plates, the cells were fixed in 10% formalin for 1–2 min and stained with 0.1% crystal violet (Sigma Aldrich) for 1–2 min. The cells were washed twice with distilled water, and left dry. Cellular morphology was observed using light microscope (Nikon ECLIPSE TS100, Nikon Instruments Inc, Japan).

2.5 | Cell attachment, proliferation, and viability by MTT assay

Cell attachment and proliferation rate of DPCs was determined by MTT 3-(4, 5-dimethyl-thiazoyl)-2, 5-diphenyl-tetrazolium bromide (Sigma Aldrich). DPCs were seeded and cultured under the three different conditions (uncoated, COL1 coated and FN coated). The seeding density was 10,000 cells/well in 96 well-plates and time points for 1hr, 3hr, 24 hr, 4 days, and 7 days with five replicas were investigated. After medium was removed, cells were washed with PBS and 100 μl of MTT working solution were added to each well and incubated for 3–4 hr. After incubation period, MTT solution was discarded and cells were fixed by 10% formalin buffer for 5 min, then washed with distilled water and covered with aluminum foil in dark for overnight. DMSO with 6.25% M NaOH were added to each well and left in shaker for 20 min at room temperature. The measurement by FLUOStar OPTIMA was set to read the absorbance at 570 nm.

2.6 | Real-time RT-PCR

DPCs were cultured under the three different conditions (uncoated, COL1 coated, and FN coated) and seeded at 100,000/well in 48 well-plates for 7 days with five replicas. RNA was isolated and purified using a Maxwell 16 LEV simplyRNA Purification Kit (Promega Corporation, Madison, WI). RNA purity and quantification were measured using Nanodrop spectrophotometer (ThermoScientific NanoDrop Technologies, Wilmington, DE). The reverse transcription reaction was performed using high capacity cDNA Archive Kit system (Applied Biosystems, Foster City, CA). The reaction contained 1,000 ng total RNA was dissolved in 50 μl nuclease-free water, mixed with RT buffer, random primers, dNTPs, and multiscripe RT. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed in 10-μl reaction with five replicas for each cDNA sample, with Taqman gene expression assays (Applied Biosystems, Foster City, CA). Under standard enzyme and cycling conditions, amplification was carried out on a 96-well StepOne Plus system (Applied Biosystems). Gene expressions of Alkaline phosphatase (ALP), Osteocalcin (OC), RUNX2, Bone morphogenic protein (BMP-2) genes were detected by a comparative *Ct* or $^{\Delta\Delta}Ct$ method by StepOne software. GAPDH served as house-keeping gene and was used for normalization of expression levels (Table 2).

TABLE 2 Genes used for real-time PCR

Gene	Assay ID
GAPDH	Hs03929097_g1
ALP	Hs01029144_m1
OC	Hs00609452_g1
BMP-2	Hs00154192_m1
Runx-2	Hs01047973_m1

2.7 | Alkaline phosphatase staining

DPCs were cultured in the three different conditions (uncoated, COL1 coated, and FN coated) and were seeded at 100,000/well in 48 well-plates for 7 days using three replicas. ALP was detected by BCIP/NBT as a substrate: stained cells were shown blue–violet when ALP was present. The substrate solution was prepared by dissolving one BCIP/NBT tablet (Sigma Aldrich) in 10 ml distilled water in the dark room and used within 2 hr. The 0.05% Tween 20 was added to Dulbecco's PBS without (Ca^{2+}/Mg^{2+}) to prepare the washing buffer. Cells were fixed using 10% formalin for 1 min then washed with a washing buffer. Carefully the washing buffer was removed and BCIP/NBT substrate solution was added to cover the cellular monolayer. Then, the cells were incubated at room temperature in dark for 5–10 min. The imaging for the cells was taken using light microscope (Nikon ECLIPSE TS100).

2.8 | Alizarin red staining

DPCs were cultured in the three different conditions (uncoated, COL1 coated, and FN coated) and were seeded at 100,000/well in 48 well-

plates for 21 days with three replicas. Calcium deposits were detected by slight reddish to orange stain. The ARS staining solution was prepared by dissolving 2 g Alizarin Red substrate in 100 ml distilled water, then adjustment for pH between 4.1 and 4.3 with 0.1% NH_4OH, and then filtering in dark room. Cells were fixed using 10% formalin for 1 min then rinsed with distilled water. ARS solution was added to cover the cellular monolayer and incubated at room temperature in dark room for 45 min. The working solution was removed and the cells were washed with distilled water. PBS was added and images were taken by light microscope.

2.9 | Western blot analysis

For isolation of total protein, cells were collected and washed twice with ice-cold PBS. Then 60 μl RIPA buffer (THERMO Scientific) supplemented with 1× protease inhibitor was added in each well and transferred onto ice for 10–15 min. Cell scraper was used to scrape off cells and pipetted cells around 10 times to form homogeneous lysate. The lysates were collected and then centrifuged at 16,000 rpm at 4°C for 20 min. This separated the total protein (supernatant) from the cellular debris (pellet). 20 μg of total protein samples by BCA assay were loaded on a Mini-PROTEAN TGX SDS-polyacrylamide gel (Bio-Rad) for electrophoresis, and transferred onto transfer membranes (BIO-RAD). Membranes were blocked at 4°C overnight with blocking solution (TBS containing 2% tween 20 and 5% nonfat dry milk). Membranes were incubated overnight at 4°C with the following antibodies: RUNX2 (1:1000: Santa Cruz, CA) and GAPDH (1:1000: Santa Cruz, CA). Secondary antibodies were mouse anti-rabbit IgG (BIO-RAD) diluted in TTBS and Precision protein StrepTactin (HRP) and incubated for 1 hr in room temperature. Immunoblot bands were visualized by Immun-Star chemiluminescence kit, mixture of 4 ml lumino/enhancer with 4 ml peroxidase solution and incubate for 5 min in dark.

3 | STATISTICS

All values in bar diagrams were presented as mean ± SD. Data were tested for homogeneity and normal distribution and analyzed by one-way ANOVA-Tukey test. Differences were considered significant when $p < .05$.

4 | RESULTS

4.1 | DPCs characterization and evaluation of osteogenic capacity

The analyses performed by flow cytometry showed that DPCs express the stromal-associated markers CD90 (24.4%), CD105 (98%) and mesenchymal precursor cell marker STRO-1 (7.3%) as shown in Figure 1. After 21 days, we found that DPCs under osteoinductive media were able to differentiate into osteogenic phenotype compared to the untreated cells (Figure 2).

4.2 | COL1 and FN coating change cell attachment and proliferation

A Crystal Violet staining was utilized to describe the morphology of the cells using a light microscope (Figure 3). At 1 hr of incubation, DPCs exhibit rounded shape or spherical. At 3 hr, the cells have become more flat and getting more cuboidal. At 24 hr incubation, the cells shown to be more elongated and exhibited fibroblast-like spindle shape. The morphological observation disclosed no obvious difference between the noncoated and coated with COL1 and FN after 1 and 3 hr. Normal cell spreading was observed in all groups at 24 hr.

Attachment assays were performed using MTT to measure the cellular viability and attachment after 1, 3, and 24 hr of incubation. The absorbance between coated plates and the corresponding control group was not statistically significant (Figure 4). And the proliferation was measured using MTT at 4 and 7 days. After 4 days, DPCs were proliferated better on the culture plates coated with COL1 and FN than on the noncoated plates (**$p < .05$ and ***$p < .01$, respectively). After 7 days, cellular proliferation on the coated plates were significantly higher than noncoated plates (***$p < .01$). However, after 4 and 7 days, there were no significant intergroup differences between COL1 and FN groups (Figure 5).

4.3 | ALP staining and alizarin red staining (ARS) shows that COL1 and FN coating affect the osteogenesis related pathway

ALP positive staining was detected in all the groups (uncoated, COL1 coated, and FN coated) after 7 days incubation. The blue areas which were obvious observed from the coated plates indicated osteogenic differentiation (Figure 6). The ARS analyses for calcium deposition (mineralization) of DPCs revealed slight weak staining in all groups after 21 days. Although more mineralization areas could be observed in coated plates compared to noncoated (Figure 7), further quantified image analysis will be needed to get statically results for both ALP and ARS staining.

4.4 | COL1 and FN coating affect the osteogenesis related gene expression and protein production

For ALP, RT-PCR revealed that there was higher mRNA expression in both COL1 and FN groups than control group at day 7, however, with no significant difference between the coated groups. The mRNA expression of RUNX2, OC, and BMP-2 was not statistically significant between the coated and uncoated groups (Figure 8). The results from Western Blot revealed that DPCs grown on the various culture plates

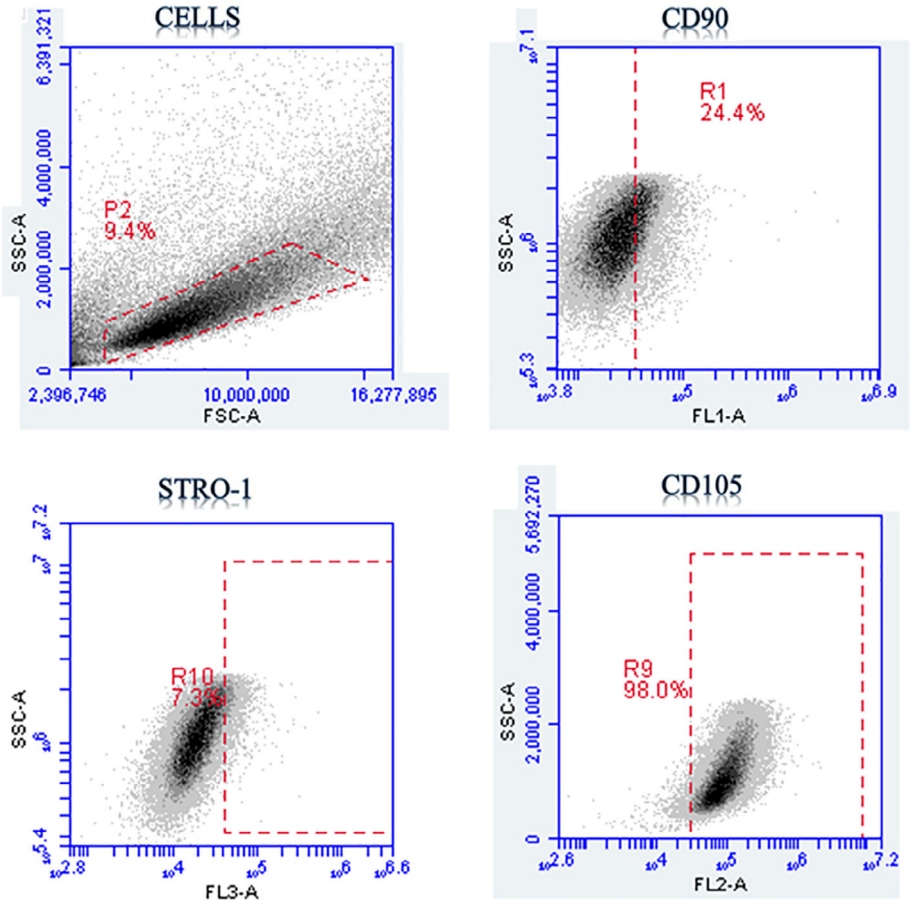

FIGURE 1 The human DPCs showed high expression for surface markers CD105, but low expression for CD24, CD90. Stro-1 was moderately expressed

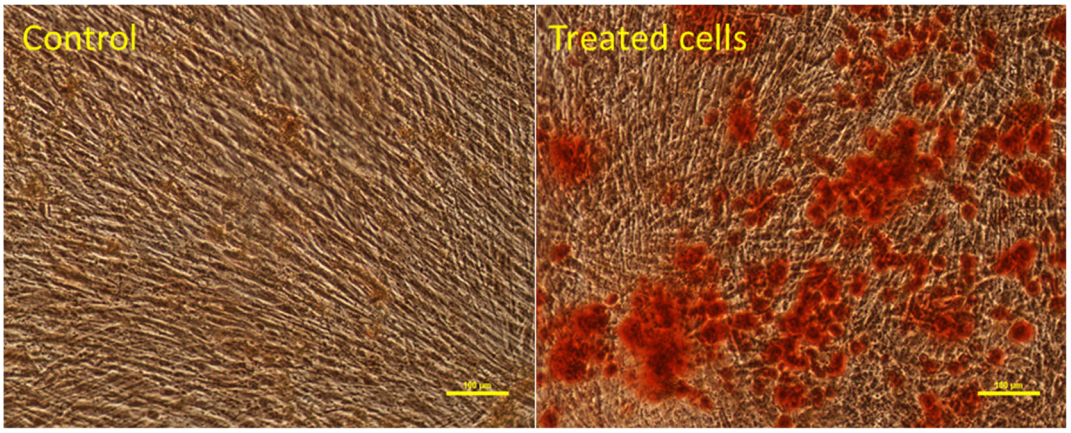

FIGURE 2 Alizarin red staining at day 21. More calcium nodules formation (red staining) was observed in the OM treated DPCs than in the control group (×10 magnification, scale bar = 100 μm)

expressed RUNX2 after 7 days and GAPDH was used as an endogenous control (Figure 9).

5 | DISCUSSION

In the current study, our characterization results using the flow cytometry showed that hDPCs expressed positively the following mesenchymal surface markers (STRO-1:7.3%, CD90: 24.4%, and CD105: 98%). Furthermore, the DPCs formed calcium nodules indicating osteogenic potential after 21 days incubation. The data revealed no obvious differences in cell morphology between the coated and noncoated plates at 1 and 3 hr. However, the cells showed more spreading in coated plates compared to control at 24 hr. Regarding cell attachment, the findings demonstrated no significant difference on cell attachment between the coated plates and noncoated at 1, 3, and 24 hr, although there was a tendency of higher cell attachment on the coated plates. In this study, the data showed that cells proliferated significantly higher

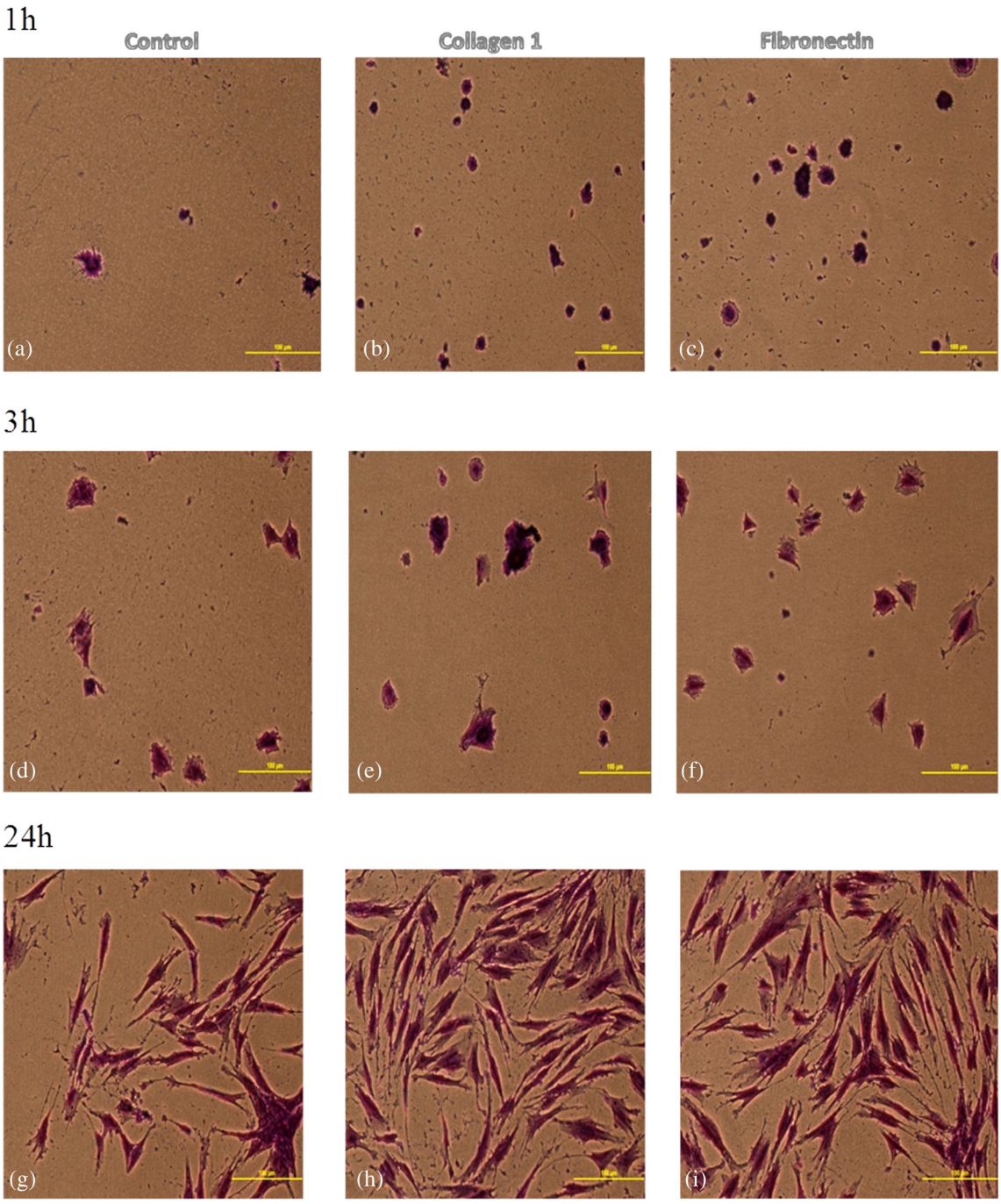

FIGURE 3 DPCs stained with crystal violet and morpohology was analyzed at three time points: 1, 3, and 24 hr. (a) DPCs control group at 1 hr. (b) DPCs, COL1 coated plates at 1 hr. (c) DPCs, FN coated plates at 1 hr. (d) DPCs control group at 3 hr. (e) DPCs, COL1 coated plates at 3 hr. (f) DPCs, FN coated plates at 3 hr. (g) DPCs control group at 24 hr. (h) DPCs, COL1 coated plates at 24 hr. (i) DPCs, FN coated plates at 24 hr (×10 magnification, scale bar = 100 μm)

on the coated plates compared to the control group after 4 and 7 days. Within the limitation of seeding cells using normal medium without osteoinductive medium, our findings suggested that DPCs on the coated plates with COL1 and FN did not reach the terminal state of differentiation which is essential for mineralization.

In the recent years, research has focused on the improvement of scaffolds, in order to be used as bone grafting substitutes (Al-Munajjed, Gleeson, & O'Brien, 2008). Like other porous scaffolds, polymer scaffolds are only osteoconductive which means lack osteoinductive properties (Liao, Guo, Nelson, Kasper, & Mikos, 2010). Incorporation of osteoinductive materials such as peptides, on polymer scaffolds might help to provide osteogenesis-stimulating signals for stem cells. This study proposed to test the influence of modifying biomaterials by COL1 and FN on the initial responses of DPCs.

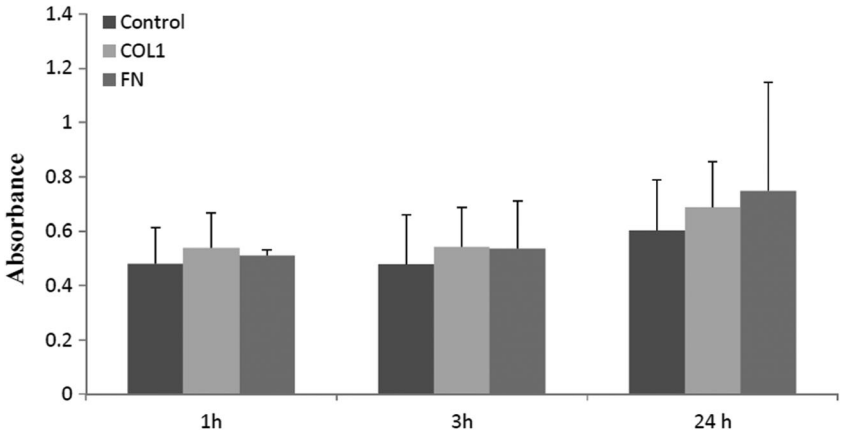

FIGURE 4 Evaluation of cell attachment using MTT at three time points 1, 3, and 24 hr for DPCs under different treatment conditions. COL1, collagen type-1; FN, fibronectin

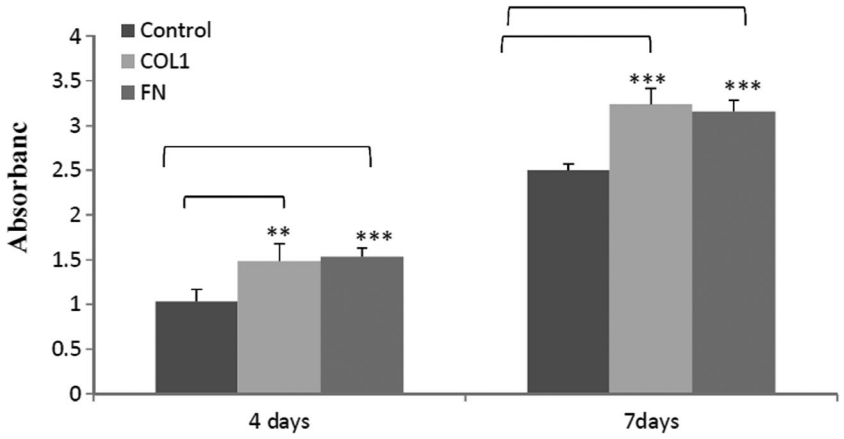

FIGURE 5 MTT measured cell proliferation of DPCs under the three culturing conditions, tested at 4 and 7 days, (**$p < .05$ and ***$p < .01$, respectively indicating significant difference). COL1, collagen type-1; FN, fibronectin

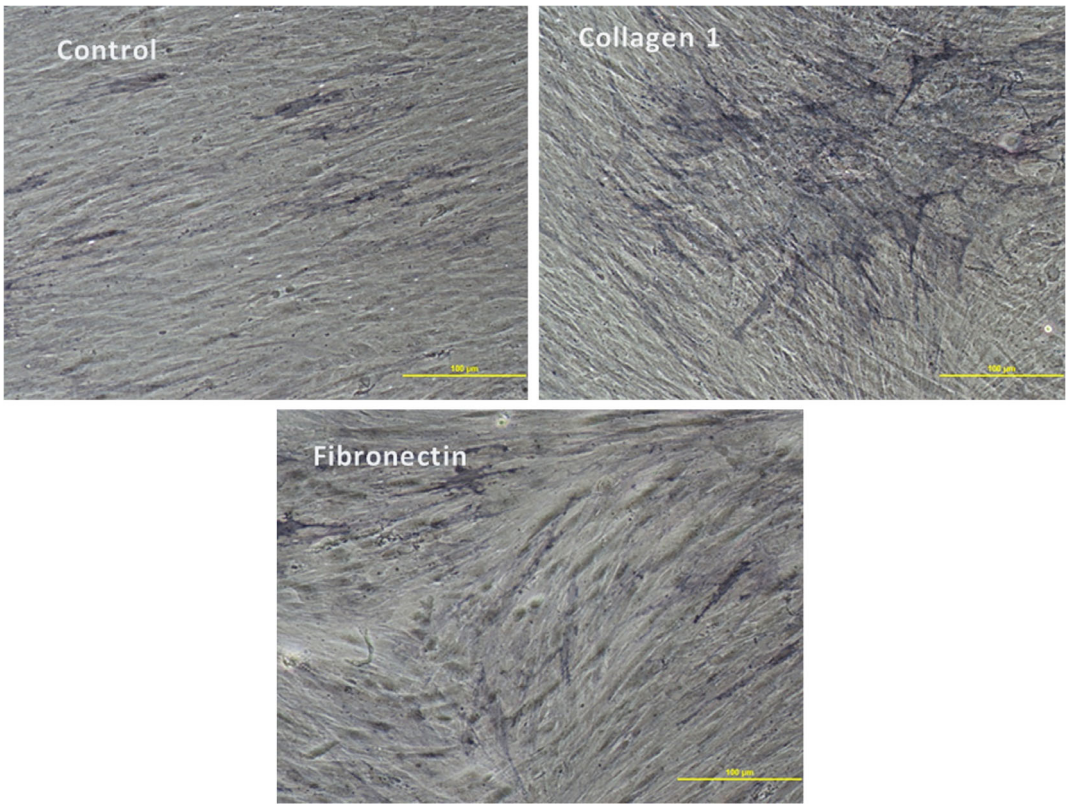

FIGURE 6 ALP staining for osteogenic differentiation potential at day 7. Control group, COL1 coating and FN coating, (×10 magnification, Scale bar = 100 μm)

Until now, no marker has been used to specifically identify the stem cells from dental pulp tissue. However, the flow cytometric results obtained from the present study showed that human dental pulp cells were positive for mesenchymal antigens (STRO-1, CD90, and CD105). There were approximately 7.3% STRO-1-positive cells in the isolated DPCs. In previous study, it has been demonstrated lower level expression of STRO-1 (5%) by dental pulp cell (Zhang et al., 2005). Another previous experiment verified that STRO-1 antigen is considered an early marker of mesenchymal stem cells (MSCs) and extremely important in selecting DPSCs (Yang et al., 2007). The finding in this study documented an expression of CD 90 (24.4%) (Known as Thy1) and CD 105 (98%) (Known as endoglin). According to previous studies, it has been confirmed that CD90 and CD105 are expressed on MSCs(Dominici et al., 2006).

It has been demonstrated that DPSCs cultured in osteoinductive medium were able to differentiate into osteoblast-like cells producing mineralized matrix and expressing typical osteoblastic markers (Mori et al., 2011). In our study, DPCs stained with Alizarin Red staining showed calcium deposit after 21 days incubation with osteoinductive materials, which considered a reliable phenomenon for bone differentiation. It has been proved that DPCs still exhibit capacity for nodule formation after several passages in vitro (Laino et al., 2005). These

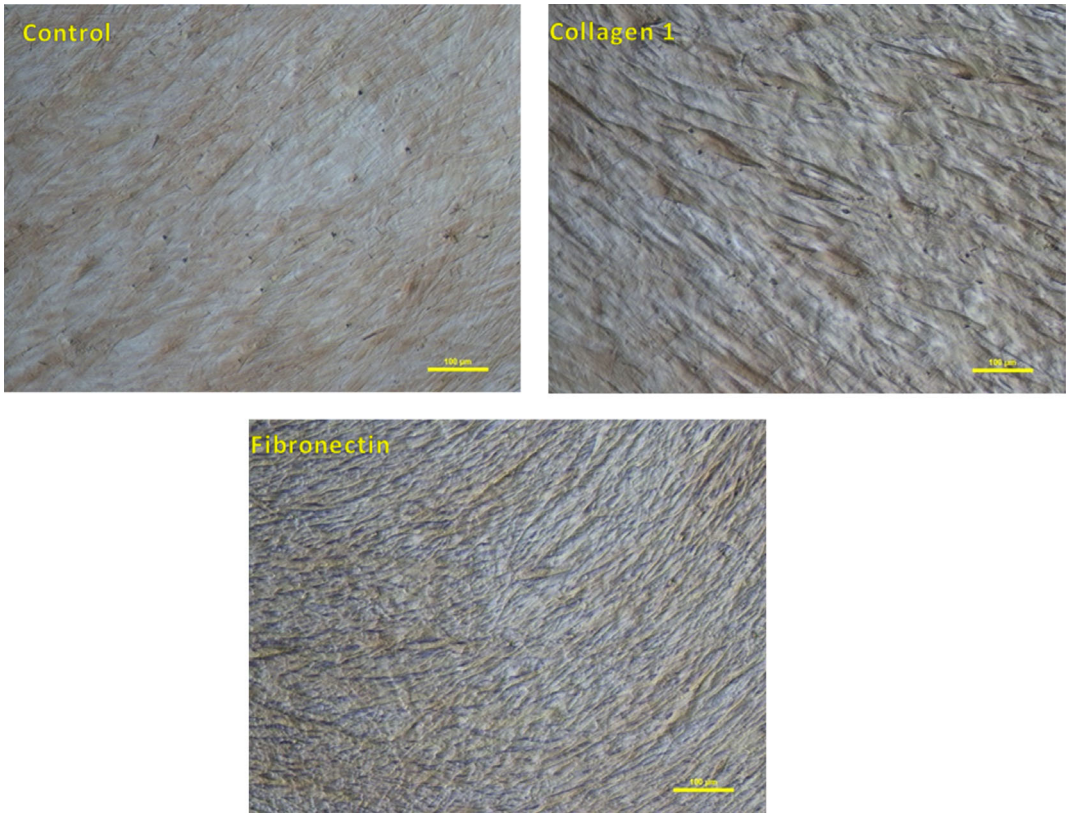

FIGURE 7 ARS for calcium deposition (mineralization) after 21 days. Control group, COL1 coating and FN coating (scale bar = 100 μm)

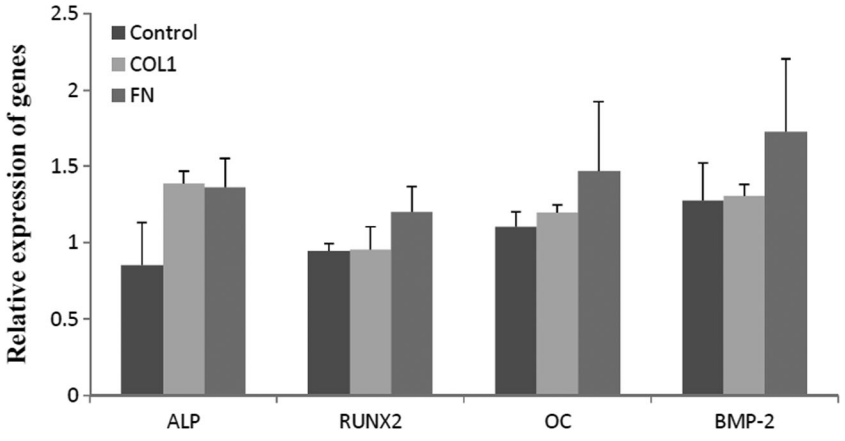

FIGURE 8 RT-PCR results after 7 days incubation of DPCs in the three conditions. No significant difference among the groups could be detected for the following genes: ALP, RUNX2, OC, and BMP-2. ALP, alkaline phosphatase; BMP-2, bone morphogenic protein 2; COL1, collagen type-1; FN, fibronectin; OC, osteocalcin; RUNX2, Runt related transcription factor 2

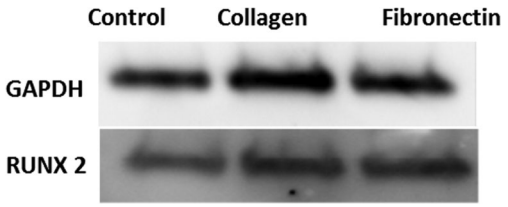

FIGURE 9 Western blot after 7 days incubation under the three different culture conditions revealed synthesis of RUNX2 protein in the three groups. GAPDH, glyceraldehyde-phosphate dehydrogenase; RUNX2, Runt related transcription factor 2

obtained data from our study are in agreement with previous studies showing the isolated DPCs are mesenchymal stem-like since they expressed the relevant surface markers and have osteogenic potential.

The attachment assays with 24 hr of culture revealed no significant differences between the cells grown the various culture substrates investigated in the present study (coated and noncoated culture plates). Previous Studies showed that cell membrane proteins, such as integrin, can bind to ECM proteins including FN and COL1 (Hidalgo-Bastida & Cartmell, 2010). Another study reported that FN can be used to preferentially isolate progenitor stem cell populations from dental pulp (Waddington, Youde, Lee, & Sloan, 2009). In our investigation, DPCs were cultured in normal culture plates which were designed to promote cellular attachment and have been reported to be effective for rapid expansion of MSCs. Therefore, the profound effect of the surfaces of culture plates used in the study might mask the effect of the COL1 and FN coating on cellular attachment. Another possibility might be raised that COL 1 and FN used to coat the culture plates in this study might exhibit different behavior and interaction than those found in physiological environment. Even though the number of attached cells did not show significant changes there might be variations in cell attachment of specific cell types, which might have represented in different percentage of the total number of cells population. Actually, the DPCs contained different cells types with different morphologies that might have different affinities to the various ECM proteins such as FN and COL1 used in this study (Gronthos et al., 2000).

It has been illustrated that cell attachment to FN coated-dishes can enhance stem cells proliferation and differentiation (Ogura et al., 2004). The cells bind to the ECM by integrins, and this binding can activate signaling networks by nucleating signaling proteins on the cytoplasmic side of the plasma membrane (Chastain, Kundu, Dhar, Calvert, & Putnam, 2006). In the present study, our results showed cells proliferated more on the coated surfaces by COL1 and FN compared to noncoated surfaces after 4 and 7 days. This can be possibly explained either by the direct contact with ECM which enhance cell signaling transduction, or by the influence of COL1 and FN in enhancing mitogenesis (Kundu & Putnam, 2006; Larivière, Rouleau, Picard, & Beaulieu, 2003; Sudhir, Wilson, Chatterjee, & Ives, 1993).

The findings obtained from the RT-PCR showed that there was expression of the various gene markers (ALP, OC, RUNX2, and BMP-2) indicating the osteogenic differentiation potential of the cells grown on the three different types of surfaces, but the difference was not statistically significant. These findings were also confirmed by the results from ALP staining which showed positive staining but without obvious difference between the groups. In the present study, the differentiation assays performed on cells grown on the coated and noncoated substrates were performed in culture environment using osteogenic-free medium. The data suggested that DPCs grown on the coated and noncoated surfaces in osteogenic-free medium did not reach the terminal state of differentiation. The study design has precluded using high cell density or longer incubation time.

In contrast to our study, previous reports revealed that ECM proteins enhance mineralization of MSCs from bone marrow, even in the absence of osteoinductive signals (Salasznyk, Williams, Boskey, Batorsky, & Plopper, 2004). This was possibly due to the difference between the two types of cells used. Furthermore, BMSCs were reported to have higher osteogenic, chondrogenic, and adipogenic potential compared to DPSCs (Gronthos et al., 2000; Huang, Gronthos, & Shi, 2009; Sonoyama et al., 2008). The enhancement for differentiation could be due to mimic physiological environment necessary such as mechanical strain and oxygen tension for signal transduction, or by other word it could possibly increase the sensitivity toward signaling molecules (He, Genetos, Yellowley, & Leach, 2010; Karbanova, Soukup, Suchanek, & Mokry, 2010; Zhang, Wan, & Wang, 2019).

The enhancement of differentiation could be provided by ECM signal molecules involved in the chemical groups present on the surface of ECM. It has been documented that these reactive surface groups, such as methyl and carboxyl can affect the transition of cells from mitotic status to differentiation (Curran, Chen, & Hunt, 2006). Particularly, FN integrated with scaffold reported to enhance stem cells differentiation in vitro (Sogo et al., 2007). COL1 also has reported to enhance osteogenic differentiation of MSCs (Salasznyk et al., 2004).

The mechanism of osteogenic differentiation has been described by activating mitogen-activated protein kinase (MAPK) cascade. One of the most important kinase that has been investigated in MAPK cascade is the activation of extracellular signal-regulated kinase (ERK) by growth molecule. ERK is regulated by Ras-dependent signaling pathway, which is vital in regulation of cell growth, migration, and differentiation. In particular, the adhesion of cells to ECM components for example COL1 and FN can enhance activation of ERK (Chen, Kinch, Lin, Burridge, & Juliano, 1994; Zhu & Assoian, 1995). FN has been shown to increase specificity for $\alpha 5\beta 1$ which significantly stimulate osteogenic differentiation of MSCs. It also has been shown how integrin-dependent cellular interactions with the ECM components can be engineered to control stem cell behavior (Martino et al., 2009).

In the current study, we believed that the heterogeneous nature of the cell population had strong contribution in cell behavior and activity. It could be useful in the future study to characterize the cell types found in the cell population and its proportion. Elucidating the proportion of each cell type can give better insight in their contribution in the whole cell population behavior.

The influence of the two ECM proteins (COL1 and FN) alone in coated plates could be different from the influence of the same

proteins in natural ECM. Understanding the architecture model of ECM components in nature could emphasize the structural building of each molecule. This structure might be different from the isolated structure to the same molecule. The osteogenic inductive materials are different in nature and effect intensity. This means difference between those materials can be time related. COL1 and FN used in this study might have a different role but in long term.

6 | CONCLUSIONS

The expression of stem cell marker indicated that DPCs are mesenchymal stem-like cell phenotype. The DPCs used in this study were shown to have the capacity to differentiate into osteoblastic linage. These data indicate that in vitro culture of DPCs in osteoinductive materials environment provides comparable properties and stem cell-like characteristics. Our data revealed that COL1 and FN coating did not significantly enhance cell attachment throughout the cultivation periods compared to the untreated group under the currently used experimental condition. The modification of biomaterial substrates with COL1 and FN resulted in increased DPCs proliferation. In the absence of osteogenic supplements, COL1 and FN did not significantly promote the differentiation of DPCs.

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