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

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Summary

Collagen 1 and fibronectin are extracellular matrix proteins that contribute in cell activity and involve in regulating dental pulp stem cells. The purpose of this *in vitro* study was to investigate the effect of collagen 1 and fibronectin on the behavior of dental pulp stem cells.

Material and Methods: Polystyrene multiwell culture plates were coated with collagen 1 and fibronectin according to the manufactures. Dental pulp stem cells (DPSCs) were isolated from third molar teeth of adult patients undergoing routine surgery at the Dental Clinic in the department of oral surgery at the Faculty of Medicine and Dentistry, University of Bergen, Norway. The cells were cultured and characterized using the following surface markers:
Stro-1, CD90 and CD105. Characterization and analysis was performed using a flow cytometer. DPSCs were grown under three different conditions: collagen 1 coating, fibronectin coating and control group without coating. Cellular morphology and spreading were investigated by Crystal Violet staining under a normal light microscope at 1, 3 and 24 h. Cell attachment at 1, 3 and 24 h, and cell proliferation at 4 and 7 days were assessed using 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium assay (MTT) assay.

Furthermore, the mRNA expressions of alkaline phosphatase (ALP), osteocalcin (OC), runt-related transcription factor 2 (RUNX2) and bone morphogenic protein-2 (BMP-2) were determined after 7 days using real-time RT-PCR. The production RUNX2 by the cells cultured on the various plates was determined by Western Blot analysis. Furthermore, cell mineralization was assessed by ALP staining at day 7 and by Alizarin Red staining after 21 days of cultures.

Results: In general, immunophenotypical characterization showed that, DPSCs expressed CD90 (24.4 %), CD105 (98 %) and STRO-1(7.3 %). Furthermore, DPSCs in osteoinductive 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 non-coated culture plates with slight more spreading in the coated plates after 24 h. The MTT analysis did not demonstrate a significant difference at early time points among the groups but interestingly, the analysis disclosed more cells on the coated plates after longer cultures indicating a higher proliferative capacity in response to collagen 1 and fibronectin.
RT-PCR, Western Blotting and mineralization assays did no revealed significant differences between the coated and non-coated surfaces in relation to osteogenic differential potential.

Conclusion: In the present *in vitro* study, the isolated dental pulp cells expressed cell surface markers comparable to the expression from bone marrow mesenchymal stem cells and

demonstrated osteogenic differentiation potential. Our data revealed that fibronectin and collagen 1 were able to promote cellular proliferation.

Abbreviations

ALP	Alkaline phosphatase
BMP-2	Bone morphogenic protein 2
cDNA	Complementary deoxy ribonucleic acid
COL1	Collagen type-1
DMEM	Dulbecco's modified Eagle's medium
DMP-1	Dentine Matrix Protein 1
DMSO	Dimethyl sulfoxide
DPSC	Dental pulp stem cell
ECM	Extracellular matrix
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FN	Fibronectin
MTT	3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium assay
MSC	Mesenchymal stem cells
OC	Osteocalcin
RT-PCR	Reverse transcription polymerase Chain reaction
RNA	Ribo nucleic acid
RUNX2	Runt related transcription factor 2

1. Introduction

1.1 Bone tissue engineering

1.1.1 The concept of tissue engineering

Bone defects have been reported as one of the most common challenges in clinical dentistry [1]. The periodontal diseases, tumors, trauma and congenital anomalies are the main causes of bone defects. Substantial efforts have been made using different types of bone transplantations as remedies treatment of bone defects among these are autogenic graft, allogenic graft and xenograft**.** The autogenic grafts: is the bone harvested from one site to another in the same individual. These grafts have good mechanical and biological function [2]. However, the availability of autografts is limited and often associated with donor-site morbidity [3]. The other type is the allograft: bone transplanted from one individual to another with the same species, but there is always a risk for diseases transmission, immune rejection and the lack of osteogenesis which remain the main disadvantages of using allograft [4]. The third type is xenograft in which the bone transplanted from one spices to another, this type has the same limitations of allograft such as immune rejection and the risk for diseases transmission [5]. However, allografts and xenografts are still considered as clinical alternatives to autograft [6, 7].

The needs for engineered functional tissue become necessary to overcome the obstacles faced by using different types of bone transplantations. The concept of tissue engineering was firstly introduced in 1993 by Langer and Vacanti, relays on three main components (1) cells (2) cell carrier: scaffold and (3) growth factors [8].

1.1.2 Scaffolds in bone tissue engineering

The main task for the scaffold in tissue engineering is to create an environment that provides the cells with mechanical support required for 3D proliferation and growth, highly porous to provide flow transport of the necessary growth factors and nutrients. Scaffolds martial should be biocompatible, and it should be transplanted to host without provoking any immune reaction. In addition scaffold material should be bioresorbable, and the degradation rate should be consistent to the growth rate of cells/tissue *in vitro* and *in vivo*.

The scaffold material should also be osteoconductive and if possible osteoinductive. Osteoconduction means the ability of osteoblasts to grow on the scaffold surface [9]. Osteoinduction is the ability for recruitment of oteoproginator –and stem cells and stimulation of these cells to differentiate into osteoblasts [9].

Surface properties of scaffolds as topography and bioactivity are crucial and important determining cellular attachment, proliferation and differentiation [10]. It has been reported that nano-particles can be used for surface biomodification demonstrating numerous advantages [11, 12]. Nano-particles can be used for coating biomaterial surfaces and improving the bioactivity of the material and the mechanical support without losing the chemical properties [12]. It has been reported that the extracellular matrix proteins such as COL1 and FN molecules contribute in cellular activity [13].

1.1.3 Cells for bone tissue engineering

Successful bone tissue engineering requires osteogenic cells stimulating bone regeneration. Stem cells were reported to be appropriate for this purpose [14, 15].

Types of Stem cells:

1. Embryonic Stem cells

These cells are pluripotent cells derived from the inner cell mass of the blastocytes in early embryo [16]. Embryonic stem cells have the ability for differentiating into tissue of all the three embryonic germ layers [17]. It has been reported that embryonic stem cells in combination with 3D structures have capacity to regenerate 3D vascularized human cardiac tissue, thus can be used in studying tissue replacement therapy [18]. Despite the fact that these properties have useful application on drug discovery and transplantation medicine, the ethical issues are still debatable [19, 20]. Besides, there is a risk for tumor formation since the control of the differentiation process is still a challenge [21].

2. Adult Stem Cells

These cells can be taken from all the three embryonic germ layers: ectoderm, mesoderm and endoderm. The main differences between adult stem cells and embryonic stem cells are their proliferation capacity and the differentiation ability is limited. However, adult stem cells are less oncogenic since the ability to transdifferentiation (differentiation from one embryonic line to another, for example mesenchymal stem cells to ectodermal) is limited [22].

In addition availability and the accessibility to be obtained from different tissues made adult stem cells as the first choice in research and for clinical application in bone tissue engineering [15]. It has been reported that tissue engineering osteogenic material contains platelet-rich plasma and mesenchymal stem cells were used in induction the osteogenic potential in bone augmentation procedures as a replacement for autologous bone grafts [23].

2.1 Mesenchymal stem cells

These cells are available and can be obtained in a large numbers since they can be taken from different tissues (bone marrow, muscle, adipose tissue and synovial membrane) and cultured *in*

vitro [24]. Furthermore, they have the ability to differentiate to osteoblasts, adipocyte and chondroblast *in vitro* [25]. These cells are currently been characterized by their adherent properties to plastic substrates when maintained under normal standard culture and they express the following surface markers: CD105, CD73 and CD90. These cells lack of the expression of CD45, CD34, CD14 or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules [26].

2.2 Dental Stem cells

Several types of stem cells can be isolated from extracted teeth. Dental pulp stem cells (DPSC) can be isolated using enzymatic digestion either from adult teeth [27] or from exfoliated human deciduous teeth (SHED) [28]. Those cells have the capacity to self-renewal, and the ability to differentiate to multiple lineages: neurons, adipocyte, osteoblasts and dentin like structure with mineralized complex structure with dentinal tubules. They can also differentiate to fibrous like structure containing blood vessels [29]. Another population can also be obtained from the apical papilla of root to human teeth (SCAP) [30]. Periodontal ligament (PDL) tissue is another source for mesenchymal stem cells (PDLSC) [31]. These cells have high regenerative ability and high rate of self-renewal after exposing to trauma, suggesting that the PDL can be a reliable source for stem cells (figure 1) [31, 32].

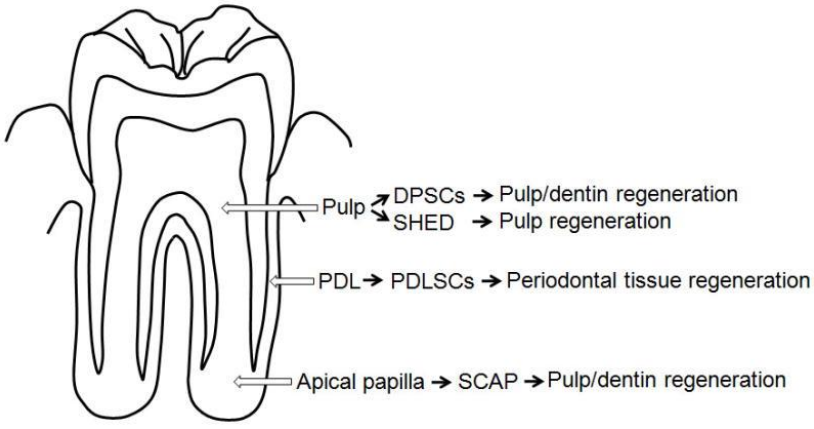

Figure 1. Sources of dental stem cells used in tissue engineering. The figure cited from Huang GT. (2011).

1.2 Human Dental Pulp as a source of stem cells

1.2.1 Normal physiology of the Pulp

Human dental pulp is a special organ developed during the sixth week of embryonic life. The ectoderm covers the stomodaeum and starts proliferation. This forms the dental lamina. The reciprocal interaction between the mesodermal layer and the ectodermal layer will give rise to a placode formation. This thick ectodermal structure will continue developing to tooth germ, while the neural crest will differentiate into dental papilla and dental follicle (dental organ). The dental pulp has an ectomesenchymal origin which emphasizes the plasticity and the multipotentail capability [33]. Dental pulp is resilient unique soft tissue of mesenchymal origin surrounded by dentin. This rigid chamber provides mechanical support in addition to the protection from microorganism in oral cavity. Odontoblasts and the undifferentiated stem cells (may differentiate to dentin-forming cells), are able to produce dentin during life, which enables the pulp to compensate the hard tissue defect like defect in dentin partially. Therefore its referred to the dentin-pulp complex [34].

1.2.2 Histology of dental pulp

Classical histological section of the pulp reveals 4 different zones:

(1) Odontoblastic zone: on the pulp periphery, adjacent to calcified dentin, lays columnar odontoblasts cells.

(2) Cell free zone of Weil: it lies centrally to odontoblasts. Plexus of capillaries and small nerve fibers run in this layer.

(3) Cell-rich zone: this zone contains fibroblasts, and undifferentiated cells which may preserve the population of odontoblasts by differentiation.

(4) Central pulp zone: characterized by the presence of vessels and nerves. The main cells are fibroblasts, and the principal extracellular elements are ground substance and collagen.

Types of pulp cells:

Odontoblasts: dentin forming cells.

Undifferentiated cells: they are multipotent cells that are capable of differentiation according to certain stimulus and on demand of the mature cells type. These cells have fibroblasts like shape, and they can produce little collagen. This is evidence that they are not mature fibroblasts. It has been revealed that there are cytoplasmic connections between these cells and the odontoblasts, by this connections cellular signaling can be performed in case of odontoblasts injury or death enhancing these cells to differentiate to odontoblasts like cells as needed [35].

Fibroblasts: the majority of the cells inside pulp are fibroblasts, they are spindle shape cells with ovoid nuclei, and they produce extracellular matrix components, like collagen [36].

Defense cells: Histocytes and Macrophages, Polymorphonuclear Leukocytes, Lymphocytes and Plasma Cells, Mast Cells [37].

1.2.3 Functions of dental pulp tissues

(1) Protection: pulp tissue protects the tooth from bacterial invasion through the dentinal tubules, this is due to the presence of fluid and odontoblastic processes, and those have positive charged hydrogel which prevents the bacteria to enter the pulp [38]. Moreover, antimicrobial agents can be found in the dentinal fluid [34]. Also the high molecular weight proteins found in the dentinal fluid such as fibrinogen, have the ability in reduction the dentin permeability by reducing the radius of dentinal tubules [39]. The cellular components of the pulp are also able to provoke immune defense reaction [37].

(2) Sensation: the pulp is a sensory tissue: it registers the different forms of stimuli such as thermal, trauma, etc. This pain sensation is considered as a part of the defense mechanism [40]. The proprioceptive receptors give the pulp also the capability to adjust the load on teeth during mastication [41].

(3) Dentin formation: Odontoblasts are the cells that are responsible for formation of dentin. The inner dental epithelium induces the ectomesenchymal cells to differentiate into odontoblasts in the dental papilla [42]. The process involves TGF-β growth factors family secreted by epithelial cells [43]. The odontoblasts have 4 different morphologies reflect the functionality, which are pre-odontoblasts, secretory, transitional and aged [44, 45]. With the pre-odontoblasts the cells prepare for protein synthesis intracellularly. In the secretory stage turned to active primary dentin production. The transitional phase characterized by degradation of some intracellular organelles and limiting the secretory phase activity. The last stage the cells turned to be small and with reduced secretory activity.

So the compensation for the lost cells during life needs progenitor cells which can differentiate to odontoblasts-like cells which play a role in dentinogenesis process [46]. After differentiation, odontoblasts start the secretion of unmineralized organic matrix of pre-dentin. The organic matrix of pre-dentin consists of COL1 and other ECM proteins.

Odontoblasts mineralize the pre-dentin by releasing vesicles near the basement membrane, within these vesicles crystal nucleation occurs. After that the crystals will rupture the membrane–bound vesicles and fuse with other crystals in ECM [47]. The mineralization at this stage may illustrate that the odontoblasts are unable to perform fully mineralization, at the level the odontoblasts are fully differentiated they are able to perform mineralization by secreting two matrix proteins which characterize dentin. Those are dentin phosphoprotein (DPP), and dentin sialoprotein (DSP) [48]. In parallel to the secretion of organic matrix, the odontoblasts

migrate to the pulp direction and leave their cytoplasmic extensions embedded in the newly formed dentin, a process which that will form dentinal tubules [49]. Secondary dentin formed by the same odontoblasts that formed the primary dentin, but the secretion rate is much slower. Tertiary dentin forms in response to stimuli. It is secreted from odontoblasts-like cells in response to stimulus that affects the odontoblasts responsible for primary and secondary dentin formation [46]. Variety of pulp cells populations may be involved in the process of tertiary dentin formation: undifferentiated mesenchymal stem cells found in the cell-rich layer may be considered a source for this progenitor cells. Other populations as perivascular cells (pericytes), and fibroblasts can also be considered as a source for progenitor cells [50]. The dental pulp stem cells (DPSCs) which are highly proliferative cells are also considered a source [51]. A recent analysis to the type of cells found in an area of dental tissue damage showed the presence of a variety of theses populations [52].

1.2.4 Proliferation and differentiation potential of dental pulp stem cells

DPSCs are able to differentiate to odontoblasts-like cells which are responsible for dentin formation and mineralization when exposed to EDTA [53]. Stem cells from other sources than dental source as bone marrow derived cells (BMSCs) can also exhibit differentiation to dental cells, for example can give rise to ameloblasts-like cells and express odontogenic genes [54]. Although stem cells from dental and bone marrow sources have dental regenerative capacity and express similar gene profile, DPSCs have higher proliferation rate [55]. The BMSCs can produce calcified nodules within the adherent cell layer and been shown to exhibit higher osteogenic, chondrogenic, and adipogenic potential in comparison with DPSCs [56]. However, DPSCs have higher neurogenic potential than BMSCs [30, 32, 51].

1.3 Extracellular Matrix Protein (ECM)

The ECM is connective tissue built up by proteins, polysaccharides and basement membrane produced by cells and organized in a meshwork [17]. The ECM 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 [23, 55]. The main contribution of a scaffold in tissue engineering is to provide the same environment provided by ECM [57].

The structure of eukaryotic cell composed of a complex network of protein filaments known as cytoskeleton. Three types of cytoskeletal filaments found in the cell: the actin filaments which determine the shape of cell's surface and necessary for the whole-cell locomotion. The second type is microtubules which determine the positions of membrane-enclosed organelles and direct intracellular transport. The third type is the intermediate filaments which provide mechanical strength and resistance to shear stress. Those connections are critical for the signaling pathway

and cellular transduction. ECM plays a significant role in determining the physical properties of the tissue [57].

The two major components of ECM are (1) glycosaminoglycan (GAG), or proteoglycan which is gel-like material fill up most of extracellular space in which the protein integrated. (2) Proteins: elastin, collagen, fibronectin, vitronectin and laminin. This unique structure gives the necessary strength and mechanical support at the same time allows the nutrients and signaling molecule to pass through. Each molecule has its own contribution to structural building. Collagen provides the network with strength, elastin provides the flexibility. The basement membrane separate connective tissue from epithelial tissue and act as a selective membrane or filter which controls diffusion of molecules between two tissues.

1.3.1 The role of ECM during cell adhesion, migration and proliferation

As mentioned previously, connections of cells are very important for the functionality of the tissue. Two forms of connections can be illustrated, cell-cell connection, and cell-ECM, both known as cell junctions. The process by which the cells connect to each other or to ECM is known as cell adhesion. There are three types of cell junctions: (1) Occluding junctions: they are selective permeable barriers which regulate the movement of nutrients and water molecules depending on chemical composition and size, charge and polarity. (2) Communicating junctions, gap junction: its special form composed of six connexin proteins create a channel-like structure connecting the cytoplasm of cells together allowing inorganic ions, metabolites and water soluble molecules to move directly. (3) Anchoring junction: composed of two types of protein, one intracellular, and the other is transmembrane adhesion protein. Anchoring junction containing of adherens junction (a continuous band of protein known as cadherins binds to actin filament) and desmosome (by which the same type of protein, cadherins, attached to intermediate filament). Anchoring junction by which the cells bind to ECM exhibits also two types: focal adhesion (protein called integrin binds ECM to actin filament inside the cell) and the other is hemidesmosome (integrin binds ECM to intermediate filament).

The actin filaments bounded by adherents junction forms trans-cellular network, with myosin motor protein, this network can contract and helping morphogenesis. Focal contacts play an important role in signaling pathway by connecting ECM to the cells. The signaling process can be performed by the help of a kinase known as focal adhesion kinase (FAK). ECM can regulate cell growth, morphology and proliferation using this FAK connection.

Another kinase that enhances the cell signaling is activating mitogen-activated protein kinase (MAPK) cascade. One of the most important kinases has been investigated in MAPK cascade is the activation of extracellular signal-regulated kinase (ERK). ERK is regulated by Ras-dependent signaling pathway, which is vital in regulation of cell growth, migration and differentiation. An example of how the ECM regulates the cell activity is that the cells can secrete extracellular proteolytic enzyme (protease) which are able to degrade the ECM components partially which enhance cell migration by facilitating cell movement.

1.3.2 Collagen type 1

Collagen is the most abundant protein in human, it comprises one third of total amount of protein in human body, and is the most prevalent protein in extracellular matrix (ECM). Collagens consist of a family of proteins (more than 28 molecular types have been identified) which have the same triple helical structure in a form of an extended rod (figures 2) [58]. This monomer structure is characterized by a sequence of amino acids, with glycine in every 3rd location and a high density of proline and hydroxyproline. The structure gives the functionality of collagen, specifically the self-assembly into fibrils: a process known as fibrillogensis [59]. The vital importance of this structure lies in the thermal stability, mechanical support and the involvement in structural building of other molecules. In addition, COL1 has been shown to has a crucial role in osteogenic differentiation of MSC [60].

In vitro studies were conducted assuming that COL1 in the form of triple helix will aggregate in normal physiological condition. This aggregate will form in the fibers and eventually fibrils that will adhere to cells by interaction with cell surface α2β1 integrin [61]. It was also reported that the interactions between COL1 and α2β1 integrin receptors stimulate osteoblastic differentiation of rat MSCs and incorporate bone matrix building [61, 62].

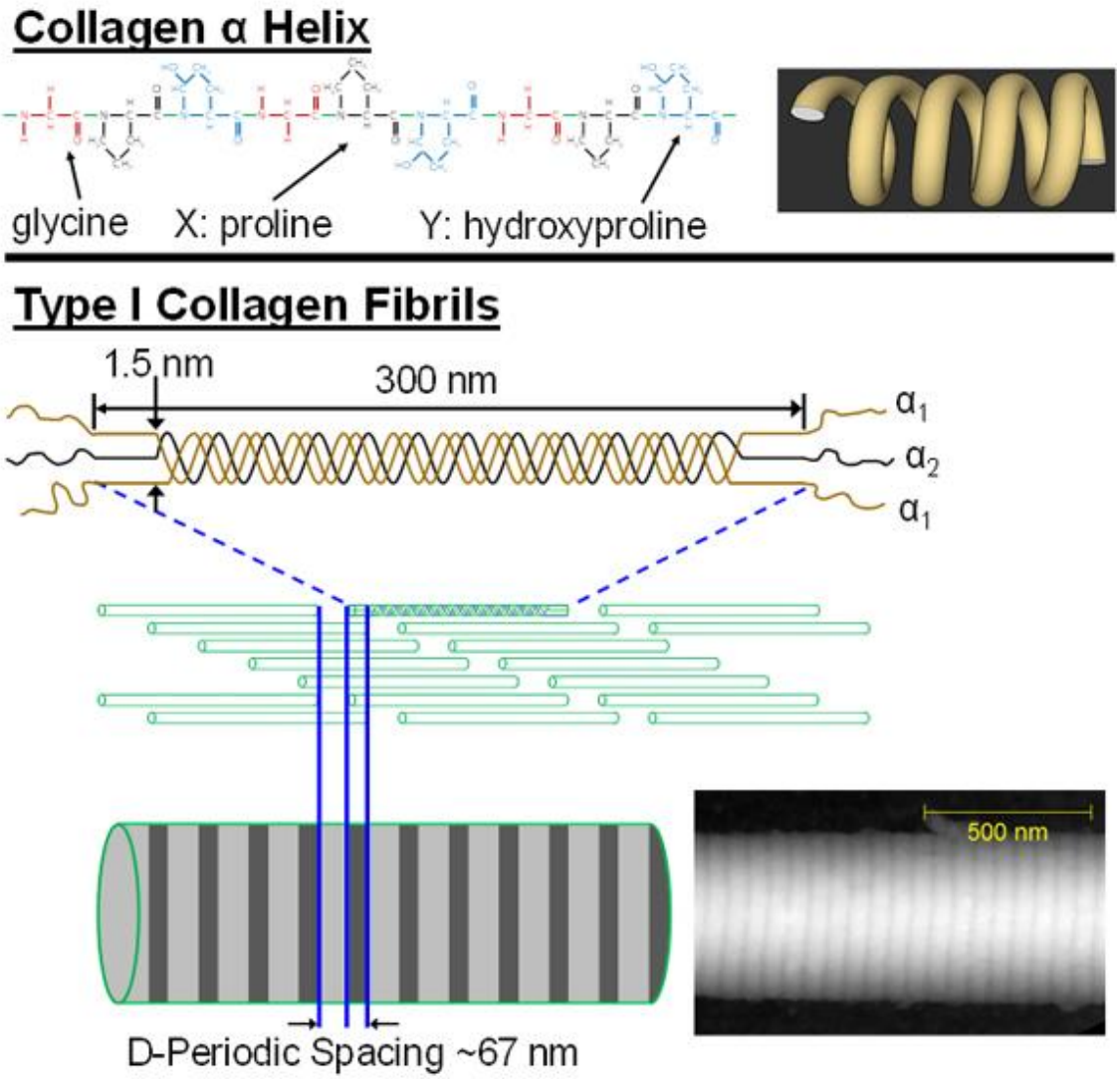

Figure 2. Collagen type 1 triple helical structure in the form of extended rod, the figure cited from Orgel JP, et al. (2006).

COL1 has been used in tissue engineering in approaches involved 3D scaffolds fabrication. 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/osteo-inductive material to enhance more functional engineered tissue [63]. However, collagen is a protein and the process of sterilization without changing in its structure still considered as a challenge [9].

1.3.3 Fibronectin

Fibronectin (FN) is one of the most important components in the extracellular matrix. Its glycoprotein contains repeating units of amino acids which can enhance cell attachment to biomaterial surface with its central-binding domain RGD sequence (figure 3) [64, 65].

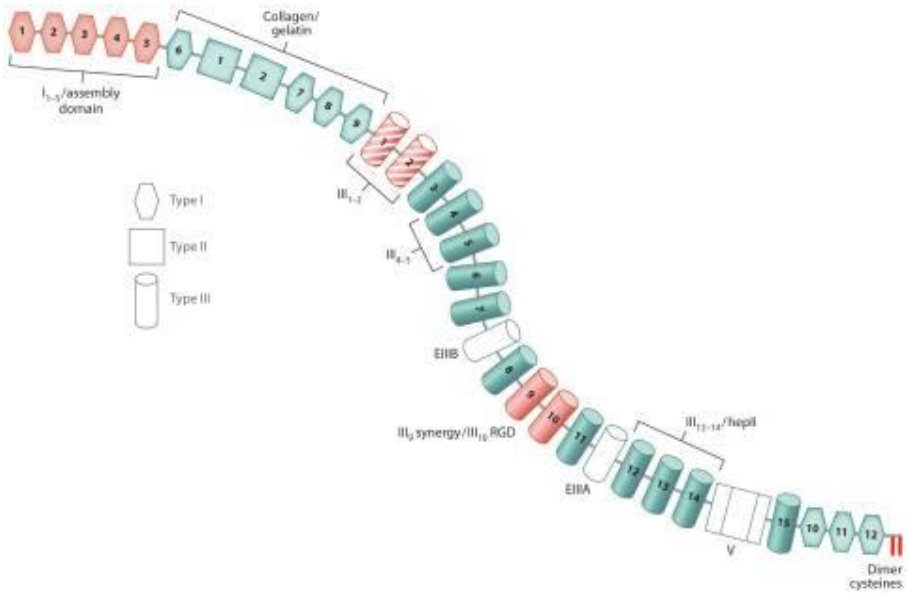

Figure 3. Structure of fibronectin, cited from Singh, et al. (2010).

FN forms dynamic domains that can undergo conformational changes which are critical for the functionality. The main role of FN is associated with cell migration during healing process and development, regulation of cell proliferation and growth, differentiation and hemostasis/thrombosis. Different cells can synthesize and secret FN in a form of plasma soluble disulfide-linked dimer of two large polypeptides chains. FN assembles in the form of fibrillar network [18, 63]. The network can interact with integrin and non-integrin receptors. It has a role in cell migration, proliferation and differentiation. It can also integrate with other molecules on the cell surface.

In tissue engineering several approaches have been done after the discovery of the essential cell adhesion peptide sequence arginine–glycine–aspartic acid (RGD) in FN in order to conjugate FN to synthetic polymers surfaces [12, 52]. Cell culture studies using human coronary artery smooth muscle cells illustrated that FN-conjugated scaffolds enhanced cell attachment [14].

2. The aim of the study

The collagen 1 and fibronectin coating in the 2D might induce cellular changes and differentiation down the osteogenic lineage even without the addition of osteoinductive media. This might be used as a substrate to modify scaffold materials used in tissue engineering. This in vitro study was aimed to investigate the influence of modalities of a substrate on the cellular activity of dental pulp stem cells.

Specific aims

- Evaluate the effect of collagen 1 and fibronectin coating-modalities on morphology of dental pulp stem cells.
- Investigate the influence of collagen 1 and fibronectin coating on attachment and proliferation of dental pulp stem cells.
- Elucidate the effect of collagen 1 and fibronectin on osteogenic differentiation of dental pulp stem cells at gene and protein levels.

3. Materials and Methods

3.1 Isolation of dental pulp cells

Human dental pulp cells 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. Dental pulp stem cells were isolated by enzyme dissociation method according to earlier reported protocol [51]. 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 were 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 h at 37 °C. The digested tissue was centrifuged at 1400 rpm for 10 min, and thereafter filtered through a 70 µm strainer. Single-cell suspension was cultured and expanded with Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, USA) 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 sub-cultured or stored in liquid nitrogen. Primary cells of dental pulp stem cells from the 2 to 4 passages were used in the follow-up experiments.

3.2 Phenotypic characterization of stem cells from dental pulp

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, USA), Fluorescein isothiocyanate (FITC) Mouse Anti-Human CD90 (BD Biosciences, New Jersey, USA) and Allophycocyanin (APC) Conjugated Mouse Anti-human CD105 (Southern Biotech, Alabama, USA). Concentrations of antibodies were based upon data obtained from a pilot study (data not shown). Cells derived-dental pulp tissue at passage 1 was applied for this analysis. Briefly, 5×10^5 cells per tube were blocked by 20 µl blocking reagent (phosphate buffered saline pH 7.4 and 0.5% BSA) for 10 min at room temperature (RT). The cells were stained by incubation with conjugated antibodies in the dark for 1 hour, washed with 200 µl of PBS (phosphate buffered saline pH 7.4) and then centrifuged at 250-300 × g for 5 min at 4°C. Supernatant was removed, and cells were re-suspended 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, USA). A total of 100.000 events were used for each sample.

To confirm the osteoblastic potential capacity, 1×10^3 cells /cm^2 in 6-well plate were cultured in either the growth media (controls) or in the osteogenic induction media containing ascorbate, dexamethasone, and β-glycerophosphate (StemCell Technologies) for 21 days (Table1).

Table 1. Osteoinductive medium supplements

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

3.3 Cell culture under different conditions

Dental pulp stem cells (DPSC) were cultured under standard condition at 37° C and in 5 % CO_2 humid atmosphere in culture flask (Nunc A/S, Denmark) with Dulbecco's modified Eagle's medium-low glucose (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin till they reached 70 % confluence. Culture medium was changed twice a week and cells were then harvested using trypsin and prepared for experiments.

The cells were seeded into three different conditions:

(1) DPSCs under standard culture condition at 37° C and in 5 % CO_2 humid atmosphere in well-plates (Nunc A/S, Denmark) with DMEM, supplemented with 10% FBS and 1% penicillin /streptomycin.

(2) DPSCs 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 by PBS, the diluted COL1 was applied on the culture plates and allowed to dry overnight in flow hood with the plate lid open. The remaining solution was removed and plates were washed twice with PBS.

(3) DPSCs 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 by PBS. The diluted FN was applied on the culture plates and allowed to dry at least 45 min at room temperature. The remaining solution was removed.

3.4 Morphological assay

DPSCs were cultured in the three different conditions and were seeded in a 24 well-plates (Nunc A/S, Denmark) at 20,000 cells/ well with three replicas. At 1 hour, 3 hours, 24 hours 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 let to dry before taking pictures under normal light microscope (Nikon ECLIPSE TS100, Nikon Instruments Inc, Japan).

3.5 Cell attachment, proliferation and viability-MTT Test

Cell attachment and proliferation rate of DPSCs was determined by MTT 3-(4, 5-dimethyl-thiazoyl)-2, 5-diphenyl-tetrazolium bromide (Sigma Aldrich). DPSCs were cultured in the three different conditions. The seeding densities was 10.000 cells/well in 96 well-plates for (1 h, 3 h, 24 h, 4 days and 7 days) with 5 replicas and were incubated in 5% CO_2 at 37°C. The culture medium was replaced twice a week. The medium was removed and the plates were washed with PBS 100 µl of MTT working solution were added to each well and covered with aluminum foil for 3-4 h. After incubation period, MTT solution was sucked and the cells were fixed by MTT Tris-formalin fixative buffer for 5 min, then washed with distilled water and covered overnight with aluminum foil in dark. DMSO with 6.25 % M NaOH were added to each well and left in shaker for 20 min at room temperature. FLUOStar OPTIMA was used to read the absorbance at 570 nm.

3.6 RNA extraction

DPSCs were cultured in the three different conditions and were seeded at 100.000/ well in 48 well-plates for 7 days with 5 replicas. RNA was isolated and purified using a Maxwell® 16 LEV simplyRNA Purification Kit (Promega Corporation, Madison, WI, USA). RNA purity and quantification was measured using Nanodrop spectrophotometer (ThermoScientific NanoDrop Technologies, Wilmington, DE).

3.7 Reverse Transcriptase

The revers transcription reaction was performed using high capacity cDNA Archive Kit system (Applied Biosystems, Foster City, CA, USA). 1.000 ng RNA was dissolved in 50 µl nuclease -free water, mixed with RT buffer, random primers, dNTPs and Multiscripe RT.

3.8 Real-time RT-PCR

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed in 10-µL reaction with 5 replicas for each cDNA sample, with Taqman® gene expression assays (Applied Biosystems, Foster City, CA, USA). Under standard enzyme and cycling conditions, amplification was carried out on a 96-well StepOne Plus™ system (Applied Biosystems). Levels 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

3.9 Alkaline phosphatase staining

DPSCs were cultured in the three different conditions and were seeded at 100,000/ well in 48 well-plates for 7 days using 3 replicas. ALP was detected by BCIP/NBT as a substrate: stained cells were shown blue-violet when ALP present. The substrate solution was prepared according to manufacture by dissolving one BCIP/NBT tablet (Sigma Aldrich) in 10 ml distilled water in the dark room and used within 2 hours. The 0.05% Tween 20 was added to Dulbecco's PBS without (Ca^{+2}/ Mg^{+2}) to prepare the washing buffer. The cells were taken from the incubator and medium was removed and then washed with PBS. After that, the cells were fixed using formalin (10%) 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 pictures for the cells were taken using normal light microscope (Nikon ECLIPSE TS100, Nikon Instruments Inc, Japan).

3.10 Alizarin Red staining

DPSCs were cultured in the three different conditions and were seeded at 100,000/ well in 48 well-plates for 21 days with 3 replicas. Calcium deposits were detected by slight reddish to orange stain. The ARS staining solution was prepared by dissolving 2g Alizarin Red substrate in 100 ml distilled water, then adjustment for pH between 4.1 – 4.3 with 0.1% NH_4OH, and then filtering in dark room. The cells were taken from the incubator and the medium was removed and washed with PBS without (Ca^{+2}/ Mg^{+2}) after that the cells were fixed using

formalin (10%) for 1 min then cells were 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 pictures for the cells were taken by normal light microscope.

3.11 Western Blot Analysis

For isolation of total protein fractions, cells were collected, washed twice with ice-cold PBS, and lysed using cell lysis buffer RIPA buffer (THERMO Scientific, USA) and adding 1× protease inhibitor to the buffer. 60 µl RIPA buffer was added in each well in 48 well-plates. The culture plates were transferred onto ice for 10 – 15 min. Cell scraper was used to scrape off cells and pass cell lysate through pipette around 10 times to form homogeneous lysate.

The lysates were collected by scraping from the plates and then centrifuged at 16.000 rpm at 4 °C for 20 min. This separated the total protein (supernatant) from the cellular debris (pellet). Total protein samples (20 µg) according to BCA assay were loaded on a Mini-PROTEAN ®TGX SDS-polyacrylamide gel for electrophoresis, and transferred onto transfer membranes (BIO-RAD, USA). 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, USA) and GAPDH (1:1000: Santa Cruz, CA, USA). Secondary antibodies were mouse anti-rabbit IgG (BIO-RAD) diluted in TTBS and Precision protein StrepTactin (HRP) and incubated for 1h in room temperature. Immunoblot bands were visualized by Immun-Star™ chemiluminescence kit, mix with 4ml lumino/enhancer with 4ml peroxidase solution and incubate for 5 min in dark.

3.12 Statistics

All values in bar diagrams were presented as mean ± standard deviation. Data were tested for homogeneity and normal distribution and analyzed by one way ANOVA- Tukey test. Differences were considered significant when $P<0.05$.

4. Results

4.1 Cell characterization and osteogenic capacity

The analyses performed by flow cytometry showed that DPSCs express the stromal-associated markers CD90 (24.4 %) and CD105 (98 %) and the perivascular marker STRO-1 (7.3 %) (Figure 4).

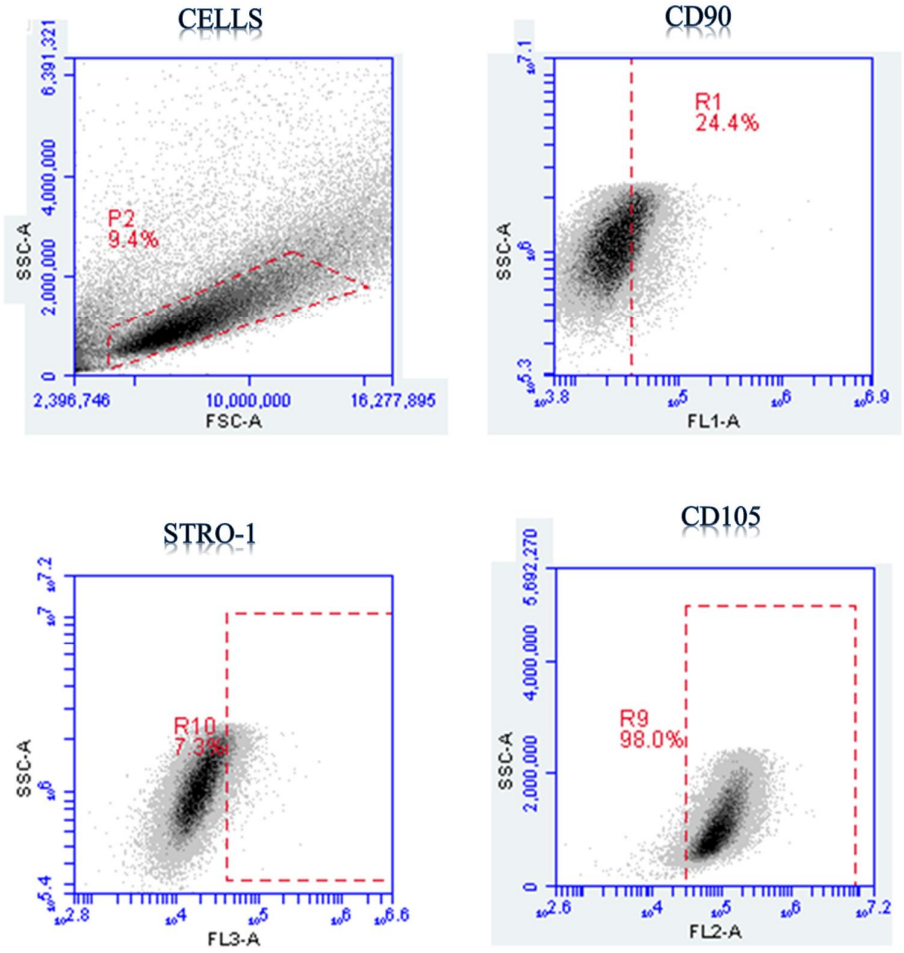

Figure 4. The human DPSCs showed high expression for surface markers CD105, but low expression for CD24, CD90. Stro-1 was moderately expressed.

After 21 days, we found that DPSCs under osteoinductive media were able to differentiate into osteoblastic phenotype compared to the untreated cells (Figure 5).

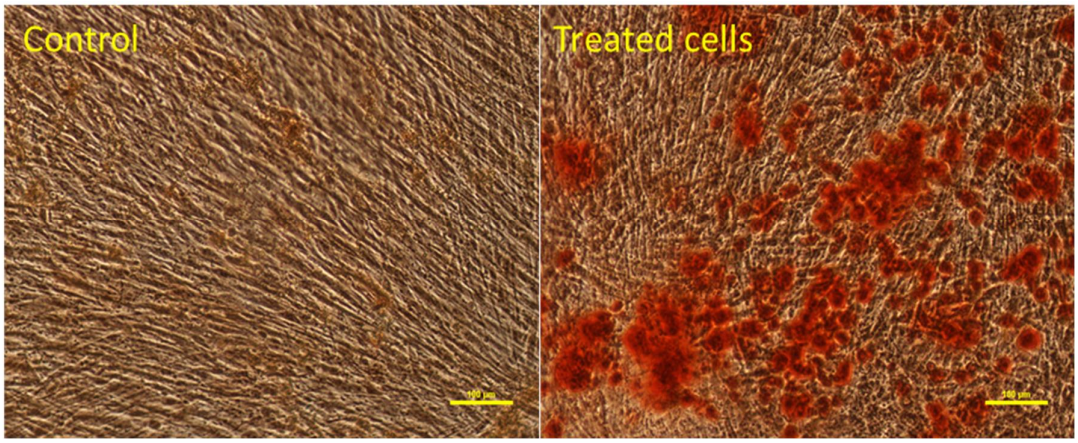

Figure 5. Alizarin red staining at day 21. More calcium nodules formation (Red staining) was observed in the OM treated DPSCs than in the control group. (10× magnification, scale bar =100 µm).

4.2 Cell Morphology

A Crystal Violet staining was utilized to describe the morphology of the cells using a light micrscope (figure 6). At 1 h of incubation, DPSCs exhibit rounded shape or spherical. At 3 h incubation, the cells have become more flat and getting more cuboidal. At 24 h incubation, the cells shown to be more elongated and exhibited fibroblaste-like spindle shape. The morphological observation disclosed no obvious difference between the non-coated and those coated with COL1 and FN after 1 and 3. However, the cells showed more spreading in coated plates compared to non-coated at 24 h.

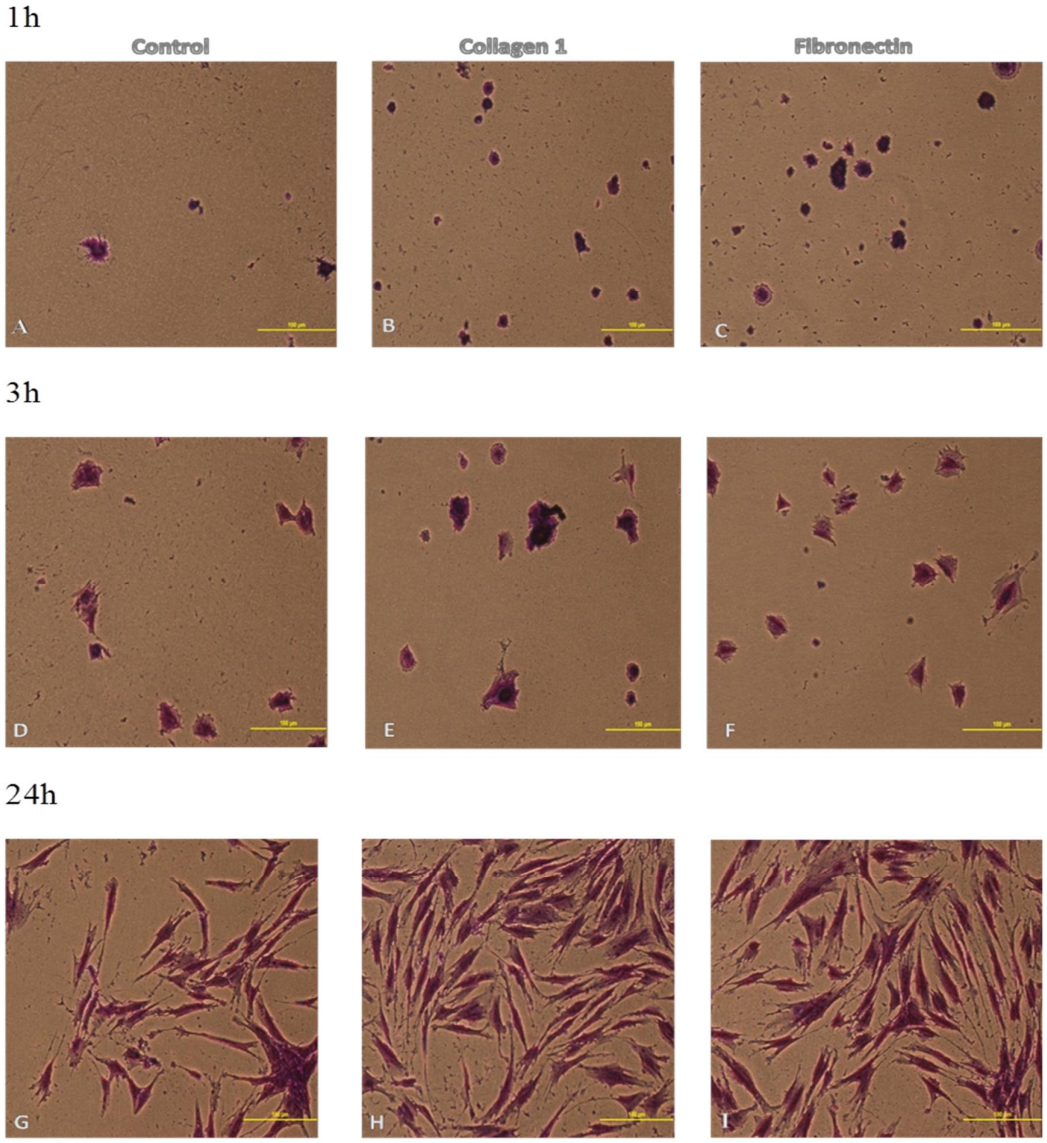

Figure 6. DPSCs stained with Crystal Violet and morpohology was anlaysed at 3 time points: 1, 3 and 24 h. (A) DPSCs control group at 1 h. (B) DPSCs, COL1 coated plates at 1h. (C) DPSCs, FN coated plates at 1 h. (D) DPSCs control group at 3 h. (E) DPSCs, COL1 coated plates at 3 h. (F) DPSCs, FN coated plates at 3h. (G) DPSCs control group at 24 h. (H) DPSCs, COL1 coated plates at 24 h. (I) DPSCs, FN coated plates at 24 h (10 × magnification, scale bar =100 μm).

4.3 Cell Attachment

At harvest, the unattached cells were washed away by PBS. Attachment assays were performed using MTT to measure the cellular vaibility and attachment after 1, 3 and 24 h of incubation. The absorbance was higher in COL1 and FN coated plates than the corresponding control group, although it was not statiticaly significant (figure 7).

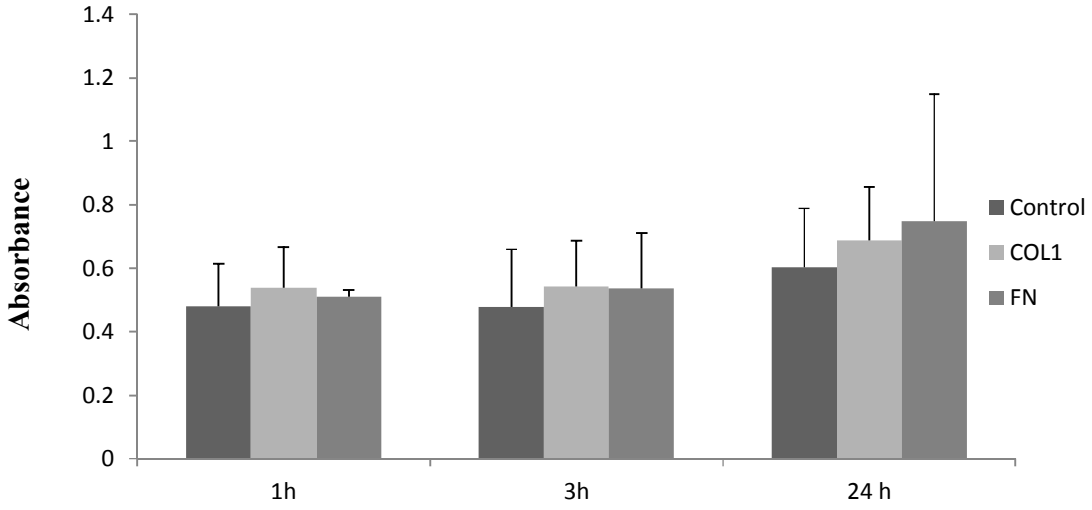

Figure 7. Evaluation of cell attachment using MTT at three time points 1, 3 and 24 h for DPSCs under different treatment conditions.

4.4 Proliferation assay

DPSCs were cultured in the three different conditions and the proliferation was measured using MTT at 4 and 7 days. At 4 days, DPSCs were proliferated better on the culture plates coated with COL1 and FN than on the non-coated plates (**p<0.05 and ***p<0.01, respectively). At 7 days, cellular proliferation on the coated plates were signuficantly higher than those non-coated plates (***p<0.01). However, after 4 and 7 days, there were no significant intergroup differences between COL1 and FN groups (figure 8).

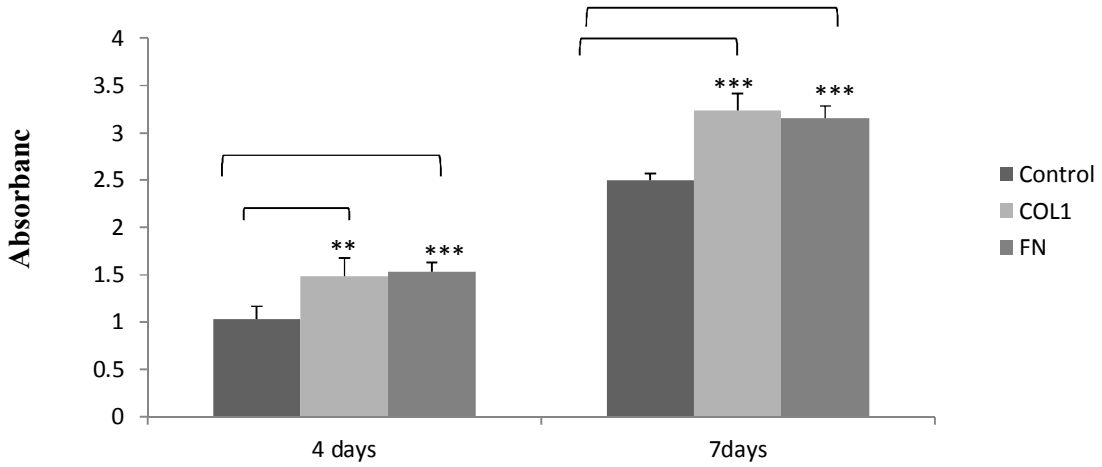

Figure 8. MTT measured cell proliferation of DPSCs under the three culturing conditions ,tested at 4 and 7 days, (**p<0.05 and ***p<0.01, respectively indicating significant difference).

4.5 RT-PCR

For ALP, RT-PCR revealed that there was higher mRNA expression in both COL1 and FN groups than on the control group at 7 days of incubation, however with no significant difference between the coated groups. The mRNA expression of RUNX2, OC and BMP-2 was higher by the cells grown on the plates coated with FN. However, the differences was not statistically signifiant. (figure 9).

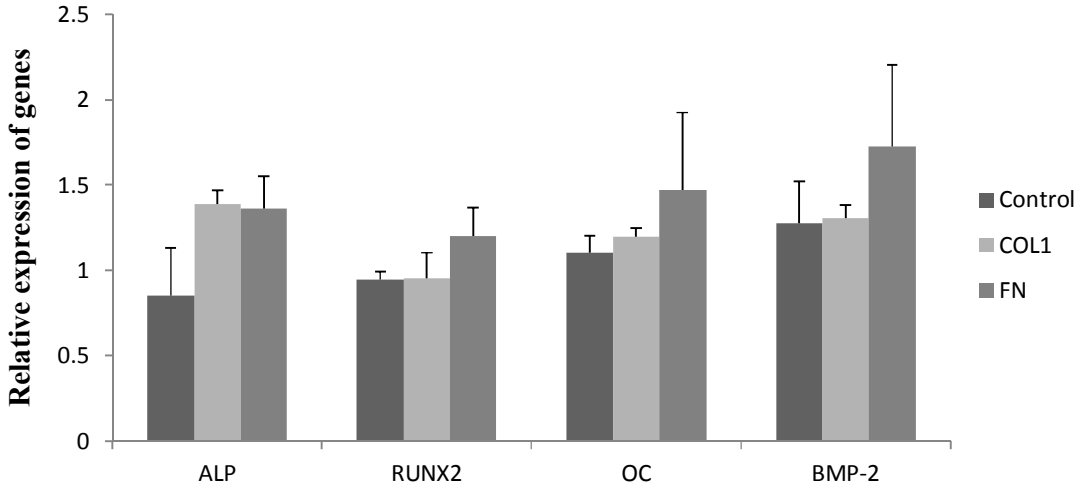

Figure 9. RT-PCR results after 7 days incubation of DPSCs in the three conditions. No significant difference among the groups could be detected for the following genes: ALP, RUNX2, OC and BMP-2.

4.6 ALP staining

Positive staining could be detected in all the groups (COL1, FN and control) after 7 days incubation. The blue staining is indicating osteogenic differentiation (Figure 10).

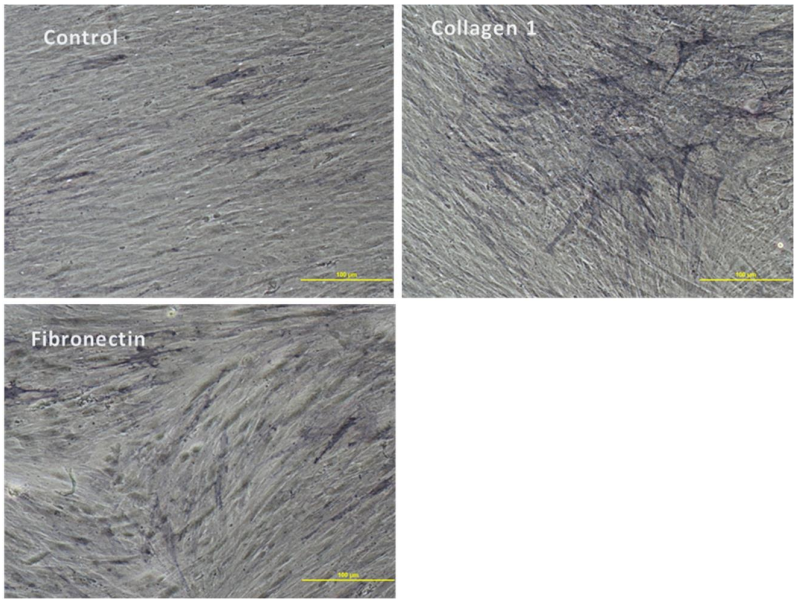

Figure 10. ALP staining for osteogenic differentiation potential at day 7. Control group, COL1 coating and FN coating, (10× magnification, Scale bar =100 μm).

4.7 Western Blot

The results from protein analysis revealed that DPSCs grown on the various culture plates expressed RUNX2 after 7 days incubation. GAPDH was used as a reference (Figure 11).

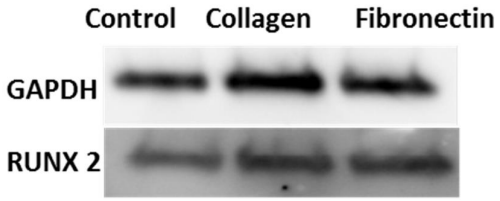

Figure 11. Western blot after 7 days incubation under the three different culture conditions revealed synthesis of RUNX2 protein in the three groups.

4.8 Alizarin Red Staining (ARS)

The ARS analyses for calcium deposition (mineralization) of DPSCs revealed slight weak staining in all groups after 21 days incubation. However, no obvious difference between the groups was detected (figure 12).

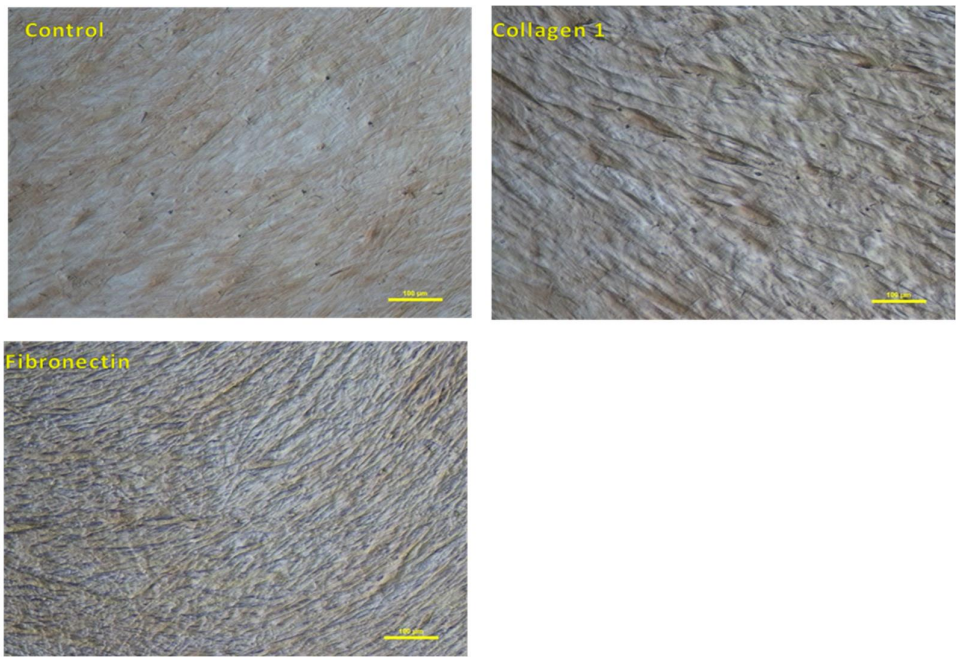

Figure 12. ARS for calcium deposition (mineralization) after 21 days. Control group, COL1 coating and FN coating. (Scale bar =100 μm).

5. Discussion

In the current study, our characterization results using the flow cytometry showed that human dental pulp cells (DPCs) expressed positively the following mesenchymal surface markers (STRO-1: 7.3 %, CD90: 24.4 % and CD105: 98%). Furthermore, the DPSCs formed calcium nodules indicating osteogenic potential after 21 days incubation. The data revealed no obvious differences in cell morphology between the coated and non-coated plates at 1 and 3 h. However, the cells showed more spreading in coated plates compared to control at 24 h. Regarding cell attachment, the findings demonstrated no significant difference on cell attachment between the coated plates and non-coated at 1, 3 and 24, although there was more tendency for cell attachment on the coated plates. In this study, the data showed that cells proliferated significantly more 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 dental pulp stem cells on the coated plates with COL1 and FN did not reach the terminal state of differentiation which is essential for mineralization.

Elucidation of dental pulp stem cells in an environment that is more physiologically relevant will afford an enhanced understanding of their behaviors and consequently, afford better vision into how these cells that can be used in regenerative therapies. Fibronectin plays an important role the cell adhesion attaching the cells to COL1 or proteoglycan substrates. It mediates the cell-cell interaction and cell – ECM by binding to different components of ECM. Another significance function of FN is its role in cell migration.

COL1 protein plays an important role in cell behavior by binding to specific integrin cell surface receptors, which activate intracellular signaling and further control gene expression, and cell morphology.

In the recent years, research has focused on the improvement of scaffolds, in order to be used as bone grafting substitutes [66]. However, for tissue engineering, several properties should be ideally integrated on the scaffold to facilitate bone regeneration such as: degradable over time, provide structural support to the repair region, allow cell attachment, induce osteogenic differentiation of stem and progenitor cells, and biocompatibility. Recently, three types of materials such as metals, ceramics and polymers are shown to be applicable in the tissue engineering. In particular, a bone healing such as a fracture is dependent on osteoinduction. For examples, tricalcium phosphate scaffold (TCP) acts as osteoinductive materials through recruitment of immature cells and cytodifferentiation into preosteoblasts [67]. On the other side, osteoconductive materials have been used in many treatment modalities for fractures and bone

defects. Polymer scaffold, copper, silver and bone cement are examples for these osteoconductive materials that are currently used.

Polymer scaffolds can be produced with a multiplicity of chemical and physical properties and have been shown to support osteogenesis of mesenchymal stem cells (MSCs) with dexamethasone [68]. Like other porous scaffolds, polymer scaffolds are only osteoconductive which means lack osteoinductive properties [69]. 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 dental pulp stem cells. The findings obtained from this simple model might provide important clues regarding the biological effect behind these extracellular matrix proteins and shed light on efforts which are devoted to improvement, modification and enhancement of biomaterials synthesized by increasing the bioactivity of the material.

Dental pulp contains mixture of progenitor, mesenchymal stem cells and other connective tissue cell-types. Stem cells exist in dental pulp tissues (DPSCs) considered as a precursor for dentin formation cells, and also can differentiate into many cell types under certain inductive molecules. From this point of view, progenitor cells, endothelial cells, fibroblast-like cells and pericytes could be those cells that cultured and propagated [70]. 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 %) of STRO-1–positive cells in the isolated DPSCs. In previous study, it has been demonstrated lower level expression of STRO-1 (5 %) by dental pulp cell [71]. Another previous experiments verified that STRO-1 antigen is considered an early marker of mesenchymal stem cells and extremely important in selecting dental pulp cells [72]. It is well known that this antibody identified a cell surface antigen expressed by the multilineage stem cells derived from bone marrow [73, 74].

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 mesenchymal stem cells [25]. It has been reported that different expression of cell markers can be influenced by variety of tissue condition, composition of cell culture medium, cell confluence-dependent manner and variations among different donors [75], different cell surface receptor expressions in different studies have been reported [25, 76].

Osteogenic differentiation for MSCs can be induced by using osteoinductive materials: 50μg/mL-ascorbic acid, 3.5mM β-glycerophosphate and 10^{-8} M dexamethasone [77]. Osteoblastic phenotype cells showed calcium deposits after 21 days incubation with inductive materials [78]. In our study, DPSCs stained with Alizarin Red staining showed calcium deposit after 21 days incubation with osteoinductive materials, which considered a reliable marker for bone differentiation. It has proved that DPSCs after several passages still exhibit capacity for nodule formation vitro [79].

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

Undifferentiated DPSCs cells exhibit round to oval shape, within time the cells start taking star-like shape by getting more flat with clearer cytoplasm then cells exhibit spindle shape, fibroblasts-like cells, with cytoplasmic extensions and adhere to the surface with microvilli and filopodia, and the cells have a tendency to grow and spread in colonies [80]. However, during differentiation, the cells undergo a morphological transition from an elongated, spindle shape to a polygonal shape with long extension of cytoplasm [81]. There are extensive evidences in the studies that the functional cell activity, including differentiation, is associated with cell morphology and spreading [51]. Cell spreading associated with cytoskeletal modifications have influence in functionality of cell and its fate. MSCs allowed to spread out more on a large area (10.000 μm^2) showed higher osteogenic differentiation compared with small area (1.024 μm^2), which turned to be more adipogenic [15].

The attachment assays with 24 hours of culture were revealed no significant differences between the cells grown the various culture substrates investigated in the present study (coated and non-coated culture plates). Previous Studies showed that cell membrane proteins, such as integrin, can bind to ECM proteins including FN and COL1 [82]. Another study reported that FN can be used to preferentially isolate progenitor stem cell populations from dental pulp [83]. However, in our investigation, DPSCs were cultured in normal culture plates (Nunc A/S, Denmark). This type of normal culture plates were designed to promote cellular attachment and have been reported to be effective for rapid expansion of MSCs. It was also been documented that those surfaces do not compromise the osteogenic and adipogenic potential of MSCs. Therefore, the profound effect of the surfaces of culture plates used in the study might mask the effect of the collagen and fibronectin coating on cellular attachment. Another possibility might raise that COL 1 and FN used to coat the culture plates in the present study might exhibit different behavior and different interaction than those found in physiological environment. In

other words the coated surfaces may not completely mimic the natural environment that permit the exact interaction between cell surface receptors and ECM components. 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. Therefore, each cell type inside the population can has its own contribution to the total cell number. Actually, the dental pulp cells used in this study have been described to be heterogeneous in nature by Gronthos, et al. [51]. The authors described that the dental pulp cells contained different cells types with different morphologies that may have might have different affinities to the various ECM proteins such as FN and COL1 used in this study [51].

In a previous study used microarray of different ECM proteins revealed that cell-type specific adhesion profiles exist [84]. In the present work, although the cell attachment was not significantly changed, the proportions of different cell types found in the population might be changed as a result of different specific interactions with ECM proteins [84].

The binding of ECM components to the dental pulp stem cells can activate a variety of cellular activities through different signaling transduction pathways, this may include proliferation, differentiation and cell migration [85]. It has been illustrated that cell attachment to FN coated-dishes can enhance stem cells proliferation and differentiation [86]. The cells bind to the ECM by integrins, which are heterodimeric cell surface receptors that make a physical linkage between the ECM and the cytoskeleton. This binding can activate signaling networks by nucleating signaling proteins on the cytoplasmic side of the plasma membrane [87]. In the present study, our results showed cells proliferated more on the coated surfaces by COL1 and FN compared to non-coated 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 [88-90]. The mechanism by which FN enhance mitogenic activity has been reported by the recruitment of integrin action in activation of mitogen-activated protein kinase (MAPK) which regulate many cell functions [91]. Others suggested that FN bonds at the cell surface, directly increase levels of extracellular signal-regulated kinase and focal adhesion kinase phosphorylation involved in cell cycle progression [92, 93].

It has been demonstrated that DPSCs cultured on osteoinductive medium were able to differentiate into osteoblast-like cells producing mineralized matrix and expressing typical osteoblastic markers such as ALP, osteopontin (OPN) and OC [94]. This can lead to a more

confident in using DPSCs in replacement tissue therapy of bone defects as a source of osteoblasts.

The findings obtained from the RT-PCR showed that there was a significant 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 type of surfaces. Although the expression was higher on the COL1 and FN coated plates, 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 non-coated substrates were performed in culture environment using osteogenic-free medium.

The data suggested that dental pulp stem cells grown on the coated and non-coated surfaces in osteogenic-free medium did not reach the terminal state of differentiation. The study design also precluded using high cell density on 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 [95]. This could be possibly 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 [30, 32, 51].

The enhancement for differentiation could be due to mimic physiological environment necessary for signal transduction, or by other word it could possibly increase the sensitivity toward signaling molecules [96]. It has been described that COL1 alone couldn't enhance mineralization while observed in the same condition with presence of BMP-2 [97].

The differentiation of dental pulp stem cells to another specific cell type is vital aspect of tissue regeneration. Many factors can enhance stem cell differentiation such as mechanical forces [98]. The mechanical forces has been tested in as scaffold model seeded by mesenchymal progenitor cells and exposed to cyclic, mechanical compression, this eventually enhance cell differentiation. Another stimulation factor is oxygen tension which plays a significant role in the process of osteogenic differentiation [99].

Cell-cell interaction and cell-matrix signaling molecules such as cytokines and growth factors can influence stem cells differentiation [100]. A previous study found that dentin matrix protein-1 induced differentiation of DPCSs into dentine-forming cells [101]. Specifically, osteogenesis is an organized developmental process which is regulated by hormones and growth factors, trigger osteoblast-specific signaling proteins and transcription factors [102]. The

literatures showed that ECM components have influence on the differentiation potential of stem cells [103, 104].

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 [105]. Particularly, FN integrated with scaffold reported to enhance stem cells differentiation *in vitro* [106]. COL1 also has reported to enhance osteogenic differentiation of mesenchymal stem cell [95].

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. Extracellular signal-regulated kinase (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 [107, 108]. FN has been shown to increase specificity for α5β1 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 [103].

Some collagenous and non-collagenous extracellular proteins express during osteogenesis [62]. For examples, ALP which is metaloenzyme expressed in early stage of osteogenic differentiation. Moreover, OC is a non-collagenous protein, has a role in mineralization process. RUNX2 is a transcriptional molecule which has a role in osteogenic differentiation. BMP-2 is a member of transforming growth factors-β (TGF- β) which enhance osteogenesis and bone formation.

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. Elucidation the proportion of each cell type can give better insight in their contribution in the whole cell population behavior. Moreover, it can give comprehensive understanding in cell-cell interaction.

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 dental pulp cells are mesenchymal stem –like cell phenotype.

- The dental pulp cells used in this study were shown to have the capacity to differentiate into osteoblastic linage. These data indicate that *in vitro* culture of dental pulp cells in osteoinductive materials environment provides comparable properties and stem cell-like characteristics.

- Our data revealed that collagen 1 and fibronectin 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 collagen 1 and fibronectin resulted in increased dental pulp stem cells proliferation.

- In the absence of osteogenic supplements, collagen and fibronectin did not significantly promote the differentiation of dental pulp stem cells.

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