

Adhesion and growth of dental pulp stem cells on enamel-like fluorapatite surfaces

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Abstract: To study how apatite crystal alignment of an enamel-like substrate affects DPSC cellular adhesion and growth as a precursor to produce an *in vitro* enamel/dentin superstructure for future studies. The cells were subcultured in 10% FBS DMEM up to seven weeks on the two surfaces. Specimens were observed under SEM, counted, and analyzed using the human pathway-focused matrix and adhesion PCR array. After three days, the cell number on ordered FA surface was significantly higher than on the disordered surface. Of the 84 focused pathway genes, a total of 20 genes were either up or down regulated in the cells on ordered FA surface compared to the disordered surface. More interestingly, of the cell-matrix adhesion molecules, integrin alpha 7 and 8 (ITGA 7 and 8), integrin beta 3 and 4 (ITGB3 and 4),

and the vitronectin receptor-integrin alpha V (ITGAV) and the key adhesion protein-fibronectin1 (FN1) were up-regulated. In SEM, both surfaces showed good biocompatibility and supported long term growth of DPSC cells but with functional cell-matrix interaction on the ordered FA surfaces. Significance: The enhanced cellular response of DPSC cell to the ordered FA crystal surface involves a set of delicately regulated matrix and adhesion molecules which could be manipulated by treating the cells with a dentin extract, to produce a dentin/enamel superstructure. © 2011 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 96A: 528–534, 2011.

Key Words: fluorapatite, stem cell, PCR array, matrix, adhesion

INTRODUCTION

In primary dentinogenesis during tooth development, epithelial signals are essential to induce the dental papilla ectomesenchymal cells to terminally differentiate into odontoblasts. After tooth eruption, the dental pulp still retains its defensive and regenerative potential. Under certain physical and pathological stimuli, bound bioactive molecules, including growth factors and some noncollagenous proteins, may be released from the dentin matrix and recapitulate developmental events allowing reparative dentin to form.^{1–3} Recently, a distinct population of human postnatal dental pulp stem cells (DPSCs) have been described^{4,5} and similar cells have been isolated from shed deciduous teeth.⁶ These postnatal stem/progenitor cells have offered us promising possibilities either for the regeneration from resident populations in the pulp or for the engineering tissue constructs. Most recently, an enamel-dentin-like complex has been generated *in vivo* by transplantation of the odontogenic epithelial cells and dental mesenchymal cells co-culture construct⁷ and the odontogenic epithelial cells and dental pulp cells co-culture construct.⁸ For many years, the methodology to “grow a tooth” for transplantation into humans has been the subject of much conjecture and a large number of studies. The complex nature of the tooth may preclude this being done in an *in vitro* situation. The ability to synthesize a fluorapatite superstructure *in vitro* which has the

prismatic appearance of normal enamel will make the biological situation less complex and perhaps, a tooth grown in culture a reality.

We have described the *in vitro* induction of these DPSCs into odontoblast-like cells after treatment with a mineralization supplement (MS) containing AA and β -GP and an EDTA-soluble dentin extract (DE), which includes a cocktail of growth factors and bio-active molecules sequestered in the dentin.⁹ In a more recent study the gene profile changes during the DPSC cell mineralization process induced in the same experimental conditions as above were also analyzed¹⁰ which lead us to speculate whether the DPSC adhere to and grow long term on an enamel-like substrate *in vitro*.

Promisingly, an ordered enamel-like fluorapatite (FAP) film via a hydrothermal method has been established in our laboratory. The crystals are very well aligned over a large area after being deposited on metal surface and the rods spontaneously formed bundles (prism-like structures).¹¹ It should be possible, therefore, that under the control of the appropriate signaling molecules and the DPSC culture conditions to induce dentin formation on the synthetic FA layer *in vitro* without using the epithelial cells, thus, producing a model enamel/dentin superstructure formation.

In this study we used fluorapatite (FA) substrates, with ordered (enamel-like) or disordered surface organization, to study their effect on the biocompatibility, initial cellular

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response and long-term growth of the DPSC cells. The underlying mechanisms during these processes were also investigated.

MATERIALS AND METHODS

Synthesis of the FA apatite surfaces

For a typical synthesis of FA crystals, 9.36 g ethylenediaminetetraacetic acid calcium disodium salt (EDTA-Ca-Na₂) and 2.07 g NaH₂PO₄·H₂O were mixed with about 90 mL distilled water. The suspensions were stirred continuously until the powder dissolved. The pH was adjusted to 6.0 using NaOH. Prior to mixing 0.21 g NaF in 90 mL of the EDTA-Ca-Na₂ and NaH₂PO₄ solution, it was dissolved in 10 mL water (pH 7.0) and stirred continuously. The FA crystal growth on the substrates (15 mm 316 stainless steel discs) was achieved by adding the plates to 100 mL of newly prepared EDT-Ca-Na₂/NaH₂PO₄/NaF mixture and then autoclaving at 121°C at pressure of 2.4×10^5 Pa for 10 h. Ordered and disordered films were produced individually on the undersurfaces and upper surfaces of the stainless steel discs respectively.

Cell culture and cell attachment assay

DPSC cells (kindly donated by Dr. S. Shi, USC) were subcultured on the FA films in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin, at a seeding density of 0.5×10^5 , in 12-well plates. The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Before cell seeding, the ordered and disordered FA substrates were equilibrated with 10% FBS culture media for 2 h. After being counted, harvested cells were then seeded onto 12-well cell culture plates containing different FA samples under the above serum adsorption conditions. Cells were seeded on the two sterilized coating surfaces at a density of 1×10^5 cells/mL and cultured for three days. At the end of culture, the samples were taken out and removed to new 12-well plates. After being washed twice with phosphate buffered saline (PBS) solution, cells were detached with trypsin/EDTA, stained with trypan blue and then counted using a haemocytometer.

RNA isolation and reverse transcription

Total cellular RNA was isolated from DPSC cells grown for three days on the two surfaces using the RNeasy Mini kit (Qiagen, CA) according to the manufacturer's instructions. The RNA was treated with the RNase-free DNase Set (Qiagen, CA) during RNA isolation. The cDNA samples were prepared from the isolated RNA using the RT first strand kit (Cat. No. C-03, Qiagen, CA) according to the manufacturer's protocols. An average of six to eight replicates of each FA crystal substrate on which the cells were grown has been used for the total cellular RNA isolation and cDNA sample preparation.

RT² profiler PCR array analysis

Specimens were analyzed using the human pathway-focused matrix and adhesion PCR array (Qiagen, CA), which com-

bins the PCR sensitivity and the multigene profiling capability of a microarray. Briefly, the above prepared cDNA samples were added to the RT² qPCR master mix containing SYBR Green and reference dye. The above mixture was then aliquoted across the PCR array templates which contain 84 pathway-specific genes plus controls. The real-time PCR analysis was carried out using an ABI 7700 sequence detector (Applied Biosystems, Foster, USA). Relative gene expression values were analyzed using the Superarray web-based software package performing all $\Delta\Delta C_T$ -based fold-change calculations. Duplicate 96-well plates were used in this PCR array analyses.

Scanning electron microscopy

After 1, 3, 5, and 7 weeks of culture, the DPSC cells grown on the two FA surfaces were rinsed and fixed in 2.5% glutaraldehyde in distilled water, serially dehydrated and critical point dried. SEM analysis was conducted on a Phillips XL30FEG Scanning Electron Microscope (SEM) FEI company, Hillsboro, OR) operated at 10 kV (Resolution: 2.0 nm at 30 kV, 5.0 nm at 1 kV). The SEM specimen was coated with Au/Pd film to prevent specimen charging.

Statistical analysis

The cell counting results were analyzed statistically using GraphPad Prism 5 for unpaired *t* test of an average of three to five replicates and significance was considered at $p < 0.05$. Data are expressed as means \pm standard deviation.

RESULTS

Hydrothermal synthesis of ordered and disordered FA crystal surfaces

The ordered rod-like FA crystals were very well aligned along the *c*-axis after being grown on the undersurfaces of the 316 stainless steel discs. The densely packed growth mode along the *c*-axis forced the rod-like crystals to align parallel to each other [Fig. 1(A)]. On the upper surfaces of the discs the FA crystals are randomly arranged without forming the well-aligned structure [Fig. 1(B)].

Cell density on ordered and disordered FA surfaces

The initial attached cell numbers on the ordered FA surfaces were significantly higher than that on the disordered FA surfaces (unpaired *t* test, $p < 0.001$, $n = 3$) (Fig. 2).

Human matrix and adhesion pathway-focused PCR array analysis

Of the 84 focused pathway genes, a total of 20 genes were either up or down regulated in the cells on ordered FA surface compared to the disordered surface. Of the cell-matrix adhesion molecules, secreted phosphoprotein 1 (SPP1), thrombospondin 3 (THBS3), integrin alpha 7 and 8 (ITGA 7 and 8), integrin beta 3 and 4 (ITGB3 and 4), and more interestingly, the vitronectin receptor-integrin alpha V (ITGAV) were up-regulated. Other adhesion molecules for example collagen type VII alpha 1 (COL7A1), COL8A1, COL12A1, laminin alpha1 (LAMA1) and the key adhesion protein-fibronectin1 (FN1) were also up-regulated. Among the ECM proteases, the

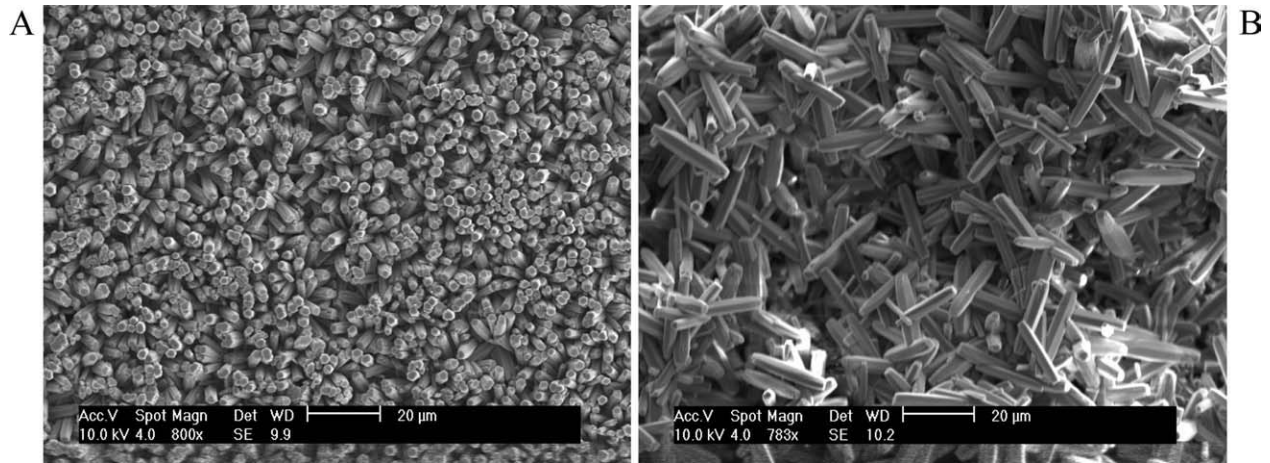


FIGURE 1. Scanning Electron Micrograph of synthesized ordered (A) and disordered (B) FA crystal surfaces. Ordered and disordered films were produced individually on the undersurfaces (A) and upper surfaces (B) of the stainless steel discs.

up-regulation of the matrix metalloproteinase 13 (MMP13) was coordinated with the down-regulation of 3 other proteases (Fig. 3, Table I). The expression change of at least 1.5-fold was set as the “cutoff” value for the transcripts being differentially expressed on the two crystal surfaces. In our preliminary studies, this “cutoff” value for the detection of the differentially regulated individual genes was further confirmed by the single real-time PCR amplifications. A similar methodology was adopted in our earlier publication.¹²

SEM observation

Scanning Electron Microscopy (SEM) of the DPSC cells grown on both the ordered and disordered FA crystal surfaces showed good biocompatibility. The cells spread and flattened on both surfaces; however, a greater number of cells were identified on the ordered FA crystal surface compared with the disordered surface after one week of cell growth [Fig. 4(A)]. After culturing for three weeks, dense cell nodule formation was observed on the ordered FA surface with more cellular extensions and extracellular matrix; whereas isolated or fused cell nodules were seen on the disordered FA surface [Fig. 4(B)]. At five weeks, the cells were fused together to completely cover the FA surfaces with denser matrix formation on the ordered FA surfaces than on the disordered [Fig. 4(C)]. After seven weeks, multilayers of cells with cell bodies extensively elongated and polarized were seen on the ordered FA surfaces. Further a much denser matrix was seen on the ordered surface as opposed to the disordered surfaces [Fig. 4(D)]. At greater magnification of the DPSC cells grown on ordered FA crystal surfaces, cytoplasmic extensions of the cells adhering to the individual FA crystals can be seen (Fig. 5).

DISCUSSION

In this study, we focused on the initial cellular response of DPSC cells to ordered enamel-like and disordered FA surfaces and explored the mechanisms underlying this process, which will provide valuable information in understanding of the biocompatibility of these newly developed biomedical

materials and for the future application of these materials in creating a functional enamel-dentin superstructure.

Correlations among surface properties, protein adsorption, and cell response will dictate the value of the synthetic FA material to be used in a model for the creation *in vitro* of the enamel/dentin superstructure. Proteins will be adsorbed onto FA substrates in different quantities, densities, conformations, and orientations depending on the chemical and physical characteristics of the surface and involves van der Waals, hydrophobic and electrostatic interactions, and hydrogen bonding. Adsorbed proteins may act as mediators for cell adhesion if they have the correct geometry (i.e. exposed RGD sequence in fibronectin) to mediate cell attachment.¹³ In this study, the initial cellular attachment of the DPSC cells was significantly higher on the ordered than the disordered FA surfaces, though both surfaces showed excellent biocompatibility and support the long term growth of the cells. Thus, the composition and conformation of the adsorbed protein layer might be responsible for the enhanced DPSC cellular response on the ordered FA surfaces. Surface

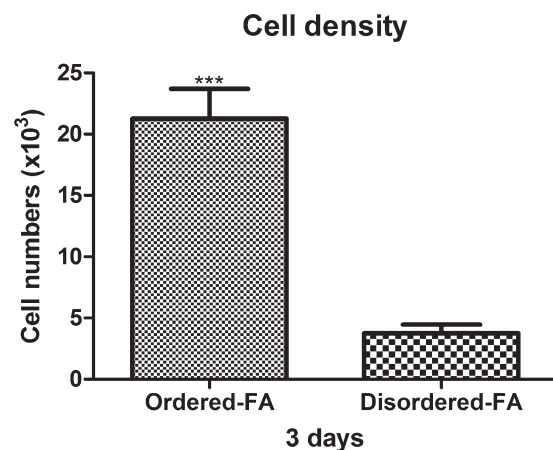


FIGURE 2. Cell density on ordered and disordered FA surfaces. The attached cell numbers on the ordered FA surfaces were significantly higher than that on the disordered FA surfaces (***) $p < 0.001$.

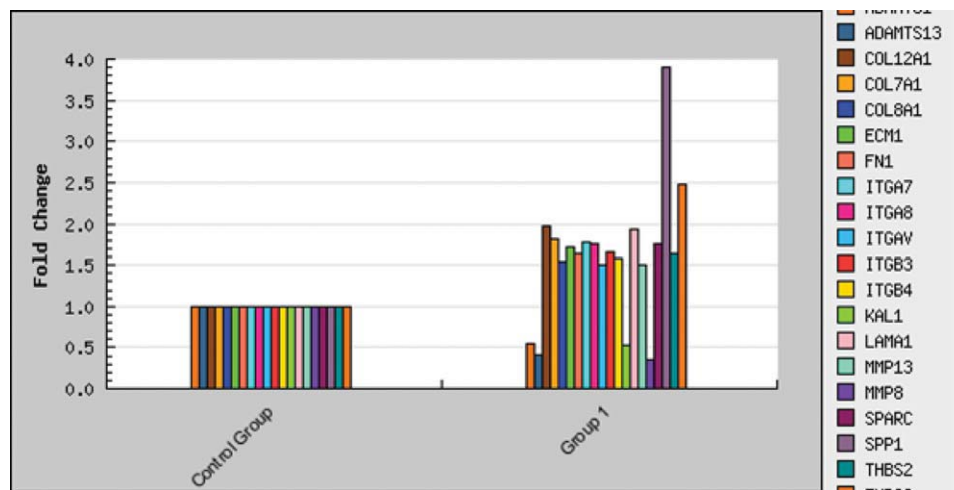


FIGURE 3. Plot bar of the relative expression of human extracellular matrix and adhesion molecules. The boundary value is 1.5. Bar graph of the expression of human extracellular matrix and adhesion molecules on ordered FA surfaces (Group 1) compared to disordered FA surfaces (Control group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

characteristics such as chemistry affect cell-surface interactions, whereas neither the structural or topographical effects on the cell adhesion have been fully elucidated.¹³

Following protein adsorption, the cell-matrix interactions control further cell adhesion, cell survival and the cellular proliferation and differentiation process. Adsorption of adhesion proteins, such as fibronectin (FN) and vitronectin has been reported to mediate cell adhesion.¹⁴ During tooth development, FN has been found in the dental pulp and predentin at different stages of dentinogenesis; FN has also been observed in the basement membrane which is between the inner enamel epithelium and the underlying dental mesenchyme, and in the mantle predentin.¹⁵ In bone,

it is involved in the early stages of osteogenesis and the structural organization of its fibrillogenesis has been suggested to be essential in mineral nucleation.¹⁶

It has been suggested that studies of the gene regulation responsible for cell adhesion selectivity in cell-surface interactions is critical at an early stage (three days). However, if applied too early before three days or too late after three days, the cells will either not sense the related environment or have already remodeled their micro-environment to give inaccurate adhesion information.¹⁷ Therefore, in this study, after the growth of DPSC cells on the two surfaces for three days, the expression of human pathway-focused matrix and adhesion molecules of the cells were analyzed using PCR

TABLE I. Fold Change of the Human Extracellular Matrix and Adhesion Molecules Expressed by the DPSC Cells Grown on the Ordered FA Surface Relative to the Disordered Surface

Symbol	Unigene	Refseq	Gname	Fold Change
SPP1	Hs.313	NM_000582	BNSP/BSPI	3.91
THBS3	Hs.169875	NM_007112	TSP3	2.47
COL12A1	Hs.101302	NM_004370	COL12A1L	1.97
LAMA1	Hs.270364	NM_005559	LAMA	1.94
COL7A1	Hs.476218	NM_000094	EBD1/EBDCT	1.82
ITGA7	Hs.524484	NM_002206	FLJ25220	1.77
ITGA8	Hs.171311	NM_003638	Integrin α 8	1.76
SPARC	Hs.111779	NM_003118	ON	1.75
ECM1	Hs.81071	NM_004425	ECM1	1.71
ITGB3	Hs.218040	NM_000212	CD61/GP3A	1.65
FN1	Hs.203717	NM_002026	CIG/DKFZp686F10164	1.64
THBS2	Hs.371147	NM_003247	TSP2	1.63
ITGB4	Hs.632226	NM_000213	CD104	1.58
COL8A1	Hs.134830	NM_001850	MGC9568	1.54
ITGAV	Hs.436873	NM_002210	CD51/MSK8	1.51
MMP13	Hs.2936	NM_002427	CLG3	1.50
MMP8	Hs.161839	NM_002424	CLG1/HNC	-2.88
ADAMTS13	Hs.131433	NM_139028	C9orf8/DKFZp434C2322	-2.45
KAL1	Hs.521869	NM_000216	ADMLX/HHA	-1.92
ADAMTS1	Hs.643357	NM_006988	C3-C5/METH1	-1.83

After the real-time PCR amplification, relative gene expression values were analyzed using the Superarray web-based software package performing all $\Delta\Delta C_t$ based fold-change calculations.

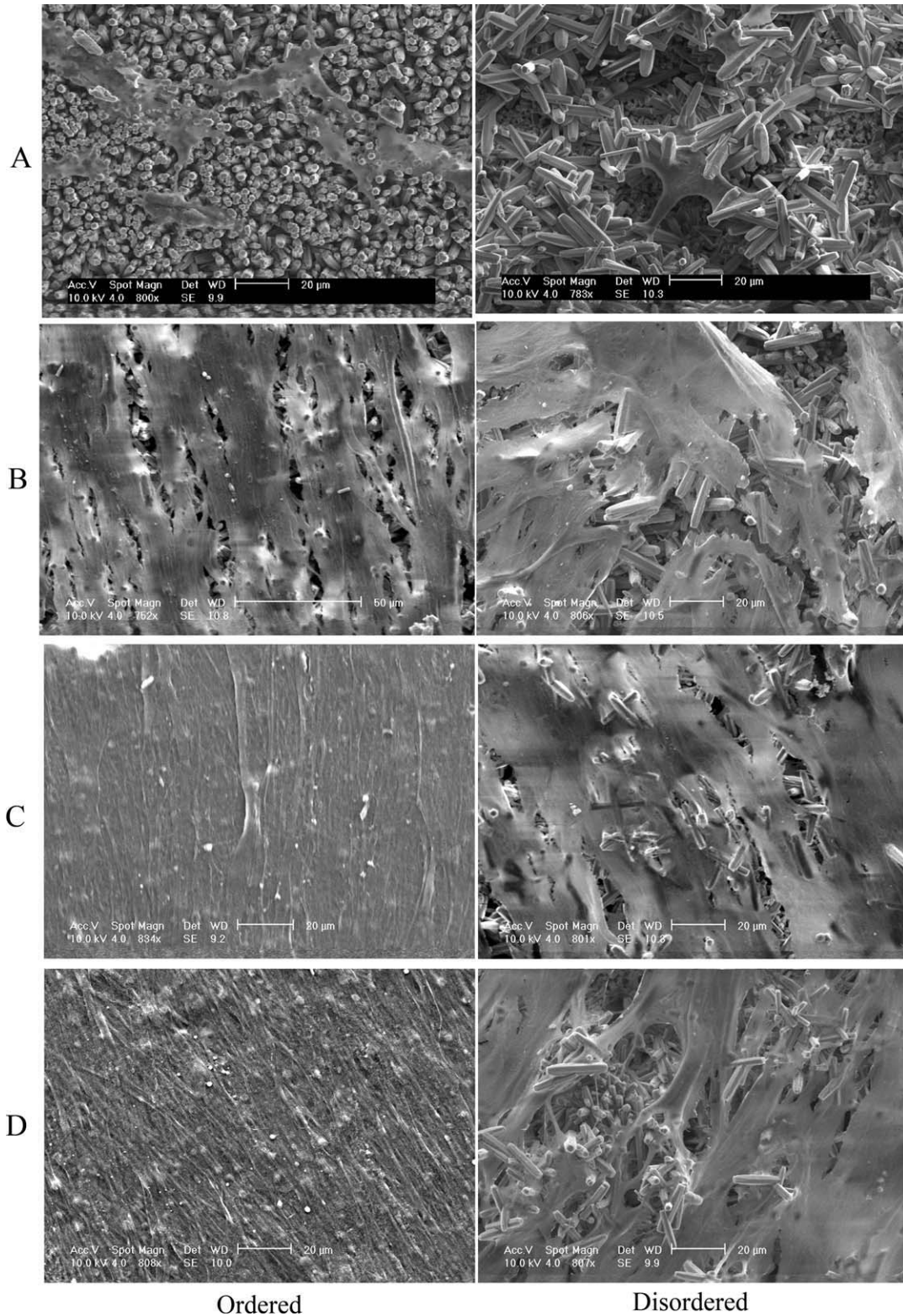


FIGURE 4. Scanning Electron Micrograph of DPSC cells grown on FA surfaces for 1 week (A), 3 weeks (B), 5 weeks (C) and 7 weeks (D).

array. Of the cell-matrix adhesion molecules, secreted phosphoprotein 1 (SPP1), thrombospondin 3 (THBS3), integrin alpha 7 and 8 (ITGA 7 and 8), integrin beta 3 and 4 (ITGB3 and 4), and more interestingly, the vitronectin recep-

tor-integrin alpha V (ITGAV) were up-regulated on the ordered enamel-like FA surfaces. Other adhesion molecules for example collagen type VII alpha 1 (COL7A1), COL8A1, COL12A1, laminin alpha1 (LAMA1) and importantly, the key



FIGURE 5. Scanning Electron Micrograph of DPSC cells grown on FA surface. At greater magnification shows cytoplasmic extensions of the cells adhering to the individual FA crystals.

adhesion protein-fibronectin1 (FN1) were also up-regulated on the ordered FA surfaces. As key adhesion molecules, FN and vitronectin along with fibrinogen, collagens, laminin, osteopontin, and some other trace proteins, mediates the specific interaction between cells and surfaces via cell integrin receptors.^{18,19} Thus, the differential expression of adhesion and matrix related molecules especially the up regulation of vitronectin receptor-integrin alpha V and FN itself may play important role in the enhanced initial cellular response of DPSC cells to the ordered enamel-like FA surfaces. Among the ECM proteases, the up-regulation of the matrix metalloproteinase 13 (MMP13) was coordinated with the down-regulation of 3 other proteases which also appears to be important in establishing the favorable micro-environment (i.e. favorable focal adhesions) for the DPSC cells grown on the ordered FA surfaces.

The long term differential growth pattern of the DPSC cells on the two FA surfaces may be closely related to the differential initial cellular adhesion characteristics of these surfaces. It has been suggested that the first phase of cell-material interaction involving attachment, adhesion and spreading of the cell may probably influence the second phase of the interaction involving proliferation and differentiation of the DPSCs into odontoblast-like cells. As we had shown earlier the induction of DPSCs into odontoblast-like cells after treatment with a mineralizing supplement and a dentin extract, we did not attempt to recapitulate this process.^{9,10}

Similarly, it has been shown that surface characteristics not only affect the focal adhesion and extracellular matrix formation of the human bone marrow mesenchymal stem cell but also the differentiation of these cells.²⁰ In a most recent study, it has been reported that surface characteristics such as mechanical strength or rigidity of biomaterials not only affected the cell focal adhesions, cytoskeletal contractility but also stem cell differentiation.²¹ Therefore it would not be surprising that the different topographical characteristics of the crystal surface might affect the DPSC differentiation and mineralization process. Thus, experiments marrying an enamel-like synthetic fluorapatite (FA)

coated with basement membrane component (FN, collagen type IV etc) and the induction of DPSC cells to create a dentin-like structure together with a functional enamel/dentin junction will be our future line of investigation. In the future application, it should be possible to synthesize these FA superstructures on the inner surface of metal crowns in order to obtain the appropriate anatomical crown shapes. Then we would generate the “dentin” on the inner surface of these synthetic enamel-like crown shaped superstructures.

CONCLUSION

From this study, the newly created ordered FA crystal surface provides a favorable environment for the long term growth and the functional cell-matrix interaction of the DPSC cells. The enhanced cellular response of DPSC cell to the ordered FA crystal surface involves a set of delicately regulated matrix and adhesion molecules. As a consequence of this study a biomimetic model for the formation of the enamel/dentin junction will be developed.

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