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Differentiation of Human Embryonic Stem Cells on Periodontal Ligament Fibroblasts In Vitro

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Abstract: Human embryonic stem cells (hESCs) are pluripotent cells with unlimited proliferation potential and differentiation capacity to all types of somatic cells. Periodontal tissue engineering based on in vitro expanded cells holds the promise to overcome the limitations associated with contemporary regenerative techniques. The aim of this study was to investigate the differentiation patterns of hESCs under the influence of periodontal ligament cells in vitro. hESCs (HUES-9) were expanded and characterized for their pluripotency. Then they were transfected with green fluorescent protein-carrying plasmid, and cocultured with human periodontal ligament fibroblastic cells for 21 days. Two experimental groups were established with different medium constituents. Specimens were fixed at days 7, 14, and 21 and were analyzed morphologically under inverted light microscope, and by immunohistochemistry using antibodies against collagen types I and III, fibronectin, fibroblast surface protein, vimentin, and pancytokeratin. Our results demonstrate different patterns of cell differentiation between groups, with about one-fifth of cells in colonies acquiring characteristics similar to periodontal ligament fibroblastic progenitors while others proceed toward distinctive lineages. This indicates the feasibility to direct the differentiation of hESCs toward the periodontal ligament fibroblastic progenitors to some extent. These findings support the notion that hESCs may become a cell source with unlimited supply for periodontal tissue engineering applications. **Key Words:** Human embryonic stem cells—Periodontal ligament fibroblast—Directed differentiation—Periodontal tissue engineering—Coculture.

First established as lines in 1998 by Thomson et al. (1), human embryonic stem cells (hESCs) are pluripotent cells with virtually unlimited proliferative potential in undifferentiated state and possess the ability to differentiate into derivatives of all three embryonic germ layers (2). These two exquisite features make them candidates for various applications in life sciences, like elucidation of early human developmental processes, determining the teratogenic effects of new developed drugs, providing insight into causes of birth defects, and utilization in prospective cell and tissue replacement therapies (3). Significant advances have been made in elucidating gene expression profiles (4), proteomic signature (5), and cell-signaling pathways (6), giving further insights into the mechanisms controlling self-renewal and differentiation of hESCs. Although differentiation of hESCs begins spontaneously and proceeds stochastically in vitro when allowed to aggregate in three-dimensional (3-D) structures as embryoid bodies (EBs) (7), numerous attempts have been made to direct the differentiation preferentially to certain cell types and lineages. Neural progenitors (8) and keratinocytes (9) of the ectodermal lineage, cardiomyocytes (10), hematopoietic cells (11) and osteoblast progenitors (12) as mesodermal cells, and pancreatic β-cells (13) of endodermal origin are some representatives of successful directed differentiation of hESCs into some somatic cell types. Different approaches have been applied like allowing the cells to begin

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spontaneous differentiation as EBs followed by culturing in the presence of various growth factors, differentiation in solely two-dimensional (2-D) environment, or coculture with the cell type of intended differentiation or different one, providing inductive stimuli through soluble and trophic factors (8–13).

The development of the periodontium is a part of tooth development, a process involving reciprocal inductive interactions between the ectoderm of the stomadeum and neural crest-derived ectomesenchyme, beginning at week 6 of human embryonic development and continuing until the completion of tooth eruption and root formation (14). The regeneration of the periodontal structures damaged by inflammatory or degenerative disorders affecting the periodontium involves cellular and molecular mechanisms closely resembling the developmental processes (15). Thus, it has been postulated that overcoming the limitations of periodontal regenerative procedures in many instances and successful implementation of tissue engineering approaches to achieve optimal regeneration can be possible on the basis of better understanding of cellular and molecular mechanisms of cell differentiation and tissue development, resulting in therapeutic strategies recapitulating these processes (16).

Application of periodontal tissue engineering for improved periodontal regeneration can involve cell-based, protein-based, or genetic engineering approaches with advantages and drawbacks of their own (16). Of these, the cell-based approach will require the ex vivo expansion of appropriate cells and their transplantation via different vehicles (17). However, the proliferation potential of human periodontal cells in vitro is limited (18), as is the case for most adult cells, including adult stem cells (19), thus posing considerable difficulties when large amounts of cells are needed. Given the recurrent nature of inflammatory periodontal disease and associated periodontal tissue destruction (16), the need for extensive cell quantities is obvious. With proven capacity of virtually unlimited proliferation in pluripotent state, while retaining the differentiation potential to cells of all germ layers (1), hESCs may become suitable cell types for successful cell-based periodontal tissue engineering applications.

The aim of this study was to investigate the differentiation patterns of hESCs under the influence of periodontal ligament cells in vitro, as well as the effects of coculture on directing differentiation of hESCs toward periodontal ligament progenitors, which could serve as an unlimited cell source for prospective periodontal tissue engineering-based therapies.

MATERIALS AND METHODS

Culture of hESCs and human periodontal ligament fibroblastic cells (hPDLFs)

hESC line (HUES-9) was a kind gift of Dr. D.A. Melton (Harvard University, Cambridge, MA, USA). The experiments were performed according to the institutional ethical committee approval. Isolation and characterization of the HUES-9 line was previously described; thus, the cells were expanded accordingly with minor modifications (20). Briefly, ~4×10^5 cells were thawed and seeded on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells in 6-well plates. The cells were cultured in KnockOut Dulbecco's modified Eagle's minimal essential medium (KO-DMEM)-containing 20% KnockOut Serum Replacement (KO-SR), 1% nonessential amino acid stock solution, 1-mM L-glutamine, 100-U/mL penicillin, 100-µg/mL streptomycin (all from Gibco, Invitrogen, Paisley, UK) and 8-ng/mL basic fibroblast growth factor (bFGF) (Sigma, St. Louis, MO, USA). Henceforth, this will be stated as the hESC-expansion medium (ES-EM). Resultant colonies with undifferentiated morphologies were mechanically passaged every 5th day, using a micropipette and were split 1:3.

hPDLFs were isolated, cultured, and expanded, essentially as described (21). The procedures were performed according to the institutional review board approval and informed consent was taken from the patients. Periodontal ligament tissue was obtained from the middle third of the roots of premolar teeth designated for extraction due to orthodontic treatment needs. The medium used for periodontal ligament fibroblastic cell (PDLF) expansion and culture was the same as described (21) and will be referred in the text as PDLF-expansion medium (PDLF-EM) unless otherwise indicated. Cells between the second to fifth passages obtained from four individuals were pooled and used in coculture experiments. All cultures were maintained in a CO_2 incubator at 37°C, 5% CO_2, 95% humidity.

Characterization of hESCs and hPDLFs

Morphological characterization of hESCs was performed under an inverted light microscope (Nikon TS 100, Nikon, Tokyo, Japan). To confirm the undifferentiated status of ES cells on MEF feeders after expansion and before differentiation experiments, tissue culture-treated coverslips were fixed at day 5 of passage in ice-cold methanol (Fluka, Buchs, Switzerland) and were examined immunohistochemically for pluripotency, using goat polyclonal antibody against

stage-specific embryonic antigen (SSEA)-4 (1:50) (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA).

Human PDLFs at confluent state were characterized immunohistochemically using the mouse monoclonal antibody against fibroblast surface protein (1:200) (Sigma). Species and isotype-specific secondary biotinylated rabbit antigoat IgG (1:100) (Zymed, San Francisco, CA, USA) and goat antimouse IgG (1:100) antibodies were used, respectively, to bind the primary antibodies. Enzymatic reaction was carried out with streptavidin horseradish peroxidase, and AEC chromogen (Lab Vision, Fremont, CA, USA) was used to develop red staining.

Transfection of hESCs with green fluorescent protein (GFP)-encoding plasmid

To be able to track the progeny of differentiating hESCs throughout the experimental period, hESCs were transfected with GFP-encoding plasmid (pQBI pgk, Quantum, Q-Biogene, Montreal, Canada) (22). Transfection procedure was carried out using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) as described with some modifications (23). Briefly, the expansion culture medium was removed from all the wells of the 6-well culture vessel containing hESCs on MEF feeders at day 3 following passage, and the cells were washed twice with KO-DMEM and kept in 400-μL KO-DMEM or ES-EM for 15 min in the incubator. Six microliters of FuGENE (Roche Diagnostics) was added to 97 μL of KO-DMEM in a sterile DNA-free 0.6-mL vial at room temperature and was stirred gently to allow for thorough mixing of the components for 5 min. A 20-microliter carrier containing 1 μg of plasmid DNA was added and mixed thoroughly, kept for 20 min at room temperature, then added to KO-DMEM- or ES-EM-containing wells of hESCs. After 6 h, ES-EM was added until reaching 3-mL/well volume. The whole medium was changed after 24 h and the cells were observed under fluorescent microscope (Eclipse C-SHG, Nikon) with excitation peak set at 488 nm and emission at 509-nm wavelength. The transfection percent on representative samples was determined for 24, 48, and 72 h after transfection, based on counting GFP-positive colonies under fluorescent microscope, and all hESC colonies in the same well under phase-contrast microscope. To obtain stably transfected cells maintaining the expression of GFP during experimental course, some GFP-positive colonies were mechanically picked up with a micropipette and transferred to nonresistant MEFs for antibiotic selection. The expansion culture of GFP+ hESCs was maintained in the presence of 600-μg/mL G418 (Sigma) for 14 days, and the cells were passaged every other day on newly prepared MEFs. Clones that had high levels of GFP expression by fluorescence microscopy were selected and used in the coculture experiments with PDLFs.

Coculture experiments

hPDLF cells at the second to fifth passages were dissociated using 0.05% trypsin/0.53-mM EDTA in Hank's balanced salt solution (Invitrogen) for 5 min at 37°C under 95% CO_2 and seeded on 26-mm tissue culture-treated Thermanox coverslips (Nalge Nunc International, Rochester, NY, USA), one in each well of 6-well tissue culture plates (Corning, Schiphol-Rijk, The Netherlands) at an approximate density of 5×10^4 cells/cm^2 and cultured in PDLF-EM until reaching confluence. Then, PDLF-EM was removed and the cells were washed three times with PBS, and 2 mL of ES-EM was added to the wells. GFP+ hESCs from passages 9–12, on 5th days after passage, were mechanically collected and separately transferred at approximately 20 colonies per well. Alternatively, hESC colonies were collected mechanically into 15-mL centrifuge tubes (Corning), washed three times with PBS, and trypsinized for 5 min at 37°C under 95% CO_2. Colonies were dissociated into single-cell suspension by gentle pipetting for 1–2 min with a micropipette. Cell suspension containing approximately 6×10^4 hESCs was added to hPDLFs containing 6-well plates at a concentration of approximately 10^4 cells/well. Two different media were used during the experimental period: (i) ES-EM containing 8-ng/mL bFGF and (ii) fetal calf serum (FCS)-containing differentiation medium (ES-DM), same as ES-EM except that 20% FCS was used instead of KO-SR and no bFGF was added. The experiments lasted for 21 days and the samples were processed for immunohistochemistry (IHC) at the end of weeks 1–3, as described further.

Morphological evaluation and determination of cell viability

Changes in cell morphology and colony shapes of hESCs during coculture with hPDLFs were evaluated under an inverted light microscope and photographed at days 7, 14, and 21. Cell viability at the beginning and at the end of the experiments was determined using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT)-based assay evaluating the mitochondrial dehydrogenase activity at days 1 and 21, as previously described (21).

Immunohistochemistry (IHC)

Three different areas in cell coculture specimens were evaluated for every marker: (i) hPDLF induc-

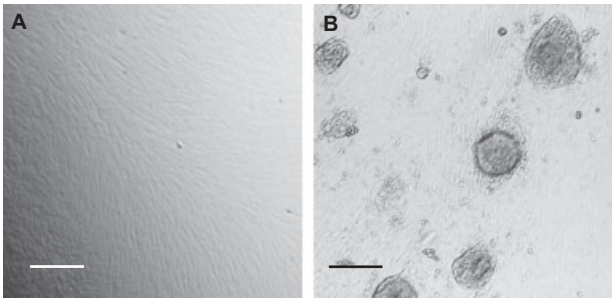

FIG. 1. Representative phase-contrast images of (A) confluent hPDLFs, and (B) undifferentiated hESC colonies on MEF feeder. Bars: 200 μm.

tive cells, (ii) 2-D monolayer surface of the hESC colonies, and (iii) 3-D cell clusters on the central part of the colonies. Because hESCs covered all the surface in single-cell groups, only monolayer areas of hESC were examined.

At the end of weeks 1–3, coverslips containing hESCs cultured on hPDLF monolayers were fixed in ice-cold methanol for 5 min, and then air-dried. The specimens were permeabilized with 0.01% Triton X (Sigma) for 30 min, washed three times with PBS, then endogenous peroxidase activity was blocked with peroxidase block (Zymed) for 10 min. To minimize nonspecific binding, 2% blocking serum containing PBS was applied for 10–15 min, and then the specimens were washed again with PBS and were incubated with primary antibodies for 1 h at room temperature, or overnight at 4°C. Immunohistochemical detection of differentiation markers was carried out using goat polyclonal anticollagen type I IgG (1:100) (Santa Cruz Biotech., Inc.), mouse monoclonal anticollagen type III IgG1 (1:50), mouse monoclonal antifibronectin IgM (1:100), mouse monoclonal antifibroblast surface protein IgM (1:100), mouse monoclonal antivimentin IgM (1:100) (all from Sigma), and mouse monoclonal antipan cytokeratin IgG1 (1:100) (Santa Cruz Biotech., Inc.) primary antibodies. Species and isotype-specific secondary biotinylated goat antimouse IgG (1:100), goat antimouse IgM (1:100), and rabbit antigoat IgG (1:100) (Zymed) antibodies were used to bind the primary antibodies. Enzymatic reaction was carried out with streptavidin horseradish peroxidase, and AEC (Lab Vision) chromogen was used to develop red staining. The samples were visualized under a Leica DM 4000B model digital microscope (Wetzlar, Germany).

Immunomorphometric analysis and statistics

Morphometric analyses were performed on areas of hESC colonies in each group for every marker. Images were processed and analyzed by using the Leica Q Win Plus (Leica, Wetzlar, Germany) software. Six measurements were performed on randomly selected areas on each specimen. Data were expressed as the mean ± SD from three independent experiments. One-way analysis of variance was used to determine the differences at time points inside groups, followed by Bonferroni correction for multiple comparisons, and unpaired Student's *t*-test was carried out to establish the difference for indicated parameters between groups. A value of $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Morphology and phenotype of hPDLFs and undifferentiated hESCs

Confluent PDLF cell monolayers demonstrated typical fibroblastic morphology, and varying directions of the spindle-shaped cells could be easily observed under an inverted microscope (Fig. 1A). Periodontal ligament fibroblasts demonstrated strong expression of fibroblast surface protein. Undifferentiated hESCs consisted of compact colonies, exhibiting clearly delineated borders from the feeder MEFs (Fig. 1B). Positive staining for anti-SSEA-4 was associated with the undifferentiated hESC phenotype. SSEA-4 was not detected in PDLF cells, while undifferentiated hESCs did not express any of the differentiation-associated markers used at the indicated time points in the experiments (not shown).

Transfection of hESCs with GFP-encoding plasmid

The initial transfection efficiency of undifferentiated hESCs varied between 4.84 ± 0.69% and 6.5 ± 1.97% with no statistically significant difference between KO-DMEM and ES-EM groups (Table 1). After the selection period of 14 days, ~75–80% of hESC colonies were GFP positive and the cells maintained their fluorescent expression during the 3-week experimental period.

TABLE 1. *Transfection percent of hESCs with GFP-encoding pQBI pgk plasmid in KO-DMEM and ES-EM medium conditions*

	% GFP transfection*		
	24 h	48 h	72 h
KO-DMEM	6.22 ± 2.2	4.93 ± 3.31	5.80 ± 1.56
ES-EM	4.84 ± 0.69	6.50 ± 1.97	5.88 ± 0.84

*Data expressed as the mean ± SD from three independent experiments. No statistical significant difference between the groups; $P > 0.05$.

Differentiation experiments

The change in hESC colony morphology during coculture with hPDLF cells until the end of the experiments can be seen in Fig. 2. The overall appearance of hESCs was considerably different depending on whether the cells were plated as colonies or as single-cell suspension (Fig. 2). At the end of week 1, hESC colonies consisted of cells with morphologies similar to the cells in undifferentiated state. However, colony shapes were transformed from a round compact to a prolonged one in some directions. External colony boundaries seemed to be influenced by the PDLF cells in a way resembling periodontal fibroblasts in confluence, with cells paralleling in the borders (Fig. 2A). During week 2, hESC colonies expanded further and made contact with each other at some dense areas. Between much of the closely located colonies, there remained stripes of underlying hPDLF cell surfaces. In contrast with week 1 when hESC colonies predominantly retained their flat appearance, now colonies exhibited 3-D protuberances mostly in the central parts. The borders of colonies were still largely defined, with occasional fractions of cells at the most peripheral parts acquiring fibroblast-like appearance and intermingling with the PDLFs (Fig. 2B). At the end of the experimental period (week 3), hES cell colonies had covered most of the surface area, being in contact at multiple areas. Still, borders with hPDLF could be seen, both in large swaths, and as prolonged incursion appearances of PDLF cells between hESC areas. Most of the colonies developed 3-D areas with representative cells of multiple lineages. There were areas on the colony borders with cells having fibroblast-like morphology, being intermingled, and with not readily distinguishable origins (Fig. 2C). Meanwhile, hES single cells covered the majority of the surface area on the coverslips, exhibiting more diffuse expansion at the end of week 1 compared with the hES cell colonies at the same time point. The general appearance of the cells was different from the undifferentiated hESCs. At the end of week 2, most of the cells had covered the surface area of the culture dish. The same appearance was evident at week 3, with enhanced areas of 3-D cell clusters throughout the hESC culture surface (Fig. 2D). MTT tests demonstrated overall cell viability above 95% with no statistically significant difference between the groups (data not given).

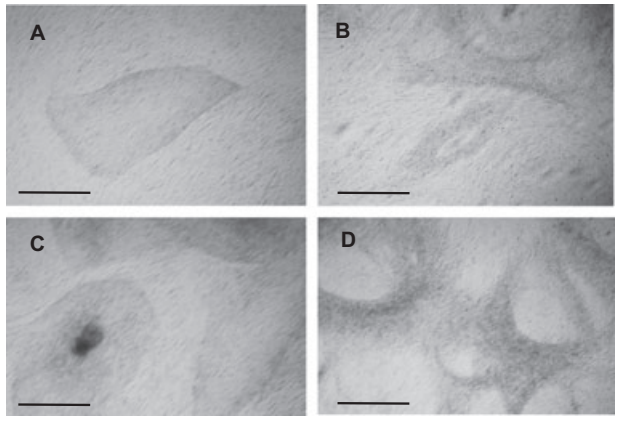

FIG. 2. Representative phase-contrast micrographs demonstrating the morphological changes in hESCs in colony (A: week 1, B: week 2, C: week 3), and in single cell (D: week 3) culture. Bars: 200 μm.

IHC and immunohistomorphometry

Figure 3 demonstrates the coexpression of GFP and intermediate filament protein vimentin in differentiated hESCs at day 21. As can be seen from Fig. 3A, differentiating hES cell clusters are positively stained for vimentin; these cells express GFP at the same time (Fig. 3B). The merged image confirms the origin of the differentiating vimentin-expressing cells (Fig. 3C).

Representative micrographs of the immunohistochemical stainings are presented in Fig. 4. The percent of positively stained areas for all markers in each group and for three time points was determined by using the Leica Q Win Plus software, and graphical representation of the results are presented in Fig. 5.

Collagen type I expression was widespread in the hPDLF layers, reflecting the well-known feature of human periodontal ligament fibroblasts, whose extracellular matrix (ECM) in the periodontium is composed primarily of collagen type I. The expression in

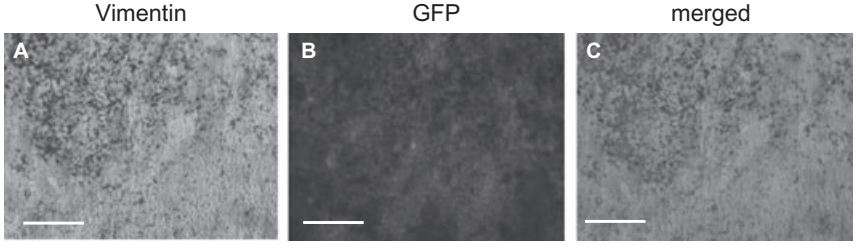

FIG. 3. Representative micrographs of (A) antivimentin staining and (B) GFP expression of differentiated hESCs cocultured with hPDLFs at day 21. C is the merged image of A and B. Bars: 100 μm.

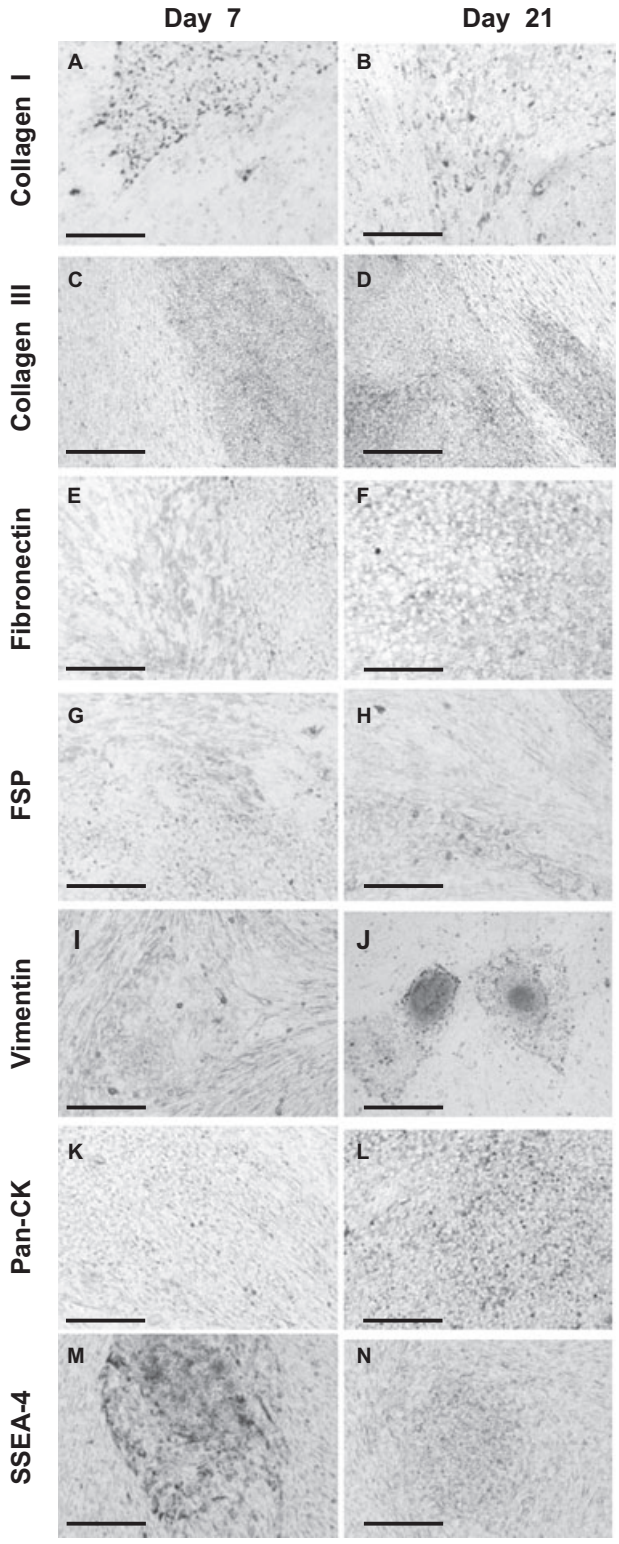

FIG. 4. Immunohistochemical stainings of hESCs cultured on human periodontal ligament fibroblasts. Anticollagen type I: (A) week 1—colony, (B) week 3—colony; anticollagen type III: (C) week 1—colony, (D) week 3—colony; antifibronectin: (E) week 1—colony, (F) week 3—colony; antifibroblast surface protein: (G) week 1—colony, (H) week 3—colony; antivimentin: (I) week 1—colony, (J) week 3—colony; antipancytokeratin: (K) week 1—colony, (L) week 3—single cell; anti-SSEA-4: (M) week 1—colony, (N) week 3—colony. Bars: 200 μm.

monolayers of hESCs in both culture conditions was weaker than central parts of the colonies where cells formed 3-D clusters, with up to two times greater staining percent (Figs. 4A,B and 5A). While the increase in 2-D parts of hESC colonies over time was also insignificant, there was significant increase at the central 3-D areas. The level of expression was lesser in single-cell cultures, also increasing with time (Fig. 5A).

Collagen type III staining was virtually absent in hPDLF cells, as well as in embryonic stem cell colonies in all specimens (Fig. 4C,D). The same was not true for the single-cell groups, where expression increased to 11.1 ± 4.78% in ES-DM at day 14, and 11.0 ± 2.86% and 21.8 ± 3.73% for ES-EM and ES-DM, respectively, at day 21 (Fig. 5B).

As an important extracellular adhesion molecule in connective tissues, fibronectin expression was uniform in hPDLF cells with staining more prominent in fibroblastic cells maintained in the ES-DM than in the ES-EM groups (Figs. 4E,F and 5C). Expression was weak at the first 2 weeks in both groups of hESCs, being slightly more pronounced in cell clusters at the colony centers (Fig. 5C). Significant elevation could be observed at the end of week 3 in all specimens, with ES-DM groups exhibiting higher levels of staining compared with their ES-EM counterparts (Fig. 5C). Single-cell cultures displayed similar patterns of increase (Fig. 5C).

Staining for fibroblast surface protein was widespread in all PDLF areas, while it remained low in embryonic stem cells (Fig. 4G,H). The increase in 2-D and 3-D areas of the colonies in ES-EM was statistically insignificant throughout the experimental period, while significant increase occurred in the ES-DM group in both areas, with elevated levels from week 2 remaining similar in week 3 (Fig. 5D). Single cells also demonstrated significant increase only in the ES-DM group (Fig. 5D).

Vimentin is an intermediate filament protein characteristic to early mesodermal differentiation, and was not detected in PDLF cells. hESCs also barely expressed it at day 7, notably on the colony peripheries (Fig. 4I). However, significant increase could be seen at the end of week 2 and remained similar at day 21 (Fig. 5E).

In order to investigate a possible ectodermal induction effect of hPDLFs upon hESCs, causing their differentiation to proceed toward epithelial

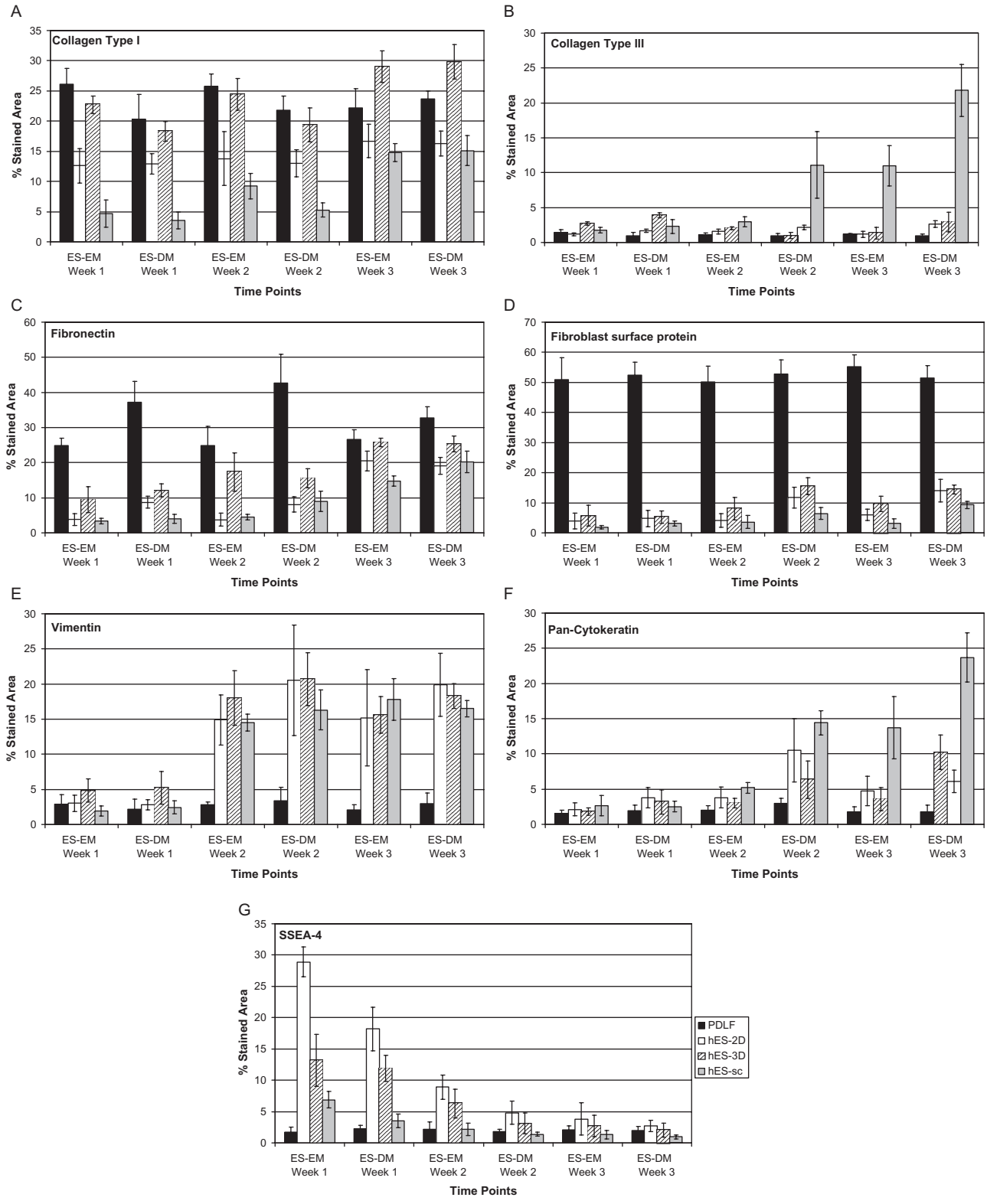

FIG. 5. Immunomorphometric data retrieved from the hESC colonies and single cells (sc) cultured on hPDLFs. (A) Anticollagen type I, (B) anticollagen type III, (C) antifibronectin, (D) antifibroblast surface protein, (E) antivimentin, (F) antipan cytokeratin, (G) anti-SSEA-4 stainings ($n = 6$ for each data point, mean $\pm$ SD; statistical significance: $P \leq 0.05$).

lineage rather than into mesodermal one, we employed pancytokeratin primary antibody, specific to all keratin types. The expression was absent in both hPDLFs and hESCs, in all groups at the end of week 1 (Figs. 4K and 5F). However, some up-regulation in monolayers of hESC colonies in ES-DM groups were observed at weeks 2 and 3 with similar levels of staining (Fig. 5F). Meanwhile, single-cell hESCs displayed increase for that marker at week 2 in the ES-DM group, and further at week 3 in both groups (Figs. 4L and 5F).

As the highly specific marker for pluripotent hESCs, SSEA-4 was strongly expressed in undifferentiated hESCs before experiments (Fig. 4M). The expression was highest in the 2-D areas of hESC colonies in ES-EM at week 1 with 28.9 ± 2.4%, followed by the 18.2 ± 3.48% in the corresponding ES-DM specimens (Fig. 5G). It sharply decreased in the following time points (Fig. 4N), was weaker in the 3-D areas of colonies, and nearly undetectable in single-cell groups (Fig. 5G).

DISCUSSION

hESCs are pluripotent cells with unlimited proliferative potential, obtained from the inner cell mass of preimplantation-stage blastocysts (2). As long as they are maintained in undifferentiated state, hESC lines possess characteristics typical to the parental cells of origin at the corresponding developmental stage (4,7). Because cellular differentiation is a natural process during embryonic development, contribution of mouse embryonic stem cells to all tissues in created chimeras and also hESC differentiation into cells from three germ layers both in EBs and formed teratomas in severe combined immunodeficiency (SCID) mice reflect the propensity of embryonic stem cells (ESCs) to differentiate in every life-supporting environment other than in optimal conditions for undifferentiated embryonic stem cell expansion culture (1,7). However, embryonic stem cell differentiation begins spontaneously and has a stochastic pattern of development when cells are allowed to aggregate in 3-D EB structures, resulting in a mix of heterogeneous cells representing all three embryonic germ layers (2). Directing the differentiation to a particular cell type is especially important when studying the development of specific lineage or cell-based tissue engineering therapy development is considered. hESCs were successfully directed to differentiate into lineages of ectodermal, mesodermal, and endodermal origin through various growth factors and coculture with different cell types (8–13). Interestingly, fibroblast-like cells with confirmed fibroblastic phenotype according to specific marker expression have been obtained as a result of spontaneous differentiation of hESCs and have been used as an autogenic feeder cell system for maintaining the undifferentiated growth of the parental cell line (24). Similar cells have been differentiated from another hESC line and used as media conditioners after immortalization through human telomerase reverse transcriptase (hTERT) transfection. Additionally, these HEF1-hTERT cells had the capacity to differentiate into cells of osteogenic lineage, further proving their mesodermal characteristics (25). As principal cells in connective tissues, fibroblasts have widespread distribution in mammalian organisms, fulfilling specific functions according to the requirements of the particular localization. This renders them distinct characteristics at genetic expression and protein synthesis level, despite morphological similarity in appearance (15). Because cementoblasts, periodontal ligament fibroblasts, and osteoblasts in the alveolar bone have common developmental ancestry, the ectomesenchyme of neural crest origin possessing the capability to develop into both soft and hard connective tissues, our goal was to investigate the effects of coculture with hPDLFs on the differentiation of hESCs particularly into mesenchymal lineage and periodontal ligament progenitor-like cells. We have investigated the differentiation of hESCs in two different culture media and when plated as colonies as well as single cells, thus seeking to gain further insight into the effects of cell–cell interaction and integrity of colonies to the developmental behavior of the cells. Given that tooth development is a process involving reciprocal inductive interactions between the stomadeal ectoderm and ectomesenchyme of the jaws (14), pancytokeratin expression as a marker of epithelial cells has also been investigated along with the expression of fibrogenic and mesenchymal markers, to determine the possible effects of coculture with hPDLF on ectodermal and particularly epithelial differentiation of ESCs. Rapid down-regulation of SSEA-4, specific marker for undifferentiated hESCs, in all groups during coculture at the end of week 1 indicates that differentiation process at this time point leaves the minority of hESCs undifferentiated. The most prominent expression remained in colonies cultured in ES-EM with 28.9 ± 2.4% followed by 18.2 ± 3.48% in ES-DM. However, when hESCs were plated as single cells, the expression levels of SSEA-4 for ES-EM and ES-DM at day 7 were 6.9 ± 1.31% and 3.5 ± 1.08%, respectively, confirming that cellular interactions have an important role during hESC expansion and differentiation, and in line with the

observations that dissociating into single cells quickly triggers embryonic stem cell differentiation (26).

Collagen type I is the principal type of collagen and is the most abundant protein in the ECM of periodontal ligament, accounting for up to 90% of all collagen content in the area, while collagen type III is predominant during wound healing and regeneration processes (15). The expression of both fibrogenic markers during embryonic stem cell differentiation demonstrated different characteristics. Collagen type I staining was nearly twofold greater in 3-D central cell clusters in hESC colonies than in monolayer parts; however, there was no statistically significant difference between time points. Expression seems unaffected by the culture medium difference, but it was lower in single-cell cultures, gradually increasing and reaching the levels comparable with monolayer areas of colonies at the end of the experimental period. In contrast, collagen type III was nearly absent in both PDLF and embryonic stem cell colonies in all specimens, except in single-cell groups where prominent increase could be noted in the ES-DM group at the end of week 2 and in both groups at week 3. The expression pattern of these two markers seems to be influenced rather by cellular contacts than by medium constituents, and early expression could imply that they may have a wider role in embryonic development and early differentiation, not limited to the ECM composition of fibroblast-like cells. Fibronectin is an important extracellular adhesion protein in connective tissues, and its expression was elevated during coculture in hESC colonies as well as in single-cell cultures. A similar pattern of increase was observed in ES-EM and ES-DM groups, with increase beginning earlier in 3-D areas of colonies and in the ES-DM group in single cells. Along with abundant staining in hPDLFs, the expression of fibroblast surface protein remained low in hESCs, with slight increase in ES-DM groups only after week 2. At the end of week 1, overall staining in hESC colonies for intermediate filament protein vimentin remained low at day 7, but increased significantly at the following time points. Prominent staining at week 1 appeared on the colony borders, where ESCs were in close contact with PDLFs. This is the area where ESCs first changed their characteristic morphology and acquired fibroblast-like appearance probably as a result of earlier differentiation induced by the proximity of periodontal ligament fibroblasts. However, there is a possibility that these mesodermal lineage cells have developed as a result of cell fusion between neighboring PDLFs and embryonic stem cells, as has been demonstrated by Yu et al. for hESCs and hESC-derived myeloid precursors, where the former had a dedifferentiation effect on the latter, with no loss in redifferentiation capacity of tetraploid hybrids after aggregation in EBs (27). A work by Driesen et al. also demonstrated cell fusion through functional gap junctions and partial cell fusion mediated by intercytoplasmic contacts between fibroblasts and neonatal cardiomyocytes after coculture of the two cell types in vitro, where fibroblasts induced cardiomyocytes to dedifferentiate (28). It was not in the scope of this study; however, there is a need for further work to determine whether cell fusion, as well as dedifferentiation in PDLFs, takes place in areas where hESCs are in direct contact with periodontal ligament fibroblasts.

ESCs mainly remained as colonies during the whole experimental period, whose borders were clearly distinguishable at the end of week 1 and gradually disappearing, particularly in the ES-DM groups. When hESCs were plated as single cells, they rapidly proliferated and covered the entire PDLF surface even at day 7. Plated as colonies, 3-D cell clusters developed at the central parts with different expression patterns in most of the studied markers. This may have happened as a result of the different cell proliferation rates inside the colonies, either due to migration of slowly proliferating cells toward the periphery or need for time to second replication of the daughter cells in these areas (29). Heng et al. have reported such a difference in cell proliferation rates between central and peripheral parts of hESC colonies cultured on mitotically inactivated MEF feeders (29). Unlike MEFs, hPDLF cell layers used during coculture were confluent and with established ECM, which could further limit the expansion of hESC colonies and explain partially the change in colony shapes, losing spherical appearance and acquiring fusiform extensions in various parts. Along with the expected induction of hESCs into fibroblastic lineage of mesodermal origin, pancytokeratin expression was investigated to determine possible induction toward ectodermal lineage and particularly epithelial cells. There was a marked increase of this marker predominantly in hESCs plated as single cells and to a lesser degree in 2-D areas of colonies, while nearly the absence in 3-D cell clusters implies that monolayers may favor epithelial-like cell development to a certain degree.

CONCLUSIONS

Overall, the results indicate feasibility to direct differentiation of hESCs into periodontal ligament progenitor-like cells in some areas of hESC colonies and also single-cell layers through coculture with periodontal ligament fibroblasts in vitro. Cell-to-cell contacts and trophic factors secreted by hPDLFs may

exert the stimuli favoring specific differentiation programs in undifferentiated hESCs. Moreover, it seems that medium constituents and different intercellular relations in colonies and in single cells also have an important influence on the fibrogenic, epithelial, and stem cell-specific marker expression patterns. Multiple steps of inductive influences in longer culture periods, as well as selection of the desired cell types, could result in a more homogenous population of specifically differentiated cells of intended lineage. Findings support the notion that hESCs may become an unlimited cell source for prospective cell-based periodontal tissue engineering applications and a suitable model for gaining further insight into the earliest differentiation events during tooth and periodontium development.

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REFERENCES

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145–7.
2. Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* 2001;19:193–204.
3. Elçin YM. *Tissue Engineering, Stem Cells and Gene Therapies, Advances in Experimental Medicine and Biology (AEMB) Series, Vol. 534*. New York, Boston, Dordrecht, London and Moscow: Kluwer Academic/Plenum Publishers, 2003.
4. Bhattacharya B, Cai J, Luo Y, et al. Comparison of gene expression profiles of undifferentiated human embryonic stem cell lines and differentiating embryoid bodies. *BMC Dev Biol* 2005;5:22–7.
5. Baharvand H, Hajheidari M, Ashtiani SK, Salekdeh GH. Proteomic signature of human embryonic stem cells. *Proteomics* 2006;6:3544–9.
6. Armstrong L, Hughes O, Yung S, et al. The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. *Hum Mol Genet* 2006;15:1894–913.
7. Weitzer G. Embryonic stem cell-derived embryoid bodies: an in vitro model of eutherian pregastrulation development and early gastrulation. *Handb Exp Pharmacol* 2006;174:21–51.
8. Schulz TC, Noggle SA, Palmarini GM, et al. Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem Cells* 2004;22:1218–38.
9. Ji L, Allen-Hoffmann BL, de Pablo JJ, Palecek SP. Generation and differentiation of human embryonic stem cell-derived keratinocyte precursors. *Tissue Eng* 2006;12:665–79.
10. Wei H, Juhasz O, Li J, Tarasova YS, Boheler KR. Embryonic stem cells and cardiomyocyte differentiation: phenotypic and molecular analyses. *J Cell Mol Med* 2005;9:804–17.
11. Qiu C, Hanson E, Olivier E, et al. Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development. *Exp Hematol* 2005;33: 1450–8.
12. Bielby RC, Boccaccini AR, Polak JM, Buttery LD. In vitro differentiation and in vivo mineralization of osteogenic cells derived from human embryonic stem cells. *Tissue Eng* 2004; 10:1518–25.
13. Xu X, Kahan B, Forgianni A, et al. Endoderm and pancreatic islet lineage differentiation from human embryonic stem cells. *Cloning Stem Cells* 2006;8:96–107.
14. Miletich I, Sharpe PT. Neural crest contribution to mammalian tooth formation. *Birth Defects Res C Embryo and Today* 2004; 72:200–12.
15. Polimeni G, Xiropaidis AV, Wikesjo UM. Biology and principles of periodontal wound healing/regeneration. *Periodontol 2000* 2006;41:30–47.
16. Nakahara T. A review of new developments in tissue engineering therapy for periodontitis. *Dent Clin North Am* 2006;50: 265–76.
17. Bartold PM, Xiao Y, Lyngstaadas SP, Paine ML, Snead ML. Principles and applications of cell delivery systems for periodontal regeneration. *Periodontol 2000* 2006;41:123–35.
18. Sawa Y, Phillips A, Hollard J, Yoshida S, Braithwaite MW. The in vitro life-span of human periodontal ligament fibroblasts. *Tissue Cell* 2000;32:163–70.
19. Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 2006;7:14–21.
20. Cowan CA, Klimanskaya I, McMahon J, et al. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004;350:1353–6.
21. Inanç B, Elçin AE, Elçin YM. Osteogenic differentiation of human periodontal ligament fibroblasts under two- and three-dimensional culture conditions. *Tissue Eng* 2006;12: 257–66.
22. Lakhshmipathy U, Pelacho B, Sudo K, et al. Efficient transfection of embryonic and adult stem cells. *Stem Cells* 2004;22: 531–43.
23. Liu YP, Dovzhenko OV, Garthwaite MA, et al. Maintenance of pluripotency in human embryonic stem cells stably overexpressing enhanced green fluorescent protein. *Stem Cells Dev* 2004;13:636–45.
24. Stojkovic P, Lako M, Stewart R, et al. An autogeneic feeder cell system that efficiently supports growth of undifferentiated human embryonic stem cells. *Stem Cells* 2005;23:306–14.
25. Xu C, Jiang J, Sottile V, McWhir J, Lebkowski J, Carpenter MK. Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. *Stem Cells* 2004;22:972–80.
26. Amit M, Carpenter MK, Inokuma MS, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 2000;227:271–8.
27. Yu J, Vodyanik MA, He P, Slukvin II, Thomson JA. Human embryonic stem cells reprogram myeloid precursors following cell-cell fusion. *Stem Cells* 2006;24:168–76.
28. Driesen RB, Dispersyn GD, Verheyen FK, et al. Partial cell fusion: a newly recognized type of communication between dedifferentiating cardiomyocytes and fibroblasts. *Cardiovasc Res* 2005;68:37–46.
29. Heng BC, Cao T, Liu H, Rufaihah AJ. Reduced mitotic activity at the periphery of human embryonic stem cell colonies cultured in vitro with mitotically-inactivated murine embryonic fibroblast feeder cells. *Cell Biochem Funct* 2005;23: 141–6.