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Establishment of gene expression profiling database from human periodontal ligament.

Eisaku Nishida[1,2,3], Masahiro Saito[1,2], Takamasa Yokoi[1,2,3], Takanori Tsubakimoto[1,2], Kazutaka Kosaka[1,2], Makoto Aino[1,2,3] and Toshio Teranaka[1]

Department of Operative Dentistry and Endodontics, Kanagawa Dental College[1]
Oral Health Science Research Center[2]
Department of Periodontology, School of Dentistry, Aichi-Gakuin University[3]

ABSTRACT

Development of periodontal ligament (PDL) occurred as a series of events that requires temporal and spatial expression of numerous genes regulated differentiation of dental follicle (DF) cells. Nevertheless the genes required for PDL development have not been identified yet. To solve this problem, we used in silico approach with a human PDL database of expressed sequence tags (ESTs) named "periome" database. ESTs were obtained by partially sequencing of 5' end of 10,000 cDNA clones from human PDL cDNA library, and identified 4,384 unique EST clusters. 617 EST clusters appearing more than 3 times were collected, and classified by functional annotation to produce periome database. From periome database, we screened DF specific EST clusters by *in situ* hybridization. The result showed that *SPARC like 1, Nidogen 1, Spondin 1* specifically expressed in DF. From these findings, periome database was successfully established, and this database provides a transcriptome resource for analyzing gene clusters involved in PDL development.

Keyword: Periodontal ligament t / ESTs / cDNA library / Expression profiling

INTRODUCTION

Periodontium is a tooth supporting tissue composed of periodontal ligament (PDL), cementum, and alveolar bone. Among these, PDL plays an central role for stress- breaking capability towards occlusal force, but also acts as the sensory organ that stimulates the central nerve system(1). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, it has not yet been possible to reliably form PDL (2). Hence, there is considerable interest in the developmental mechanisms of PDL.

PDL cell is originated from the dental follicle (DF) cells that derived from neural crest derived the ectomessenchymal cells(3). After the formation of tooth root dentin, progenitor presented in DF cells migrated onto the tooth root surface to be differentiated into cementoblast. Almost at a simultaneous period, PDL progenitors within DF cells lead to differentiate into PDL cells for inserting fibers into cementum matrix, known as sharpy's fiber. Fiber insertion also occurred along the lining alveolar bone osteoblast(4). Finally both bone-derived and PDL cells derived fiber are coalescing in the PDL to form the intermediate plexus, like tendinous tissue. Each of these stages during transition of dental follicle cells to PDL cells is characterized by specific temporal and spatial gene expression patterns. It is important to clarify the cascade of these genes regulating PDL development. However most of these developmental processes are not fully understood and genes involved are only partly known.

Expressed sequence tags (ESTs), short single-pas sequence reads of randomly selected clones from cDN library, constitute a valuable source to identify the specifi genes in tissue of interest(5). Consequently, attempts a made to establish expression profiling of PDL by ES sequencing. Recently EST profile has been created fro 1,752 cDNA sequences of human PDL cDNA library, a *PLAP-1* (periodontal ligament associated protein-1) *Asporin*, a novel extracellular matrix (ECM) that involve PDL formation was identified from the database. Thus, t PDL EST dataset provide useful information f investigating the genes related to PDL formation. Howeve the dataset were comparatively small information due to t ESTs was analyzed before the human genome draft completed.

In the present study we described the expression profilir obtained through evaluation of 4,384 ESTs generated fro human PDL cDNA library. Since ECM involved in t organization of tissue specificity(6), we have focused ESTs that classified as ECM for the screening of gen involved in the PDL development.

MATERIALS AND METHODS

Construction of human PDL EST library.

After signed informed consent was obtained, third mol tooth were harvested with approval (approval number 18) Kanagawa dental college hospital. Total RNA is extracted

ISOGEN (NIPPON GENE) from PDL tissue isolated from extracted teeth. Then, mRNA was extracted from total RNA with MACS (Mitenyi Biotec). From provided mRNA, we synthesized cDNA following Superscript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Invitrogen) with some modifications. The resulting cDNAs were size- fractionated through as agarose gel electrophoresis, and cDNA fragments longer than 1kbp were extracted from the gel. The cDNA fragments then were inserted into pBluescriptSK (-) (Stratagene). The ligated DNA was electroporated into DH10B- competent cells by using E-coli Pulser (Bio-Rad). Plasmid DNA was purified after the culture of transformed bacteria for 16 hours.

DNA Sequencing and sequence data analysis.

The plasmids were amplified using TempliPhi DNA amplification kit (Amasham), and M13 primer. The 5' end of cDNA clones sequenced. Then, a total of about 10,000 cDNA sequences from human PDL library were analyzed. We obtained the ESTs information using the BLAST network client to access BLAST at the NCBI.

Probes for *in situ* hybridization.

The mouse homologue of the candidate ECM gene clusters were searched from homologene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=homologene) respectively. The cRNA probes were generated from the mouse homologue genes that were amplified by PCR using mouse specific primer: *SPARC like 1* (5'- AAT GAA CTG GAC CAG CAT CC -3' and 5'- AAA CGC AGC TGC ACA GAG TG -3'), *Nidogen 1* (5'- ACG TCA TGG GAA TCT TCA GC -3' and 5'- TGC AAA CCG AAC TTC TGA TG-3') and *Spondin-1* (5'- AGA CGG TCT ACT GGG CAC TG -3' and 5'- TGC AAA AGG ATG TGG TGG TA -3') Mouse fragments were cloned into pCR-4 TOPO vector (Invitrogen). The plasmids were linearized by *Not* I (antisense) and *Spe* I (sense). To generate antisense and sense transcripts, digoxygenin - labeled riboprobes were prepared as described previously(7) using T7 or T3 RNA polymerase.

In situ hybridization analysis on sections.

For the expression analysis on sections, the C57BL mouse head at embryonic (E) 13 days, E15, E17 and postnatal (P) 1 day were immediately frozen after embedded in OCT compound (Sakura Finetechnical Co.) and 10 μm frontal sections were prepared. *In situ* hybridization was performed as described with some modifications(8). BCIP (Boeringer Mannheim) was used for immunodetection of digoxygenin.

RESULTS

Sequence and analysis of ESTs.

In order to analyze the expression profile in human PDL cDNA library, a total of 11,520 clones were randomly selected, and sequenced from 5' ends. Among the total of 11,520 clones, sequenced clones were 9,600 (83.3%), in addition there were 255 no insert clones, and 1,665 clones were poor quality. The sequenced clones were annotated by BLAST at NCBI.

A total of 9,600 clones from human PDL library were sequenced. Of these analyses these were grouped into 4,384 clusters. In the detail of the expression frequency, 20% clusters were distributed the expression frequency of more than 3 times. Out of these 617 clusters, 481 (78%) were known function clusters. Interestingly, the 101 (16%) were unknown function clusters, and 35 (6%) were unknown transcripts.

Functional annotation of the known function clusters.

617 kinds of clusters which classified more than frequency 3 times, were collected and classified into 12 categories according to their appropriate function(9). Among the known function clusters, 481(78%) were known function clusters shown by Genbank. The remaining 101(16%) were unknown function clusters, and 35(6%) were unknown transcripts. 107(17%) clusters were secreting molecules including 39(6%) extracellular matrix, 44(7%) plasma membrane, 12(2%) proteases and protease inhibitors, 12 (2%) signaling molecules.

Expression patterning of the extracelluar matrix genes.

The ECM has been shown to play an important role in the cellular differentiation and the maintenance of the organs. (6). Thus, we screened the ESTs that specifically expressed in DF from ECM category by *in situ* hybridization. As a result, *SPARC like 1, Nidigen1, Spondin 1* found to express intensely in the DF cells. Expression pattern of these three genes during tooth morphogenesis showed that they initiate to express in DF cells of tooth germ at E15 cap stage, and became intensely expressed at P1 late bell stage.

DISCUSSION

In this study, periome database has successfully established in silico analysis revealed that periome database reflected to human PDL phenotype according to EST expression frequency within ECM category. Among these, *SPARC like 1, Nidogen 1, Spondin 1* intensely expressed during DF development, suggesting that they could serve as a marker for DF cells.

The gene expression profile was analyzed by three different parameters, frequency of gene expression, functional annotation and specific temporal and spatical gene expression patterns. Based on the top 20 ESTs within ECM list, highly expression of *Col I*,*Col III*, *SPARC*, *Periostin* and *PLAP-1 / Asporin* were observed. These genes have an important role for PDL biology. *Col I* and *Col III* are the most abundant gene in PDL(10). *SPARC* is observed strongly in the PDL(11). *Periostin* is also abundant ECM from PDL(12). *PLAP-1 / Asporin* belongs to the small leucine-rich repeat proteoglycan family, and it has been shown that it was highly expressed in the PDL(9). Periome database was similar to that of previously reported human PDL EST(9). From these findings periome database reflects human PDL phenotype. Interestingly 16% of genes are unknown function genes. This may be the reason why

developmental mechanisms of PDL have been unclear for long time.

In situ hybridization screening showed that *SPARC like 1*, *Nidogen 1*, *Spondin 1* intensely expressed in DF. *SPARC like 1* is down-regulated in many cancers and is a negative regulator of cell growth and proliferation(13). *Nidigen 1* is a basement membrane component, homozygous mutants often displayed seizure - like symptoms and loss of muscle control in the hind legs(14). *Spondin 1* is a secreted signaling molecule implicated in neuronal development and repair, binds to the conserved central extracellular domain of APP and inhibits -secretase cleavage of APP(15).

As described above, no report was found about relationship between these genes and PDL formation. On the other hand, they expressed in dermal papilla which require epithelial-mesenchymal interaction for development. These finding suggested that epithelial- mesenchymal interaction could involve in the formation of PDL (16).

In summary, we have established the periome database that provides a transcriptome resource for analyzing gene clusters involved in PDL development, and found specific ECM clusters such as *spondin-1* and *tenascin-N* that could serve as a marker for DF or PDL, respectively. Periome database has various information of human PDL, and novel molecules responsible for human PDL formation may be contained this database.

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Establishment of dental follicle cells culture system that generating periodontal ligament in vivo

Kazutaka Kosaka[1,2], Takamasa Yokoi[1,2,3], Masahiro Saito[1,2], Eisaku Nishida[1,2,3], Takanori Tsubakimoto[1,2], Makoto Aino[1,2,3], Toshio Teranaka[1]

Department of Operative Dentistry and Endodontics, Kanagawa Dental College[1]
Oral Health Science Research Center, Kanagawa Dental College[2]
Department of Periodontology, School of Dentistry, Aichi-gakuin University[3]

ABSTRACT

The dental follicle is a mesechymal tissue that surrounds the developing tooth germ. During tooth root formation, periodontal components (cementum, periodontal ligament [PDL] and alveolar bone) are created by dental follicle progenitors. Here, we report the presence of PDL progenitors in mice dental follicle (MDF) cells. MDF cells were obtained from mice incisor tooth germs, and immortalized by expression of a mutant human papilloma virus type 16 *E6* gene lacking the PDZ domain-binding motif. MDF cells expressing mutant *E6* gene ($MDF^{E6\text{-}EGFP}$ cells) had an extended life span, beyond 150 population doublings (PD). In contrast, normal MDF cells failed to proliferate beyond 10 PD. $MDF^{E6\text{-}EGFP}$ cells expressed tendon/ligament phenotype-related genes such as *Scleraxis (Scx)*, *growth and differentiation factor (GDF)-5*, *EphA4*, *Six-1* and *type I collagen*. In addition, expression of PDL-specific gene, such as *periostin*, was observed. To elucidate the differentiation capacity of $MDF^{E6\text{-}EGFP}$ cells *in vivo*, cells were transplanted into severe combined immunodeficiency (SCID) mice. At 4 weeks, $MDF^{E6\text{-}EGFP}$ cell transplants had the capacity to generate a PDL-like tissue with strong expression of *periostin*, and fibrillar assembly of type I collagen. Our findings suggest that PDL progenitors are present in $MDF^{E6\text{-}EGFP}$cells, and these cells may provide useful information for the formation of PDL for research purposes and for the development of regeneration therapies.

Keyword: Dental follicle / Regeneration / periodontal ligament Progenitor / Immortalization / Differentiation

INTRODUCTION

The periodontal ligament (PDL) surrounds the tooth root, and acts to absorb occlusal forces and function as a sense organ (1). In periodontitis, a chronic inflammatory disease, the PDL is irreversibly damaged. Despite a number of novel approaches, it has not yet been possible to predictably form PDL (2). For this reason, there is considerable interest in the developmental mechanisms of PDL.

The PDL originates from dental follicle cells formed at the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (3). Progenitors present in the dental follicle are thought to contribute to the formation of all periodontal tissues, that is cementum, PDL and osteoblasts (4). After the formation of tooth root dentin, cementoblast progenitor in the dental follicle migrate onto the tooth root surface and differentiate into cementoblasts (4). Almost simultaneously, PDL progenitors within the dental follicle cells differentiate into PDL cells. Finally both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus. Since PDL is similar to tendon with respect to its dense collagen fiber structure, it has been suggested that tendon/ligament phenotype-related genes are involved in the differentiation of PDL progenitors. Growth and differentiation factors (GDFs)-5, 6, and 7 are members of the bone morphogenetic proteins that regulate tendon/ligament formation (5), and have been shown to be expressed in both dental follicle and PDL cells (6). *Screlaxis* (*Scx*), a basic helix-loop-helix (bHLH) transcription factor that serves as a tendon progenitor marker gene has also been found to be expressed in the PDL stem cells (7). Periostin is a marker for preosteoblasts, but it is also found in the periosteum and PDL (8). During tooth germ development, periostin is initially expressed in the dental follicle cells and is then deposited in postnatal PDL cells during tooth root formation(9). Altenatively periostin -/- mice develop periodontal disease-like phenotype in 3 month after birth, suggesting that critical role for maintenance of PDL (Rios, et al. 2005). These findings suggested that both tendon/ligament phenotype-related genes and PDL-specific extracellular matrices are involved in PDL formation and maintenance. However, details of the mechanisms involved PDL formation have yet to be clarified, due to the lack of periodontal ligament progenitor culture system.

Recently, we have shown the presence of cementoblast progenitors in bovine dental follicle cells (10). Cementoblast progenitor cell line designated BCPb8 has the capacity of forming PDL and cementum-like tissue when transplanted into severe combine immunodeficiency (SCID) mice(11). Although BCPb8 is a useful clonal cell line for the study of dental follicle progenitors, there are limitations

in using these cells derived from a bovine species since there is an inadequate cDNA database and antibodies are limited. To overcome these difficulties with BCPb8, we attempted to establish immortalized mice dental follicle (MDF) cells for the study of progenitors in the dental follicle.

MATERIALS AND METHODS

In situ hybridization

To generate antisense and sense digoxygenin-labelled riboprobes, linearized *osteopontin* and *Scx* plasmids and *periostin* cDNA fragments were transcribed by T7, T3 or Sp6 RNA polymerase as described by Wilkinson (Wilkinson 1995). Heads of 1-day postnatal C57BL/6 mice were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), sagittal sections were cut at 10 μm. Mandible of 35-day postnatal were fixed in 4% paraformaldehyde at 4°C overnight, and decalcified in 12.5% EDTA containing 2.5% paraformaldehyde for 6 wks, and then embedded in OCT compound. In situ hybridization was carried out on these sections as previously described with some modification (Iseki, et al. 1999). Polyvinyl alcohol was used for buffer during color reaction buffer.

Tissue Culture

The MDF cells were isolated from mice dental follicle tissue of 1-day old (=P1) the incisor tooth germs. Briefly, mice dental follicle tissue was mechanically stripped from the lingual posterior region of mice incisor, and placed onto a 24 multi-well plate. The tissues were then incubated with α-minimum essential medium (α-MEM: Sigma, St.Louis, USA) containing 10% fetal bovine serum (FBS; BioWhittaker, Maryland, USA), 50 μg/ml of ascorbic acid, 100 units/ml of streptomycin and penicillin, in a humidified atmosphere of 5% CO_2 at 37°C. When the cells reached approximately 80% confluence, they were passaged with 0.25% trypsin/1 mM EDTA and maintained as MDF cells. These cells were plated into 6 wells at a density of 3×10^4 cells/ml, and the medium was changed every 2 days.

Infection of retrovirus constructs and establishment of $MDF^{E6\text{-}EGFP}$ cells

Construction of pCLXSN-16E6$^{\Delta 146\text{-}151}$ and production of LXSN-16E6$^{\Delta 146\text{-}151}$ retrovirus have been described previously (Kyo, et al. 2003). One milliliter of producer cell culture fluid was added to MDF (passage 1) in the presence of polybrene (8 μg/ml), and subsequently selected in the presence of G418 (100 μg/ml). Transduced cells were maintained in the medium described above. Following infection with LXSN-16E6$^{\Delta 146\text{-}151}$, MDF cells were transduced with EGFP lentivirus under the control of a CMV promoter for obtaining stably expressed EGFP ($MDF^{E6\text{-}EGFP}$).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) as described previously (10). cDNAs were synthesized from 1 μg of total RNA in a 20 μl reaction containing 10x reaction buffer, 1 mM dNTP mixture, 1 U/μl RNase inhibitor, 0.25 U/μl reverse transcriptase (M-MLV reverse transcriptase, Invitorgen Corporation, Carlsbad, CA, USA) and 0.125 μM random 9-mers (Takara, Tokyo, Japan). Amplification was performed in a PCR Thermal Cycler SP (Takara, Tokyo, Japan) for 25 cycles using the following reaction profile ; 94°C for 1 min, 60°C for 30 sec and 72°C for 30 sec. Synthesized cDNA served as a template for subsequent PCR amplification, and specific primer used for *periostin, Scx, six-1, EphA4, GDF-5, collagen I (ColI)* and *glyceraldehydes-3-phosphate dehydrogenase (GAPDH).*

In vivo differentiation assay

The differentiation potential of $MDF^{E6\text{-}EGFP}$ cells wa assessed by transplantation of the cells into SCID mice a described previously (10). Briefly, cells were inoculate subcutaneously into 5 weeks-old male CB-17 scid/sci (SCID) mice (Nihoncrea, Tokyo, Japan) after incubating 1. $\times 10^6$ cells in a mixture of 40 mg of hydroxyapatite powde (Osferion, Olympus, Tokyo, Japan) and fibrin clot (mixtur of mouse fibrinogen and thrombin: both from Sigma, S Louis, MO, USA). Mice were sacrificed after 4 weeks and implants analyzed histochemically. NIH3T3 cell were used for comparison.

Histochemical analysis

The transplants were fixed in 4% paraformaldehyde for day, decalcified with 12.5% EDTA containing 2.5% paraformaldehyde for 3 days and then embedded in OC compound to make frozen section. Subsequently, 5 μm c section were cut, and stained with hematoxylin and eosin Fluorescence was observed by fluorescence microscop (Axio imager, Carl Zeiss, Germany). Expression c *periostin* mRNA was examined by in situ hybridization a described above. For immunohistocemical analysis, th sections were blocked with 1% bovine serum albumin, an probed with goat anti-type I collagen polyclonal antibod (SouthernBiotech, Birmingham, AL, USA) for 1 hou Then sections were probed with donkey anti-goat alexa 55 (Invitrogen Corp., Carlsbad, CA, USA). After washing fluorescence was observed by fluorescence microscopy.

RESULT AND DISCUSSION

Localization of periostin mRNA in the incisor tooth

Sequential developmental process of the dental follicle v observed in a sagittal section of P1 mice incisor. Immat cells were located posteriorly and differentiation v progressed towards anterior. To obtain immature den follicle cells, we first investigated the expression pattern c PDL-specific gene, *periostin*, and a cementoblast/osteob marker, *osteopontin*, in the lower incisor tooth germ of mice. Intense expression of *periostin* was observed in dental follicle cells close to the dentin layer in the ante region. In contrast, only patchy expression of *periostin* observed in the dental follicle cells in the posterior regi *Osteopontin* was expressed intensely throughout the alveo bone. In accordance with the *periostin* expression patt *osteopontin* was expressed in the cementoblast next to dentin-forming layer in the anterior region, but not in posterior region. These data confirmed that the den follicle cells in the posterior region were at an immature st while those in the anterior region were differentiated. thus dissected dental follicle cells in the posterior region establishing a dental follicle progenitor cell culture system

Immortarization of $MDF^{E6\text{-}EGFP}$ cells

IDF cells were isolated from the posterior region of the cisor tooth germ, and their life span was extended using a trovirus expressing human papillomavirus type 16 (HPV16) 6 gene deleted with PDZ domain-binding motif ($E6^{\Delta 146\text{-}151}$), nd a lentivirus expressing *EGFP* for fluorescence detection. fter viral infection, expression of $E6^{\Delta 146\text{-}151}$ was confirmed y RT-PCR analysis. $MDF^{E6\text{-}EGFP}$ cells maintained their riginal morphology and cell proliferation activity, even hen the cells were cultured beyond population doublings PD) 150. In contrast, normal MDF cells were only able to ropagate until PD 10. We thus used $MDF^{E6\text{-}EGFP}$ cells for rther analysis.

haracterization of $MDF^{E6\text{-}EGFP}$ cells

T-PCR analysis was performed to characterize the PDL rming properties of $MDF^{E6\text{-}EGFP}$ cells. $MDF^{E6\text{-}EGFP}$ cells xpressed *periostin* and tendon/ligament phenotype-related enes encoding *Scx, EphA4, Six-1, GDF-5* and *ColI* , ggesting that they possessed PDL and tendon cell roperties. Especially, stronger expression of *Scx* and *GDF-5* ere observed in $MDF^{E6\text{-}EGFP}$ cells than with NIH3T3 cells or C3T3E1 cells. This finding suggested that $MDF^{E6\text{-}EGFP}$ cells ere similar to tendon progenitors. Since PDL is orphologically similar to the tendon/ligament *in vivo*, dental llicle cells may have a similar phenotype to tendon cells. mong these genes, we examined the expression pattern of cx in the PDL(P35 mouse molar) by in situ hybridization. tense expression of *periostin* was observed throughout the DL, and *Scx* was also expressed in the PDL, suggesting that e PDL shares some characters with the tendon. *Scx* has en revealed as a maker not only for a somitc compartment the embryo that give rise to tendinous tissue, but also for e cells of adult tendon and ligament tissue (7) (Schweitzer, al. 2001). Salingcarnboriboon et al reported that a ndon-derived cell line isolated from mouse achilles tissue owed expression of *Scx*, and that these cells were able to rm tendon-like tissue when they were implanted into a ouse tendon defect model (Salingcarnboriboon, et al. 2003). xpression of *Scx* has also been observed in PDL stem cells nich can form a ligament-like structure upon implantation eo, et al. 2004). Although the role of the *Scx* in riodontal ligament development is not known, these data ongly support our hypothesis that $MDF^{E6\text{-}EGFP}$ cells possess DL progenitors that resemble tendon progenitors.

investigate the differentiation potential of $MDF^{E6\text{-}EGFP}$ ls, they were implanted into SCID mice. After 4 weeks, $DF^{E6\text{-}EGFP}$ cell transplants formed PDL-like fibrous tissue. e PDL-like tissues resembled a PDL structure with eet-like cells. Immunohistochemical staining revealed that nse type I collagen fibers were deposited in the PDL-like sue. To validate the capacity of $MDF^{E6\text{-}EGFP}$ cells to fferentiate into PDL *in vivo*, expression of *periostin* was mined in the transplants by in situ hybridization. As pected, intense expression of *periostin* was observed in $DF^{E6\text{-}EGFP}$ cells, indicating that PDL-like tissue formed by $DF^{E6\text{-}EGFP}$ cells were indeed PDL(9)

summary, we established an immortalized mice dental licle cell culture system that possessed PDL progenitors. $DF^{E6\text{-}EGFP}$ cells could provide a new insight into the mechanisms of PDL formation, including those pertaining to PDL cell differentiation. They also provide a powerful tool for the development of therapeutic strategies for the treatment of periodontitis.

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Regulation of PLAP-1 Expression in Periodontal Ligament Cells

S. Yamada, Y. Ozawa, M. Tomoeda,
R. Matoba, K. Matsubara, and S. Murakami

RESEARCH REPORTS
Biological

S. Yamada[1], Y. Ozawa[1], M. Tomoeda[1], R. Matoba[2], K. Matsubara[2], and S. Murakami[1]*

[1]Department of Periodontology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan; and [2]Taisho Laboratory of Functional Genomics, Nara Institute of Science and Technology, 8916-5 Takayama-cho, Ikoma, Nara 630-0101, Japan; *corresponding author, ipshinya@dent.osaka-u.ac.jp

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Regulation of PLAP-1 Expression in Periodontal Ligament Cells

ABSTRACT

Periodontal-ligament-associated protein-1 (*PLAP-1*) is preferentially expressed in the periodontal ligament (PDL) and encodes a novel small leucine-rich repeat proteoglycan protein. *PLAP-1* expression was induced during the course of cytodifferentiation of PDL cells into mineralized-tissue-forming cells *in vitro*, suggesting the possible involvement of PLAP-1 in the mineralization process of PDL cells. In this study, we hypothesized that PLAP-1 expression is regulated by mineralization-related cytokines in PDL cells. *PLAP-1* expression was clearly down-regulated when the cytodifferentiation of PDL cells was reversibly inhibited by fibroblast growth factor-2 (FGF-2). In contrast, bone morphogenetic protein-2 (BMP-2) enhanced *PLAP-1* expression. Up-regulation of *PLAP-1* expression by BMP-2 was confirmed at the protein level when PDL cells were immunostained with anti-PLAP-1 polyclonal antibody. These results revealed the cytokine-mediated regulatory mechanisms of PLAP-1 expression and suggested that PLAP-1 expression may be associated with the process of cytodifferentiation of PDL cells.

KEY WORDS: PLAP-1, FGF-2, BMP-2, periodontal ligament cells, mineralization.

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INTRODUCTION

The periodontal ligament (PDL) is a connective tissue interposed between the roots of teeth and the inner wall of the alveolar bone socket. Its fibers form a meshwork that stretches out between the cementum and the bone and is firmly anchored by Sharpey's fibers. PDL is rich in extracellular matrix (ECM). The ECM provides important functions within the PDL in maintaining structural integrity and regulation of cellular activity and function. The principal elements of ECM in PDL may be considered as a collagenous fibrous network providing structural support embedded in and interacting with a non-collagenous matrix consisting of proteoglycan and various glycoproteins (Waddington and Embery, 2001).

We recently reported the gene expression profile describing quantitative aspects of the genes active in the human PDL and identified a novel gene, *PLAP-1* (*periodontal-ligament-associated protein-1*), which is frequently and predominantly expressed in the PDL tissue (Yamada *et al.*, 2001). Other groups have discovered an identical gene (Henry *et al.*, 2001; Lorenzo *et al.*, 2001). They named this gene *Asporin*, due to its unique aspartic stretch at the N terminus of the translated open reading frame. The *PLAP-1/Asporin* gene encoded a novel SLRP (small leucine-rich repeat proteoglycan) protein, which resembled Decorin and Biglycan. Interestingly, expression of the *PLAP-1* gene was enhanced during the course of the cytodifferentiation of the PDL cells into mineralized-tissue-forming cells (Yamada *et al.*, 2001). This suggests the possible involvement of PLAP-1 in the process of mineralized matrix formation in PDL tissue. PLAP-1 has no glycosaminoglycan attachment site in its predicted amino acid sequence (Yamada *et al.*, 2001), implying that PLAP-1 is not a proteoglycan and may function differently in PDL tissue compared with other SLRP proteins such as Decorin and Biglycan in the PDL.

In this study, we hypothesized that PLAP-1 expression is regulated by the mineralization-related cytokines in human PDL cells.

MATERIALS & METHODS

All experiments were performed according to institutionally approved guidelines, and informed consent was obtained from the patients (Osaka University IRB/Ethical Committee approval #1488).

Cell Culture

Human PDL cells were isolated in accordance with the method described previously (Takayama *et al.*, 1997). The cells were cultured in α-MEM supplemented with 10% FCS, 50 units/mL penicillin G, and 50 μg/mL streptomycin (standard medium) at 37°C in 5% CO_2.

Stimulation of Human PDL Cells with Cytokines

Human PDL cells were cultured in standard medium. The next day, the medium was replaced with FCS-free α-MEM. After serum deprivation for 48 hrs, the cells were stimulated with FGF-2 (Kaken Pharmaceutical, Tokyo, Japan) (100 ng/mL), platelet-derived growth factor BB (PDGF-BB) (SIGMA, St. Louis,

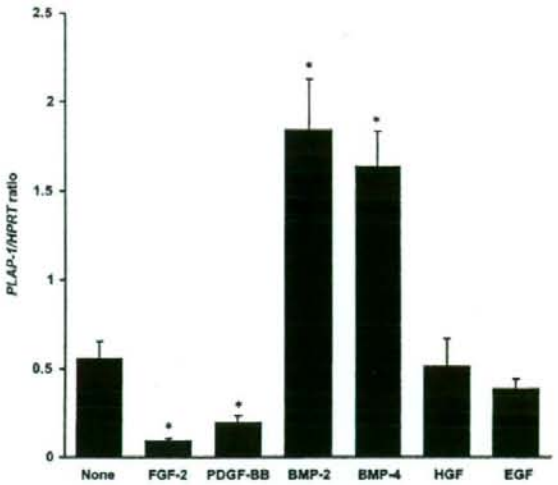

Figure 2. Effects of various cytokines on *PLAP-1* mRNA expression in PDL cells. After FCS deprivation for 48 hrs, human PDL cells were stimulated with indicated cytokines for another 48 hrs. Cells were harvested, and RNA was isolated for real-time RT-PCR analysis. Results are presented as the ratio of the amount of *PLAP-1* mRNA divided by *HPRT* mRNA. The values are given as means ± standard deviations (SD) of quadruplicate assays. *P < 0.005, n = 4, compared with None.

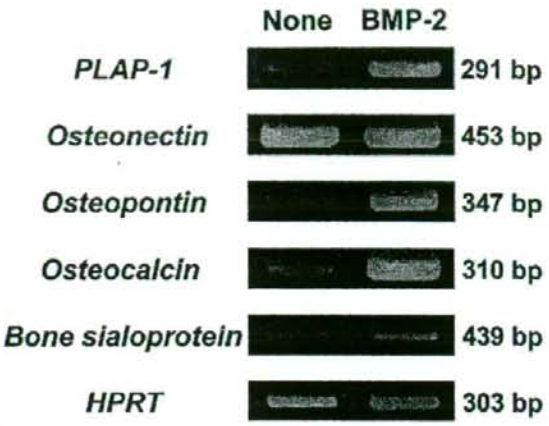

Figure 3. mRNA induction of *PLAP-1* and mineralization-related genes in PDL cells by BMP-2. After FCS deprivation for 48 hrs, human PDL cells were stimulated with 100 ng/mL of BMP-2 for another 48 hrs. Cells were harvested, and RNA was isolated for RT-PCR analysis. The numbers of PCR cycles were: 24 for *PLAP-1*, 21 for *Osteonectin*, 37 for *Osteopontin*, 37 for *Osteocalcin*, 37 for *Bone sialoprotein*, and 27 for *HPRT*. The sizes of PCR products are shown on the right of each panel. Similar results were obtained in 3 separate experiments, and representative data are shown.

differentiated into hard-tissue-forming cells, and calcified nodule formation was finally observed in the culture (data not shown). We also confirmed that the addition of FGF-2 in the late stage of this long-term culture resulted in down-regulation of ALPase activity (Takayama *et al.*, 1997) (Fig. 1A) and inhibited the calcified nodule formation (data not shown). We performed real-time RT-PCR analysis of *PLAP-1* using RNAs isolated from the PDL cells harvested on day 0 and day 20, with or without FGF-2 stimulation (Fig. 1B). Expression of *PLAP-1* was increased during the culture in mineralization media, and strong expression was detected on day 20. The transcription of *PLAP-1* was clearly decreased in the PDL cells cultured in the presence of FGF-2 from day 15 (Fig. 1B), accompanying the down-regulation of their ALPase activity (Fig. 1A.). The transcription of *Biglycan* and *Decorin* also tended to be suppressed by stimulation with FGF-2. However, the expression of *Biglycan* mRNA and *Decorin* mRNA during the culture was changed only slightly, compared with that of *PLAP-1* mRNA (Fig. 1B).

Up-regulation of *PLAP-1* mRNA Expression by BMP-2

We examined the effects of several other cytokines, assumed to be involved in the process of cytodifferentiation of PDL cells, on *PLAP-1* expression. Human PDL cells were stimulated with FGF-2, PDGF-BB, BMP-2, BMP-4, HGF, or EGF for 48 hrs, as described in MATERIALS & METHODS, and were examined for expression of *PLAP-1* mRNA by real-time RT-PCR analysis (Fig. 2). We used the optimum concentration for each cytokine, which induced proliferation of PDL cells equally (data not shown). The real-time RT-PCR analysis showed that FGF-2 and PDGF-BB significantly down-regulated the expression of *PLAP-1* mRNA in PDL cells. In contrast, BMP-2 and BMP-4, which strongly induced mineralization of PDL cells, significantly up-regulated *PLAP-1* transcription. In contrast, neither HGF nor EGF changed the expression of *PLAP-1* mRNA in PDL cells.

We then examined the regulation of the expression of *PLAP-1* mRNA by BMP-2 in detail. Human PDL cells were stimulated with various concentrations of BMP-2 for 48 hrs, and were examined by RT-PCR analysis for the expression of *PLAP-1*. Stimulation of PDL cells with BMP-2 resulted in increased expression of PLAP-1 mRNA in a dose-dependent manner (data not shown).

We then confirmed the effects of BMP-2 on the induction of the mineralization and cytodifferentiation of PDL cells by investigating the gene expression of mineralization-related proteins (Fig. 3). PDL cells were stimulated with BMP-2 (100 ng/mL) for 48 hrs, and RNA was isolated from the cells. RT-PCR analysis revealed that the transcription of PLAP-1 was induced along with *Osteopontin*, *Osteocalcin*, and *Bone sialoprotein* transcripts by the stimulation of BMP-2 (Fig. 3).

Induction of PLAP-1 Protein by BMP-2 in PDL Cells

PLAP-1 protein had a sequence similar to that of Decorin and Biglycan (Yamada *et al.*, 2001). To generate a polyclonal antibody specific for PLAP-1 protein, we carried out protein alignment of these 3 proteins to find amino acid sequences unique and antigenic for generating anti-PLAP-1 antibodies (Fig. 4A). Fig. 4A shows the peptide sequences which we selected for the immunization of rabbits. We cultured human PDL cells for 48 hrs in the presence or absence of BMP-2, and stained the cells with the polyclonal antibody (Fig. 4B). During the whole staining process, the PDL cells were alive and not fixed. The cell surface of BMP-2-stimulated PDL cells was stained with the anti-PLAP-1 polyclonal antibody in a dose-dependent manner (Fig. 4Ba~c). No staining was observed in the negative control, in which pre-immune rabbit serum was

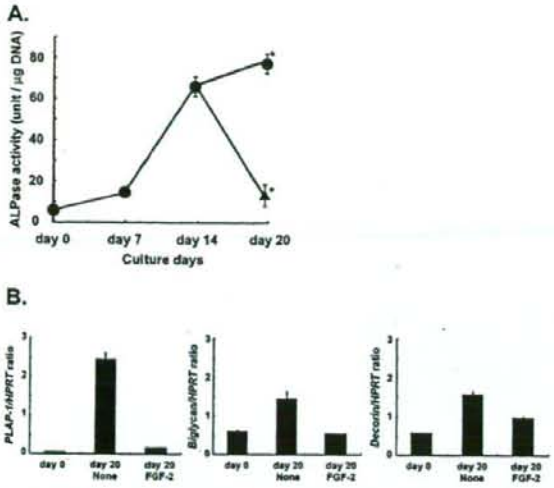

Figure 1. Real-time RT-PCR analysis of *PLAP-1* mRNA expression in the process of cytodifferentiation of human PDL cells. **(A)** ALPase activity of PDL cells. Human PDL cells were cultured in the presence of 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. Triangle shows ALPase activity of PDL cells cultured in the presence of FGF-2 (20 ng/mL) from day 15 in the cultured period. Circles show ALPase activities of PDL cells cultured without FGF-2. The values are given as means ± standard deviations (SD) of triplicate assays. *$P < 0.05$, $n = 3$ *vs.* respective PDL cells on day 20. **(B)** Down-regulation of *PLAP-1* mRNA of PDL cells by FGF-2. RNA from the same samples in panel A was reverse-transcribed into cDNA. Expression of specific mRNAs was detected by real-time PCR with primers specific for *PLAP-1*, *Biglycan*, *Decorin*, and *HPRT*. Results are presented as the ratio of the amount of each SLRP mRNA divided by HPRT mRNA. The values are given as means ± standard deviations (SD) of triplicate assays.

MO, USA) (50 ng/mL), BMP-2 (Genzyme/Techne, Minneapolis, MN, USA) (100 ng/mL), BMP-4 (Genzyme/Techne) (100 ng/mL), hepatocyte growth factor (HGF) (SIGMA) (100 ng/mL), or epidermal growth factor (EGF) (SIGMA) (100 ng/mL) and incubated for another 48 hrs. The stimulated cells were then harvested, and total RNA was isolated for RT-PCR analysis.

RT-PCR Analysis

Primers for real-time RT-PCR analysis were designed with Perfect Real Time Primer Design software (TAKARA, Shiga, Japan). Primers for *PLAP-1* were: (sense) 5′-GGGTGACGGTGTT CCATATCAG-3′ and (antisense) 5′-TGAAGCTCCAATAAA GTTGGTGGTA-3′. Primers for *Biglycan* were: (sense) 5′-CAACCAGATCAGGATGATCGAGAA-3′ and (antisense) 5′-CCCATGGGACAGAAGTCGTTG-3′. Primers for *Decorin* were: (sense) 5′-GGGAGCTTCACTTGGACAACAAC-3′ and (antisense) 5′-GGGCAGAAGTCACTTGATCCAAC-3′. Primers for *hypoxanthine-guanine phosphoribosyl transferase* (*HPRT*) were: (sense) 5′-CCAGACAAGTTTGTTGTAGG-3′ and (antisense) 5′-TCCAAACTCAACTTGAACTC-3′. Real-time RT-PCR reaction was carried out with a SYBR RT-PCR Kit (TAKARA) and performed with Smart Cycler version II (TAKARA). The amount of mRNA was calculated for each sample from the standard curve *via* the instrument software.

Semi-quantitative RT-PCR was performed according to the procedures described previously (Yamada *et al.*, 2001). RT-PCR primers for *Osteonectin* were: (sense) 5′-GGAAGAAACTGTGG CAGAGGTGAC-3′ and (antisense) 5′-TGTTGTCCTCATCCC TCTCATACAG-3′. Primers for *Osteopontin* were (sense) 5′-CCAAGTAAGTCCAACGAAAG-3′ and (antisense) 5′-GGTGATGTCCTCGTCTGTA-3′. Primers for *Osteocalcin* were: (sense) 5′-CATGAGAGCCCTCACA-3′ and (antisense) 5′-AGAGCGACACCCTAGAC-3′. Primers for *Bone sialoprotein* were: (sense) 5′-GCCTGTGCTTTCTCAATG-3′ and (antisense) 5′-TTCCTTCCTCTTCCTCCTC-3′. Primers for *HPRT* were: (sense) 5′-CGAGATGTGATGAAGGAGATGGG-3′ and (antisense) 5′-GCCTGACCAAGGAAAGCAAAGTC-3′.

In vitro Assay for Alkaline Phosphatase (ALPase) Activity

Human PDL cells were cultured with the standard medium (see above) in the presence of 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid (mineralization medium). Cellular DNA content and ALPase activity in the PDL cells were determined according to the procedures described previously (Takayama *et al.*, 1997).

Production of Anti-PLAP-1 Antibody

A peptide (EPRSHFFPFD) homologous to human PLAP-1 was synthesized with a Model 430 A peptide synthesizer (Applied Biosystems). The peptide was conjugated to keyhole limpet hemocyanin (KLH) and used for the immunization of rabbits. This animal experiment was carried out in accordance with the guidelines for animal experimentation approved by the Japanese Association for Laboratory Animal Science.

Immunocytochemical Staining

Human PDL cells grown to confluence in a 60-mm poly-L-lysine-coated glass-bottomed dish (Matsunami Glass, Osaka, Japan) were stimulated with BMP-2 for 48 hrs. Cells were washed with PBS 3 times and incubated with anti-PLAP-1 polyclonal antibody or pre-immune rabbit serum as a control at 4°C for 15 min. Cells were then incubated with biotinylated goat anti-rabbit IgG (H+L) antibody (Vector Laboratories, Burlingame, CA, USA) at 4°C for 15 min, and, finally, streptavidin-Alexa Fluor 488 (Molecular Probe, Engene, OR, USA) was added for the detection of immunoreactivity. Cells were washed 3 times with PBS after each step.

For the pre-incubation assay, we pre-incubated the anti-PLAP-1 polyclonal antibody with the antigenic KLH-conjugated PLAP-1 peptide, KLH alone, recombinant human Decorin (R&D Systems, Minneapolis, MN, USA), or recombinant human Biglycan (ABNOVA, Taipei, Taiwan) at room temperature for 15 min before staining BMP-2-stimulated PDL cells. In each pre-incubation assay, a 30-mg quantity of KLH-conjugated peptide or recombinant proteins was added to 1 mL of the anti-PLAP-1 antibody. The amount of peptide and recombinant proteins (30 mg/1 mL of anti-PLAP-1 antibody) was equivalent to the amount of total protein in the anti-PLAP-1 polyclonal antibody serum.

Statistical Analysis

Data are expressed as means ± standard deviations. The statistical significance of differences between 2 means was examined by the Mann-Whitney U test, and P values less than 0.05 were considered to indicate a significant difference.

RESULTS

Down-regulation of *PLAP-1* Gene Expression by FGF-2

When human PDL cells were cultured in the mineralization media for 20 days, ALPase activity in the PDL cells was gradually increased (Fig. 1A). The PDL cells simultaneously

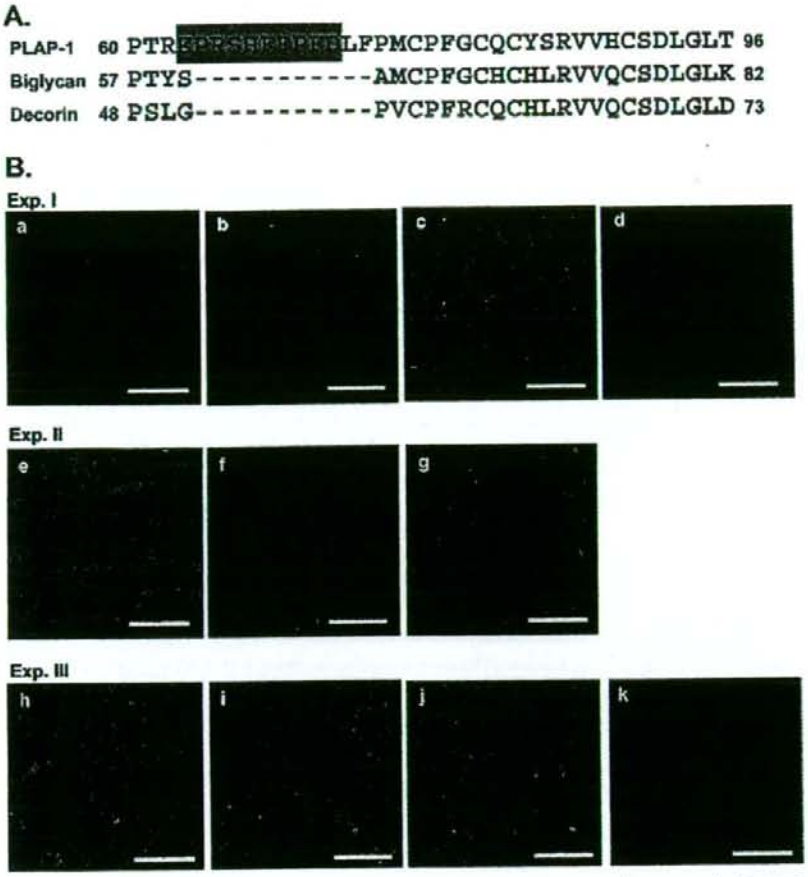

Figure 4. Induction of PLAP-1 protein on PDL cells by BMP-2. **(A)** Alignment of N-terminal of PLAP-1, Biglycan, and Decorin protein. The peptide sequence for antigen to immunize rabbits is shown in the dark-shaded box. **(B) Experiment I:** Immunocytochemical detection of PLAP-1 protein on PDL cells stimulated with BMP-2. Human PDL cells cultured in the glass-bottomed dish were stimulated with the different concentrations of BMP-2 for 48 hrs. The cells were washed with PBS and incubated with the anti-PLAP-1 polyclonal antibody or pre-immune rabbit serum, followed by the biotinylated goat anti-rabbit IgG mAb and then with streptavidin-Alexa Fluor 488. (a) No BMP-2 stimulation. (b) BMP-2 stimulation (200 ng/mL). (c) BMP-2 stimulation (400 ng/mL). (d) BMP-2 stimulation (400 ng/mL), staining with pre-immune rabbit serum. **Experiment II:** Dose-dependent inhibition of PLAP-1 staining with the PLAP-1 antigenic peptide. The anti-PLAP-1 polyclonal antibody was pre-incubated with different concentrations of the PLAP-1 peptide before BMP-2-stimulated (in 400 ng/mL) PDL cells were stained. (e) No PLAP-1 peptide. (f) Pre-incubation with the PLAP-1 peptide (30 mg/1 mL of anti-PLAP-1 antibody). (g) Pre-incubation with the PLAP-1 peptide (15 mg/1 mL of anti-PLAP-1 antibody). **Experiment III:** No cross-reaction of the anti-PLAP-1 antibody to Decorin or to Biglycan. The anti-PLAP-1 antibody was pre-incubated with either recombinant Decorin or Biglycan before BMP-2-stimulated (in 400 ng/mL) PDL cells were stained. (h) No pre-incubation. (i) Pre-incubation with recombinant Decorin (30 mg/1 mL of anti-PLAP-1 antibody). (j) Pre-incubation with recombinant Biglycan (30 mg/1 mL of anti-PLAP-1 antibody). (k) Pre-immune rabbit serum staining. Representative data of 3 independent experiments are shown. Scale bars = 50 μm.

used as the primary antibody (Fig. 4Bd). We then pre-incubated the antibody before staining the BMP-2-stimulated PDL cells with different amounts of the PLAP-1 antigenic peptide, which we utilized for immunization. Pre-incubation of the PLAP-1 antigenic peptide inhibited the binding of the antibody to BMP-2-stimulated PDL cells in a dose-dependent manner (Fig. 4Be~g). No inhibition was observed in the negative control in which only KLH carrier protein was used for the pre-incubation with the antibody (data not shown). Moreover, we pre-incubated the anti-PLAP-1 antibody with either recombinant human Decorin or recombinant human Biglycan before staining the BMP-2-stimulated PDL cells (Fig. 4Bh~k). No inhibition was observed by the pre-incubation with either recombinant Decorin or recombinant Biglycan.

DISCUSSION

In this study, we analyzed the regulation of PLAP-1 expression in human PDL cells by mineralization-related cytokines. The transcription of *PLAP-1* was up-regulated along with the cytodifferentiation process of PDL cells and down-regulated when the process was arrested by FGF-2 (Fig. 1). This suggests that *PLAP-1* expression is closely associated with the process of cytodifferentiation of PDL cells. FGF-2 and PDGF-BB, which have mitogenic and chemotactic activities (Rifkin and Moscatelli, 1989; Hoch and Soriano, 2003), clearly suppressed the expression of *PLAP-1* in PDL cells (Fig. 2). Since PDL cells proliferate after stimulation with FGF-2 or PDGF-BB, *PLAP-1* may not be essential for cell proliferation. In contrast, BMP-2 and BMP-4 clearly induced the expression of *PLAP-1* (Fig. 2). BMP-2 is one of the most potent cytokines that stimulate osteoblast differentiation and bone formation (Hogan, 1996; Canalis *et al.*, 2003). BMP-2 has also been reported to stimulate osteoblastic differentiation in human PDL cells (Kobayashi *et al.*, 1999), and to promote dental follicle cells, putative progenitor cells for the periodontium, to induce differentiation into a cementoblastic/osteoblastic phenotype (Zhao *et al.*, 2002). Given that BMP-2 induced the cytodifferentiation and mineralization of PDL cells, PLAP-1 is closely associated with the cytodifferentiation of PDL cells into hard-tissue-forming cells. BMP-2 induced the expression of *PLAP-1* in a dose-dependent manner. *PLAP-1* was then transcribed along with the induction of other BMP-2-induced mineralization-related genes (Fig. 3). These results suggest that PLAP-1 is a mineralization-related gene, and that its transcription might be driven by the BMP-2 signaling pathway.

Recently, it was demonstrated that topical application of recombinant FGF-2 or PDGF-BB enhances the healing process and accelerates periodontal tissue regeneration (Takayama *et al.*, 2001; Murakami *et al.*, 2003; Nevins *et al.*, 2003). In the *in vivo* process of periodontal tissue regeneration, FGF-2 is likely to generate a suitable micro-environment in the FGF-2-applied sites by regulating the production of extracellular matrix (Takayama *et al.*, 1997; Shimabukuro *et al.*, 2005). Interestingly, we found that both FGF-2 and PDGF-BB strongly suppressed transcription of *PLAP-1* in PDL cells (Fig. 2). Thus, the effects of FGF-2 and PDGF-BB on periodontal tissue regeneration might be partly associated with suppression of PLAP-1 expression in PDL. Temporal reduction of PLAP-1 during the early phase of wound healing might be suitable for periodontal tissue regeneration in terms of the creation of an ideal micro-environment at the site.

PLAP-1 is categorized into the same subclass of SLRP proteoglycan families as Biglycan and Decorin (Yamada *et al.*, 2001). Biglycan and Decorin were dominantly expressed in bone and connective tissue of skin, respectively (Ameye and Young, 2002). In general, both proteoglycans exist ubiquitously in mineralized tissues and connective tissues. In oral tissues, Biglycan is expressed in odontoblasts and ameloblasts and regulates the cytodifferentiation of those cells (Iozzo, 1997). Decorin is expressed ubiquitously in tissues of the periodontium (Hakkinen *et al.*, 1993). However, we could not detect the *PLAP-1* transcript in bone tissue by Northern blot analysis, but its expression was specifically revealed in PDL by *in situ* hybridization (Yamada *et al.*, unpublished observations). Changes in *Decorin* and *Biglycan* were slight in the course of cytodifferentiation of PDL cells, compared with *PLAP-1* expression (Fig. 1). These findings suggest that PLAP-1 has unique function(s) in the PDL, compared with those of Decorin and Biglycan, and may play different roles in the process of the cytodifferentiation of the PDL cells.

The present findings demonstrated that the *PLAP-1* transcript is tightly regulated by FGF-2 and BMP-2 and closely associated with the process of cytodifferentiation and mineralization of human PDL cells. Thus, PLAP-1 is expected to be useful for our understanding of the molecular basis of periodontal ligament functions.

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Menstrual Blood-derived Cells Confer Human Dystrophin Expression in the Murine Model of Duchenne Muscular Dystrophy via Cell Fusion and Myogenic Transdifferentiation [D]

ChangHao Cui,*† Taro Uyama,* Kenji Miyado,* Masanori Terai,* Satoru Kyo,‡ Tohru Kiyono,§ and Akihiro Umezawa*

*Department of Reproductive Biology and Pathology, National Institute for Child Health and Development, Tokyo, 157-8567, Japan; †Department of Basic Medical Science, Mudanjiang Medical College, Mudanjiang, 157011, China; ‡Department of Obstetrics and Gynecology, Kanazawa University, School of Medicine, Kanazawa, 920-8640, Japan; and §Virology Division, National Cancer Center Research Institute, Tokyo, 104-0045, Japan

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Duchenne muscular dystrophy (DMD), the most common lethal genetic disorder in children, is an X-linked recessive muscle disease characterized by the absence of dystrophin at the sarcolemma of muscle fibers. We examined a putative endometrial progenitor obtained from endometrial tissue samples to determine whether these cells repair muscular degeneration in a murine mdx model of DMD. Implanted cells conferred human dystrophin in degenerated muscle of immunodeficient mdx mice. We then examined menstrual blood–derived cells to determine whether primarily cultured nontransformed cells also repair dystrophied muscle. In vivo transfer of menstrual blood–derived cells into dystrophic muscles of immunodeficient mdx mice restored sarcolemmal expression of dystrophin. Labeling of implanted cells with EGFP and differential staining of human and murine nuclei suggest that human dystrophin expression is due to cell fusion between host myocytes and implanted cells. In vitro analysis revealed that endometrial progenitor cells and menstrual blood–derived cells can efficiently transdifferentiate into myoblasts/myocytes, fuse to C2C12 murine myoblasts by in vitro coculturing, and start to express dystrophin after fusion. These results demonstrate that the endometrial progenitor cells and menstrual blood–derived cells can transfer dystrophin into dystrophied myocytes at a high frequency through cell fusion and transdifferentiation in vitro and in vivo.

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INTRODUCTION

Skeletal muscle consists predominantly of syncytial fibers with peripheral, postmitotic myonuclei, and its intrinsic repair potential in adulthood relies on the persistence of a resident reserve population of undifferentiated mononuclear cells, termed "satellite cells." In mature skeletal muscle, most satellite cells are quiescent and are activated in response to environmental cues, such as injury, to mediate postnatal muscle regeneration. After division, satellite cell progeny, termed myoblasts, undergo terminal differentiation and become incorporated into muscle fibers (Bischoff, 1994). Myogenesis is regulated by a family of myogenic transcription factors including MyoD, Myf5, myogenin, and MRF4 (Sabourin and Rudnicki, 2000). During embryonic development, MyoD and Myf5 are involved in the establishment of the skeletal muscle lineage (Rudnicki *et al.*, 1993), whereas myogenin is required for terminal differentiation (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). During muscle repair, satellite cells recapitulate the expression program of the myogenic genes manifested during embryonic development.

Dystrophin is associated with a large oligomeric complex of glycoproteins that provide linkage to the extracellular membrane (Ervasti and Campbell, 1991). In Duchenne muscular dystrophy (DMD), the absence of dystrophin results in destabilization of the extracellular membrane-sarcolemma-cytoskeleton architecture, making muscle fibers susceptible to contraction-associated mechanical stress and degeneration. In the first phase of the disease, new muscle fibers are formed by satellite cells. After depletion of the satellite cell pool in childhood, skeletal muscles degenerate progressively and irreversibly and are replaced by fibrotic tissue (Cossu and Mavilio, 2000). Like DMD patients, the mdx mouse lacks dystrophin in skeletal muscle fibers (Hoffman *et al.*, 1987; Sicinski *et al.*, 1989). However, the mdx mouse develops only a mild dystrophic phenotype, probably because muscle regeneration by satellite cells is efficient for most of the animal's life span (Cossu and Mavilio, 2000).

Myoblasts represent the natural first choice in cellular therapeutics for skeletal muscle because of their intrinsic myogenic commitment (Grounds *et al.*, 2002). However, myoblasts recovered from muscular biopsies are poorly expandable in vitro and rapidly undergo senescence (Cossu and Mavilio, 2000). An alternative source of muscle progenitor cells is therefore desirable. Cells with a myogenic potential are present in many tissues, and these cells readily

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Address correspondence to: Akihiro Umezawa (umezawa@1985.jukuin.keio.ac.jp).

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form skeletal muscle in culture (Gerhart *et al.*, 2001). We report here that human dystrophin expression in the mdx model of DMD is attributed to cell fusion of mdx myocytes with human menstrual blood–derived stromal cells.

MATERIALS AND METHODS

Isolation of Human Endometrial Cells from Menstrual Blood

Menstrual blood samples (n = 21) were collected in DMEM with antibiotics (final concentrations: 100 U/ml penicillin/streptomycin) and 2% fetal bovine serum (FBS), and processed within 24 h. Ethical approval for tissue collection was granted by the Institutional Review Board of the National Research Institute for Child Health and Development, Japan. The centrifuged pellets containing endometrium-derived cells were resuspended in high-glucose DMEM medium (10% FBS, penicillin/streptomycin), maintained at 37°C in a humidified atmosphere containing 5% CO_2, and allowed to attach for 48 h. Nonadherent cells were removed by changing the medium. When the culture reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and plated to new dishes. After 2–3 passages, the attached endometrial stromal cells were devoid of blood cells. Human EM-E6/E7/hTERT-2 cells, endometrium-derived progenitors, were obtained from surgical endometrial tissue samples and were immortalized by E6, E7, and hTERT-2 (Kyo *et al.*, 2003). C2C12 myoblast cells were supplied by RIKEN Cell Bank (The Institute of Physical and Chemical Research, Japan).

Flow Cytometric Analysis

Flow cytometric analysis was performed as previously described (Terai *et al.*, 2005). Cells were incubated with primary antibodies or isotype-matched control antibodies, followed by additional treatment with the immunofluorescent secondary antibodies. Cells were analyzed on an EPICS ALTRA analyzer (Beckman Coulter, Fullerton, CA). Antibodies against human CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD50, CD54, CD55, CD59, CD73, CD90, CD105, CD117 (c-kit), CD133, HLA-ABC, and HLA-DR were purchased from Beckman Coulter, Immunotech (Marseille, France), Cytotech (Hellebaek, Denmark), and BD Biosciences PharMingen (San Diego, CA).

In Vitro Lentivirus-mediated Gene (EGFP) Transfer into EM-E6/E7/hTERT-2 Cells

Infection of EM-E6/E7/hTERT-2 cells with lentivirus having a CMV promoter and EGFP reporter resulted in high levels of EGFP expression in all cells. Cells were analyzed for EGFP expression by flow cytometry (Miyoshi *et al.*, 1997, 1998).

In Vitro Myogenesis

Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells were seeded onto collagen I–coated cell culture dishes (Biocoat, BD Biosciences, Bedford, MA) at a density of 1×10^4/ml in growth medium (DMEM, supplemented with 20% FBS). Forty-eight hours after seeding onto collagen I–coated dishes, cells were treated with 5-azacytidine for 24 h. Cell cultures were then washed twice with PBS and maintained in differentiation medium (DMEM, supplemented with either 2% horse serum (HS) or 1% insulin-transferrin-selenium supplement [ITS]). The differentiation medium was changed twice a week until the experiment was terminated.

RT-PCR Analysis of EM-E6/E7/hTERT-2 Cells and Menstrual Blood–derived Cells

Total RNA was prepared using Isogen (Nippon Gene, Tokyo, Japan). Human skeletal muscle RNA was purchased from TOYOBO (Osaka, Japan). RT-PCR of Myf5, MyoD, desmin, myogenin, myosin heavy chain-IIx/d (MyHC-IIx/d), and dystrophin was performed with 2 μg of total RNA. RNA for RT-PCR was converted to cDNA with a first-stand cDNA synthesis kit (Amersham Pharmacia Biotechnology, Piscataway, NJ) according to the manufacturer's recommendations. The sequences of PCR primers that amplify human but not mouse genes are listed in Supplementary Table 1. PCR was performed with TaKaRa recombinant Taq (Takara Shuzo, Kyoto, Japan) for 30 cycles, with each cycle consisting of 94°C for 30 s, 62°C or 65°C for 30 s, and 72°C for 20 s, with an additional 10-min incubation at 72°C after completion of the last cycle.

Immunohistochemical and Immunocytochemical Analysis

Immunohistochemical analysis was performed as previously described (Mori *et al.*, 2005). Briefly, the sections were incubated for 1 h at room temperature with mouse mAb against vimentin (Cone V9, DakoCytomation, Fort Collins, CO). After washing in PBS, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin, diluted, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H_2O_2 in 0.05% M Tris-HCl buffer, pH 6.7. Slides were counterstained with hematoxylin. In the cases of fluorescence, frozen sections fixed with 4% PFA were used. The antibodies against human dystrophin (NCL-DYS3; Novocastra, Newcastle upon Tyne, United Kingdom) or anti-human nuclei mouse mAb (clone 235-1, Chemicon, Temecula, CA) was used as a first antibody, and goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-mouse IgG antibody conjugated with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used as a second antibody.

Immunocytochemical analysis was performed as previously described (Mori *et al.*, 2005), with antibodies to skeletal myosin (Sigma, St. Louis, MO; product no. M 4276), MF20 (which reacts with all sarcomere myosin in striated muscles, Developmental Studies Hybridoma Bank, University of Iowa, IA), α-sarcomeric actin (Sigma, product no. A 7811), and desmin (BioScience Products, Bern, Switzerland; no. 010031, clone: D9) in PBS containing 1% bovine serum albumin. As a methodological control, the primary antibody was omitted. In the cases of fluorescence, slides were incubated with Alexa Fluor 546–conjugated goat anti-mouse IgG antibody.

Western Blotting

Western blot analysis was performed as previously described (Mori *et al.*, 2005). Blots were incubated with primary antibodies (desmin, myogenin [Clone F5D, Santa Cruz Biotechnology], and dystrophin [NCL-DYSA, Novocastra]) for 1–2 h at room temperature. After washing three times in the blocking buffer, blots were incubated for 30 min with a horseradish peroxidase–conjugated secondary antibody (0.04 μg/ml) directed against the primary antibody. The blots were developed with enhanced chemiluminescence substrate according to the manufacturer's instructions.

Fusion Assay

EM-E6/E7/hTERT-2 cells (2500/cm²) or EGFP-labeled EM-E6/E7/hTERT-2 cells (2500/cm²) were cocultured with C2C12 myoblasts (2500/cm²) for 2 d in DMEM supplemented with 10% FBS and then cultured for 7 additional days in DMEM with 2% HS to promote myotube formation. The cultures were fixed in 4% paraformaldehyde and stained with a mouse anti-human nuclei IgG1 mAb and the mouse anti-human dystrophin IgG2a mAb (or anti-myosin heavy chain IgG2b mAb MF-20). The cells were visualized with appropriate Alexa-fluor–conjugated goat anti-mouse IgG1 and IgG2a (or IgG2b) secondary antibodies (Molecular Probes). Total cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

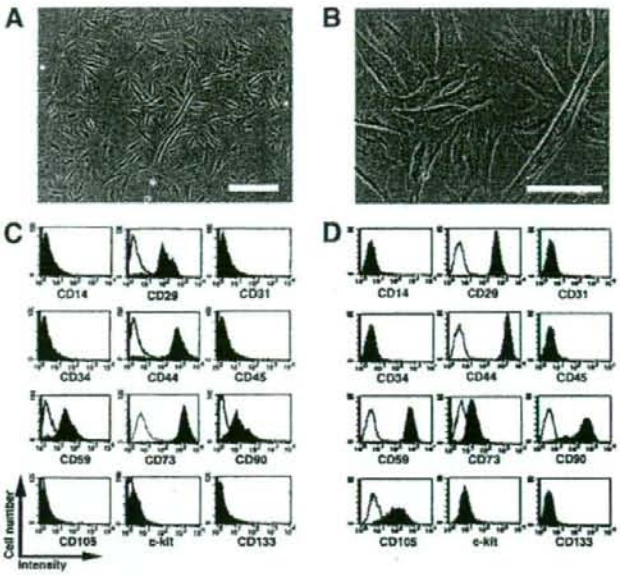
In Vivo Cell Implantation

Six- to 8-wk-old NOD/Shi-scid/IL-2 receptor −/− (NOG, CREA, Shizuoka, Japan) mice and 6- to 8-wk-old mdx-scid mice were implanted with EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells in seven independent experiments. The cells (2×10^7) were suspended in PBS in a total volume of 100 μl and were directly injected into the right thigh muscle of NOG mice or mdx-scid mice. The mice were examined 3 wk after cell implantation, and the right thigh muscle was analyzed for human vimentin and dystrophin by immunohistochemistry. The antibodies to vimentin and dystrophin (NCL-DYS3) react with human vimentin and dystrophin-equivalent protein, but not murine protein.

RESULTS

Surface Marker Expression of Endometrium-derived Cells

We investigated myogenic differentiation of primary cells without gene introduction from menstrual blood, because menstrual blood on the first day of the period is considered to include endometrial tissue. We successfully cultured a large number of primary cells from menstrual blood. Menstrual blood–derived cells showed at least two morphologically different cell groups: small spindle-like cells and large stick-like cells, regarded as being passage day (PD) 1 or 2 (Figure 1, A and B, respectively). Surface markers of the menstrual blood–derived cells were evaluated by flow cytometric analysis. Surface markers of EM-E6/E7/hTERT-2 cells (Figure 1C) and menstrual blood–derived cells (Figure 1D) were evaluated by flow cytometric analysis (Figure 1E). In these experiments, the cells were cultured in the absence of any inductive stimuli. EM-E6/E7/hTERT-2 cells were positive for CD13, CD29 (integrin β1), CD44 (Pgp-1/ly24), CD54, CD55, CD59, CD73, and CD90 (Thy-1), implying that EM-E6/E7/hTERT-2 cells expressed mesenchymal cell–related antigens in our experimental setting. Menstrual blood–derived cells were positive for CD13, CD29, CD44, CD54, CD55, CD59, CD73, CD90, and CD105, implying that prolif-



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Surface marker	EM-E6/E7/ hTERT-2 cells	Menstrual blood cells	Surface marker	EM-E6/E7/ hTERT-2 cells	Menstrual blood cells
CD13	+	++	CD55	++	++
CD14	-	-	CD59	++	+++
CD29	++	+++	CD73	+++	+
CD31	-	-	CD90	++	+++
CD34	-	-	CD105	-	++
CD44	+++	+++	c-kit	-	-
CD45	-	-	CD133	-	-
CD50	-	-	HLA-ABC	++	++
CD54	+	++	HLA-DR	-	-

Figure 1. Surface marker expression of endometrium-derived cells. (A and B) Morphology of menstrual blood–derived cells, regarded as being PD 1 or 2. Scale bars, 200 μm (A), 100 μm (B). (C and D) Flow cytometric analysis of cell surface markers of EM-E6/E7/hTERT-2 cells (C) and menstrual blood–derived cells (D). (E) Further phenotypic analysis in EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells are summarized. Peak intensity was estimated in comparison with isotype controls. +++, strongly positive (>100 times the isotype control); ++, moderately positive (<100 times but more than 10 times the isotype control), weakly positive (<10 times but more than twice the isotype control), −, negative (less than twice the isotype control).

erated and propagated cells express mesenchymal cell-related cell surface markers. Unlike EM-E6/E7/hTERT-2 cells, the menstrual blood–derived adherent cells were positive for CD105. EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed neither hematopoietic lineage markers, such as CD34, nor monocyte-macrophage antigens such as CD14 (a marker for macrophage and dendritic cells), or CD45 (leukocyte common antigen). The lack of expression of CD14, CD34, or CD45 suggests that EM-E6/E7/hTERT-2 cells and the menstrual blood–derived cell culture in the present study is depleted of hematopoietic cells. The cells were also negative for expression of CD31 (PECAM-1), CD50, c-kit, and CD133. The cell population was positive for HLA-ABC, but not for HLA-DR. These results demonstrate that almost all cells derived from endometrium are of mesenchymal origin or stromal origin.

Implanted Endometrium-derived Cells Induce De Novo Myogenesis in Immunodeficient NOG Mice

EM-E6/E7/hTERT-2 cells originate from the endometrial gland and are considered as endometrial progenitor cells or bipotential cells capable of differentiating into both glandular epithelial cells and endometrial stromal cells (Kyo *et al.*, 2003). To determine whether EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells generate complete endometrial structure in vivo, like endometriosis, the cells without any treatment or induction were injected into the right thigh muscle of immunodeficient NOG mice. PBS without cells was injected into the left thigh muscles as a control. We failed to detect any endometrial structure in the cell-injected site. Immunohistochemical analysis using an antibody specific to human vimentin, an intermediate filament associated with a mesenchymal cell, revealed that the injected EM-E6/E7/hTERT-2 cells (Figure 2, A–F) or menstrual blood–derived cells (Figure 2, G–L) extensively migrated or infiltrated between muscular fibers (Figure 2, arrowheads). To investigate if the donor cells between muscular fibers occur as a result of cell migration, we performed a time-course analysis of implanted cells, as probed by human-specific antibody to vimentin (Supplementary Figure 1). Donor cells at 3 h after implantation are observed at the injection site, which is considered to be due to just injection of cells. Cells at 1–3 wk after implantation are detected between myocytes in the muscle bundle or muscular fascicle as well as in the interstitial tissue, implying that the donor cells between myotubes result from cell migration. Interestingly, some of the vimentin-positive implanted cells exhibited round-shaped structure (Figure 2, D, F, and J, arrows), suggesting that endometrium-derived cells are capable of differentiating into myoblasts/myotubes, and can contribute to skeletal muscle repair in patients suffering from genetic disorders such as DMD, similar to previous reports for marrow stromal cells (Dezawa *et al.*, 2005) and synovial membrane cells (De Bari *et al.*, 2003).

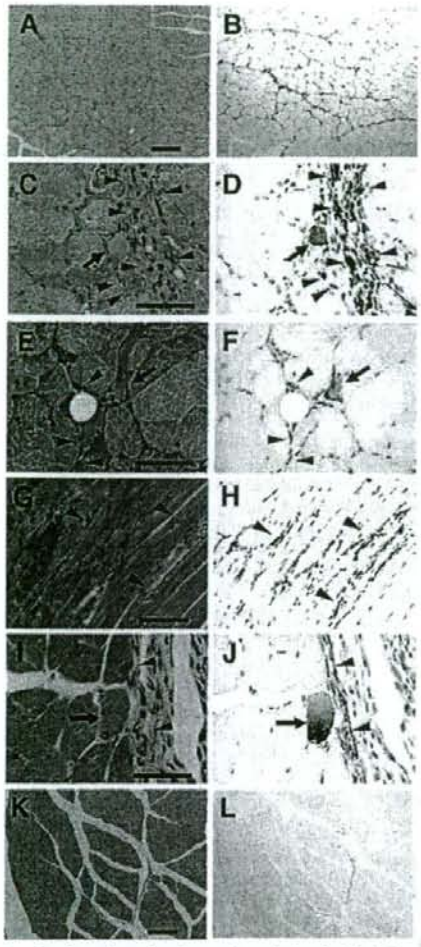

Figure 2. Implantation of endometrium-derived cells-derived cells into the muscle of NOG mice. EM-E6/E7/hTERT-2 cells (A–F) or menstrual blood–derived cells (G–J) cultured in absence of any stimuli were directly injected into the right thigh muscle of NOG mice. Immunohistochemical analysis was performed using antibody that reacts to human vimentin but not to murine vimentin. (A, C, E, G, I, and K) hematoxylin and eosin stain (HE; B, D, F, H, J, and L) immunohistochemistry. Note that vimentin-positive EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells with a spindle morphology (C–J, arrowheads) extensively migrated into muscular bundles at 3 wk after injection, and some of the injected cells exhibited round structure (D, F, and J, arrows). Isotype mouse IgG1 served as a negative control (L). Scale bars, 100 μm (A, B, K, and L), 50 μm (C–F, I, and J), 90 μm (G and H).

Induction of Myogenic Differentiation in Endometrial Progenitor Cells In Vitro

EM-E6/E7/hTERT-2 cells at 2 wk (cultured in the DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μM) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (Figure 3, A–F). The number of desmin-positive cells was significantly higher in experimental groups with 5 or 10 μM 5-azacytidine than in untreated control groups ($p < 0.05$). To investigate whether EM-E6/E7/hTERT-2 cells are capable of differentiating into skeletal muscle cells in vitro, the cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in the DMEM supplemented with 2% HS (Figure 3, G–J) or serum-free ITS for up to 21 d (Figure 3K). Skeletal myoblastic differentiation of the cells was analyzed by evaluating expression of MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. The MyoD, desmin, myogenin, and dystrophin genes were constitutively expressed, but MyHC-IIx/d and Myf5 genes were not. The decline of MyoD was observed in both the 2% HS (Figure 3, G and H) and the serum-free ITS (Figure 3K). The expression of MyHC, as determined by RT-PCR and immunocytochemistry, significantly increased with 2% HS (Figure 3, G–J) and serum-free ITS (Figure 3K). Immunocytochemical analysis indicated that α-sarcomeric actin (Figure 3I) and MyHC (Figure 3J) were detected in the cells incubated with 2% HS for 21 d.

In Vitro Myogenic Differentiation of Menstrual Blood-derived Cells

Menstrual blood–derived cells at 3 wk (cultured in DMEM supplemented with 20% FBS) after exposure to different concentrations (5, 10, and 100 μM) of 5-azacytidine were analyzed by immunostaining using anti-desmin antibody (data not shown). The number of desmin-positive cells was significantly higher in experimental groups with 5 or 10 μM 5-azacytidine than with 100 μM 5-azacytidine; for further in vitro experiments, the menstrual blood–derived cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with low serum (2% HS) or serum-free ITS for up to 21 d (Figure 4). Myogenic potential of human menstrual blood–derived cells was analyzed by evaluating the expression of Myf5, MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin by RT-PCR. MyoD, desmin, and dystrophin genes were constitutively expressed in menstrual blood–derived cells, but MyHC-IIx/d and Myf5 were not (Figure 4A). For cells treated with 2% HS or serum-free ITS, the mRNA level of desmin and myogenin significantly increased after 3 d, and desmin steadily increased until day 21 (Figure 4, C and D). MyHC-IIx/d started to be expressed at a low level at day 21 of induction (Figure 4C). We then analyzed desmin expression by immunocytochemistry. Menstrual blood–derived cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 20% FBS for up to 2 wk. Desmin was readily detected in colonies of the menstrual blood–derived cells (Figure 4B). Western blot analysis indicated that desmin, myogenin, and dystrophin were highly expressed in the cells incubated for 3 wk (Figure 4, E–G). These results suggest that menstrual blood–derived cells are, like the EM-E6/E7/hTERT-2 cells, able to differentiate into skeletal muscle.

Regeneration of Dystrophin by Cell Implantation in the DMD Model mdx-scid Mouse

To investigate whether human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells can generate muscle tissue in vivo, cells without any treatment or induction were implanted directly into the right thigh muscles of mdx-scid mice (Supplementary Figure 2). The left thigh muscles were injected with PBS as an internal control. After 3 wk, myotubes in the muscle tissues injected with human EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells expressed human dystrophin as a cluster (Figure 5, A, C, and D, EM-

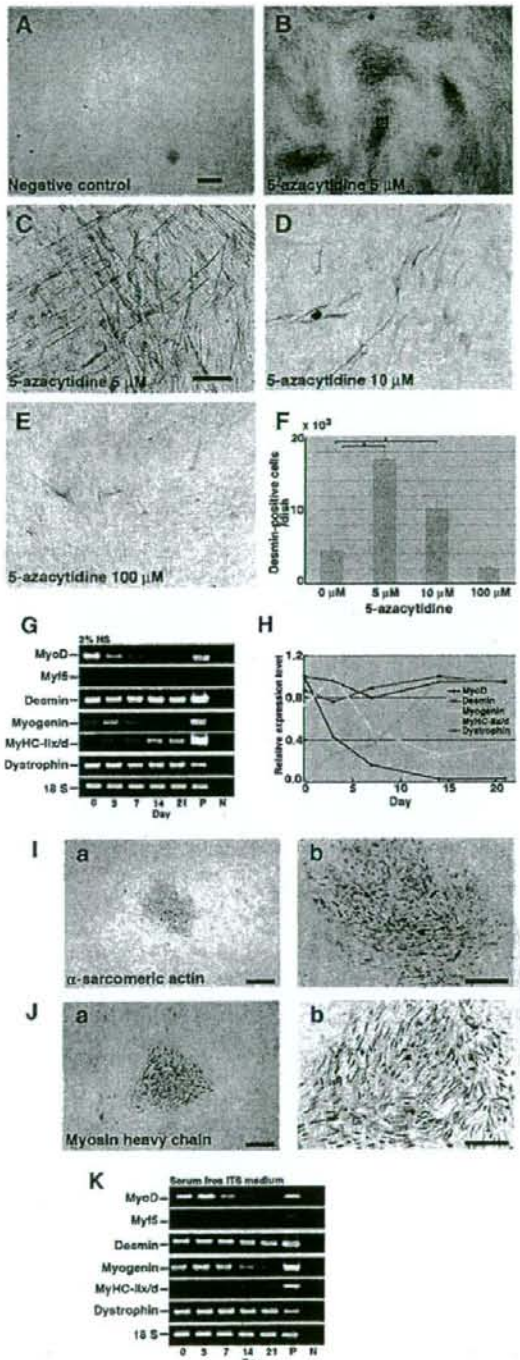

Figure 3. Expression of myogenic-specific genes during myogenic differentiation of EM-E6/E7/hTERT-2 cells. (A–F) Immunocytochemical analysis of EM-E6/E7/hTERT-2 cells using an antibody to desmin. (A) Omission of only the primary antibody to desmin serves as a negative control. (C) Higher magnification of inset in B. (F) Myogenic differentiation of EM-E6/E7/hTERT-2 cells with exposure to different concentrations (B, 5 μM; C, 5 μM; D, 10 μM, E, 100 μM) of 5-azacytidine. To estimate myogenic differentiation, the number of all the desmin-positive cells was counted for each dish (n = 3). Data were analyzed for statistical significance using ANOVA. EM-E6/E7/hTERT-2 cells were cultured in the DMEM supplemented with 2% HS (G–J; horse serum), and serum-free ITS (K). (G and K) RT-PCR analysis with PCR primers allows amplification of the human MyoD, Myf5, desmin, myogenin, myosin heavy chain type IIx/d (MyHC-IIx/d), and dystrophin cDNA (from top to bottom). RNAs were isolated from EM-E6/E7/hTERT-2 cells at the indicated day after treatment with 5-azacytidine. RNAs from human muscle and H_2O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (H) Time course of MyoD, desmin, myogenin, MyHC-IIx/d, and dystrophin expression in the cells incubated with 2% HS for up to 21 d after 5-azacytidine treatment. Relative mRNA levels were determined using Multi Gauge Ver 2.0 (Fuji Film). The signal intensities of MyoD, desmin, and dystrophin mRNA at day 0, myogenin mRNA at day 3, and MyHC-II/d mRNA at day 21 were regarded as equal to 100%. (I and J) The cells were exposed to 5 μM 5-azacytidine for 24 h and then subsequently cultured in DMEM supplemented with 2% HS for 21 d. α-Sarcomeric actin (I) and skeletal myosin heavy chain (J) was detected by immunocytochemical analysis. Scale bars, 2 mm (A and B), 300 μm (C–E), 900 μm (Ia and Ja), 425 μm (Ib and Jb).

E6/E7/hTERT-2 cells, and 5B, menstrual blood–derived cells). Quantification analysis revealed that the percentage of dystrophin-positive myofibers after implantation of menstrual blood–derived cells was high, compared with that after implantation of EM-E6/E7/hTERT-2 cells (Figure 5E). Donor cells with EGFP fluorescence participated in myogenesis 3 wk after implantation (Supplementary Figure 3). EGFP-labeled EM-E6/E7/hTERT-2 cells became positive for human dystrophin (Figure 5C). Dystrophin was not detected in the muscle of mdx-scid mice and NOG mice without cell implantation because the antibody to dystrophin used in this study is human-specific, implying that dystrophin is transcribed from dystrophin genes of human donor cells but not from reversion of dystrophied myocytes in mdx-scid mice.

To determine if dystrophin expression in the donor cells is due to transdifferentiation or fusion, immunohistochemistry with an antibody against human nuclei (Ab-HuNucl) and DAPI stain was performed. If dystrophin expression is explained by fusion, dystrophin-positive myocytes must be demonstrated to have both human and murine nuclei. We examined almost all the 7-μm-thick serial histological sections parallel to the muscular bundle (longitudinal section) of the muscular tissues by confocal microscopy and found that dystrophin-positive myocytes have nuclei derived from both human and murine cells in the longitudinal section of the myocytes (Figure 5D), implying that dystrophin expression is attributed to fusion between murine host myocytes and human donor cells, rather than myogenic differentiation of EM-E6/E7/hTERT-2 cells and menstrual blood–derived cells per se.

Detection of Human Endometrial Cell Contribution to Myotubes in an In Vitro Myogenesis Model

To simulate in vivo phenomena, human endometrial cells were cocultured in vitro with murine C2C12 myoblasts for 2 d under proliferative conditions and then switched to differentiation conditions for an additional 7 d. Figure 6A

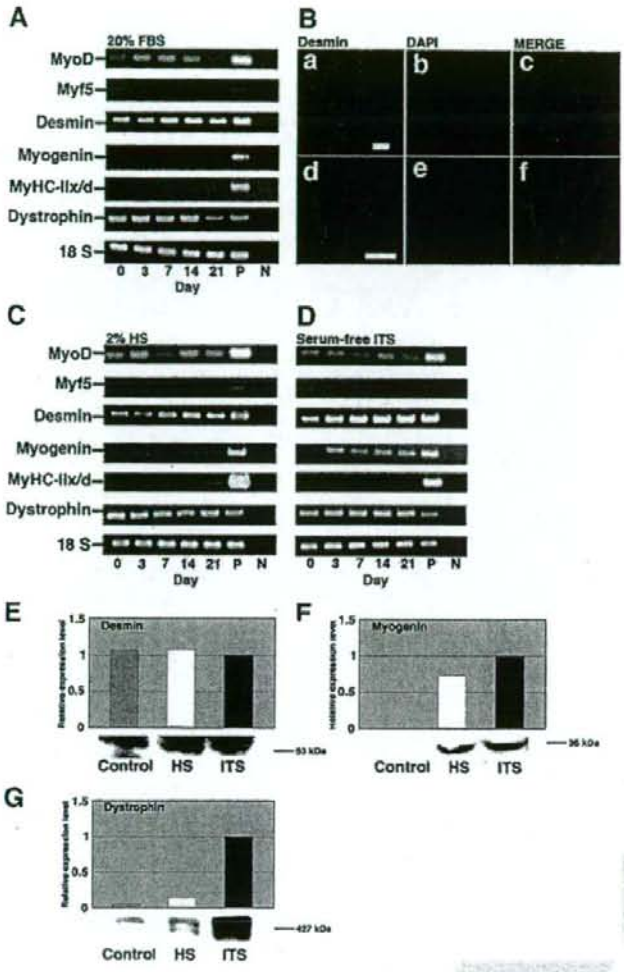

Figure 4. Expression of myogenic-specific genes in differentiated menstrual blood–derived cells. Menstrual blood–derived cells were cultured in DMEM supplemented with 20% FBS, 2% HS, or serum-free ITS medium. (A) RT-PCR analysis with PCR primers that allows amplification of the human MyoD, Myf5, desmin, myogenin, MyHC-IIx/d, and dystrophin cDNA (from top to bottom). RNAs were isolated from menstrual blood–derived cells at the indicated day after treatment with 5 μM 5-azacytidine for 24 h. RNAs from human muscle and H_2O served as positive (P) and negative (N) controls. Only the 18S PCR primer used as a positive control reacted with the human and murine cDNA. (B) Immunocytochemical analysis using an antibody to desmin (a–f) was performed on the menstrual blood–derived cells at 2 wk after exposure to 5 μM of 5-azacytidine for 24 h. The desmin-positive cells are shown at higher magnification (d–f). Merge of a and b is shown in c, and merge of d and e is shown in f. The images were obtained with a laser scanning confocal microscope. Scale bars, 200 μm (a–c) and 75 μm (d—f). (C and D) RT-PCR analysis of menstrual blood–derived cells on DMEM supplemented with 2% HS (C) or serum-free ITS medium (D) after exposure to 5 μM 5-azacytidine for 24 h. (E–G) Western blot analysis was performed on the cells cultured in myogenic medium indicated for 21 d. The blot was stained with desmin (E), myogenin (F), and dystrophin (G) antibodies followed by an HRP-conjugated secondary antibody.

provides an example of how human and mouse nuclei in the EGFP-positive myotubes were detected. Multinucleated myotubes were revealed by the presence of specific human dystrophin (Figure 6, B and C) and myosin heavy chain (Figure 6D). Dystrophin was detected in cytoplasm in culture condition (Figure 6, B and C) despite evidence of cell surface localization in vivo. Human dystrophin and human nuclei were unequivocally identified by staining with antibodies to human dystrophin and human nuclei, whereas the numerous mouse nuclei present in this field, as shown by DAPI staining, are negative (Figure 6, B and C).

DISCUSSION

Skeletal muscle has a remarkable regenerative capacity in response to an extensive injury. Resident within adult skeletal muscle is a small population of myogenic precursor cells (or satellite cells) that are capable of multiple rounds of proliferation (estimated at 80–100 doublings), which are able to reestablish a quiescent pool of myogenic progenitor cells after each discrete regenerative episode (Mauro, 1961; Schultz and McCormick, 1994; Seale and Rudnicki, 2000; Hawke and Garry, 2001). Although muscle regeneration is a highly efficient and reproducible process, it ultimately is exhausted, as observed in senescent skeletal muscle or in patients with muscular dystrophy (Gussoni *et al.*, 1997; Cossu and Mavilio, 2000). In the present study, we investigated the myogenic potential of human endometrial tissue-derived immortalized EM-E6/E7/TERT-2 cells and primary cells derived from human menstrual blood. Human menstrual blood–derived cells proliferated over at least 25 PDs (9 passages) for more than 60 d and stopped dividing before 30 PDs. This cessation of cell division is probably due to replicative senescence or shortening of telomere length. Cell life span of menstrual blood cells is relatively short when compared with human fetal cells (Imai *et al.*, 1994; Terai *et al.*, 2005), and this shorter cell life span may be attributed to shorter telomere length of adult cells (i.e., endometrial stro-

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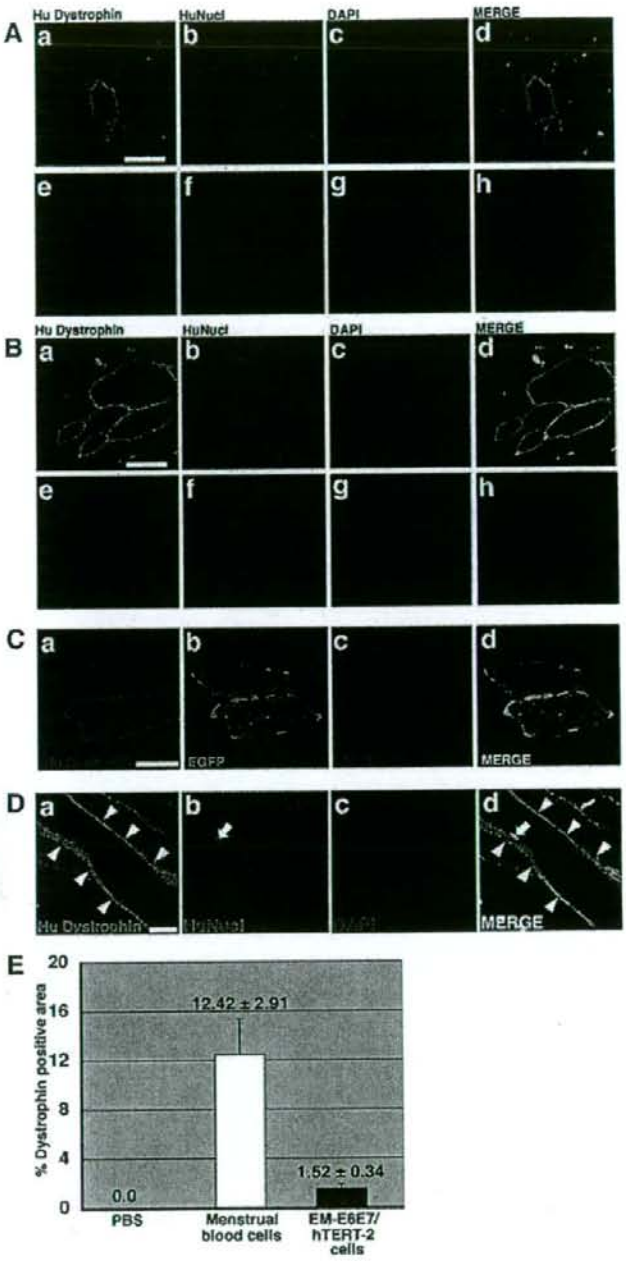

Figure 5. Conferral of dystrophin to mdx myocytes by human endometrial cells. (A and B) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green), human nuclei (HuNucl, red), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of EM-E6/E7/hTERT-2 cells (A) or menstrual blood–derived cells (B) without any treatment or induction. (C) EGFP-labeled EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into the thigh muscle of mdx-scid mice. Immunohistochemistry revealed the incorporation of implanted cells into newly formed EGFP-positive myofibers, which expressed human dystrophin 3 wk after implantation. (A and B) As a methodological control, the primary antibody to dystrophin was omitted (e and f). (D) Immunohistochemistry analysis using an antibody against human dystrophin molecule (green, arrowheads), human nuclei (HuNucl, red, arrow), and DAPI staining (blue) on thigh muscle sections of mdx-scid mice after direct injection of human EM-E6/E7/hTERT-2 cells without any treatment or induction. (A and B) Merge of a–c is shown in d, and merge of e–g is shown in h. (C and D) Merge of a–c is shown in d. Scale bars, 50 μm (A and B), 20 μm (C and D). (E) Quantitative analysis of human dystrophin-positive myotubes. Menstrual blood–derived cells or EM-E6/E7/hTERT-2 cells without any treatment or induction were directly injected into thigh muscle of mdx-scid mice. The percentage of human dystrophin–positive-myofiber areas was calculated 3 wk after implantation of the EM-E6/E7/hTERT-2 cells or menstrual blood–derived cells. Injection of PBS without cells into mdx-scid myofibers was used as a control.

mal cells) at the start of cell cultivation, as is the case with hematopoietic stem cells (Suda *et al.*, 1984).

Menstrual blood–derived cells had a high replicative ability similar to progenitors or stem cells that display a long-term self-renewal capacity and had a much higher growth rate in our experimental conditions than marrow-derived stromal cells (Mori *et al.*, 2005). In addition, the myogenic potential of menstrual blood–derived cells, i.e., a high frequency of desmin-positive cells after induction, is much greater than expected. The higher myogenic differentiation ratio can be explained just by alteration of cell characteristics from epithelial and mesenchymal bipotential cells or heter-