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Establishment of porcine pulp-derived cell lines and expression of recombinant dentin sialoprotein and recombinant dentin matrix protein-1

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The major non-collagenous proteins in dentin have extensive post-translational modifications (PTMs) that appear to be odontoblast-specific, so expression of recombinant dentin proteins in other cell types does not achieve the *in vivo* pattern of PTMs. We established cell lines from developing porcine dental papillae and used them to express recombinant dentin sialoprotein (DSP) and dentin matrix protein-1 (DMP1). Pulp cells were immortalized with pSV3-neo and clonally selected. Cell lines were characterized by reverse transcription–polymerase chain reaction (RT–PCR) and assayed for alkaline phosphatase activity and mineralized nodule formation. One of the five cell lines (P4-2) exhibited an odontoblastic phenotype, as determined by expression of tooth-specific markers, response to cytokines, and ability to form mineralized nodules. DSP and DMP1 expression constructs were transiently transfected into various cell lines. DSP, expressed by P4-2 cells, contained chondroitin 6-sulfate, which is a defining modification of the DSP proteoglycan. DMP1 was secreted and cleaved by proteases, even in human kidney 293 cells, which normally do not express DMP1, demonstrating susceptibility to non-specific proteolysis. Both recombinant proteins enhanced P4-2 cell attachment in a dose-dependent manner. We conclude that we have immortalized porcine odontoblast-like cells which express recombinant dentin extracellular matrix components with post-translational modifications that closely resemble those produced *in vivo*.

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Odontoblast-like cell lines have proved to be a versatile research tool. Pulp-derived cell lines are considered to be odontoblast-like if they express alkaline phosphatase (ALP) and dentin extracellular matrix molecules, and form mineralized nodules when incubated with β-glycerophosphate (βGP). A number of different cell lines have been generated from rat (1, 2), mouse (3, 4), bovine (5), and human (6–8) pulp tissues. These lines have served well in many applications, including the study of normal and pathological mineralization; the repair and regeneration of dental tissues; in tooth development, gene regulation and promoter analyses; and in toxicity and biocompatibility testing. In this study, we have established a porcine odontoblast-like cell line for the expression of recombinant dentin extracellular matrix proteins that are as identical as possible to those secreted *in vivo*.

Dentin and enamel proteins can be obtained in quantity from pigs because of their availability from stockyards and the large size of their developing teeth. For this reason, the porcine animal model has proved important for the discovery and characterization of dentin and enamel extracellular matrix molecules. Most of the porcine amelogenin amino acid sequence was determined by protein methods (9, 10), even before the landmark cloning and characterization of the first amelogenin cDNA (11, 12). Enamelin protein was first discovered (13, 14) and extensively characterized (15–18) from pig, which led directly to the cloning and characterization of the first enamelin cDNA (19). Ameloblastin protein was isolated and characterized from pig enamel (13, 16, 20) and described as a 'sheath protein' (21) before the first cDNA clones were isolated from rat teeth using molecular biology techniques (22, 23). Kallikrein 4 (KLK4), originally designated enamel matrix serine proteinase 1 (EMSP1), was first isolated from porcine enamel (24); and the first KLK4 cDNA was cloned from developing pig teeth (25, 26), as was enamelysin (MMP-20) (27). The porcine animal model continues to yield important discoveries concerning the structure and function of enamel proteins.

More recently, the porcine animal model has been used to identify and characterize dentin extracellular matrix components, such as osteonectin (28), dentin matrix protein 1 (DMP1) (29), and dentin sialoprotein (DSP) (30). The 'middle' portion of the dentin sialophosphoprotein (DSPP) chimera, designated dentin glycoprotein (DGP), was recently discovered in pig dentin and

completely sequenced by protein methods (31). Porcine DSP was used to demonstrate that DSP is a proteoglycan having chondroitin 6-sulfate attachments, forms covalent dimers, and may assemble into large proteoglycan aggregates by binding hyaluronan (32).

All dentin and enamel extracellular matrix molecules have post-translational modifications, and these modifications are typically extensive (33). Furthermore, all of the enamel and dentin non-collagenous proteins are processed by proteases, so that the uncleaved proteins can be difficult, or impossible, to isolate. To advance our understanding of the structures and biological properties of extracellular matrix proteins, it is desirable to express recombinant proteins that closely approximate the structures of the native proteins. Recombinant proteins expressed in bacteria have been used extensively to characterize the functional properties of tooth proteins, but results obtained using molecules that lack the extensive post-translational modifications of the native proteins may lead to erroneous conclusions about the roles of these proteins *in vivo*. Expression in any type of eukaryotic cell is also not always a reliable solution. Recombinant amelogenins expressed in insect cells using the baculovirus system, for instance, were not phosphorylated (34,35). Here we report the establishment of porcine pulp-derived cell lines that are odontoblast-like and express recombinant porcine dentin proteins with the native pattern of post-translational modifications.

Material and methods

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Program at the University of Michigan.

Isolation of primary tooth germs and cell culture procedures

Tooth germs of permanent molars were surgically extracted from the mandibles of 6-month-old pigs at the Michigan State University Meat Laboratory (East Lansing, MI, USA). Enamel organ epithelia (EOE) and pulp tissue were carefully separated from the crown and digested in a solution of collagenase/dispase (Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at room temperature. The released cells were passed through a 100-μm cell strainer (BD Biosciences Discovery Labware, Bedford, MA, USA) and pelleted by centrifugation. For pulp cells, the pellet was resuspended and maintained in the alpha modification of Eagle's medium (MEM; Gibco-BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco-BRL) and 1% antibiotics (100 U ml^{-1} of penicillin-G and 100 μg ml^{-1} of streptomycin sulfate; Gibco-BRL) at 37°C in a humidified 5% CO_2 atmosphere. Primary EOE cells were maintained in keratinocyte-SFM (Gibco-BRL) at 37°C in a humidified 5% CO_2 atmosphere.

Transfection and establishment of pulp cell lines

The primary pulp cells, at 80% confluence, were transfected with the pSV3-neo plasmid (ATCC 37150) by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. This plasmid expresses the SV40 virus large T antigen for immortalization and expresses neomycin phosphotransferase for selection (36). Forty-eight hours after transfection, the cells were plated at low density (1 : 6) and selected with 0.35 mg ml^{-1} of G418 sulfate (Invitrogen). The cells were maintained under selection until the colonies were visible (10 d). Individual clones were isolated with cloning cylinders and maintained in alpha MEM containing 10% fetal bovine serum (FBS) for at least 150 d. The population doubling level was more than 140.

Teleomeric repeat amplification protocol (TRAP) assay

A TRAP assay was performed using the TRAPeze telomerase detection kit (Chemicon, Temecula, CA, USA). Porcine pulp cells that were grown in culture for more than 45 passages were washed twice with phosphate-buffered saline (PBS). Protein extracts were prepared using CHAPS lysis buffer and then extracted for 30 min on ice. The extracts were centrifuged at 12,000 *g* for 20 min, and the supernatants were collected, aliquoted, and frozen at −80°C. The protein concentration in the extracts was determined by a protein assay (Bio-Rad, Hercules, CA, USA). Telomerase activity was detected by the TRAP assay using the TS oligonucleotide (5′-AATCCGTCGAGCAGAGTT), which served both as the telomerase template and the forward primer for PCR, and the CXa oligonucleotide (5′-GTGTAACCCTAACCCTAACCC) was used as the reverse primer (37). After incubation for 30 min at 30°C, PCR was performed for 33 cycles, with 1 min of denaturation at 94°C, 30 s of annealing at 58°C, and 30 s of elongation at 72°C. Products were resolved by electrophoresis in non-denaturating 12% polyacrylamide gels (acrylamide/*N*,*N*′-methylenebisacrylamide, 19 : 1, w/w) in ×0.5 TBE (Tris-Borate-EDTA). The gels were stained with ethidium bromide.

Preparation of RNA and the polymerase chain reaction (PCR)

Total RNA of each cell line at passage 30 was obtained with TRIzol reagent (Invitrogen). Thereafter, cDNA was synthesized from 1 μg of the total RNA using Superscript 3 (Invitrogen). The PCR reactions proceeded as follows: 5 min of denaturation at 94°C, followed by repeated cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 30 s. The final elongation was at 72°C for 5 min. The PCR products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide.

ALP assay

Pulp cells were spread on 96-well plates, at a density of 5×10^3 cells/well, and incubated for 24 h. The growth medium was changed to growth medium with various concentrations of recombinant-human bone morphogenetic protein 2 (rhBMP2) (R & D Systems, Minneapolis, MN, USA), or recombinant-porcine transforming growth factor-β1 (rpTGF-β1) (R & D Systems). After an additional 72 h of incubation, the cells were washed once with PBS. ALP activity was determined by incubation for 8 min at 37°C in 100 μl of 10 mM *p*-nitrophenylphosphate (substrate) in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer

(pH 10.0) containing 5 mM $MgCl_2$. The reaction was quenched with 50 μl of 0.2 M NaOH, and the absorbance at 405 nm was read by using a plate reader.

DMP1 and DSP expression vector constructs

The DMP1 (acc. no. AY963261) (29) and DSP (acc. no. AF332578) (30) coding regions were amplified from pulp tissue cDNA using the primer pairs 5′-CAGCTATGAAGACCAGCATCC/5′-GTAGCCGTCCTGGCAGTCATT and 5′-TCCTGGATTTTCAAAGTCCC/5′-GCGGGGAGCTGCAATTTCTA, respectively, and the amplification products were cloned into pEF6/V5-His-TOPO (Invitrogen). The resulting constructs expressed recombinant dentin proteins with a 45 amino acid combined V5-epitope and poly histidine tag at the C terminus: KGNSADIQHSGGRSSLEGPRFEGKPIP NPLLGLDSTRTGHHHHHH, which adds $\approx$5 kDa to a protein's apparent molecular weight on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The DNA sequences of the PCR insert and the adjacent vector at the junctions were confirmed by DNA sequence analyses.

Transfection of expression vectors

P4-2 cells, at 90% confluence, were transfected with 4 μg of the expression vector, per 35-mm tissue culture dish, using lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For transient expression studies, cells were processed for protein production 24 h post-transfection. For the establishment of stable lines, cells were subcultured 1 : 4 at 48 h following transfection and selected in growth medium containing 2.5 μg ml^{-1} of blasticidin (Invitrogen). The cells were maintained under selection until colonies were visible (10 d). Individual clones were isolated with cloning cylinders and amplified under selection in complete growth medium containing 2.5 μg ml^{-1} of blasticidin, and individual clones were processed for protein production.

Protein production and purification

The cells used for recombinant protein expression included porcine EOE primary cultures, a mouse osteoblast-like cell line (MC3T3-E1, subclone no. 14, ATCC CRL-2594), a human kidney epithelial cell line (HEK293, ATCC CRL-1573), and the porcine odontoblast-like cell line P4-2 (this work). Primary EOE cells were maintained in keratinocyte-SFM. MC3T3-E1 and P4-2 were maintained in alpha-MEM containing 10% FBS. HEK293 cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% FBS. Twenty-four hours after transfection, growth medium was removed from the cell cultures, after which the cell layers were washed twice with PBS and incubated in serum-free alpha-MEM at 37°C. In some experiments, tunicamycin (7 μg ml^{-1}; Sigma, St Louis, MO, USA) was added to block the glycosylation of newly synthesized proteins. After 24 h, the conditioned medium was harvested, cell debris was removed by centrifugation, and the His-tagged recombinant proteins were purified with Talon Resin (Clontech, Palo Alto, CA, USA). To obtain recombinant protein from cells, the cells were washed with five times with PBS, then a cell lysate was generated using RIPA lysis buffer (sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer's instructions. To test for glycosaminoglycan chains (32), recombinant DSP (50 μg) was digested with 0.2 units of protease-free chondroitinase ABC (Seikagaku America, East Falmouth, MA, USA) in 0.1 ml of 40 mM Tris-HCl/40 mM sodium acetate buffer (pH 8.0).

Mineralization assay

The P4-2 line of immortalized pulp cells, P4-2/DMP1 (P4-2 cells and stable transfectants of pEF6/DMP1), and P4-2/DSP (P4-2 cells and stable transfectants of pEF6/DSP), were plated in 24-well plates at an initial density of 9.0×10^3 cells/well. After incubation for 24 h, the medium was changed to growth medium, with or without ascorbic acid (AA; 50 μg ml^{-1}; Wako, Tokyo, Japan), βGP (10 mM; Sigma), and dexamethasone (DEX; 10 nM; Sigma). Pulp cells were cultured for up to 10 d. The medium was changed every 72 h. Mineralization was visualized by alizarin red S staining. Following fixation with 100% methanol, the cells were stained with alizarin red S (Sigma) solution for 10 min, and then washed with dH_2O and photographed. The stained area was quantified using NIH IMAGE (NIH, Bethesda, MD, USA).

Western blot analysis

Samples were applied to precast 4–20% Tris–glycine sodium dodecyl sulfate–polyacrylamide gels or to 4–12% bis-(2-hydroxylethyl) aminoTris (hydroxymethyl) methane (bis–Tris) NuPAGE gels (Invitrogen), separated by electrophoresis, and electrotransferred onto a Hybond-P membrane (GE Healthcare Biosciences, Little Chalfont, Bucks., UK). Blocking was performed with 5% non-fat dry milk for 1 h, followed by incubation, for 1 h, with a 1 : 10,000 dilution of antibody to V5-horseradish peroxidase (V5-HRP) (Invitrogen), or a 1 : 20,000 dilution of polyclonal antibody to porcine DSP (30) in TTBS (50 mM Tris-HCl, 154 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% milk. The blots were washed three times, for 20 min each wash, in TTBS. When DSP antibody was used, the membrane was incubated with anti-chicken immunoglobulin G (IgG) secondary antibody (Bio-Rad) at a dilution of 1 : 20,000, and washed three times, for 20 min each wash, in TTBS. The immunoreactive proteins were detected by enhanced chemiluminescence (ECL; GE Healthcare Biosciences).

Cell adhesion assays

Cell adhesion assays were performed in Nunc MaxiSorp flat-bottom 96-well plates (eBioscience, San Diego, CA, USA). The lyophilized purified test protein was weighed and dissolved in PBS (pH 7.4). Successive dilutions of each test protein solution were plated in triplicate, on a 96-well plate, at 4°C overnight and the wells were blocked with 3% bovine serum albumin (BSA) for 1 h at 37°C. The test proteins used were rDMP1, rDSP, rat-tail collagen I (BD Biosciences, San Jose, CA, USA), as positive controls, and BSA (Fisher Scientific, Fair Lawn, NJ, USA) as a negative control. Subconfluent immortalized porcine odontoblasts (P4-2) were detached with trypsin, counted using a hemocytometer, and 2×10^4 cells were added to each well and incubated for 1 h. The unattached cells were removed by washing three times with PBS. The attached cells were quantified using two separate color assays: the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), in which viable cells bioreduce MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-ZH-tetrazolium, inner salt], allowing it to react with

PES (phenazine ethosulfate) to form a colored product that is quantified by measuring the absorbance at 485 nm; and by staining the cells with crystal violet and quantifying by measuring the absorbance at 595 nm. All experiments were repeated in triplicate ($n = 3$).

Effect of DSP deglycosylation on cell adhesion activity

Porcine DSP was sequentially deglycosylated with glycopeptidase A (Seikagaku America, East Falmouth, MA, USA) and protease-free chondroitinase ABC (Seikagaku America), as reported previously (31). Then, 5 μg ml^{-1} from each sample was used for cell adhesion assays, as described above.

Statistical analyses

All values were represented as the mean ± standard error (SE). Statistical significance was determined using the unpaired Student's *t*-test, with a *P*-value of < 0.05 being the threshold for statistical significance.

Results

Immortalization of porcine pulp cells

During the immortalization procedures, 12 G418-resistant colonies were transferred to separate Petri dishes and grown to confluence. At confluence, the cells were counted, detached, diluted 1 : 8 to 1 : 16, and grown on fresh plates. The cells were passaged in this way for at least 150 d and their cell population doubling levels were determined and plotted (Fig. 1). Five (P1-1, P2-1, P4-2, P4-6, and P4-7) of the 12 clones were immortalized, based upon their continued proliferation after 50 d. The histological appearances of both cultured and immortalized pulp cells were fibroblast-like (Fig. 1). Telomerase activity was demonstrated in the P4-2 and P4-7 cell lines by using a TRAP assay, and this activity was inhibited by heat treatment (Fig. 2A). We also checked the telomerase activities in the P1-1, P2-1, and P4-6 cells, and observed similar results in all of the cell lines (data not shown). Stock cultures were judged free of contamination using the VenorGeM Mycoplasma Detection Kit (Sigma). All cell lines were positive for the expression of SV40 mRNA. Expression of a panel of dentin and enamel markers was assayed by reverse transcription (RT)–PCR (Fig. 2B). Messenger RNA for DMP1, DSPP, osteocalcin, enamelin, and nestin were expressed in all of the five cell lines, whereas the expression of amelogenin, KLK4, and MMP-20 was limited to selected clones.

ALP expression and mineralized nodule formation

We investigated the effects of BMP2 and TGF-β1 on ALP activity in the P4-2 cell line, as BMPs are believed to induce the differentiation of pulp cells into odontoblasts (38). ALP activity was enhanced by BMP treatment, but inhibited by TGF-β1 treatment in a dose-dependent manner (Fig. 3A,B). These results are consistent with earlier findings showing that TGF-β decreased the ALP activity of a rat pulp cell line (RPC-C2A) (39). In the presence of AA and βGP, the P4-2 cell line formed mineralized nodules after 10 d in culture. A higher concentration of serum (10%) induced more mineralized nodules, and these were significantly reduced

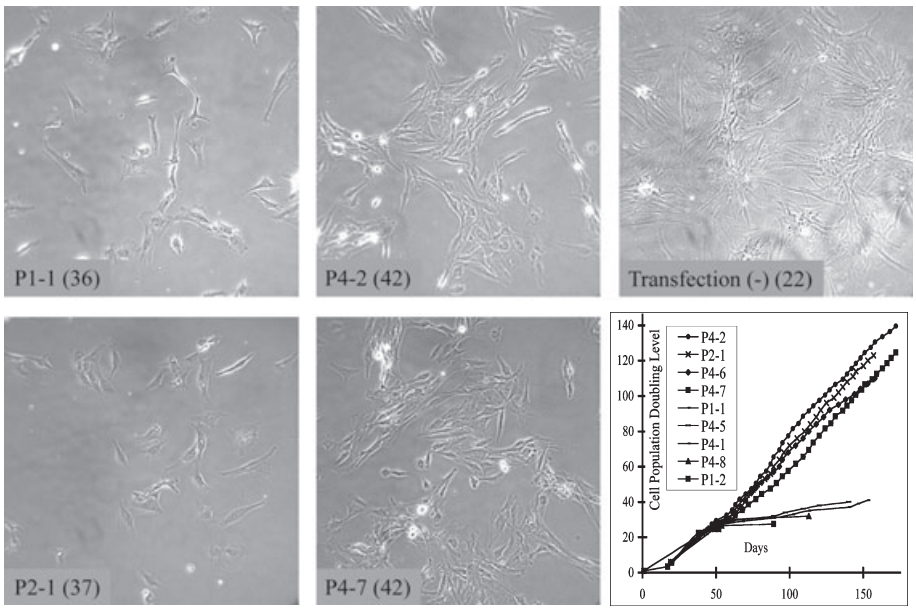
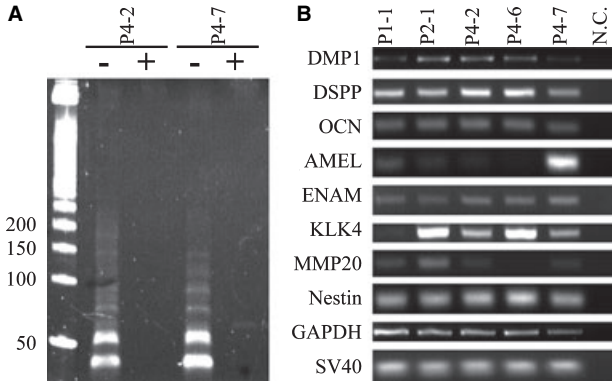


Fig. 1. Morphology and proliferation of porcine pulp-derived cell lines. Four pulp-derived cell lines (P1-1, P4-2, P2-1, P4-7) and the untransfected pulp culture are shown (original magnification ×40). The number in parentheses corresponds to the number of passages at the time of the imaging. A plot of the cell population doubling level against days in culture following transfection is shown on the lower right of the figure. The key accompanying the plotted growth lines is in the same order, from top to bottom, as the lines themselves. Note that only the cell population doubling level of successfully immortalized cells (P4-2, P2-1, P4-6, P4-7, and P1-1) continued to increase after 50 d.



C

DMP1	310 bp	F: tgtcctgtgctctcccagt	R: tcgcccaaggtgtcatctcc
DSPP	203 bp	F: gcatgccattgacaaaagtg	R: aactgttccctcccacgtct
OCN	188 bp	F: tggctgatcacatcggctt	R: ttattttggagcagctggga
AMEL	236 bp	F: acccctctgaagtggtaccag	R: tgttgggttggagtcatggag
ENAM	433 bp	F: aagaactgctggccttactcc	R: tccccaaagaatgtttggttg
KLK4	478 bp	F: tggcaggcggcactgttc	R: aggagtccttttggtcctg
MMP20	290 bp	F: atacgtgcagcgaatagatgc	R: ctatttagcaaccaatccagg
Nestin	150 bp	F: ggcttctctcagcatcttgg	R: aaggctggcataggtgtgtc
GAPDH	452 bp	F:accacagtccatgccatcac	R: tccaccaccctgttgctgta
SV40	200 bp	F: catgaacagactgtgaggac	R: tgtggctatgggaattggag

Fig. 2. Teleomerase activity and expression of dental markers in pulp-derived cell lines. (A) Teleomeric repeat amplification protocol (TRAP) assay. The sizes of the bands (in bp) in the marker lane are shown on the left. Samples without (–) or with (+) heat inactivation were amplified with specific polymerase chain reaction (PCR) primers. (B) Agarose-gel electrophoresis of reverse transcription (RT) products from the five cell lines (P1-1, P2-1, P4-2, P4-6, P4-7) and a control without cDNA (N.C.) amplified using primer sets for dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), osteocalcin (OCN) amelogenin (AMEL), enamelin (ENAM), kallikrein 4 (KLK4), enamelysin (MMP20), nestin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the SV40 large T antigen (SV40). (C) Predicted sizes of the amplification products and sequences of the oligonucleotide primers used to generate them.

in number by adding DEX in culture with 10% FBS (Fig. 3C,D).

Recombinant expression of DMP1 and DSP

We expressed recombinant porcine DMP1 and DSP in several types of cells (Fig. 4). The extent of DMP1 post-translational modifications (based upon the mobility of the largest band) was different in 293, MC3T3, EOE, and P4-2 cells (Fig. 4A). The two rDMP1 bands recovered from the media of 293 cells had the same apparent size ($\approx$ 98 kDa and $\approx$60 kDa), with (ME+) or without (ME-) β-mercaptoethanol, demonstrating that the upper band was not a covalent dimer of the lower band. Only the upper band was isolated from the cells themselves, suggesting that the lower band observed in the media was a C-terminal cleavage product generated outside the cells, following its secretion. DMP1 was transiently transfected in MC3T3 and cultured EOE cells, and in the P4-2 cell line. In each case, the recombinant DMP1 showed multiple bands, with proteolysis and variations in its post-translational modifications contributing to its heterogeneity (Fig. 4A, right).

Recombinant DSP, expressed in P4-2 cells, presented as a proteoglycan smear migrating between 150 and 300 kDa on 4–20% gradient SDS–PAGE, which is similar in apparent molecular weight to native porcine DSP (32), and as a smear of 95–150 kDa on 4–12% NuPageMOPS gels (Fig. 4B). The mobility of recombinant DSP varied depending on the gel type. The expression of DSP in EOE, MC3T3, and 293 cells generated recombinant protein with a lower apparent molecular weight. Treatment, with chondroitinase ABC, of recombinant DSP expressed in P4-2 reduced its apparent molecular weight. These findings are consistent

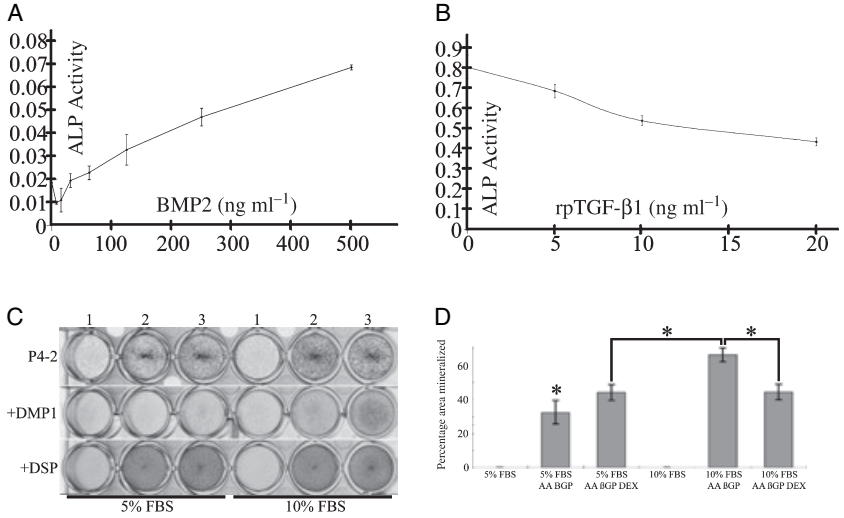


Fig. 3. Alkaline phosphatase (ALP) activity and mineralized nodule formation in the P4-2 cell line. ALP activity increased following the addition of bone morphogenetic protein 2 (BMP2) (A) and decreased with increasing concentration of transforming growth factor-β1 (TGF-β1) (B). Both of these responses were dose dependent. The mineralization of P4-2 cultures was visualized with alizarin red staining (C). P4-2 was grown in 5 or 10% fetal bovine serum (FBS), without supplements (wells 1); with ascorbic acid and β-glycerophosphate (βGP) (wells 2); or with βGP and dexamethasone (wells 3). NIH IMAGE quantifications of independent experiments, and the average percentage area mineralized, was plotted (D). Data are presented as the mean $\pm$ standard error (SE) ($n = 4$). *Significantly different from the group of 10% FBS with ascorbic acid (AA) and βGP ($P < 0.05$).

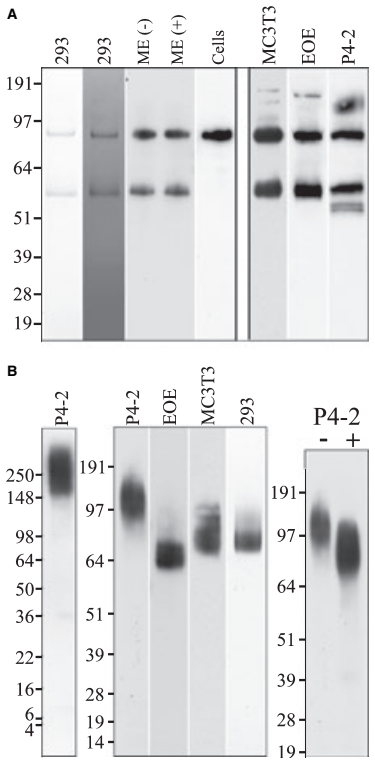


Fig. 4. Transient transfection of dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP). Recombinant porcine DMP1 was transiently expressed in human kidney 293 cells, affinity purified, separated on a NuPageMOPS gel and visualized by CBB, Stains-All, and by western blot analysis using the C-terminal V-epitope antibody (A, left). Two DMP1 bands were recovered from the media, and the apparent sizes of these bands were the same ($\approx$ 90 kDa and $\approx$60 kDa), with (ME+) or without (ME–) β-mercaptoethanol. Only the upper band was isolated from the 293 cell lysate (Cells), suggesting that the lower band was a C-terminal cleavage product generated outside the cells. DMP1 was transiently transfected in MC3T3 cells, cultured enamel organ epithelial cells (EOE) and in the P4-2 cell line, isolated and visualized on western blots (A, right). Recombinant porcine DSP was expressed in P4-2, EOE, MC3T3, and 293 cells (B). Digestion of recombinant DSP expressed in P4-2 cells with chondroitinase ABC (+) significantly reduced its mobility when compared with the undigested (–) sample, indicating that the recombinant protein is a proteoglycan.

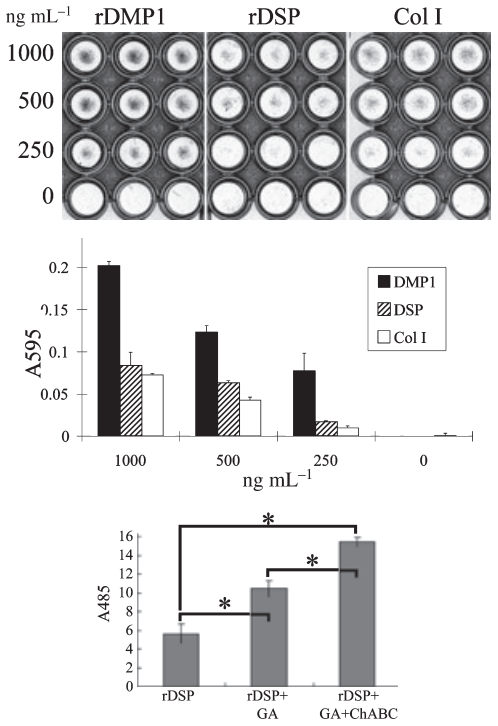


Fig. 5. Cell adhesion assays. The top panel shows triplicate wells of crystal violet-stained cells adhering to plates containing 0, 250, 500, or 1000 ng ml^{-1} of recombinant dentin matrix protein 1 (rDMP1), recombinant dentin sialoprotein (rDSP), or type I collagen (Col I). The middle panel graphs the absorbance, at 595 nm, of the cells after crystal violet staining. The bottom panel shows the effect of deglycosylation of DSP on cell adhesion activity in the P4-2 cell line. Porcine DSP was sequentially deglycosylated with glycopeptidase A (GA) and then with protease-free chondroitinase ABC (ChABC). The MTS assay was performed and the absorbance of each well was then measured at 485 nm using a microplate reader. Data are presented as the mean $\pm$ standard error (SE) ($n = 3$). $^*P < 0.05$.

with a previous report that DSP is a proteoglycan that can exist as a covalent dimer connected by a disulfide bridge (32), and that the pattern of DSP post-translational modifications is significantly affected by the type of cell hosting the expression construct.

Effects of recombinant DMP1 and DSP on cell attachment

Porcine DSP and DMP1 both contain RGD sequences and belong to the small integrin-binding ligand N-linked glycoproteins (SIBLING) family of dentin and bone extracellular matrix molecules (40). Recombinant porcine DSP and DMP1, expressed and purified from P4-2 cells, promoted the attachment of P4-2 cells in a dose-dependent manner (Fig. 5, top and middle), and the attachment activity of rpDMP1 was up to twice that of rpDSP and type I collagen (Fig. 5, middle). Digestion with glycopeptidase A significantly enhanced the cell-adhesion activity of DSP compared with untreated DSP. Furthermore, sequential digestion with glycopeptidase A followed by chondroitinase ABC significantly enhanced the cell-adhesion activity of DSP compared with glycopeptidase A-treated DSP.

Discussion

The major non-collagenous proteins secreted by odontoblasts are the DSPP-derived proteins, which include DSP, DGP, and DPP. The unique importance of DSPP for normal dentin formation is demonstrated by the many *DSPP* mutations identified in kindreds with inherited dentin defects (41–47), and by the tooth defects observed in *Dspp*$^{-/-}$ mice (48). Although no human kindreds with *DMP1* mutations have yet been characterized, *Dmp1*$^{-/-}$ mice show severe defects in virtually all

mineralized tissues (49, 50). DSPP and DMP1 are highly glycosylated, highly phosphorylated proteoglycans. Despite the proven importance of these proteins in the formation of tooth dentin, the full extent of their post-translational modifications (PTMs) and their attachment sites are only poorly characterized. We have been using the porcine animal model to characterize the PTMs of the *in vivo* proteins, and seek to express recombinant proteins as identical to them as possible.

Dental pulp cell lines have been generated and characterized from various mammalian species, but none have been used to compare the way these cells add PTMs to the major extracellular matrix proteins they secrete relative to the situation *in vivo*. To investigate pulp cell-specific PTMs, we established porcine pulp-derived cell lines as a first step. We isolated 12 clones after neomycin selection; however, only five of the clones survived for as many as 100 generations. We performed RT–PCR profiling to determine which, if any, of the cell lines naturally expressed the extracellular matrix proteins previously shown to be synthesized by odontoblasts (51). All of the clones expressed DMP1, DSPP, osteocalcin, enamelin, and nestin mRNA. Nestin is an intermediate filament in the cytoskeleton, thought to be a marker of odontoblast progenitor cells (52, 53). The expression of amelogenin, KLK4, and MMP-20 was limited to selected clones. Although generally associated with enamel formation, amelogenin, KLK4, and MMP-20 are expressed by odontoblasts at some point in their life cycles. KLK4 expression by odontoblasts has been implicated in the formation of a highly mineralized zone of enamel near the dentin–enamel junction (54), and MMP-20 (along with MMP-2) cleaves DSPP to generate DSPP-derived proteins during odontogenesis (55). Among the five clones, P4-2 was judged to be most similar to odontoblasts and was further characterized. Its expression of ALP was increased by BMP2 and decreased by TGF-β1. P4-2 was the only cell line able to form mineralized nodules when cultured with βGP.

Recent studies have shown that both DSP and DMP1 are expressed not only by odontoblasts but also by ameloblasts transiently (56) and by osteoblasts (57, 58). To check the PTMs of DSP and DMP1, we overexpressed both proteins in three types of cells: the odontoblastic cell line (P4-2), the osteoblastic cell line (MC3T3-E1), and in primary EOE-derived cells. The molecular sizes of DMP1 expressed in the three types of cells were almost the same on SDS–PAGE (Fig. 4A), whereas those of DSP varied on SDS–PAGE analyses (Fig. 4B). Only the P4-2 cell line expressed recombinant DSP with appropriate post-translational modifications, considered to be chondroitin 6-sulfate, based upon the results of chondroitinase ABC digestion. DMP1 appeared to be attacked by proteases in the extracellular compartment. Recombinant DSP, however, was expressed as a large proteoglycan, and did not appear to suffer from proteolytic degradation. An important criteria in the future for the selection of cell lines to express recombinant matrix proteins may be the isolation of immortalized cells that do not secrete proteases.

In normal bone and dentin, DMP1 is expressed in too low abundance to be isolated in quantity. As a consequence, recombinant DMP1 expressed in bacteria without any post-translational modifications has been used for structural and functional analyses (59). Recombinant rat DMP1, isolated from bacteria, was able to promote the attachment of dental pulp cells (RPC-C2A) and calvarial cells (MC3T3-E1) *in vitro* (61, 62). The DMP1-mediated attachment was cell-type specific and involved the DMP1 RGD sequence (60). We have confirmed these findings using recombinant porcine DMP1 expressed in dental pulp cells (P4-2), suggesting that post-translational modifications do not interfere with the ability of DMP1 to bind integrins and promote cell attachment. The context of the DMP1 RGD sequence is in a region away from predicted PTMs, and antipeptide antibodies raised against the deduced amino acid sequence centering around the porcine RGD sequence were able to detect, specifically, DMP extracted from teeth (29). On the other hand, our data showed that deglycosylation of DSP enhances its cell-adhesion activity. Perhaps the many carbohydrate attachments on DSP, such as its N-linked glycosylations and chondroitin 6-sulfate attachments, obscure the RGD sequence.

The expression of recombinant porcine DSP and DMP1 in immortalized porcine pulp cells that naturally express these proteins is an important step towards gaining sufficient amounts of these proteins for functional studies. An important additional parameter in determining if a cell line is truly 'odontoblast-like' may be its ability to introduce the appropriate post-translational modifications on the matrix proteins its secretes. We have shown that this is a sensitive criterion that is not met by most cell lines. The production of recombinant proteins suitable for functional studies may require the overexpression of matrix proteins through the stable transfection of cell lines that produce native post-translational modifications, while at the same time showing minimal expression of secreted proteases that degrade the matrix.

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