empty MSCV plasmid. NC, negative control.

Figure 4 Characterization of Runx3 binding to the *Osterix* promoter by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay

(A) Interaction of nuclear extract of mouse DPCs with *Osterix* promoter -713 to -707 sequence (site 3) in EMSA. Arrowhead indicates the retarded protein-DNA complexes. Arrow indicates the super-shift band in the reaction with the addition of Runx3 antibody (Lane 5). Lane 1 shows a binding reaction with no protein, and lane 2 is a control reaction with no competing oligonucleotides. Lane 3 and 4 represent competition reactions with wild type (WT) or mutation (MT) unlabelled oligonucleotides. Lane 5 is the reactions with Runx3 antibody. Lane 6 shows that only nuclear extract loaded. (B) ChIP Assay was carried out to investigate that both Runx3 and Runx2 could bind to *Osterix* promoter *in vivo*. A 120-bp band could be detected by RT-PCR both in Runx3 antibody and Runx2 antibody added sample but no band with rabbit or goat IgG. Ab, antibody; NC, negative control.

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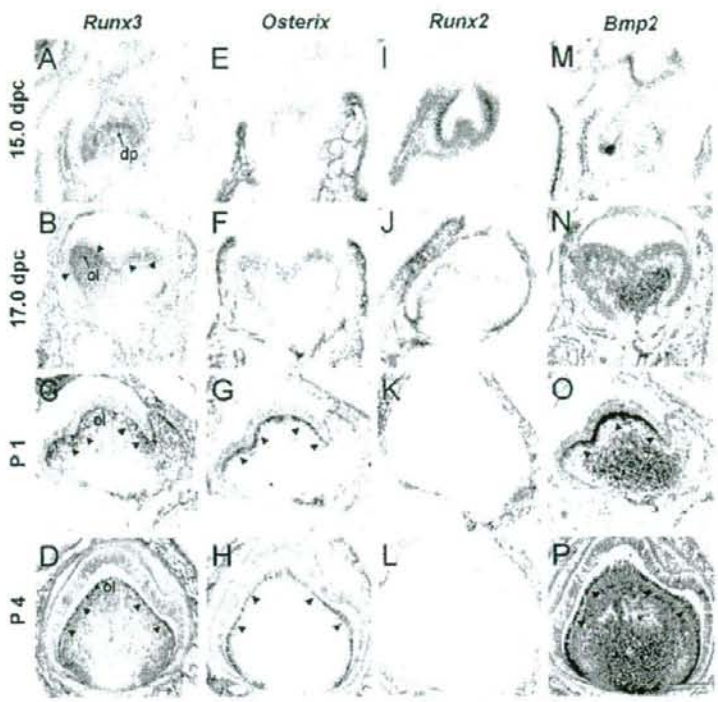

Figure 1

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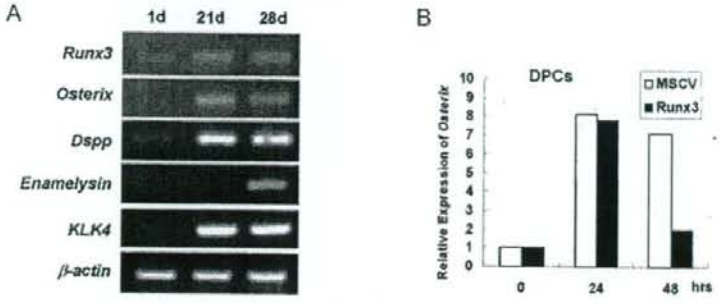

Figure 2

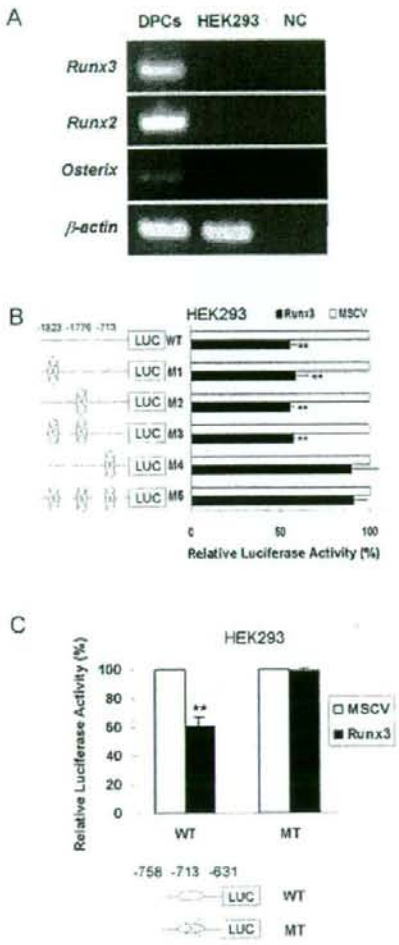

Figure 3

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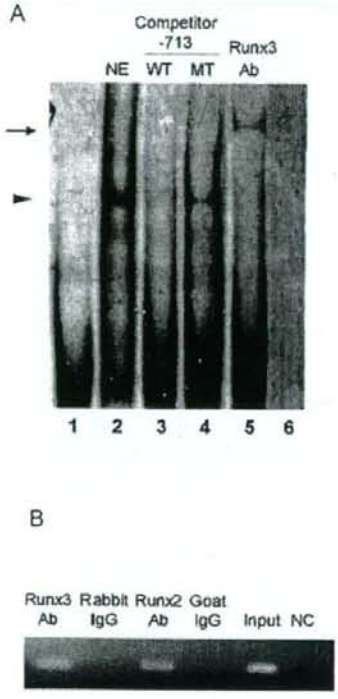

Figure 4

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Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis

Koichiro Iohara, Li Zheng, Masataka Ito, Atsushi Tomokiyo, Kenji Matsushita and Misako Nakashima

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Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis

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Key Words. Dental pulp stem cells • Side population cells • Dentin regeneration • Bone morphogenetic proteins • Neurogenesis Chondrogenesis • Adipogenesis

ABSTRACT

Dental pulp has the potential to form dentin as a regenerative response to caries. This regeneration is mediated by stem/progenitor cells. Thus, stem cell therapy might be of potential utility in induction of reparative dentin. We isolated side population (SP) cells from dental pulp based on the exclusion of the DNA binding dye Hoechst 33342 by flow cytometry and compared its self-renewal capacities and multipotency with non-SP cells and primary pulp cells. The cumulative cell number of the SP cells was greater than the non-SP cells and primary pulp cells. *Bmi1* was continuously expressed in SP cells, suggesting longer proliferative lifespan and self-renewal capacity of SP cells. Next, the maintenance of the multilineage differentiation potential of pulp SP cells was investigated. Expression of type II collagen and aggrecan confirmed chondrogenic conversion (30%) of SP cells. SP cells expressed peroxisome proliferator-activated receptor γ and adaptor protein 2, showing adipogenic conversion. Expression of mRNA and proteins of neurofilament and neuromodulin confirmed neurogenic conversion (90%). These results demonstrate that pulp SP cells maintain multilineage differentiation potential. We further examined whether bone morphogenetic protein 2 (BMP2) could induce differentiation of pulp SP cells into odontoblasts. BMP2 stimulated the expression of dentin sialophosphoprotein (*Dspp*) and enamelysin in three-dimensional pellet cultures. Autogenous transplantation of the Bmp2-supplemented SP cells on the amputated pulp stimulated the reparative dentin formation. Thus, adult pulp contains SP cells, which are enriched for stem cell properties and useful for cell therapy with BMP2 for dentin regeneration. STEM CELLS *2006;24:2493–2503*

INTRODUCTION

Caries is a common problem in dentistry, characterized by damaged dentin and the consequent exposure of the dental pulp. Regeneration and repair of the damaged dentin to protect the pulp is the goal of operative dentistry and endodontology. Regenerative dentistry is based on the triad of stem or progenitor cells, and morphogens and a scaffold of extracellular matrix [1–3]. The potential utility of pulp stem cells for cell therapy and gene therapy of caries and endodontic treatment is immense. The pulp cells have the potential to differentiate into dentin-forming odontoblasts [4–11]. In view of this, there is a growing interest in isolating stem cells from the dental pulp both of adult teeth [12–14] and deciduous teeth [15]. The unequivocal isolation and identification of dental pulp stem cells, however, have not been achieved yet, since there are no specific cell surface markers for dental pulp stem cells. The isolation and enrichment of a side population (SP) fraction based on the efflux of fluorescent dye Hoechst 33342 with stem cell properties from hematopoietic bone marrow was a key advance [16]. SP cells have also been isolated from various adult tissues such as skeletal muscle [17, 18], brain [19], liver [20], pancreas [21], lung [22], heart [23], and kidney [24], indicating the common features of SP phenotype. SP cell population is highly enriched for stem cell activity [25, 26]. The dye efflux property of the SP cells is

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based on the ATP binding cassette transporter ABCG2 (breast cancer-resistant protein [BCRP] 1) [26]. The goal of this investigation was to isolate SP cells from the adult dental pulp tissue and to evaluate the self-renewal capacity and multilineage differentiation potential including dentin-forming odontoblast differentiation. The role of bone morphogenetic proteins (BMPs) as morphogenetic signals for tooth development and reparative/regenerative dentin formation is well known [27–29]. BMPs play critical roles in regulating fate direction during stem cell differentiation [30]. Recombinant human BMP2 induced differentiation of pulp stem/progenitor cells into odontoblasts in three-dimensional pellet cultures [31].

The results of the present investigation demonstrated that SP cells isolated from porcine dental pulp tissue have the plasticity to differentiate into adipogenic, chondrogenic, and neurogenic lineages. Their potential to differentiate into odontoblasts in response to BMP2 was also demonstrated. Furthermore, autogenous transplantation of the pulp SP cells treated with BMP2 resulted in regenerative dentin formation on the amputated dental pulp.

Materials and Methods

Isolation of Pulp SP Cells

The pulp cells from human, bovine, canine, and porcine pulp tissues were isolated enzymatically with slight modification of the described method in bovine pulp cells [5]. Normal human adult third molars were extracted from patients 16–25 years old under approved guidelines set by Kyushu University, Faculty of Dental Science. Red blood cells were lysed with IOTest3 lysing solution (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com). The cells passing through 40-μm nylon mesh (Cell Strainer; BD Biosciences, San Jose, CA, http://www.bdbioscience.com) were suspended at 1×10^6cells per milliliter in prewarmed Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) to 37°C with 2% fetal calf serum containing 5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) and labeled for 70 minutes at 37°C. Cells were resuspended in Hanks' balanced saline solution (Invitrogen) with 2% fetal calf serum and 10 mM HEPES buffer containing 2 μg/ml propidium iodide (PI) (Sigma-Aldrich) for the discrimination of dead cells. Analysis/sorting of cells was performed on JSAN (Bay Bioscience, Kobe, Japan, http://www.baybio.co.jp) equipped with laser lines of 375 nm (for UV), 488 nm, and 635 nm. Hoechst/PI red and Hoechst blue fluorescence signals were displayed on a linear, dual-fluorescence dot plot. A rectangular gate was drawn to exclude PI^+ dead cells and unstained debris. In parallel, 10^6 cells were stained in the presence of 50 μM verapamil (Sigma-Aldrich), an inhibitor of ABCG2/BCRP involved in dye efflux of Hoechst 33342 to set the gate for the isolation of SP cells. A non-SP gate was drawn that excluded SP cells. Part of the freshly sorted cells were analyzed for mRNA expression of Bcrp1, Stat3, Bmi1, and Telomerase reverse transcriptase (Tert) by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Characterization of Porcine Pulp SP Cells

After flow cytometry of the primary pulp cells from porcine tooth germ, isolated SP cells, non-SP cells, and the original primary pulp cells without flow cytometry were plated into 35-mm collagen type I-coated dishes (Asahi Technoglass Corp., Funabashi City, Japan, http://www.atgc.co.jp) in proliferation medium: DMEM high-glucose (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Inc., Lenexa, KS, http://www.jrhbio.com), penicillin-streptomycin (Invitrogen), and basic fibroblast growth factor (bFGF) (5 ng/ml; Invitrogen). At subconfluence, the cells were passaged by enzymatic digestion with 0.2% trypsin containing 0.02% EDTA. Total RNA were extracted from these cells at each passage using Trizol (Invitrogen). First-strand cDNA syntheses were performed from 1 μg of total RNA by reverse transcription using the SuperScript II preamplification system (Invitrogen). Real-time RT-PCR amplifications were performed at 95°C for 10 seconds, 62°C for 15 seconds, and 72°C for 8 seconds using porcine *Bcrp1*, *Stat3*, *Bmi1*, *Tert*, and *β-actin* primers (Table 1) labeled with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics, Pleasanton, CA, http://www.rochediagnostics.com) in Light Cycler (Roche Diagnostics). The RT-PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) and confirmed by sequencing on the basis of published cDNA sequences.

The expression of cell-surface antigen markers for tissue stem cells, CD31, CD34, CD45, CD90, CD105, CD146, and CD150 mRNAs, in SP cells and non-SP cells was examined by real-time RT-PCR. The number of SP and non-SP cells was normalized to 5×10^4 cells in each experiment. The mRNA expression of the vascular pericyte markers smooth muscle (SM) α-actin, desmin, and NG2 proteoglycan and the endothelial cell marker vascular endothelial growth factor receptor 2 (Vegfr2) was also analyzed (Table 2). The design of the oligonucleotide primers was based on published cDNA sequences of porcine. If porcine sequences were not available, human or mouse sequences were used (Table 1). The RT-PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, http://www.promega.com) and confirmed by sequencing. The absence of the differentiation markers for odontoblasts alkaline phosphatase (ALP), α1(I) collagen, α1(III) collagen, dentin sialophosphoprotein (Dspp), and enamelysin/MMP20 indicated the stemness of isolated SP cells. The expression in SP cells and non-SP cells were compared with porcine tooth germ control after normalizing with β-actin.

In situ hybridization for RNA in sections was carried out as described previously [32] with minor modification using the tyramide signal amplification (TSA) biotin system (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com) for incorporation of horseradish peroxidase (HRP). Porcine Bcrp1 (196 base pairs [bp]) cDNA linearized with NcoI was used as a probe.

Fifteen micrometers of frozen porcine tooth germ sections were dried and fixed in 100% acetone at −20°C for 5 minutes. After being treated with 2% peroxidase in methanol for 20 minutes and 1% blocking reagent (PerkinElmer) for 2 hours at room temperature, they were incubated with primary anti-mouse BCRP1 monoclonal antibody (BXP-53; 1:50; ABCAM Plc., Cambridge, U.K., http://www.abcam.com) for 2 hours at room temperature. Anti-rat IgG (1:100; HRP-conjugated; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, http://www.amersham.com) enhanced with TSA system Alexa Fluor 488-conjugated tyramide (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) was used as

Table 1. Porcine primers for real-time reverse transcription-polymerase chain reaction

Gene		5′ DNA Sequence 3′	Product size (base pairs)	Accession number
Bcrp1/ABCG2	Forward	GGCTTAGACTCCAGCACAGC	196	AJ420927
	Reverse	TGGAGGCAAAGTATCCCAAG		
Bmi1	Forward	ATATTTACGGTGCCCAGCAG	179	CK451985
	Reverse	GAAGTGGCCCATTCCTTCTC		
Stat3	Forward	GTGGTGACAGAGAAGCAGCA	191	CK453710
	Reverse	TTCTGCCTGGTCACTGACTG		
Tert	Forward	CAGGTGTACCGCCTCCTG	180	DQ400924
	Reverse	CCAGATGCAGTCTTGCACTT		
Collagen α1(II)	Forward	CAGGGGTGAACGAGGTTTC	190	AF201724
	Reverse	AATACCAGCAGCTCCCCTCT		
Aggrecan	Forward	CCCACTAGTGCAGCAACAGA	191	AF314813
	Reverse	AGGGTAGATGGCTGCTCTGA		
PPARγ	Forward	CATGCTGTCATGGGTGAAAC	188	AB097930
	Reverse	TCAAAGGAGTGGGAGTGGTC		
aP2	Forward	AACCCAACCTGATCATCACTG	192	AF102872
	Reverse	TCTTTCCATCCCACTTCTGC		
Sox2	Forward	AATGCCTTCATGGTGTGGTC	203	DQ400923
	Reverse	CGGGGCCGGTATTTATAATC		
Neuromodulin	Forward	CACTGATAACTCGCCGTCCT	187	DQ471296
	Reverse	CTCTTCAGCTTGGCTGCTCT		
Neurofilament	Forward	ACCCGACTCAGTTTCACCAG	216	DQ471295
	Reverse	CTCATCCTTGGCTTCCTCAG		
CD31	Forward	CATTTCCAAAGTCAGCAGCA	172	X98505
	Reverse	CATCATCATGCCTCCCTTCT		
CD34	Forward	TTCTGTCCAGCCTCAGACCT	184	NM_214086
	Reverse	GCTACCTGGGGTAGGAGGAG		
CD45	Forward	GGACATGTGACCTGGAAACC	191	AY444866
	Reverse	CCATTACGCTCTGCTTTTCC		
CD90	Forward	GACCCGTGAGACAAAGCAGC	171	DQ400919
	Reverse	TGGCCAGAGTGGTGGAGT		
CD105	Forward	TGCTCCTGATCCTCAGTGTG	204	NM_214031
	Reverse	GCTCAGCAGCAGAGATGATG		
CD146	Forward	GTCATCGTGGCTGTGATAGT	185	DQ400926
	Reverse	CCCATCTCTTCTGGGAGCTT		
CD150	Forward	AGCATGAACAAAAGCATCCA	190	DQ400925
	Reverse	ACCATCCCTCATCCTCCTTC		
Vegfr2/Flk-1	Forward	GTGACCAACATGGAGTCGTG	218	DQ400921
	Reverse	TGCTTCACAGAAGACCATGC		
α-Smooth muscle actin	Forward	AATGGCTCTGGGCTCTGTAA	219	DQ400922
	Reverse	CTTTTCCATGTCGTCCCAGT		
NG2	Forward	TGAACTTCACTCAGGCAGAGG	171	DQ400920
	Reverse	GACAGGCAGCCTCAAAAGAC		
Desmin	Forward	CAGGAACAACAGGTCCAGGT	182	AF363284
	Reverse	CGCAGTGCATCATTGTTCTT		
Alkaline phosphatase	Forward	CCAAAGGCTTCTTCTTGCTG	195	AY145131
	Reverse	TGTACCCGCCAAAGGTAAAG		
Collagen α1(I)	Forward	AAGGACAAGAGGCACGTCTG	166	BI233976
	Reverse	CGCTGTTCTTGCAGTGGTAG		
Collagen α1(III)	Forward	TTCAAAATCAACACCGACGA	180	AB050301
	Reverse	TTTGCAGCCTTGGTTAGGAT		
Dentin sialophosphoprotein	Forward	GGAATGGAGAGAGGACTGCT	174	AF332578
	Reverse	AGGTGTTGTCTCCGTCAGTG		
Enamelysin/MMP20	Forward	CACTGTTGCTGCTCACGAAT	182	SSU54825
	Reverse	CAGTGGGCTTTCCTGTGAAT		
Dentin matrix protein 1	Forward	TGGGGATTATCCTGTGCTCT	177	AY524986
	Reverse	GCTGTCACTGGGGTCTTCAT		
β-Actin	Forward	CTGGGGCCTAACGTTCTCAC	198	BI118314
	Reverse	GTCCTTTCTTCCCCGATGTT		

Abbreviations: aP2, adaptor protein 2; Bcrp1, breast cancer-resistant protein 1; MMP20, matrix metalloproteinase 20; PPARγ, peroxisome proliferators-activated receptor γ; Vegfr2, vascular endothelial growth factor receptor 2.

Table 2. mRNA expression of cell surface antigen markers for stem cells and odontoblast differentiation markers by real-time reverse transcription-polymerase chain reaction analysis in porcine pulp SP cells and non-SP cells compared with tooth germ

Marker	SP/tooth germ	Non-SP/tooth germ	SP/non-SP
CD31	9.0	1.0	9.0
CD34	0.9	0.1	9.0
CD45	0.1	1.3	0.1
CD90	0.5	2.2	0.2
CD105	9.5	1.1	8.6
CD146	10.0	3.0	3.3
CD150	36.0	0.1	360.0
Vegfr2/Flk-1	9.2	0.3	30.7
α-Smooth muscle actin	0.3	5.0	0.1
NG2	1.2	1.8	0.67
Desmin	0.8	3.5	0.2
Alkaline phosphatase	0.04	0.5	0.08
Colα1(I)	0.05	0.09	0.56
Colα1(III)	0.2	1.0	0.2
Dspp	0.0	0.01	0.0
Enamelysin	0.0	0.01	0.0

The experiment was repeated five times, and one representative experiment is presented.
Abbreviations: Dspp, dentin sialophosphoprotein; SP, side population; Vegfr2, vascular endothelial growth factor receptor 2.

secondary antibody. The stained sections were observed under a fluorescence microscope IX 71 (Olympus, Tokyo, Japan, http://www.olympus.co.jp) after being counterstained with Hoechst 33342.

Differentiation of Pulp SP Cells into Odontoblast Lineage in Three-Dimensional Pellet Culture

Pulp SP cells were cultured in a three-dimensional pellet (cellular aggregates) supplemented with and without recombinant human BMP2 (kindly provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan, http://informagen.com) at a final concentration of 50 ng/ml, 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), and 50 μg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan, http://www.wako-chem.co.jp/english), and penicillin-streptomycin. The medium was changed twice a week. To determine the effect on proliferation, the pellets were digested by trypsin and total cell numbers were calculated on days 0, 1, 3, 6, 10, and 14. On days 1, 3, 7, 10, and 14, the pellets was sonicated in 100 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 at 20 kHz for 5 minutes. Alkaline phosphatase activity was assayed by the method of Lowry et al. [33]. Pellets were fixed on days 14 and 35, and the paraffin-embedded sections were stained in Masson trichrome for extracellular matrix formation and alizarin red for calcification, respectively. Total cellular RNA was isolated at each point on days 10, 14, and 28, and real-time RT-PCR was performed using porcine Dspp, enamelysin/MMP20, dentin matrix protein 1 (Dmp1), and α1(I) collagen as differentiation markers of odontoblasts.

Induced Chondrogenic, Adipogenic, and Neurogenic Differentiation of Pulp SP Cells

The differentiation of pulp SP cells into adipogenic, chondrogenic, or neurogenic cells was determined by the method of Pittenger et al. [34]. For chondrogenic differentiation, 2.5×10^5 porcine pulp SP cells, non-SP cells, and primary pulp cells at the third passage were centrifuged in 15-ml conical polypropylene tube (Asahi Techno Glass) at 1,000 rpm for 5 minutes. The pellets were maintained in DMEM supplemented with 10% heat-inactivated bovine calf serum, 10 μg/ml insulin-transferin-selenite X (Invitrogen), 5.35 μg/ml linoleic acid (Sigma-Aldrich), 1.25 μg/ml bovine serum albumin (Sigma-Aldrich), 1.0 μg/ml dexamethason, 10 μg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries), and 10 ng/ml transforming growth factor β3 (Peprotech, London, http://www.peprotechec.com). Medium was changed every 3 days, and cultures were maintained for 28 days. Pellets were fixed in 4% paraformaldehyde overnight, and the paraffin-embedded sections (4–5 μm thick) were stained by Alcian Blue. The total cellular RNA of the pellets was isolated on day 28 for analysis of α1(II) collagen and aggrecan (Table 1) by RT-PCR.

For adipogenic differentiation, cells were seeded at 2×10^5 cells per milliliter in 35-mm collagen type I-coated dishes in DMEM high-glucose supplemented with 10% heat-inactivated fetal calf serum. The third-passage culture of the SP cells was grown to confluence in DMEM low-glucose supplemented with 10% heat-inactivated fetal calf serum and 10 μg/ml L-glutamine (Invitrogen). Thereafter, adipogenic differentiation was induced by subjecting confluent monolayers to three rounds of adipogenic treatment. Each round had two steps: incubation with induction medium of Poietics hMSC Differentiation Media Bullet Kit-Adipogenic (Cambrex, Walkersville, MD, http://www.cambrex.com) for 3 days and incubation with maintenance medium for 3 days. Cells were cultured for another 7 days in maintenance medium. As a control, cells were cultured only in maintenance medium. The cell layers were stained with oil red O, and the mRNA levels of adaptor protein 2 (aP2) and peroxisome proliferator-activated receptor γ (PPARγ) (Table 1) were analyzed by RT-PCR.

For neurogenic differentiation, pulp SP cells were cultured in noncoated 35-mm dishes in neurosphere medium (Neurobasal A; Invitrogen) containing B27 supplement (Invitrogen), penicillin-streptomycin, L-glutamine, endothelial growth factor (EGF) (Invitrogen), and bFGF (Invitrogen) for 15 days, designated for neurosphere formation. The neurospheres were triturated using polished glass pipettes, and the obtained single-cell suspension was cultured in gelatin-coated 35-mm dishes in neurodifferentiation medium one (Neurobasal A) containing 1 μg/ml laminin (Invitrogen), 5 μg/ml fibronectin (Nitta Gelatin, Osaka, Japan, http://nitta-gelatin.co.jp), 2 mM L-glutamine, 10 μg/ml N_2 supplement (Invitrogen), 20 ng/ml bFGF, and 40 ng/ml EGF. The medium was changed to neurodifferentiation medium 2 (Neurobasal A) containing 1 μg/ml laminin, 5 μg/ml fibronectin, 2 mM L-glutamine, 10 μg/ml N_2 supplement, 20 ng/ml neurotrophin-3 (Peprotech) after 24 hours of cultivation. The medium was changed every 3 days. Immunocytochemical analysis was performed 28 days after cultivation. The cells were fixed for 30 minutes in cold 4% paraformaldehyde in PBS, treated with 0.1% Triton-X for optimal penetration of cell membrane, and incubated at room temperature in a blocking solution (protein block Dako, Glostrup, Denmark, http://www.dako.com) for 30 minutes. After an overnight incubation at 4°C with a primary antibody anti-neuromodulin (GAP-43) (Clone91E12) (1:100 Roche Diagnostics), the cells were incubated for 30 minutes a

room temperature with a secondary antibody (Envision+; Dako) and stained with diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). The mRNA expression of neuromodulin and neurofilament (Table 1) was analyzed by RT-PCR.

Canine Pulp SP Cells and In Vivo Transplantation on Amputated Pulp

The ultimate utility of the SP cell-derived stem cells is the in vivo differentiation into dentin-forming odontoblasts. A switch was made to an experimental model of canine pulp partial removal and transplantation of SP cell-derived pulp stem cell population. Adult dogs were obtained from Narc (Chiba, Japan, http://www.narc.co.jp). The canine SP pulp cells were isolated from the pulp tissues surgically extracted from the incisors by flow cytometry as performed in porcine pulp tissue. Canine autogenous pulp pellets (cellular aggregates) were prepared from third-passage SP cells supplemented with and without recombinant human BMP2. Anesthesia for surgery was performed by intravenous administration of pentobarbital sodium. Surgical amputation was carried out in the canine teeth of the dog, and the pellets were implanted autogenously on the amputated pulp. The cavity was sealed with zinc phosphate cement and composite resin with the caution of using minimal pressure. The teeth were obtained by extraction 1 month after transplantation, fixed in 4% paraformaldehyde at 4°C overnight, and demineralized in 10% formic acid. Reparative dentin formation was evaluated by histology in serial paraffin sections. For in situ hybridization in sections, after dewaxing and inhibition of endogenous peroxidase activity by treatment with 0.3% H_2O_2 in methanol solution for 30 minutes, the procedure described above was performed. The first-strand cDNA was synthesized from 2 μg of total RNA isolated from canine primary pulp pellet cultures on day 28, and RT-PCR amplifications were performed at 95°C for 10 seconds, 58°C for 15 seconds, and 72°C for 8 seconds using canine *Dspp* primers (forward, 5′-GTCCTAGTGGGAATGGAGCA-3′; reverse, 5′-TCTTCAGGGCCATCATCTTC-3′), and *enamelysin* primers (forward, 5′-TATTCACCGTTGCTGCTCAC-3′; reverse, 5′-TACAATGCCTGGATCCCTTT-3′). RT-PCR products of *Dspp* (190 bp) and *enamelysin* (151 bp) were subcloned into pGEM-T Easy vector (Promega) and confirmed by sequencing based on published cDNA sequences. The canine Dspp and enamelysin cDNA, linearized with SpeI and NcoI, respectively, were used for making RNA probes. The animal experiments were conducted using the strict guidelines of the Kyushu University Animal Protocol Committee and DNA Safety Program.

RESULTS

Isolation of SP Cells from Dental Pulp from Various Species

Pulp cells isolated from human, bovine, canine, and porcine adult pulp tissues and stained with Hoechst 33342 identified approximately 0.2% of the population with relatively lower Hoechst 33342 fluorescence (SP cells) (Fig. 1A–1D). Although some markers (CD90 and CD117) reacted with human pulp cells (data not shown), the markers required to subfractionate pulp SP cells are not well-defined due to small yields of isolated human SP cells. Therefore, sensitivity to verapamil, an inhibitor

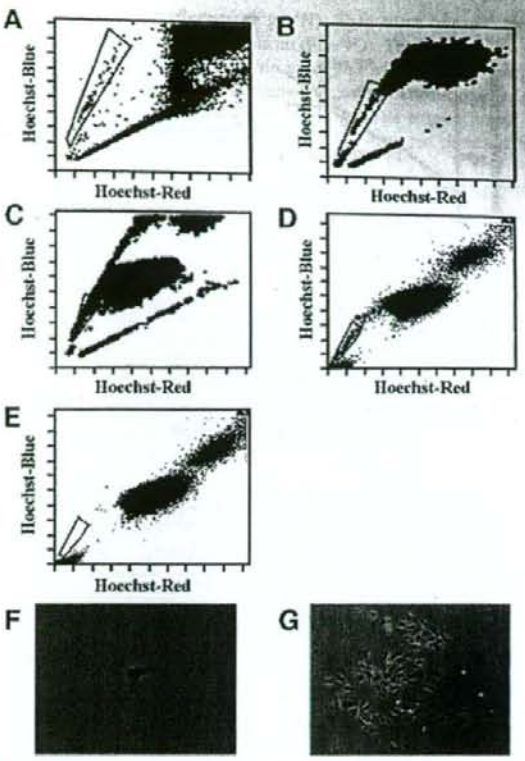

Figure 1. Isolation of side population (SP) cells from dental pulp from various species. SP cells were isolated from human (**A**), bovine (**B**), canine (**C**), and porcine (**D**) dental pulp. It is noteworthy that in the presence of 50 μM verapamil, few SP cells were obtained from porcine (**E**) (compare [**D**] and [**E**]). The growth of a cell colony derived from single porcine SP cell is shown on day 1 (**F**) and on day 7 (**G**). SP cells could form a colony at a rate of 0.2%. Scale bars = 50 μm.

of multidrug resistance (MDR) and MDR-like transporter, was used to set the SP gate (Fig. 1D, 1E). This SP subpopulation has been shown to be a more homogeneous population than primary pulp cells and non-SP cells. Some of the single SP cells plated in a 35-mm, collagen type I-coated dish formed a colony in 7 days (Fig. 1F, 1G).

Self-Renewal Capability of Pulp SP Cells

The cumulative cell number of porcine pulp SP cells was much higher, and the proliferative life span of SP cells was longer than that of pulp non-SP cells and primary pulp cells (Fig. 2A). At first, the non-SP cells and primary cells were passaged every 3–4 days from the second to the 15th passage. The rate of proliferation progressively became slower. The cells became senescent by passage 26, on day 116 and by passage 27, on day 132, respectively. Most of SP cells proliferated and maintained a spindle-like appearance until the 42nd passage. The molecular basis for the SP phenotype has been attributed to multidrug-resistance transporter (ABCG2/Bcrp 1). To characterize pulp SP cells, the expression of the markers associated with other stem cells (Bcrp1, Stat3, Bmi1, and Tert mRNA) was analyzed by the

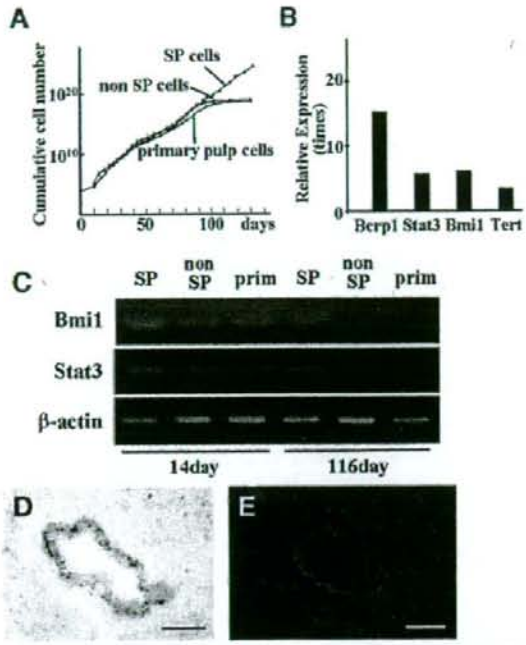

Figure 2. Characterization of porcine pulp SP cells from dental pulp. **(A):** The cumulative cell number of pulp SP cells, non-SP cells, and primary pulp cells up to 140 days. **(B):** The relative expression (fold increases) of Bcrp1, Stat3, Bmi1, and Tert mRNA in porcine SP cells compared with that of non-SP cells. **(C):** The expression of Stat3 and Bmi1 mRNA on days 14 and 116 in SP cells, non-SP cells, and primary porcine dental pulp cells. Note that SP cells demonstrate Stat3 and Bmi1 mRNA up to 116 days. **(D, E):** The in situ expression of Bcrp1 mRNA **(D)** and BCRP1 protein **(E)** in vascular region in porcine dental pulp sections. Counterstain (blue) is Hoechst 33342. Scale bars = 50 μm. Abbreviations: Bcrp1, breast cancer resistant protein 1; prim, primary; SP, side population; Tert, telomerase reverse transcriptase.

real-time RT-PCR. Those levels of expression were much higher in SP cells compared with non-SP cells (Fig. 2B), suggesting that this population was highly enriched for stem cells. Stat3 and Bmi1 mRNA were still expressed in SP cells on day 116 and disappeared in non-SP cells and primary pulp cells (Fig. 2C), suggesting the self-renewal and replicative capacity of SP cells. The expression of Bcrp1 mRNA and BCRP1 protein was detected by in situ hybridization and immunohistochemistry in the perivascular region of porcine dental pulp tissue (Fig. 2D, 2E).

Cell Surface Antigen Markers for Stem Cells

To examine the "stemness" of the porcine pulp SP cells, mRNA expression of cell surface antigen markers for stem cells were examined by real-time RT-PCR, since antibodies cross-reacting against CD are not available for porcine cells. CD105 and CD150 mRNA were highly expressed in SP cells compared with non-SP cells like other tissue stem cells (Table 2). Much lower expression of CD45, a gene typical of hematopoietic cells, was detected in SP cells than in non-SP cells. Expression of the differentiation markers Colα1(I) and Colα1(III) was much lower in SP cells than in non-SP cells. Markers of odontoblast differentiation Dspp and enamelysin were not detected in SP cells. These results suggest that SP cells contain an undifferentiated population, as do other tissue stem cells. CD146 is known to be highly expressed in smooth muscle cells and endothelial cells, as are CD31 and Vegfr2 in endothelial cells and NG2, desmin, and α-smooth muscle cell actin in microvascular pericytes. The expression of α-smooth muscle cell actin in SP cells was much lower than in non-SP cells. Endothelial markers CD31 and Vegfr2 were expressed in SP cells (Table 2).

Differentiation of SP Cells into Odontoblast Lineage

Total cell numbers were rapidly decreased in BMP2-supplemented pellets and control non-supplemented pellets of pulp SP cells by day 1. They gradually decreased until day 10 and did not change thereafter. There was no difference in cell number between BMP2 pellets and control pellets (Fig. 3A). The alkaline phosphatase activity gradually increased after day 7 and was significantly higher on day 10 in BMP2 pellets than in control pellets (Fig. 3B). The collagenous extracellular matrix stained by Masson trichrome was stronger in the BMP2-supplemented pellets than those in control pellets starting on day 14 (Fig. 3C, 3D). Alizarin red staining showed mineralization in the BMP2 pellets on day 35 (Fig. 3E, 3F). We next investigated the functional effect of BMP2 application on the expression of α1(I) collagen and odontoblast markers *Dspp*, *enamelysin*, and *Dmp1* on days 10, 14, and 28. Quantitative analysis by real-time RT-PCR showed that the expression of α1(I) collagen, Dspp, and enamelysin was significantly increased in the BMP2-supplemented pellet cultures compared with the control on days 14 and 28 (Fig. 3G). These results indicated that BMP2 supplement enhanced differentiation of pulp SP cells into odontoblasts.

Multidifferentiation Capability of Pulp SP Cells

The chondrogenic potential of SP cells was examined. The porcine pulp SP cells obtained from the fourth passage culture were maintained in pellet cultures for 45 days. The amount of cartilage proteoglycan stained with Alcian Blue was much greater in the pellets induced from SP cells compared with those from non-SP cells, primary pulp cells (Fig. 4A–4C), and non-induction pellet (data not shown). The expression of aggrecan and type II collagen mRNA was much stronger in the SP cells than that in the primary pulp cells. Almost 30% of SP cells were converted into chondrocytes. No expression was seen in the non-SP cells and uninduced SP cells (Fig. 4D).

Next, the adipogenic potential of pulp SP cells was investigated in the third-passage cultures, which were cultured under the adipogenic condition for 28 days. The pulp SP cells showed intense staining with oil red O compared with non-SP and primary pulp cells (Fig. 4E–4G). aP2 and PPARγ mRNA was expressed in SP cells on day 28, but not in uninduced SP cells, non-SP cells, or primary pulp cells (Fig. 4H).

Finally, the neurogenic potential of SP cells was examined. The neurospheres, which were proliferating clusters of cells detached, were seen 15 days after neuronal induction of the SP cells from third-passage culture (Fig. 4I, 4J). Ninety percent of SP cells formed neurospheres. Sox2 mRNA was expressed in the neurospheres induced from SP cells but not in cells from non-SP cells or primary cells (Fig. 4M). Those neurosphere cells from SP cells were dissociated and seeded to the gelatin-coated dish to adhere and immunostained with antibody to neuromodu-

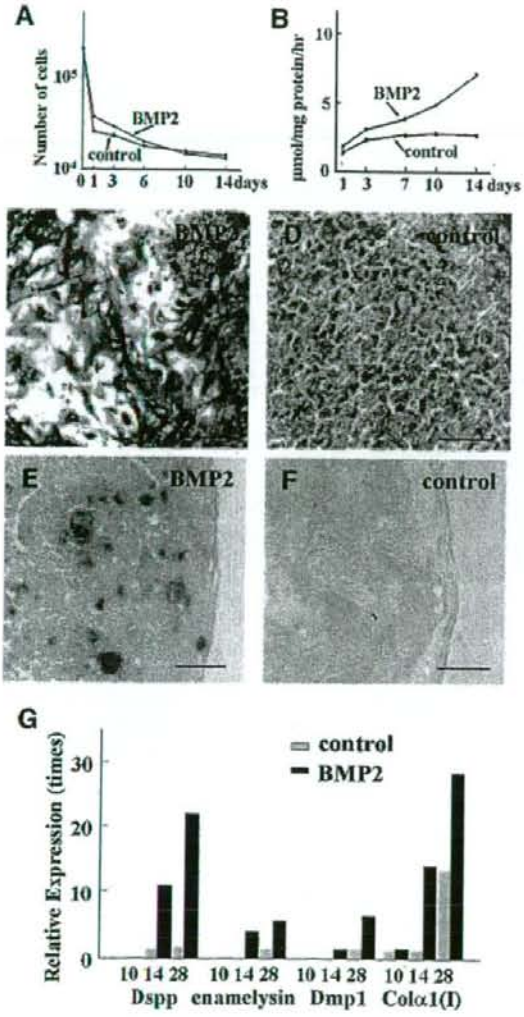

Figure 3. Differentiation of pulp SP cells into odontoblast lineage in the three-dimensional pellet culture. **(A):** The changes in cell numbers of porcine pulp SP cells in pellet culture treated with BMP2 compared with control. Each point is expressed as the mean ± SD of six determinations. **(B):** The changes in alkaline phosphatase activity in pellet culture treated with BMP2 and control. Each point is expressed as the mean ± SD of six determinations. In pellet cultures treated with BMP2, there was increased alkaline phosphatase activity compared with control. Morphological changes in the pellet were observed on day 14 (Masson trichrome stain). Shown are results of treatment with BMP2 (**C**) and untreated control (**D**). Scale bars = 50 μm. Pellets treated BMP2 had increased collagen matrix compared with control. Alizarin red staining shows mineralization of BMP2 supplemented pellet on day 35. Shown are BMP2-treated pellet (**E**) and control pellet (**F**). Scale bars = 50 μm. Note that mineralization was increased in the pellet supplemented with BMP2 compared with control. **(G):** Real-time reverse transcription-polymerase chain reaction, analysis of Dspp, enamelysin/matrix metalloproteinase 20, Dmp1, and α1(I)collagen expression in the pellet culture treated with BMP2 compared with control. The experiment was repeated four times, and one representative experiment is presented. Abbreviations: BMP, bone morphogenetic protein; Dmp1, dentin matrix protein 1; Dspp, dentin sialophosphoprotein; prim, primary; SP, side population.

lin after subconfluence (Fig. 4N). They were immunoreactive for neuromodulin in cytoplasm (Fig. 4O). The cells had rounded soma and a few of long, thin processes with neuronal morphology. These processes contacted each other at the end or soma and showed synapse-like morphology. The expression of neuromodulin and neurofilament mRNA was also detected in SP cells but not in uninduced SP cells (Fig. 4P).

Dentin Regeneration by Transplantation of Bmp2-Transduced Cells In Vivo

The autologous transplantation of the pellet (cell aggregate) of SP cells was performed on the amputated pulp of canine teeth 14 days after three-dimensional culture supplemented with BMP2. The pulp SP cells differentiated into osteodentinoblasts and secreted osteodentin matrix around them 1 month after surgery (Fig. 5A, 5B). A higher amount of osteodentin was observed in the BMP2 pellet than in the control pellet (Fig. 5A–5D). The in situ hybridization in serial sections showed mRNA expression of Dspp and enamelysin (Fig. 5E, 5F) in odontoblasts/osteodentinoblasts confined in osteodentin matrix stained with eosin (Fig. 5G) and Masson trichrome (Fig. 5H). There was no expression of Dspp or enamelysin in the control pellet (data not shown). These results suggest that the pulp SP cells responded to BMP2 to differentiate into odontoblasts/osteodentinoblasts.

Discussion

The present work describes the isolation and characterization of SP cells from dental pulp and their multilineage differentiation. SP cells were detected in adult pulp tissue from several species, including human, bovine, canine, and porcine species. They were purified by flow cytometry on the basis of efficient efflux of vital fluorescent dye Hoechst 33342 [35]. The dye efflux property of SP cells is mediated by the multidrug resistance transporter of ATP binding cassette G2 (ABCG2)/breast cancer resistance protein 1 (Bcrp1). In the porcine dental pulp, the SP cell fraction was approximately 0.2% of the gated population of primary pulp cells. The SP cells were sensitive to verapamil and expressed a higher level of Bcrp1 compared with non-SP and primary pulp cells. The three key characteristics of the SP cells are self-renewal capacity, expression of stem cell surface markers, and multilineage differentiation. The cumulative cell number was higher and proliferative life span was longer in pulp SP cells compared with non-SP cells. The polycomb gene was highly expressed in the SP cells. The *Bmi1* gene has been implicated in self-renewal [36]. Similarly, *Oct4* and *Stat3*, implicated in maintenance of self-renewal and pluripotency [37], were highly expressed in SP cells compared with non-SP cells. Finally, telomerase reverse transcriptase (Tert) mRNA was also highly expressed in pulp SP cells. TERT has a crucial function of maintenance of telomere length, preserving genomic stability and the long-term viability of highly proliferative organs [38, 39]. These results suggest that the pulp SP cells have self-renewal capacity.

The human pulp mesenchymal stem cells are currently demonstrating the similarity in positive expression of mesenchymal progenitor-related antigens, such as SH2, SH3, SH4, CD29, and CD166, to bone marrow mesenchymal stem cells [14]. The presence of cell surface markers in porcine pulp SP cells was examined by RT-PCR, as there is a paucity of specific CD antibodies for porcine cells. It is well known that SP cells

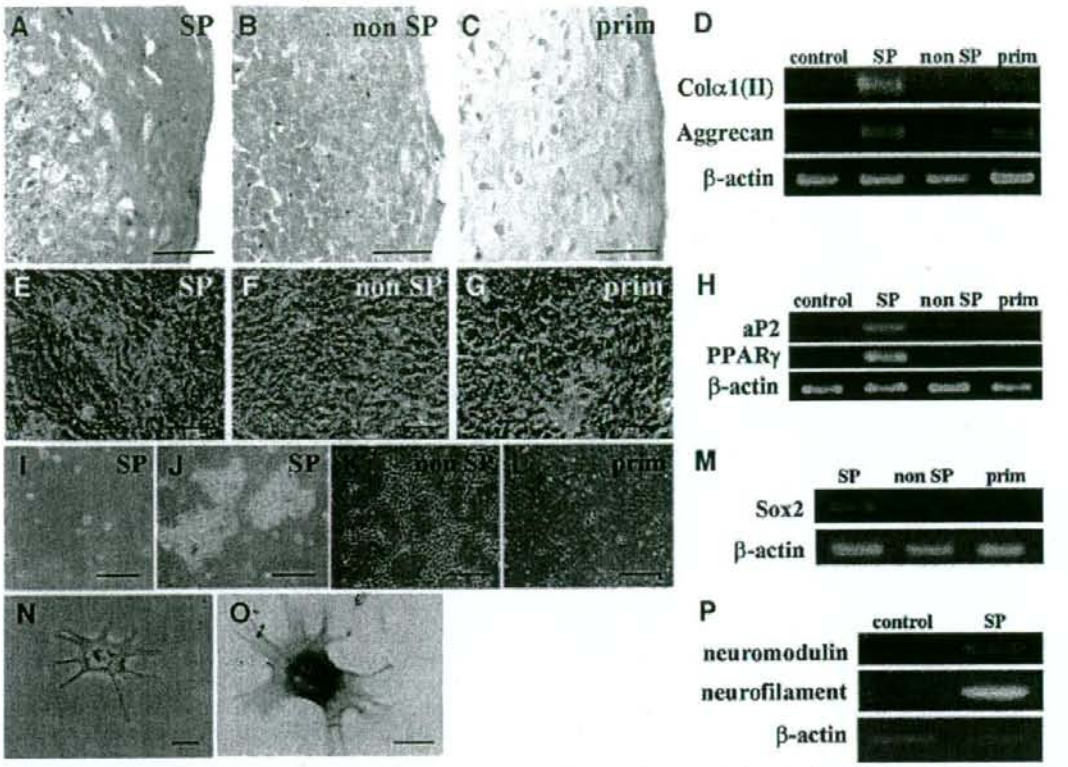

Figure 4. Induced differentiation of chondrogenic, adipogenic, and neurogenic lineages of dental pulp SP cells. Histological appearance of the pellet cultures of SP cells on day 28 (**A**), non-SP cells (**B**) and primary cells (**C**) stained by Alcian blue. Scale bars = 50 μm. (**D**): The expression of Colα1 (II) and Aggrecan mRNA, markers of chondrogenic differentiation is prominent in SP cells compared with non-SP cells. (**E**): Oil red O staining of adipogenic differentiation on day 28. Note the presence of adipocytes in the SP cell fraction (**E**) but not in non-SP cells (**F**) and a few in primary cells (**G**). Scale bars = 100 μm. Markers of adipogenic differentiation aP2 and PPARγ mRNA were observed only in SP cells (**H**). (**I**): Appearance of SP cells on day 3. Neurosphere formation on day 15 was seen only in SP cells (**J**) and not in non-SP cells (**K**) or primary cells (**L**). Scale bars = 500 μm. The expression of Sox2 mRNA confirms the neurosphere formation from SP cells only (**M**). A neuron-like cell induced after dissociation of the neurosphere from SP cells and plating on the gelatin-coated dish for 13 days (**N**). Immunostaining of neuromodulin (**O**) and the expression of neuromodulin and neurofilament mRNA were observed in SP cells (**P**). Scale bars = 5 μm. Abbreviations: aP2, adaptor protein 2; Colα1, collagen α1; PPARγ, peroxisome proliferator-activated receptor γ; prim, primary; SP, side population.

isolated from bone marrow express stem cell markers such as Sca-1 (stem cell antigen), Thy1 (CD90), and the panhematopoietic markers CD45 and c-kit (CD117) [16, 40, 41]. However, they do not express differentiated markers characteristic of hematopoietic lineage, as in other tissue stem cells, such as skeletal muscle, pancreas, cardiac muscle, and intestine [42]. Pulp SP cells expressed mRNA for stem cell marker mRNA, *CD105* (*endoglin*) [43] and *CD150* [44]. CD150 has recently been demonstrated to be a useful marker for distinguishing stem cells from other progenitors. The primitiveness of pulp SP cells could thus be predicted based on the expression of CD150. On the other hand CD45 expression was very low compared with non-SP cells. The pulp SP cells expressed markers of endothelial cells, CD31 and Vegfr2. They lacked differentiated odontoblast markers Dspp and enamelysin and expressed low levels of Colα1(I) and Colα1(III). The origin and lineage of pulp SP cells are not clear. The presence of endothelial markers in isolated dental pulp SP cells and the localization of BCRP1 in perivascular region are suggestive of potential perivascular origin of some pulp SP cells. This is noteworthy in view of the implication of perivascular niche in mesenchymal stem cells of human dental pulp [45].

Accumulating evidence indicates that tissue stem cells have the potential to differentiate into other unrelated organs. For example, the multipotency of bone marrow stem cells to engraft into cardiac muscle, vascular endothelium, liver, and skeletal muscle has been described [46–49]. Mesenchymal stem cells of the bone marrow have the potential to differentiate into adipocytes, muscle, and bone [50, 51]. SP cells isolated from porcine dental pulp were multipotent, giving rise to adipogenic, chondrogenic, and neurogenic tissues in addition to odontoblasts. The porcine SP cells were chondrogenic, whereas unfractionated human dental pulp "stem" cells [13, 14] were not. It is possible that the SP cells were more in the hierarchy of stem cell lineage. The expression of odontoblast differentiation markers *Dspp* and *enamelysin* was increased in the BMP2-treated pellet

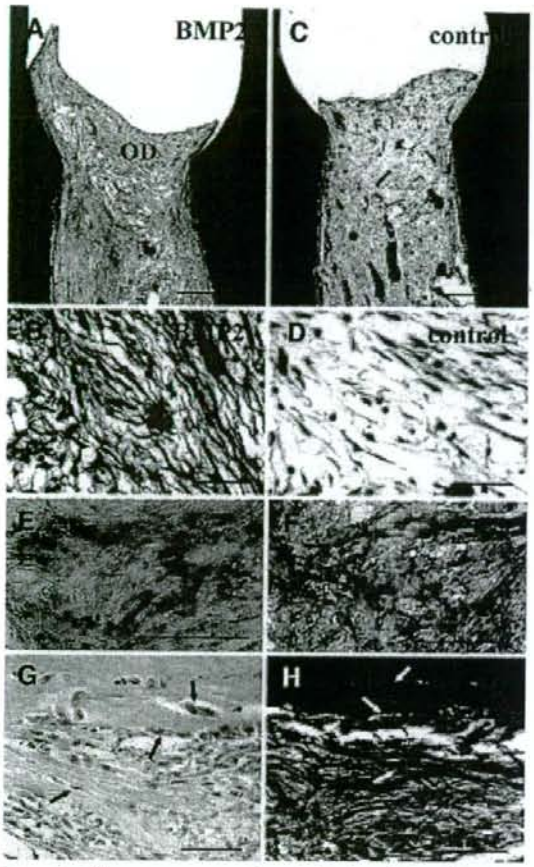

Figure 5. The autogenous transplantation in vivo of the pellet culture of canine side population (SP) cells on the canine amputated pulp (Masson trichrome stain). **(A, B):** Formation of OD in amputated pulp 1 month after autogenous transplantation of SP cell pellet treated with BMP2. **(C, D):** Small amount of osteodentin formation (OD) in the control. Scale bars = 500 μm **(A, C)**, 50 μm **(B, D)**. Shown is in situ hybridization analysis of canine tooth transplanted with SP cell pellet treated with BMP2 dentin sialophosphoprotein **(E)** and enamelysin **(F)**. Adjacent sections were stained with H&E **(G)** and Masson trichrome **(H)**. Note the odontoblasts/osteodentinoblasts (arrows) confined in osteodentin matrix. Scale bars = 50 μm. Abbreviations: BMP, bone morphogenetic protein; OD, osteodentin.

cultures (cellular aggregates) compared with the untreated control, indicating that BMP2 enhanced differentiation of pulp SP cells into odontoblasts. Osteodentin matrix formation was also increased in the BMP2-treated pellet cultures compared with the control.

Finally, odontoblastic differentiation in vitro from pulp SP cells has to be extended to in vivo experiments to demonstrate osteodentin and tubular dentin formation. To experimentally investigate the dentinogenesis in vivo, a switch was made to the dog, as previously our laboratory has demonstrated the BMP-induced dentinogenesis in the canine teeth of dogs [27, 52]. An experimental model of surgical amputation to expose the dental pulp of canine teeth in dogs was used. Since the dogs were outbred, autogenous SP cells isolated from the individual dogs were surgically implanted in the same dog. Pellet cultures of BMP2-treated dog SP cells induced osteodentin formation in surgically-created defects on amputated dental pulp. The terminal differentiation of odontoblasts was demonstrated by in situ hybridization of *Dspp* and *enamelysin*. Induction of osteodentin by BMP2-treated SP cells extends the previous work by our team and others of BMP-dependent osteodentin and tubular dentin in dogs [27, 52–54]. Thus, the response of the SP cells to BMP2 was similar to that of the mixed population of cells in the canine dental pulp.

The utility of pulp SP cells to induce in vivo dentin formation in dogs deserves additional comments with special reference to regeneration of tissues. SP cells derived from the liver and bone marrow were successfully used to regenerate liver [49]. Bone marrow-derived SP cells contributed to repair of lungs and tracheal epithelium [55, 56]. Transplantation of SP cells from adult mouse skin into *Mdx* mice with a mutation in the dystrophin gene resulted in expression of normal dystrophin by homing to and fusion with mutated myofibers [57]. The results in the present investigation of dentin formation by SP cells in surgically amputated teeth, along with earlier results in liver, lung, and skeletal muscle, demonstrate the regenerative potential of SP cells. In conclusion, pulp-derived SP cells induced to differentiate with morphogens such as BMP2 may be of potential clinical utility in the cell therapy for endodontics and dentistry.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Side Population Cells Isolated from Porcine Dental Pulp Tissue with Self-Renewal and Multipotency for Dentinogenesis, Chondrogenesis, Adipogenesis, and Neurogenesis

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ORIGINAL ARTICLE

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Production of IL-7 is increased in ovariectomized mice, but not RANKL mRNA expression by osteoblasts/stromal cells in bone, and IL-7 enhances generation of osteoclast precursors in vitro

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Abstract Osteoclastogenic cytokines produced by T and B lineage cells and interleukin (IL)-7-induced expansion of the pool size of osteoclast precursors have been suggested to play an important role in acceleration of osteoclastogenesis induced by estrogen deficiency. However, the contribution of increased RANKL produced by osteoblasts/stromal cells to increase osteoclastogenesis in a mouse model of estrogen-deficient osteoporosis and in vitro effects of IL-7 on osteoclast precursor generation remain controversial. Thus, we investigated the effect of ovariectomy (OVX) of mice on production of RANKL, osteoprotegerin (OPG), and IL-7 in bone and the effect of IL-7 on osteoclast precursor generation in vitro. OVX did not significantly stimulate mRNA expressions of RANKL and OPG in whole femurs. Because the epiphysis, but not the femoral shaft (diaphysis) or bone marrow, is the main site of osteoclastogenesis, it is important to specifically analyze mRNA expression by osteoblasts/stromal cells at these parts of the femur. Therefore, we isolated RNA from bone marrow cell-free epiphysis, diaphysis, and flushed-out bone marrow and examined mRNA expression. The results showed no significant changes of RANKL and OPG mRNA expression in any part of the femur. In addition, OVX did not significantly affect RANKL and OPG mRNA expression by the adherent stromal cells isolated from flushed-out bone marrow cells but did stimulate RANKL mRNA expression by $B220^+$ cells in the nonadherent cell fraction. On the other hand, OVX increased IL-7 mRNA expression in the femur as well as IL-7 concentrations in bone fluid. In cultures of unfractionated bone cells isolated by vigorous agitation of minced whole long bones to release the cells tightly attached to the bone surfaces, but not in cocultures of clonal osteoblasts/stromal cells and flushed-out bone marrow cells, IL-7 stimulated generations of osteoclasts as well as osteoclast precursors. These data suggest that increased RANKL production by osteoblasts/stromal cells is unlikely to play a central role in acceleration of osteoclastogenesis in estrogen deficiency of mice and that IL-7 stimulates osteoclast precursor generation, presumably through an action of IL-7 on the cells attached to bone rather than on cells contained in the bone marrow cell population.

Key words estrogen · RANKL · osteoprotegerin · interleukin-7 · osteoclasts

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Introduction

Estrogen deficiency causes bone loss mainly as a consequence of excessive bone resorption by osteoclasts. Because estrogen deficiency is accompanied by accelerated osteoclastogenesis, regulation of osteoclastogenesis is central to the understanding of the pathogenesis and treatment of estrogen deficiency-induced osteoporosis [1–3]. In addition, estrogen directly regulates the bone-resorbing activity of osteoclasts [4].

Accumulated evidence has suggested that osteoclast differentiation is principally stimulated by an increase in the ratio of RANKL to osteoprotegerin (OPG), a soluble decoy receptor of RANKL, in bone [5–7]. Recent studies have revealed that RANKL is produced by at least three types of cells: osteoblasts/stromal cells, T cells [8,9], and pre-B cells [10]. In addition, T cells produce another osteoclastogenic cytokine, tumor necrosis factor (TNF)-α [11–13]. Recent reports have shown that ovariectomy (OVX) increases the production of interleukin (IL)-7 [14], which stimulates pro-

duction of RANKL and/or TNF-α by T cells [13,15], and that neutralization of IL-7 by administration of its specific antibody prevents bone resorption induced by OVX [14]. OVX-induced bone loss was absent in T-cell-deficient nude mice [11,12], and was restored by adoptive transfer of T cells from normal mice, but not by T cells from TNF-α knockout mice [12]. Production of RANKL by $B220^+$ pre-B cells was also upregulated in ovariectomized mice and by addition of isolated $B220^+$ cells from ovariectomized mice, but not cells from sham mice, to $B220^-$ cell cultures enhanced in vitro osteoclastogenesis in the presence of prostaglandin $(PG)E_2$ [10]. These data suggest that TNF-α and/or RANKL produced by T and B lineage cells plays an important role in acceleration of osteoclastogenesis caused by estrogen deficiency.

On the other hand, the involvement of increased RANKL produced by osteoblasts/stromal cells in accelerated osteoclastogenesis caused by estrogen deficiency is controversial. It has been reported that the expression of RANKL mRNA by osteoblasts/stromal cells is stimulated after estrogen withdrawal [16]. Upregulation of RANKL expression on osteoblasts/stromal cells as well as on immune cells in human bone marrow isolated from postmenopausal women has been demonstrated [17]. However, there also exists compelling evidence demonstrating that estrogen deficiency does not alter substantially RANKL [18,19] and OPG [19] mRNA expression in bone. In addition, estrogen has failed to consistently regulate osteoclastogenesis induced by osteoblast/stromal cell-derived RANKL in vitro. Treatment of ovariectomized mice with the pro-drug of 1α, 25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], which upregulates RANKL expression by osteoblasts/stromal cells in vitro, suppressed osteoclastogenesis without significantly altering RANKL and OPG mRNA expression in bone [19]. Because osteoblasts/stromal cells have been considered to play a pivotal role in regulation of osteoclastogenesis under physiological conditions, it is essential to establish whether osteoblasts/stromal cells would be involved in the increase of RANKL production by estrogen deficiency.

Alternatively or in addition, recent studies have implicated that estrogen deficiency stimulates osteoclastogenesis by increasing the pool size of osteoclast precursors [19–21]. Although it is a widespread belief that osteoclast precursors are of the monocyte/macrophage lineage, it has been consistently reported that osteoclasts can be generated from a subfraction of $B220^+$ pre-B cells [20–23]. Because proliferation of $B220^+$ pre-B cells is stimulated by IL-7, it raises the intriguing possibility that IL-7 induced by OVX stimulates osteoclastogenesis by expanding the pool size of $B220^+$ osteoclast precursors and that estrogen deficiency selectively augments the number of osteoclast precursors in the $B220^+$ cell fraction but not in the $B220^-$ fraction [21]. However, the effect of IL-7 on generation of osteoclast precursors in vitro has remained controversial [24].

The objective of our study was to determine precisely at mRNA levels whether estrogen deficiency would affect production of RANKL and OPG by osteoblasts/stromal cells in vivo and to investigate whether IL-7 would stimulate the generation of osteoclast precursors in vitro.

Materials and methods

Ovariectomy

Seven-week-old ddy mice were purchased from Nipp SLC (Shizuoka, Japan) and sham-operated or ovariec mized. The mice were allowed free access to water a commercial standard rodent chow (CE-2) under standa laboratory conditions at 24° ± 2°C and 50%–60% humidi After surgery, blood samples were collected for enzyn linked immunoabsorbent assay (ELISA). Femurs were i lated and immediately stocked in RNlator (Qiagen, Tok Japan) at –80°C. In some experiments, femurs were us for bone marrow cell preparations. These animal stud were carried out in accordance with the ethical guideli for animal care and approved by the Animal Care Comn tee of Meikai University.

Separation of adherent cells and nonadherent cells in bone marrow cells

The epiphysis of each femur was separated from the fer ral shaft (diaphysis) by cutting with scissors, and bone m row cells were isolated from the diaphysis by flushing w α-minimal essential medium (α-MEM) by means of a 26 needle. The bone marrow cells were seeded at 1×10^6/ into culture dishes and cultured in α-MEM with 5% fe bovine serum (FBS) for 24 h. Nonadherent cells were se rated by harvesting culture medium and were further h vested by gentle washing with PBS. The nonadherent c were pelleted by centrifugation, and adherent cells t remained on the dishes were used for further analyses [

Isolation of $B220^+$ cells

$B220^+$ cells were isolated from nonadherent cells by us a magnetic cell-sorting system (MACS; Miltenyi Bio Gladbach, Germany), as previously described [10,20].

Preparation of total RNA

Total RNA of separated cells was isolated by using RNeasy Mini Kit (Qiagen), in accordance with the inst tion manual. Total RNA of femurs from each mouse isolated by using a Concert Micro-to-Midi Total RNA p fication System (Invitrogen, Tokyo, Japan). Briefly, the physis, diaphysis, flushed-out bone marrow cells, and wh femur were homogenized in lysis buffer and then used total RNA purification as described in the instruct manual. Total RNA was treated with RNase-free DNa (Takara Bio, Tokyo, Japan) at 37°C for 90 min, puri with an RNeasy Mini kit, and used for cDNA synthesis

Real-time RT-PCR

cDNA was synthesized from total RNA (5 μg) of e sample by random priming with Super Script-II reve