Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

ACCACA), -1776 bp to -1771 bp (site 2, ACCACT) and -713 bp to -707 bp (site 3, AGTGGTT) from the cap site by computer analysis of the *Osterix* promoter (Fig. 3B). A wild type-luciferase reporter plasmid containing all the three putative Runx binding sites were compared in the *Osterix* promoter activity with five different mutant plasmids (M1-M5) in which some of the three putative sites were mutated (Fig. 3B). The wild type-reporter transfected with Runx3 reduced the *Osterix* promoter activity to nearly 55%. Transfection of the mutant reporters in which the site 1 and/or site 2 were mutated resulted in almost the same reduced activity as that of the wild type. In contrast, when used the mutant reporters in which the site 3 were mutated (M4 and M5), only a weak repression (90% activity) was detected (Fig. 3B). These results suggest that the site 3 is essential for *Osterix* promoter activity. To confirm this, shorter wild type- and mutant plasmids only containing the site 3 were used. The *Osterix* promoter activity was significantly reduced by the wild type, while not affected by the mutant (Fig. 3C). These results suggest that the site 3 is essential for *Osterix* promoter activity.

Characterization of the Runx3 protein binding to the site 3

To determine whether transcriptional repression of *Osterix* is due to direct binding of Runx3 to the site 3, electrophoretic mobility-shift assays using nuclear extracts from the mouse DPCs were performed. As shown in Fig. 4A, 38 bp end-labelled oligonucleotide containing the site 3 (-713 bp to -707 bp) of the *Osterix* promoter formed a DNA/protein complex (lane 2, arrowhead). The complex was completely competed by a 125-fold excess of unlabelled wild type-oligonucleotide (lane 3). An oligonucleotide in which the site 3 was mutated did not affect this binding (lane 4). Furthermore, antibody against Runx3 bound to the DNA/protein complex (lane 5, arrow), indicating the specificity of the DNA/protein complex. No band was not be detect when only nuclear extract loaded (lane 6).

Next, we carried out ChIP assay to test if Runx3 specifically binds to the element *in vivo*. The mouse DPCs were cross-linked and immunoprecipitated by Runx3

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

antibody. The presence of the *Osterix* promoter DNA was detected by PCR using primers flanking the site 3 (-713 bp to -707 bp) (Fig.4B), indicating that Runx3 binds to the site 3 of *Osterix* promoter specifically and functionally. The use of Runx2 antibody resulted in the similar result (Fig.4B), suggesting Runx3 and Runx2 both bind to the site 3 *in vivo*.

DISCUSSION

During systematic in situ hybridization study of tooth development *Osterix* mRNA was first detected in terminally differentiating odontoblasts, and colocalized with *Runx3*, suggesting a potential role for both in odontoblast differentiation. *Runx3* over-expression resulted in down-regulation of *Osterix* in the mouse dental pulp cells (DPCs). This suggests that *Osterix* might be a downstream target of *Runx3* in tooth development. *Osterix* null mice [18] have a similar phenotype to the *Runx2* null mice [9, 10], in which both intramembranous and endochondral bone are not formed due to the lack of osteoblast differentiation. Whereas *Osterix* is not expressed in the *Runx2* null mutants, *Runx2* expression is not changed in the *Osterix* null mutants [18]. These genetic studies have placed *Osterix* downstream to *Runx2* [18]. The precise regulatory role of Runx2 in *Osterix* expression is not clear. A recent study has shown that -737 bp fragment of *Osterix* promoter is up-regulated upon *Runx2* over-expression in ATDC5 chondroprogenitor cells and the function of -737 bp fragment was confirmed by site-directed mutagenesis experiments [19]. Furthermore, this functional binding site is conserved among mouse, rat and human, showing conservation of the DNA binding site [19]. However, no information is available on the regulation of *Osterix* expression by Runx3. Therefore, we have performed transient co-transfection, electrophoretic mobility shift and ChIP assays to investigate the relationship between Runx3 and Osterix in dental pulp cells. Structural dissection of the proximal regulatory region of the *Osterix* gene revealed the presence of three putative Runx-binding sites. Only the site 3 (-713 bp to -707 bp) of these sites, was preferentially and functionally occupied by Runx3. The disruption of site 3 leads to increased *Osterix* promoter activity in HEK293 cells, in which both Runx2 and Runx3 are not expressed endogenously. These results indicate that *Osterix* expression is negatively regulated by Runx3. Furthermore, our electrophoretic mobility shift and ChIP assays confirmed that Runx3 directly down-regulates *Osterix* expression in

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

dental pulp cells prior to terminal differentiation into odontoblasts. It is noteworthy that Runx3 negatively regulates *CD36* expression in myeloid cells [25] and suppresses gastric epithelial cell growth [26], implying a general role for Runx3 in transcriptional repression.

The distinct roles of Runx2 and Runx3 in odontoblast differentiation are not clear. Previous research indicated that tooth development was disrupted in the cap/early bell stages in the *Runx2* null mice and no overt differentiation of odontoblasts was observed [11, 27]. There was no conspicuous phenotype in teeth of *Runx3* null mice [17]. In *Runx2* null mice *Runx3* expression was dramatically enhanced in the mesenchyme of upper molars, and they differentiated into odontoblasts [27]. Our electrophoretic mobility shift and ChIP assays have shown that not only Runx2 but also Runx3 binds to the site 3 of *Osterix* promoter. *Runx3* over-expression resulted in down-regulation of *Osterix* in dental pulp cells. The *Osterix* promoter activity was down-regulated by *Runx3* transfection in HEK293 cells. These results suggested that the *Osterix* expression is cooperatively regulated by Runx2 and Runx3 sharing the same binding site on *Osterix* promoter. Thus, Runx3 might cooperate with Runx2 to regulate *Osterix* expression during odontoblast differentiation. The role of Osterix in tooth development is not clear. In skeletal development, Runx2, Runx3 and Osterix play a pivotal role in osteoblast differentiation and hypertrophic chondrocyte maturation [28, 29]. Osterix may play a role in segregation of osteoblast and chondrocyte lineages [29, 30]. *Runx2* and *Runx3* are co-expressed in early stage of tooth development. There is overlapping expression of *Osterix with Runx3* but not *Runx2* in terminal differentiation of odontoblasts. Therefore, *Osterix* in tooth development may play a role in lineage commitment of odontoblasts. The diverse transcriptional outcomes of Runx activity are dependent on context [1]. Runx family acts as organizing factors on the promoter of target genes where they associate with coactivators and other DNA-binding transcription factors including Smads [1]. Repression of *Osterix* by Runx3 in dental pulp cells is an example of context dependent regulation of lineage commitment. Thus, there might be cooperative

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

interactions among BMPs, Smads, Runx2 and Runx3 in the regulation of *Osterix* expression during dental pulp cell differentiation into odontoblasts.

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

ACKNOWLEDGEMENTS

The authors are grateful to Prof. A. Akamine for his help. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan, #17390509 and #18·06588.

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

1 Durst, K. L. and Hiebert, S. W. (2004) Role of RUNX family members in transcriptional repression and gene silencing. Oncogene **23**, 4220-4224

2 Ito, Y. (2004) Oncogenic potential of the RUNX gene family: 'overview'. Oncogene **23**, 4198-4208

3 van Wijnen, A. J., Stein, G. S., Gergen, J. P., Groner, Y., Hiebert, S. W., Ito, Y., Liu, P., Neil, J. C., Ohki, M. and Speck, N. (2004) Nomenclature for Runt-related (RUNX) proteins. Oncogene **23**, 4209-4210

4 Bangsow, C., Rubins, N., Glusman, G., Bernstein, Y., Negreanu, V., Goldenberg, D., Lotem, J., Ben-Asher, E., Lancet, D., Levanon, D. and Groner, Y. (2001) The RUNX3 gene--sequence, structure and regulated expression. Gene **279**, 221-232

5 Levanon, D., Glusman, G., Bangsow, T., Ben-Asher, E., Male, D. A., Avidan, N., Bangsow, C., Hattori, M., Taylor, T. D., Taudien, S., Blechschmidt, K., Shimizu, N., Rosenthal, A., Sakaki, Y., Lancet, D. and Groner, Y. (2001) Architecture and anatomy of the genomic locus encoding the human leukemia-associated transcription factor RUNX1/AML1. Gene **262**, 23-33

6 Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M., Lu, J., Satake, M., Shigesada, K. and Ito, Y. (1993) PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. Proc Natl Acad Sci U S A **90**, 6859-6863

7 Thirunavukkarasu, K., Mahajan, M., McLarren, K. W., Stifani, S. and Karsenty, G. (1998) Two domains unique to osteoblast-specific transcription factor Osf2/Cbfa1 contribute to its transactivation function and its inability to heterodimerize with Cbfbeta. Mol Cell Biol **18**, 4197-4208

8 Komori, T. (2005) Regulation of skeletal development by the Runx family of transcription factors. J Cell Biochem **95**, 445-453

9 Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Bronson, R. T., Gao, Y. H., Inada, M., Sato, M., Okamoto, R., Kitamura, Y., Yoshiki, S. and Kishimoto, T. (1997) Targeted disruption of

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell **89**, 755-764

10 Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. B. and Owen, M. J. (1997) Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell **89**, 765-771

11 D'Souza, R. N., Aberg, T., Gaikwad, J., Cavender, A., Owen, M., Karsenty, G. and Thesleff, I. (1999) Cbfa1 is required for epithelial-mesenchymal interactions regulating tooth development in mice. Development **126**, 2911-2920

12 Ducy, P., Zhang, R., Geoffroy, V., Ridall, A. L. and Karsenty, G. (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell **89**, 747-754

13 Mundlos, S., Otto, F., Mundlos, C., Mulliken, J. B., Aylsworth, A. S., Albright, S., Lindhout, D., Cole, W. G., Henn, W., Knoll, J. H., Owen, M. J., Mertelsmann, R., Zabel, B. U. and Olsen, B. R. (1997) Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell **89**, 773-779

14 Li, Q. L., Ito, K., Sakakura, C., Fukamachi, H., Inoue, K., Chi, X. Z., Lee, K. Y., Nomura, S., Lee, C. W., Han, S. B., Kim, H. M., Kim, W. J., Yamamoto, H., Yamashita, N., Yano, T., Ikeda, T., Itohara, S., Inazawa, J., Abe, T., Hagiwara, A., Yamagishi, H., Ooe, A., Kaneda, A., Sugimura, T., Ushijima, T., Bae, S. C. and Ito, Y. (2002) Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell **109**, 113-124

15 Levanon, D., Bettoun, D., Harris-Cerruti, C., Woolf, E., Negreanu, V., Eilam, R., Bernstein, Y., Goldenberg, D., Xiao, C., Fliegauf, M., Kremer, E., Otto, F., Brenner, O., Lev-Tov, A. and Groner, Y. (2002) The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. Embo J **21**, 3454-3463

16 Brenner, O., Levanon, D., Negreanu, V., Golubkov, O., Fainaru, O., Woolf, E.

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

and Groner, Y. (2004) Loss of Runx3 function in leukocytes is associated with spontaneously developed colitis and gastric mucosal hyperplasia. Proc Natl Acad Sci U S A **101**, 16016-16021

17 Yamashiro, T., Aberg, T., Levanon, D., Groner, Y. and Thesleff, I. (2002) Expression of Runx1, -2 and -3 during tooth, palate and craniofacial bone development. Mech Dev **119 Suppl 1**, S107-110

18 Nakashima, K., Zhou, X., Kunkel, G., Zhang, Z., Deng, J. M., Behringer, R. R. and de Crombrugghe, B. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell **108**, 17-29

19 Nishio, Y., Dong, Y., Paris, M., O'Keefe, R. J., Schwarz, E. M. and Drissi, H. (2006) Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. Gene **372**, 62-70

20 Platt, K. A., Michaud, J. and Joyner, A. L. (1997) Expression of the mouse Gli and Ptc genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. Mech Dev **62**, 121-135

21 Iohara, K., Zheng, L., Ito, M., Tomokiyo, A., Matsushita, K. and Nakashima, M. (2006) Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. Stem Cells **24**, 2493-2503

22 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res **17**, 6419

23 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**, 248-254

24 Drissi, H., Luc, Q., Shakoori, R., Chuva De Sousa Lopes, S., Choi, J. Y., Terry, A., Hu, M., Jones, S., Neil, J. C., Lian, J. B., Stein, J. L., Van Wijnen, A. J. and Stein, G. S. (2000) Transcriptional autoregulation of the bone related CBFA1/RUNX2 gene. J Cell Physiol **184**, 341-350

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

25 Puig-Kroger, A., Dominguez-Soto, A., Martinez-Munoz, L., Serrano-Gomez, D., Lopez-Bravo, M., Sierra-Filardi, E., Fernandez-Ruiz, E., Ruiz-Velasco, N., Ardavin, C., Groner, Y., Tandon, N., Corbi, A. L. and Vega, M. A. (2006) RUNX3 negatively regulates CD36 expression in myeloid cell lines. J Immunol **177**, 2107-2114

26 Chi, X. Z., Yang, J. O., Lee, K. Y., Ito, K., Sakakura, C., Li, Q. L., Kim, H. R., Cha, E. J., Lee, Y. H., Kaneda, A., Ushijima, T., Kim, W. J., Ito, Y. and Bae, S. C. (2005) RUNX3 suppresses gastric epithelial cell growth by inducing p21(WAF1/Cip1) expression in cooperation with transforming growth factor {beta}-activated SMAD. Mol Cell Biol **25**, 8097-8107

27 Aberg, T., Cavender, A., Gaikwad, J. S., Bronckers, A. L., Wang, X., Waltimo-Siren, J., Thesleff, I. and D'Souza, R. N. (2004) Phenotypic changes in dentition of Runx2 homozygote-null mutant mice. J Histochem Cytochem **52**, 131-139

28 Yoshida, C. A. and Komori, T. (2005) Role of Runx proteins in chondrogenesis. Crit Rev Eukaryot Gene Expr **15**, 243-254

29 Komori, T. (2006) Regulation of osteoblast differentiation by transcription factors. J Cell Biochem **99**, 1233-1239

30 Nakashima, K. and de Crombrugghe, B. (2003) Transcriptional mechanisms in osteoblast differentiation and bone formation. Trends Genet **19**, 458-466

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

TABLE 1 Primers for RT-PCR

Name		5'- Sequence -3'	Product size (bp)	Accession number
beta-actin	Forward	AAATCGTGCGTGACATCAAA	178	X03765
	Reverse	AAGGAAGGCTGGAAAAGAGC		
Runx3	Forward	GGTTCAACGACCTTCGATTC	180	NM_019732
	Reverse	AGGCCTTGGTCTGGTCTTCT		
Runx2	Forward	CAGACCAGCAGCACTCCATA	178	NM_009820
	Reverse	CAGCGTCAACACCATCATTC		
Osterix	Forward	GGTCCAGGCAACACACCTAC	178	AF184902
	Reverse	GGTAGGGAGCTGGGTTAAGG		
Dspp	Forward	GGAACTGCAGCACAGAATGA	199	NM_010080
	Reverse	CAGTGTTCCCCTGTTCGTTT		
Enamelysin	Forward	CGACAATGCTGAGAAGTGGA	180	NM_013903
	Reverse	CCCTTTCACATCATCCTTGG		
Klk4	Forward	TTGCAAACGATCTCATGCTC	228	NM_019928
	Reverse	TGAGGTGGTACACAGGGTCA		

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

Figure 1 Expression of *Runx3, Osterix, Runx2* and *Bmp2* by *in situ* hybridization during tooth development in the mouse

(A-D) *Runx3* was progressively restricted to the odontoblastic layer of tooth germ starting from the bell stage (17.0 dpc) to the differentiation stage (post natal stage day 1 (P1)) during terminal differentiation of odontoblasts. (E-H) *Osterix* was first detected weakly in the odontoblastic layer at 17.0 dpc, and was a more pronounced at P1, overlapping with *Runx3* expression. (I-L) *Runx2* was not expressed in odontoblast layer after P1. (M-P) *Bmp2* was strongly expressed in the odontoblasts at P1. Arrowheads indicate the positive signals in the odontoblastic layer. dp, dental papillae; ol, odontoblast layer. Bar = 200 μm

Figure 2 Down-regulation of *Osterix* expression by Runx3 in mouse dental pulp cells *in vitro*.

(A) mRNA expression of *Runx3*, *Osterix*, and differentiation markers of odontoblasts, *dentin sialophosphoprotein* (*Dspp*), *enamelysin* and *kallikrein-4* (*KLK4*) in mouse DPCs during culture. (B) *Osterix* expression was down-regulated in mouse DPCs at 48 hours after *Runx3* transfection. The experiment was repeated twice with similar results.

Figure 3 Down-regulation of *Osterix* promoter activity by Runx3 in human embryonic kidney 293 (HEK293) cells

(A) Determination of endogenous expression of *Runx3, Runx2* and *Osterix* in mouse DPCs but not in HEK293 cells. (B) Wild type (WT) and different mutation (MT) of *Osterix* promoter plasmids were analyzed 48 hours after co-transfection with *MSCV-eGFP-Flag-Runx3* into HEK293 cells. (C) Wild type (WT) or mutation (MT) with shorter *Osterix* promoter plasmids containing -713 to -707 (site 3) was co-transfected with *MSCV-eGFP-Flag-Runx3* into HEK293 cells. The activities were determined after 48 hours and normalized against co-transfected internal control plasmid (pRL-SV40). The values represent means ± S.D. of four individual samples. The experiment was repeated twice with similar results. ** $P<0.01$ compared with the

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

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.

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

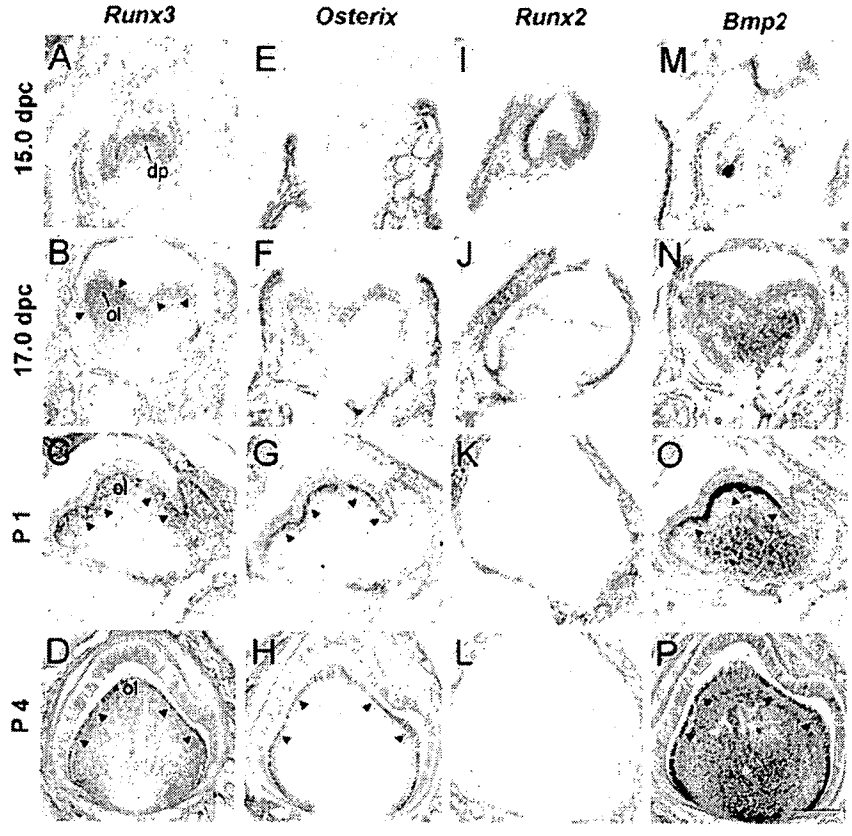


Figure 1

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

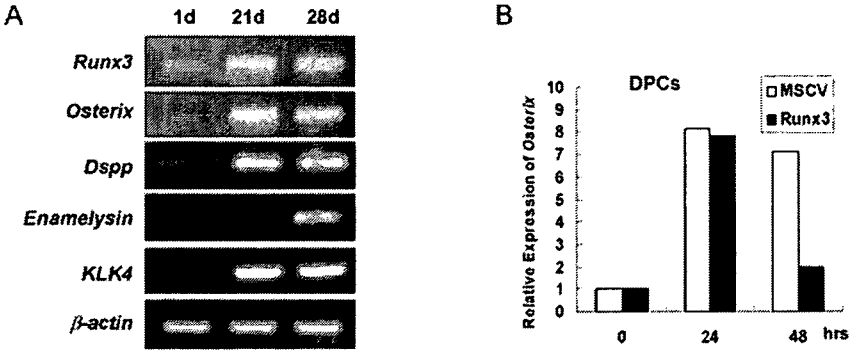


Figure 2

© 2007 The Authors Journal compilation © 2007 Biochemical Society

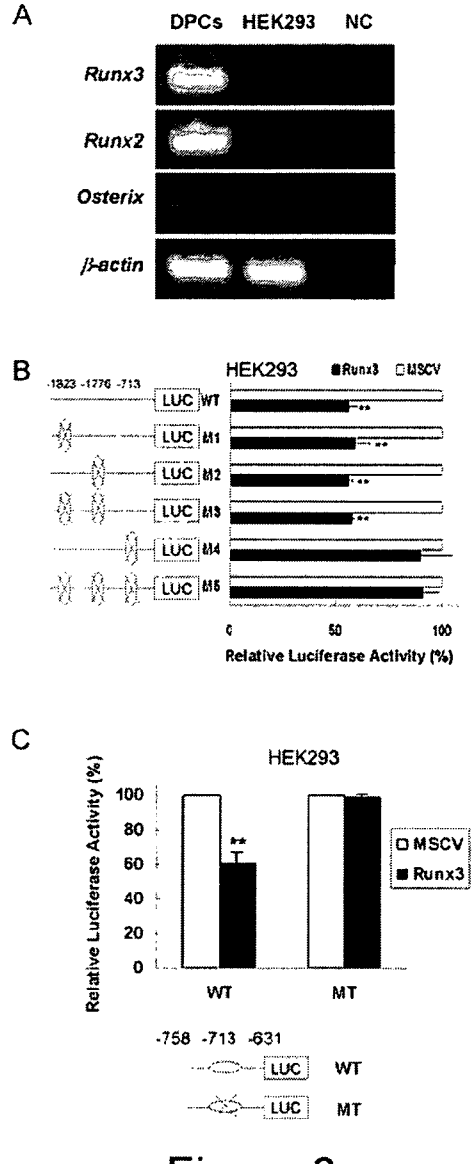


Figure 3

© 2007 The Authors Journal compilation © 2007 Biochemical Society

Biochemical Journal Immediate Publication. Published on 13 Mar 2007 as manuscript BJ20070104

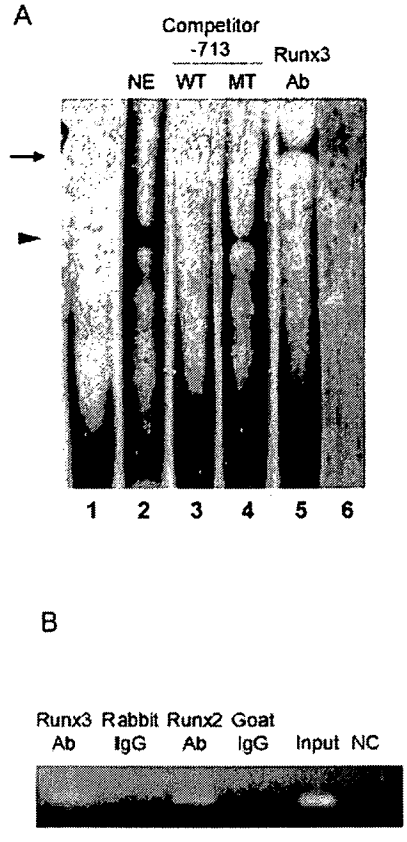
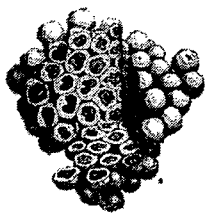


Figure 4

© 2007 The Authors Journal compilation © 2007 Biochemical Society



STEM CELLS®

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

Stem Cells 2006;24;2493-2503; originally published online Jul 27, 2006;
DOI: 10.1634/stemcells.2006-0161

This information is current as of March 28, 2007

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://www.StemCells.com/cgi/content/full/24/11/2493

STEM CELLS®, an international peer-reviewed journal, covers all aspects of stem cell research: embryonic stem cells; tissue-specific stem cells; cancer stem cells; the stem cell niche; stem cell genetics and genomics; translational and clinical research; technology development.

STEM CELLS® is a monthly publication, it has been published continuously since 1983. The Journal is owned, published, and trademarked by AlphaMed Press, 318 Blackwell Street, Suite 260, Durham, North Carolina, 27701. © 2006 by AlphaMed Press, all rights reserved. Print ISSN: 1066-5099. Online ISSN: 1549-4918.

AlphaMed Press

Downloaded from www.StemCells.com at National Institute for Longevity Sciences Library on March 28, 2007

STEM CELLS
TISSUE-SPECIFIC STEM CELLS

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

KOICHIRO IOHARA,[a] LI ZHENG,[a] MASATAKA ITO,[b] ATSUSHI TOMOKIYO,[c] KENJI MATSUSHITA,[a] MISAKO NAKASHIMA[a]

[a]Laboratory of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi, Japan; [b]Department of First Anatomy, National Defense Medical College, Tokorozawa, Japan; [c]Department of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, Fukuoka, Japan

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

Correspondence: Misako Nakashima, D.D.S., Ph.D., Laboratory of Oral Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. Telephone: 81-562-44-5651, ext. 5403; Fax: 81-562-46-8684; e-mail: misako@nils.go.jp Received March 20, 2006; accepted for publication July 19, 2006; first published online in STEM CELLS *EXPRESS* July 27, 2006. ©AlphaMed Press 1066-5099/2006/$20.00/0 doi: 10.1634/stemcells.2006-0161

Downloaded from www.StemCells.com at National Institute for Longevity Sciences Library on March 28, 2007

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

Downloaded from www.StemCells.com at National Institute for Longevity Sciences Library on March 28, 2007

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.

Downloaded from www.StemCells.com at National Institute for Longevity Sciences Library on March 28, 2007