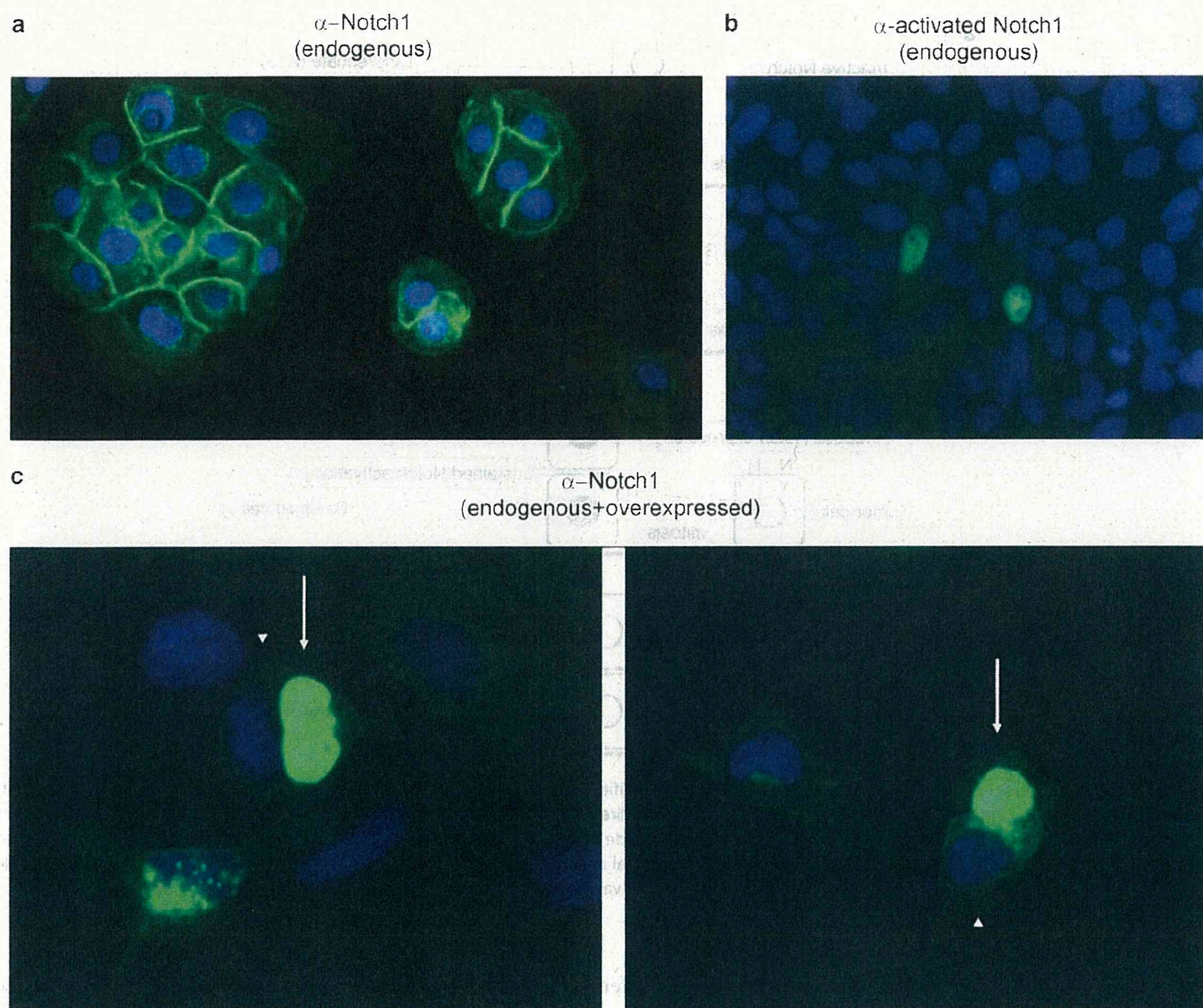

Figure 7 Nuclear translocation of NOTCH1 is rare, and it occasionally occurs asymmetrically in cultured keratinocytes. (**a**) Immunofluorostaining of HFS cells using the anti-NOTCH1 antibody. NOTCH1 protein accumulates at the cell–cell interface. Little protein was detected on the free surface or in the nuclei. (**b**) Immunofluorostaining of HFS cells using the anti-cleaved NOTCH1(Val1744) antibody. This activated form of intracellular domain of NOTCH1 is detected in the nuclei, but only in a very few cells—far $<1\%$ of the total population. (**c**) Immunofluorostaining of Ca9-22 cells transfected with wild-type mouse Notch1 using the anti-NOTCH1 antibody. The antibody crossreacts with mouse Notch1. Endogenous expression of NOTCH1 is observed as fine membranous or cytoplasmic staining. The transfected cells exhibit much stronger expression, facilitating detection. Nuclear localization of the NOTCH1 is observed in one of two neighboring cells, which appear as postmitotic daughter cells. Arrow: postmitotic cell with nuclear NOTCH1; arrowhead: postmitotic cell without nuclear NOTCH1.

normal and neoplastic epithelia, which would never be observed in nonspecific staining.

Although elevated Notch signaling has been suggested in tumor development,[13,30–32] neither an increase of NOTCH1 expression nor its nuclear translocation was observed in the cases we examined. The cDNA microarray of OSCC showed that neither *NOTCH2* nor *NOTCH3* was upregulated in OSCC, suggesting that quantitative compensation is unlikely. These results indicate that upregulation of Notch-dependent signaling may not make a major contribution to the development and progression of squamous cell carcinoma. Conversely, consistent reduction of NOTCH1 expression in squamous neoplasms was evident. Also, *NOTCH1* has recently been shown to be mutated in 11–15% of head and neck cancer, and about 40% of the mutations were predicted to generate truncated NOTCH1 proteins, whereas no apparent activating mutation was found.[33,34] These results suggest that NOTCH1 may function as a tumor suppressor gene rather than as an oncogene in squamous neoplasms.

We demonstrated that impaired Notch1 signaling led to abnormal differentiation represented by alterations of keratin subtype expression, which was commonly observed not only in cancers but also in precancers. The role of Notch in regulation of squamous epithelium differentiation has also been suggested by studies using cultured cervical[28] and esophageal keratinocytes.[35] Collectively, these results indicate that

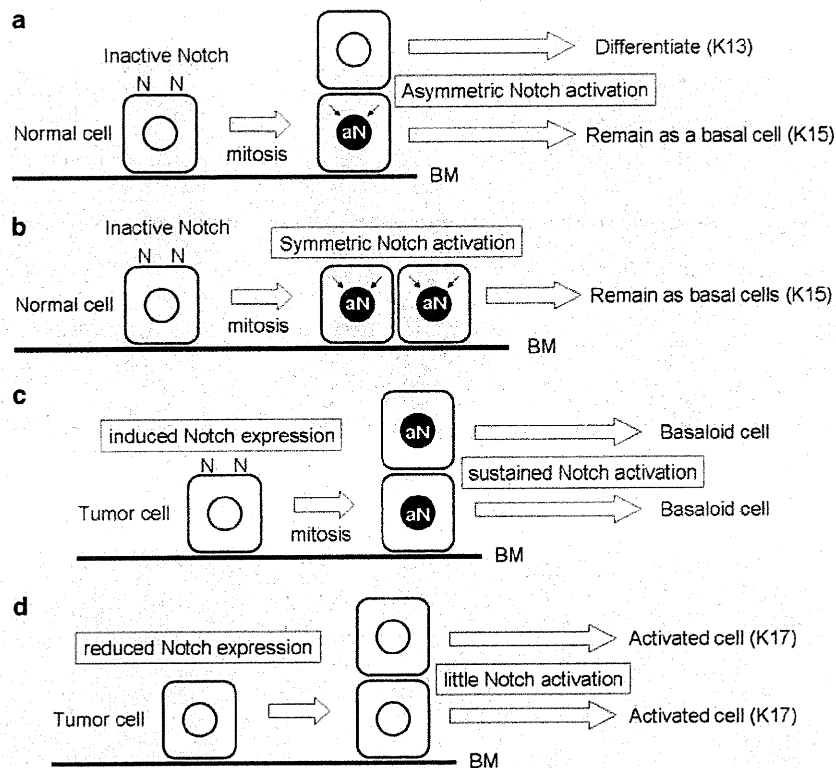

Figure 8 Proposed model of Notch-mediated mechanism of non-cornified squamous epithelium differentiation. (**a**) NOTCH1 is selectively activated in the basal cell (K15 +) when the sister cell divides basoapically, and directs it to remain as a basal cell (K15 +), whereas the apical daughter cell without Notch signal input differentiates (K13 +). (**b**) When the sister cells divide symmetrically, Notch is activated in both cells, which remain as basal cells (K15 +). (**c**) If the asymmetric Notch signaling is impaired and both basal and apical daughter cells undergo Notch activation, both remain as basaloid cells (K15 +). (**d**) The cells with reduced NOTCH1 expression convert to activated cells (K17 +) that lead to a hyperplastic phenotype.

reduced NOTCH1 expression affects the terminal differentiation, thus highlighting the essential role of NOTCH1 in maintaining normal epithelial integrity. In addition, we examined five cases of traumatic ulcer and found that NOTCH1 was downregulated in the regenerative epithelium, accompanied with loss of K13 expression and robust induction of K17 (K.S., unpublished observations). This suggests that the downregulation of NOTCH1 expression level is an inherent mechanism for switching the epithelium from a normal and mature state to an activated and immature state.

Cervical cancers tended to retain NOTCH1 expression compared with oral and esophageal cancers. We evaluated the expression in the cervical cancer cells in comparison with the neighboring vaginal squamous epithelium. However, this evaluation method might be misleading because cervical cancers arise from the reserve cells beneath the columnar epithelium of endocervix,[36] and the vaginal epithelium is not the origin of cervical cancers. As the reserve cells are almost negative for NOTCH1, it can also be said that NOTCH1 expression is increased in cervical cancer compared with its original cell type.

We hypothesized that strong NOTCH1 expression may correlate with a tendency for differentiation toward squamous epithelium. To check this hypothesis, we additionally examined five specimens of squamous metaplasia caused by obstruction of the minor salivary gland duct. As expected, NOTCH1 was significantly induced in ducts, which show ectopic K13 induction and squamous metaplasia (Supplementary Material 7). HPV infection to metaplastic epithelium in the transformation zone initiates progression to CIN or CSCC,[37] and *NOTCH1* is upregulated by HPV E6 and E7 oncoproteins, which are almost uniformly expressed in cervical cancer.[38] Virally induced NOTCH1 expression would tend to be maintained in CIN and CSCC, but would be no more upregulated. In this context, upregulation of NOTCH1 may have an essential role only in generation of metaplastic epithelium.

HPV has been detected only in a minority of OSCC cases, excluding pharyngeal cancer[39] and ESCC.[40] This is probably attributable to the difference in the NOTCH1 expression pattern between oro-esophageal and cervical cancers. The NOTCH1 expression patterns seem to underlie their histopathological differences. Expansion of a basaloid cell population is usually observed in CIN, whereas this finding is exceptional in OIN, whose basaloid cells are usually limited to the lower part of the epithelium. Remaining NOTCH1

expression appears to autonomously direct the cell to maintain the basaloid phenotype.

In our series of cell culture experiments, Notch signaling exhibited seemingly diverse effects. For example, both activation of Notch signaling and downregulation of NOTCH1 expression inhibited differentiation. To better understand such diverse effects, we have developed the following model, which is consistent with the experimental and histopathological findings, and also with the self-organizing nature of stratified epithelium. NOTCH1 is selectively activated in the basal cell when the sister cell divides basoapically, and acts to direct the basal cell to remain as a basal cell, whereas the apical daughter cell without Notch signal input is directed to differentiate (Figure 8a). When the sister cell divides laterally, Notch is activated in both cells, which directs them both to be basal cells (Figure 8b). If asymmetric Notch signaling is impaired, and both basal and apical daughter cells undergo Notch activation, both would remain as basaloid cells (Figure 8c), causing expansion of the basal-cell layer. Cells with reduced NOTCH1 expression convert to activated cells that lead to a hyperplastic phenotype (Figure 8d). In either case, impaired Notch signaling causes an immature epithelium. Although future research is required to confirm this model, it is consistent with the results of genetically engineered mouse experiments.[8,35]

Besides its role as a receptor, Notch is considered to have a function as a modulator of cell adhesion.[7] The NOTCH1 accumulation on the plasma membranes between neighboring cells and the rare observation of the activated form support this notion. Reduced Notch expression may facilitate the cell dissociation and movement that are required for regenerative epithelium and cancer invasion.

Altogether, we assume that NOTCH1 functions in two ways: it mediates the balance between populations of basal cells and differentiated cells in normal epithelium by symmetric and asymmetric activation; and in pathological conditions, such as wound healing, precancer and cancer, its expression is reduced, which converts the cells into an activated and immature state.

Impaired asymmetric cell division affects epidermal Notch signaling and results in defects in stratification and differentiation, suggesting that Notch is an effector of asymmetric cell division.[41] The mechanism of asymmetric Notch activation is unclear. JAG1, one of the canonical ligands, was expressed in the suprabasal layers (K.S., unpublished observations), which suggests that the signal is directionally transmitted from an apical cell to a basal cell because of these localizations of ligand- and receptor-expressing cells. However, JAG1 was also expressed in the basal cells, and the significance of the co-expression of receptors and ligand in the basal cells is yet to be elucidated. Accumulating evidence indicates that the canonical Notch ligands also act as a cell-autonomous repressor of Notch signaling,[26,42–46] suggesting that JAG1 expression in the basal cells may inhibit the signaling. Another possible mechanism of the asymmetric activation is the suppression of Notch signaling by protein degradation mediated by Numb, which is distributed differentially in the daughter cells and governs asymmetric cell division.[47,48]

The transcription of *NOTCH1* gene is suppressed by TP63(ΔN) in cervical keratinocytes,[28] and TP63(ΔN) inhibits differentiation in the oropharyngeal SCC cell line.[49] Our findings using Ca9-22 cells are consistent with these results, suggesting that the interplay between NOTCH1 and TP63(ΔN) in differentiation is common in non-cornified epithelia of various sites. In addition, we found that TP63(ΔN) expression was affected by Notch signaling, suggesting a feedback relationship between these factors. It should be noted that this hypothetical NOTCH1-TP63 interplay does not resemble the expression patterns of these factors in cancer tissues, in which TP63 is uniformly expressed in both basal and suprabasal layers of normal epithelium and cancer (data not shown). Thus, the NOTCH1-TP63 interplay is merely one of many mechanisms that govern the epithelial cell behaviors.

In summary, NOTCH1 is expressed predominantly in the basal cells of squamous epithelium, and it is generally downregulated in squamous neoplasms, even at early stages. Reduction of NOTCH1 expression directs the basal cells to cease terminal differentiation, resulting in an immature epithelium, which may have an essential role in the histopathogenesis of dysplastic features commonly observed in precancerous epithelium. These findings suggest that normal epithelial integrity is autonomously maintained by this evolutionarily conserved cell-to-cell signaling system.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENT
This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 21592320).

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

1. Blaumueller CM, Qi H, Zagouras P, *et al.* Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell 1997;90:281–291.
2. Logeat F, Bessia C, Brou C, *et al.* The Notch1 receptor is cleaved constitutively by a furin-like convertase. Proc Natl Acad Sci USA 1998;95:8108–8112.
3. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006;7:678–689.
4. Blanpain C, Lowry WE, Pasolli HA, *et al.* Canonical notch signaling functions as a commitment switch in the epidermal lineage. Genes Dev 2006;20:3022–3035.
5. Favier B, Fliniaux I, Thelu J, *et al.* Localisation of members of the notch system and the differentiation of vibrissa hair follicles: receptors, ligands, and fringe modulators. Dev Dyn 2000;218:426–437.
6. Pan Y, Lin MH, Tian X, *et al.* Gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. Dev Cell 2004;7:731–743.
7. Watt FM, Estrach S, Ambler CA. Epidermal Notch signalling: differentiation, cancer and adhesion. Curr Opin Cell Biol 2008; 20:171–179.

8. Nicolas M, Wolfer A, Raj K, *et al.* Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003;33:416–421.
9. Estrach S, Ambler CA, Lo Celso C, *et al.* Jagged 1 is a beta-catenin target gene required for ectopic hair follicle formation in adult epidermis. Development 2006;133:4427–4438.
10. Krebs LT, Xue Y, Norton CR, *et al.* Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 2000;14:1343–1352.
11. Krebs LT, Xue Y, Norton CR, *et al.* Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. Genesis 2003;37:139–143.
12. Robbins J, Blondel BJ, Gallahan D, *et al.* Mouse mammary tumor gene int-3: a member of the notch gene family transforms mammary epithelial cells. J Virol 1992;66:2594–2599.
13. Weng AP, Ferrando AA, Lee W, *et al.* Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004; 306:269–271.
14. Moellering RE, Cornejo M, Davis TN, *et al.* Direct inhibition of the NOTCH transcription factor complex. Nature 2009;462:182–188.
15. Veeraraghavalu K, Pett M, Kumar RV, *et al.* Papillomavirus-mediated neoplastic progression is associated with reciprocal changes in JAGGED1 and manic fringe expression linked to notch activation. J Virol 2004;78:8687–8700.
16. Zagouras P, Stifani S, Blaumueller CM, *et al.* Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc Natl Acad Sci USA 1995;92:6414–6418.
17. Daniel B, Rangarajan A, Mukherjee G, *et al.* The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. J Gen Virol 1997;78(Pt 5):1095–1101.
18. Maliekal TT, Bajaj J, Giri V, *et al.* The role of Notch signaling in human cervical cancer: implications for solid tumors. Oncogene 2008; 27:5110–5114.
19. Proweller A, Tu L, Lepore JJ, *et al.* Impaired notch signaling promotes *de novo* squamous cell carcinoma formation. Cancer Res 2006; 66:7438–7444.
20. Lefort K, Mandinova A, Ostano P, *et al.* Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. Genes Dev 2007; 21:562–577.
21. Panelos J, Tarantini F, Paglierani M, *et al.* Photoexposition discriminates Notch 1 expression in human cutaneous squamous cell carcinoma. Mod Pathol 2008;21:316–325.
22. Yoshikawa H, Kawana T, Kitagawa K, *et al.* Detection and typing of multiple genital human papillomaviruses by DNA amplification with consensus primers. Jpn J Cancer Res 1991;82:524–531.
23. Tomioka H, Morita K, Hasegawa S, *et al.* Gene expression analysis by cDNA microarray in oral squamous cell carcinoma. J Oral Pathol Med 2006;35:206–211.
24. Sakamoto K, Tamamura Y, Katsube K, *et al.* Zfp64 participates in Notch signaling and regulates differentiation in mesenchymal cells. J Cell Sci 2008;121(Pt 10):1613–1623.
25. Minamizato T, Sakamoto K, Liu T, *et al.* CCN3/NOV inhibits BMP-2-induced osteoblast differentiation by interacting with BMP and Notch signaling pathways. Biochem Biophys Res Commun 2007.
26. Sakamoto K, Ohara O, Takagi M, *et al.* Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. Dev Biol 2002;241:313–326.
27. Taylor GP. Current Protocols in Molecular Biology. Wiley, 1987.
28. Yugawa T, Narisawa-Saito M, Yoshimatsu Y, *et al.* DeltaNp63alpha repression of the Notch1 gene supports the proliferative capacity of normal human keratinocytes and cervical cancer cells. Cancer Res 2010;70:4034–4044.
29. Ma A, Boulton M, Zhao B, *et al.* A role for notch signaling in human corneal epithelial cell differentiation and proliferation. Invest Ophthalmol Vis Sci 2007;48:3576–3585.
30. Hu C, Dievart A, Lupien M, *et al.* Overexpression of activated murine Notch1 and Notch3 in transgenic mice blocks mammary gland development and induces mammary tumors. Am J Pathol 2006; 168:973–990.
31. Park JT, Chen X, Trope CG, *et al.* Notch3 overexpression is related to the recurrence of ovarian cancer and confers resistance to carboplatin. Am J Pathol 2010;177:1087–1094.
32. Zhang Z, Wang H, Ikeda S, *et al.* Notch3 in human breast cancer cell lines regulates osteoblast-cancer cell interactions and osteolytic bone metastasis. Am J Pathol 2010;177:1459–1469.
33. Agrawal N, Frederick MJ, Pickering CR, *et al.* Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 2011;333:1154–1157.
34. Stransky N, Egloff AM, Tward AD, *et al.* The mutational landscape of head and neck squamous cell carcinoma. Science 2011;333:1157–1160.
35. Ohashi S, Natsuizaka M, Yashiro-Ohtani Y, *et al.* NOTCH1 and NOTCH3 coordinate esophageal squamous differentiation through a CSL-dependent transcriptional network. Gastroenterology 2010; 139:2113–2123.
36. Christopherson WM. Dysplasia, carcinoma *in situ*, and microinvasive carcinoma of the uterine cervix. Hum Pathol 1977;8:489–501.
37. Reid R. Genital warts and cervical cancer. II. Is human papillomavirus infection the trigger to cervical carcinogenesis? Gynecol Oncol 1983;15:239–252.
38. Weijzen S, Zlobin A, Braid M, *et al.* HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation. J Cell Physiol 2003;194:356–362.
39. Machado J, Reis PP, Zhang T, *et al.* Low prevalence of human papillomavirus in oral cavity carcinomas. Head Neck Oncol 2010;2:6.
40. Syrjanen KJ. HPV infections and oesophageal cancer. J Clin Pathol 2002;55:721–728.
41. Williams SE, Beronja S, Pasolli HA, *et al.* Asymmetric cell divisions promote Notch-dependent epidermal differentiation. Nature 2011; 470:353–358.
42. del Alamo D, Schweisguth F. Notch signalling: receptor cis-inhibition to achieve directionality. Curr Biol 2009;19:R683–R684.
43. Klein T, Brennan K, Arias AM. An intrinsic dominant negative activity of serrate that is modulated during wing development in *Drosophila*. Dev Biol 1997;189:123–134.
44. Li Y, Baker NE. The roles of cis-inactivation by Notch ligands and of neuralized during eye and bristle patterning in Drosophila. BMC Dev Biol 2004;4:5.
45. Micchelli CA, Rulifson EJ, Blair SS. The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. Development 1997;124:1485–1495.
46. Miller AC, Lyons EL, Herman TG. cis-Inhibition of Notch by endogenous Delta biases the outcome of lateral inhibition. Curr Biol 2009; 19:1378–1383.
47. McGill MA, Dho SE, Weinmaster G, *et al.* Numb regulates post-endocytic trafficking and degradation of Notch1. J Biol Chem 2009; 284:26427–26438.
48. Zhong W, Jiang MM, Weinmaster G, *et al.* Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. Development 1997;124: 1887–1897.
49. Boldrup L, Coates PJ, Gu X, *et al.* DeltaNp63 isoforms regulate CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of head and neck. J Pathol 2007;213:384–391.

Anatomical Record (in press)

Canine oral mucosal fibroblasts differentiate into osteoblastic cells in response to BMP-2

Kohsuke Umehara,[1,2] Tadahiro Iimura,[1,3] Kei Sakamoto,[1] Zayar Lin,[3,4] Shohei Kasugai,[3,4] Yoshimasa Igarashi,[2] and Akira Yamaguchi[1,3*]

Sections of [1]Oral Pathology, [2]Removable Partial Denture Prosthodontics, and [4]Oral Implantology and Regenerative Dental Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, and [3]Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan

Running title: Osteoblast differentiation of oral fibroblast

*Address correspondence to: Akira Yamaguchi, D.D.S., Ph.D.
Professor and Chair
Section of Oral Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Phone: +81-3-5803-5451
Fax' +81-3-5803-0188
E-mail address: akira.mpa@tmd.ac.jp

Grant sponsors: This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 22249061 and No. 21659420) and Health and Labour Sciences Research Grants from the Ministry of Health, Labour, and Welfare (No. 21040101) to A. Y., and by the Global Center of Excellence (GCOE) Program; International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University.

ABSTRACT

Several lines of evidence show that transplantation of osteoblastic cells or genetically engineered non-osteogenic cells expressing osteoblast-related genes into bone defects effectively promotes bone regeneration. To extend this possibility, we investigated whether oral mucosal fibroblasts are capable of differentiating into osteoblastic cells by conducting *in vitro* and *in vivo* experiments. We investigated the effects of bone morphogenetic protein-2 (BMP-2) on osteoblast differentiation of cultured fibroblasts isolated from canine buccal mucosa. We also transplanted green fluorescence protein (GFP)-expressing fibroblasts with gelatin/BMP-2 complexes into the subfascial regions of athymic mice, and investigated the localization of GFP-positive cells in the ectopically formed bones. The cultured canine buccal mucosal fibroblasts differentiated into osteoblastic cells by increasing their alkaline phosphatase (ALP) activity and *Osteocalcin*, *Runx2,* and *Osterix* mRNA expression levels in response to BMP-2. Transplantation experiments of GFP-expressing oral mucosal fibroblasts with gelatin/BMP-2 complexes revealed that 17.1% of the GFP-positive fibroblasts differentiated into ALP-positive cells, and these cells accounted for 6.2% of total ALP-positive cells in the ectopically formed bone. This study suggests that oral mucosal fibroblasts can differentiate into osteogenic cells in response to BMP-2. Thus, these cells are potential candidates for cell-mediated bone regeneration therapy in dentistry.

INTRODUCTION

Bone formation and regeneration are mediated by the coordinate action of various factors. Among these factors, bone morphogenetic protein (BMP) plays crucial roles in bone formation and regeneration (Yamaguchi et al., 2000; Hirata et al., 2007). We have previously shown that BMP-2 not only stimulates osteoblastic differentiation of osteoprogenitors (Yamaguchi et al., 1991; Yamaguchi et al., 1996) but also transdifferentiates non-osteogenic mesenchymal cells to osteoblast lineage cells (Katagiri et al., 1990; Katagiri et al., 1994). Runx2 is essential for osteoblast differentiation and bone formation, because *Runx2*-deficient mice completely lacked bone formation ability due to maturational arrest of osteoblasts (Komori et al., 1997; Otto et al., 1997). Because BMP stimulates Runx2 expression during osteoblast differentiation (Yamaguchi et al., 2000; Ryoo et al., 2006), BMP is considered to play a crucial role in osteoblast differentiation, and hence bone formation and bone regeneration.

Several lines of evidence obtained from animal experiments have shown that transplantation of osteoblastic cells or genetically engineered non-osteogenic cells expressing osteoblast-related genes into bone defects effectively promotes bone regeneration (Breitbart et al., 1999; Franceschi et al., 2000; Krebsbach et al., 2000; Tamura et al., 2001; Gysin et al., 2002; Hirata et al., 2003). Although the use of such cells is a powerful therapeutic approach to cell-mediated gene therapy for critical-sized bone defects, several issues should be resolved for developing this technique and its potential clinical application. The first challenge is to identify the source of cells for genetic engineering and transplantation. Bone marrow stromal cells (Riew et al., 1998;

Musgrave et al., 2000; Cheng et al., 2001; Turgeman et al., 2001; Gysin et al., 2002; Partridge et al., 2002), periosteal cells (Breitbart et al., 1999), muscles (Riew et al., 1998; Lee et al., 2000; Cheng et al., 2001; Lee et al., 2001; Pelinkovic et al., 2001; Lee et al., 2002; Musgrave et al., 2002), and fibroblasts (Takayanagi et al., 1999; Franceschi et al., 2000; Krebsbach et al., 2000) have been successfully used as the source of transplanted cells in animal experiments. These cell types are strong candidates for clinical application, but these cause considerable challenge when applied in the dental clinics. Therefore, periodontal ligament cells (Seo et al., 2004; Hiraga et al., 2009) and dental pulp cells (Gronthos et al., 2000; Gronthos et al., 2002; Hosoya et al., 2007; Otaki et al., 2007) are being considered as candidates in the dental field. Second, before clinical application, the fate of the transplanted cells during bone regeneration should be traced in the preclinical experiments. For this purpose, green fluorescent protein (GFP) can be used to tag the source cells.

We have reported that skin fibroblasts (Hirata et al., 2003; Kadowaki et al., 2004) and osteoblastic cells (Kadowaki et al., 2004) isolated from GFP transgenic mice, which over express *BMP-2* by using an adenovirus vector, are good sources for cell-mediated bone repair. Furthermore, these cells are effective tools for tracing the fate of the transplanted cells during bone regeneration. Although the use of skin fibroblasts is a powerful therapeutic approach to cell-mediated gene therapy for critical-sized bone defects, dentists usually cannot easily procure skin fibroblasts by themselves. Therefore, we thought of oral mucosal fibroblasts as a candidate for cell-mediated bone regeneration therapy in dentistry.

In the present study, we performed *in vitro* and *in vivo* experiments to investigate whether oral mucosal fibroblasts are available as source cells for

cell-mediated bone regeneration therapy by using canine oral mucosal fibroblasts (COFs). Our *in vitro* study showed that COFs are capable of differentiating into osteoblastic cells in response to BMP-2. Furthermore, we transplanted the COFs expressing GFP with gelatin sponge/BMP-2 complexes into the subfascial regions of athymic mice; we found that oral mucosal fibroblasts retain the potency to differentiate into osteogenic cells in response to BMP-2. Thus, this study indicates that oral mucosal fibroblasts are a potential candidate for cell-mediated bone regeneration therapy in the dentistry.

MATERIALS AND METHODS

Cell culture and infection with a retrovirus expressing GFP

Canine buccal mucosal tissues, approximately 5 × 5 × 1mm, were obtained from healthy 12-month-old beagle dogs (weight approximately 10kg). To allow the COFs to adhere to the surface of culture dishes, the samples were placed on the surface of 6-well dishes for 60 min at 37°C without any culture medium. The COFs were then cultured for 7 to 10 days in α-modified minimum essential medium (α-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (100 μg/ml penicillin G and 100 IU/ml streptomycin) at 37°C in 5% CO_2/95% air. The outgrown fibroblasts from the explants were harvested by digestion with 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (trypsin/EDTA) in calcium- and magnesium-free phosphate buffered saline (PBS(−)). Next, the harvested cells were inoculated on 10-cm dishes with α-MEM containing 10% FBS and antibiotics, and eventually transfected with retrovirus vectors.

COFs were transfected with replication-deficient retroviral vectors carrying the GFP gene (pLP-LNCX-GFP). The cells at the growing phase in the 10-cm dishes were incubated with media containing retrovirus (MOI, 200) for 2h at 37°C, and the culture media were then replaced with fresh media. The transfected cells were cultured for up to 6 days to analyze their osteoblastic differentiation potential *in vitro*. For transplantation experiments, the transfected COFs with GFP were immediately removed by trypsin/EDTA digestion after washing with PBS(−), and then washed with fresh medium. These cells were used for making transplantation complexes as described below. We used 4-8 passages cells for our *in vitro* and *in vivo* experiments.

Measurement of alkaline phosphatase activity and histochemical staining

The cells were inoculated at 4×10^3 cells/cm^2 in 48-well or 24-well plates, and treated for 3 or 6 days with various concentrations of recombinant human bone morphogenetic protein-2 (rhBMP-2), which was purchased from Osteogenetics GmbH (Wuerzburg, Germany). The cultured cells were sonicated in a radioimmunoprecipitation assay (RIPA) buffer to obtain cell lysate. Alkaline phosphatase (ALP) activity was determined using *p*-nitrophenylphosphate solution (Wako Pure Chemicals, Osaka, Japan) as the substrate. The amount of *p*-nitrophenylphosphate released was estimated by measuring the absorbance at 405nm after 30 minutes of incubation at 37°C. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo, Waltham, MA). For histochemical detection of ALP activity, the cultured cells were fixed in 10% phosphate-buffered formalin for 5 minutes, washed twice with 10mM Tris-HCl (pH 7.5), and then stained with ALP. ALP staining was performed at 37°C for 20 minutes by using nitrobluetetrazolium chloride (NBT) as theformazan dye

and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the enhancer (Roche, Mannheim, Germany). To assess a capability to generate mineralized bone nodules *in vitro*, we cultured COFs with osteogenic medium (α-MEM containing 10% FBS, 10 mmol/L of β-glycerophosphate, 50 μg/ml of ascorbic acid, and antibiotics) in the presence of rhBMP-2 (200 ng/ml or 500 ng/ml) for 14 days, and then the cells were dual-stained with ALP and von Kossa.

Reverse transcriptase-polymerase chain reaction analyses

For reverse transcriptase-polymerase chain reaction (RT-PCR) analyses, total RNA was extracted from the cultured cells by using NucleoSpin (Macherey-Nagel, Duren, Germany). RNA aliquots were reverse transcribed to complementary DNAs by using an oligo(dT) primer (Roche), deoxynucleotide triphosphate (dNTP; Promega), and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, Hanover, MD). The complementary DNA products were subjected to PCR amplification with gene-specific primers for canine *Osteocalcin*, *Runx2*, and *Osterix* (Table 1). Real-time RT-PCR amplification was performed using the Light Cycler System (Roche) with a Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen, Carlsbad, CA). The relative amount of each mRNA was normalized to *β-2-microglobulin* mRNA (Brinkhof et al., 2006).

Preparation and transplantation of gelatin sponge/BMP-2/COFs Complexes into athymic mice

We used biodegradable gelatin sponges containing β-tricalcium phosphate (GS-β; MedGelCo.Ltd., Kyoto, Japan) (Takahashi et al., 2005) for a carrier of cell

transplantation. Each GS-β disk was cut into a circular fragment (diameter, 4 mm; thickness, 2 mm). After the application of 10μl of rhBMP-2 solution (200ng/μl) on the GS-β disk (GS-β/BMP-2), they were freeze-dried and kept at −4°C until further use. As control, we prepared GS-β fragments that similarly absorbed only the buffer used for BMP-2 solution (GS-β alone). All procedures were carried out under sterile conditions.

After removing the retrovirus-infected cells from culture dishes by trypsin/EDTA digestion, they were re-suspended in the culture medium, and 1.5 ml aliquots of the culture medium containing 4×10^6 cells were transferred into a sterile serum tube containing GS-β/BMP-2 or GS-β alone. Each serum tube containing the cells and these carriers was shaken on a rotator (BR-40LF, Taitec, Japan) at 37°C for 2 hours. To ensure adhesion of the cells on GS-β, each GS-β fragment was allowed to stand for 1 hour at 37°C in 5% CO_2/95% air without any culture medium. Then, the GS-β and COFs complexes with rhBMP-2 (GS-β/BMP-2/COFs) or without rhBMP-2 (GS-β/COFs) were transplanted into the subfascial region of the back muscle of BALB/c Slc-nu/nu nude mice (8-week-old males) under anesthesia with pentobarbital (40 mg/kg). We also transplanted GS-β/BMP-2 as a positive control. For histological examination, we used 7 samples of GS-β/BMP-2/COFs group (3 for 1 week after transplantation and 4 for 2 weeks after transplantation), 6 samples of GS-β/COFs group (3 for 1 week after transplantation and 3 for 2 weeks after transplantation) and 3 samples of GS-β/BMP-2 group for 2 weeks after transplantation.

Examination of the transplanted GS-β/BMP-2/COFs complexes

The transplanted complexes of each group were dissected from each athymic mice at 1 or 2 weeks after the transplantation, and fixed in 4% paraformaldehyde solution for 4

hours at 4°C. These samples were subjected to radiographic and histological analyses. Soft X-ray photographs were obtained for radiographic analyses.

For histological analyses, the fixed samples were washed with PBS containing 6.8% sucrose for 12 hours at 4°C, and then directly immersed in acetone for 1 hour at 4°C. This quick dehydration process prevented a decrease in the intensity of GFP fluorescence. The dehydrated samples were embedded in Technovit 8100 (Heraeus Kulzer GmbH & Co., Germany), and 4-μm-thick undecalcified sections were prepared. These sections were tested for GFP fluorescence by conventional fluorescence microscopy. Some sections containing GFP-positive cells were processed for both ALP and von Kossa staining after recording the morphology of GFP fluorescence.

The number of GFP-positive cells was counted in selected areas by using enlarged photographs that were obtained before staining, and the total cell number in the same areas was counted using photographs obtained after dual staining of ALP and von Kossa with counterstaining with nuclear first red. The 1000-μm^2 square area used for cell counting was randomly chosen from the GS-β/BMP-2/COFs complexes, and the cell number was counted in 3 squares in each case.

All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

Statistical analyses

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered significant. The data are presented as mean ± standard error of mean (SEM) of independent replicates.

RESULTS

Isolation of canine COFs and transfection with GFP

Numerous fibroblastic cells had migrated from the canine buccal mucosa explants after 7-10 days of culture. We isolated these outgrowing fibroblastic cells by trypsin/EDTA digestion, and cultured these cells in different culture dishes. These cultured cells showed spindle-shaped cytoplasm with elongated nuclei, which appearances typical of fibroblasts. We examined the efficiency of GFP transfection of COFs. As shown in Fig. 1A, numerous cultured COFs (over 90%) expressed GFP under the fluorescence microscope. We also investigated GFP expression in the GS-β/COFs complexes before transplantation. As shown in Fig. 1B, though GS-β exhibited strong fluorescence, GFP-positive COFs were present in the GS-β/COFs complex even before transplantation. These results indicate that the transfection efficiency of GFP is suitable for further *in vivo* transplantation experiments.

COFs differentiate into osteoblastic cells in response to BMP-2 in *in vitro*

To investigate whether BMP-2 stimulates the COFs to differentiate into osteoblastic cells, we first tested the effects of rhBMP-2 on ALP activity using GFP-transfected COFs. Treatment with various concentrations of rhBMP-2 for 3 days showed a significant increase in ALP activity at concentration of 500 ng/ml, compared to the control culture (Fig. 2A), and the 6-day treatment showed significant increase its activity over 250 ng/ml of rhBMP-2 (data not shown). Histochemical analysis for ALP also showed apparent increases in the number of ALP-positive cells after 6-day treatment with over 250 ng/ml of rhBMP-2 (Fig. 2B-G). To further confirm the effects

of rhBMP-2 on osteoblast differentiation, the expression of mRNAs related to osteoblast differentiation was examined by real time RT-PCR analyses. Treatments with rhBMP-2 (500 ng/ml) for 3 days and 6 days significantly increased *Runx2*, *Osterix* and *Osteocalcin* mRNA expression, respectively (Fig. 3A, B, C), as compared to the expression level observed in cultures without BMP-2 treatment. These results indicated that COFs differentiate into osteoblastic cells in response to BMP-2. To further confirm a capability of COFs to generate mineralized bone nodules *in vitro*, we cultured these cells for 14 days with osteogenic medium. This treatment, however, failed to generate the mineralized bone nodules in the presence of rhBMP-2 (250 or 500 ng/ml)(data not shown).

Transplantation of GS-β/BMP-2/COFs complex induces ectopic bone formation

Figure 4 summarizes the typical features of the 3 types of transplanted complexes (GS-β/COFs, GS-β/BMP-2/COFs and GS-β/BMP-2) as seen on soft X-ray radiographs. The control complex (GS-β/COFs) showed weak radiopaque structures after 1 and 2 weeks of transplantation (Fig. 4A, C), which reflects the β-tricalcium phosphate incorporated in the GS-β. Transplants in GS-β/BMP-2/COFs group showed more extensive radiopaque area after 1 week of transplantation (Fig. 4B), and exhibited more extensive and higher radiopaque regions after 2 weeks of transplantation (Fig. 4D), as compared to the areas observed in the GS-β/COFs group. The radiopaque areas observed in GS-β/BMP-2/COFs group were similar to those in GS-β/BMP-2 group after 2 weeks of transplantation (Fig. 4E).

We next performed histological analyses of the 3 types of complexes (GS-β/COFs, GS-β/BMP-2/COFs and GS-β/BMP-2) after transplantation into athymic

mice. We found scattered foci of ALP-positive cells along with GS-β after 1 week of transplantation in the GS-β/BMP-2/COFs group (Fig. 5B). Some of these foci were associated with mineralized bone formation. After 2 weeks of transplantation, extensive bone formation associated with ALP-positive cells, which covered the bone surface, was observed in the GS-β/BMP-2/COFs group (Fig. 5F). Although histological features of transplants between GS-β/BMP-2 (Fig. 5C and D) and GS-β/BMP-2/COFs (Fig. 5E and F) groups were basically similar, extensive accumulation of ALP-positive cells was observed at some areas in GS-β/BMP-2/COFs group (Fig. 5E). Decalcified paraffin sections more clearly showed numerous osteocytes embedded in bone matrix (Fig. 5G). We found a small amount of cartilage foci in GS-β/BMP-2 (Fig. 5H) and GS-β/BMP-2/COFs (Fig. 5I) groups after 2 weeks of transplantation. Histological examination of the transplanted GS-β/COFs complex revealed neither apparent ALP-positive osteoblasts nor cartilage formation after 1 and 2 weeks of transplantation (Fig. 5A).

Localization of COFs in BMP-2-induced ectopic bone formation

Next, we investigated the localization of GFP-positive cells to prove the possible role of transplanted COFs in ectopic bone formation. We first recorded the localization of GFP-positive cells under a fluorescence microscope by using Technovit 8100-embedded undecalcified sections. Then, the same sections were stained with both ALP and von Kossa, and pictures of the fields corresponding to those recorded by fluorescence microscopy were captured. We compared the 2 picture sets and confirmed the localization of GFP-positive COFs in the ectopically formed bone tissues. Because GS-β showed strong fluorescence and mineralized bone and red blood cells exhibited

weak fluorescence, we carefully observed and compared the 2 picture sets to definitively evaluate GFP-positive COFs.

GFP-positive cells were detected around bone tissues (Fig. 6 B, D). We could not find apparent GFP-positive mature osteoblasts directly covering the bone surface, but we did find cells positive for both GFP and ALP in the areas adjacent to the bones (Fig. 6, red arrows). We also observed GFP-positive and ALP-negative cells around the bones (Fig. 6, yellow arrows). To confirm these observations, we counted the number of ALP-positive and GFP-positive cells. As summarized in Table 2, ALP-positive cells comprised 59% of the total cells, and GFP-positive cells accounted for 23% of the total cells. Among the GFP-positive cells, 17.1% were also positive for ALP. Among the ALP-positive cells, 6.2% were also positive for GFP. Although a small amount of cartilage foci was observed in GS-β/BMP-2 and GS-β/BMP-2/COFs groups after 2 weeks of the transplantation (Fig. I and H), we found no GFP-positive cells in these foci.

DISCUSSION

We first investigated whether oral mucosal fibroblasts could transdifferentiate into osteoblastic cells *in vitro* in response to BMP-2. For this purpose, we choose COFs isolated from the buccal mucosa of beagle dogs, because this species is a suitable model for preclinical experiments (Ducy et al., 1997; Neff and Rine, 2006). Our study showed that BMP-2 increased the ALP activity, which is an early-stage marker of osteoblast differentiation, and the *Osteocalcin* mRNA expression, which is a late-stage marker of osteoblast differentiation. Furthermore, BMP-2 treatment increased the mRNA expression levels of *Runx2* and *Osterix*, which encode essential transcription factors

(Runx2 and Osterix) for osteoblast differentiation and bone formation (Ducy et al., 1997; Komori et al., 1997; Nakashima et al., 2002). These results suggest that COFs can differentiate into osteoblast lineage cells in response to BMP-2 by *in vitro* assay system. In addition to these osteoblast-related markers, mineralization capability is another important character to identify osteogenic potential *in vitro*. We reported successful generation of the mineralized bone-like nodules in rat calvarial primary osteoblastic cells (Wada et al., 1998). Unfortunately, we could not reproduce such typical mineralized nodules in COFs culture even in the presence of additional BMP-2 supplement. Although the exact reason why mineralized bone-like nodules were not generated in COFs culture is obscure, the differentiation capability into osteogenic lineage cells from canine oral mucosa might be more restricted than that in rodent in culture system. This phenomenon is similar to fibroblastic cells isolated from human subcutaneous and muscle tissues as we previously reported (Kawasaki et al., 1998).

To confirm whether transplanted COFs can differentiate into osteoblast lineage cells in response to BMP-2 *in vivo*, we transplanted GFP-expressing COFs by using the GS-β/BMP-2 complex as the carrier (GS-β/BMP-2/COFs complex) into the back muscle of athymic mice. We found that transplantation of the GS-β/BMP-2/COFs complex induced ectopic bone formation. We carefully examined the localization of GFP-positive cells and ALP-positive cells by using undecalcified sections. We found that 17.1% of GFP-positive COFs differentiated into ALP-positive cells, and that these cells accounted for 6.2% of the total ALP-positive cells in the ectopically formed bones. We also found that all cells positive for both GFP and ALP were located in the area adjacent to bone but not on the bone surface. This distribution pattern suggests that the transplanted COFs could differentiate into osteoprogenitor cells at ectopically formed

bones, but not into mature osteoblasts. The present study also suggested that mature bone forming osteoblasts at the ectopic bone formation regions in GS-β/BMP-2/COFs group were originated from mesenchymal stem cells of host animals as well as in the case of GS-β/BMP-2 group.

In our previous study (Hirata et al., 2003), which was conducted using the same technique applied in this study, we showed that mouse skin fibroblasts isolated from GFP transgenic mice, which overexpressed *BMP-2* by adenoviral transfection, participated in bone repair when these cells were transplanted into bone defects in wild-type mice. Transplantation of these cells also facilitated bone repair of critical-sized bone defects created in parietal bones. In the previous study (Hirata et al., 2003), we used *BMP-2*-overexpressing skin fibroblasts, but in the present study, we did not use COFs that overexpressed this gene because we wanted to investigate whether COFs could differentiate into osteoblastic cells in response to exogenous BMP-2. This study revealed that COFs could differentiate into osteoblastic cells in response to exogenous BMP-2, but failed to differentiate into mature osteoblasts. These results suggest that simple application of non-osteogenic cells is not enough to involve these cells in active bone formation, and overexpression of BMP-2 in transplanted COFs is necessary to effectively induce more mature osteoblasts that possess the bone forming ability after transplantation. Our study to further test this possibility is underway.

BMP-2 is a strong inducer for bone formation as well as cartilage formation. In the present study, a small amount of cartilage foci was observed in GS-β/BMP-2 and GS-β/BMP-2/COFs groups after 2 weeks of the transplantation (Fig. I and H). We previously reported that transplantation of skin fibroblasts transduced with *BMP-2* gene generated similar cartilage foci (Hirata et al., 2003), and showed that a small number of

chondrocytes in these foci were originated from the transplanted fibroblasts by assessment of GFP expression. In this study, however, we could not found GFP signals in cartilage foci, suggesting that chondrocytes observed in the present study were originated from the host cells as observed in GS-β/BMP-2 group. The differences between our previous study and the present study were an application technique of BMP-2; we overexpressed *BMP-2* gene in skin fibroblasts by using adenovirus vector in the former study and we applied exogenous recombinant BMP-2 using GS-β as a carrier in the latter study. Taken together, it is suggested that overexpression of BMP-2 in transplanted COFs might be necessary to directly induce the trans-differentiation of COFs to chondrocytes.

In summary, we demonstrated that COFs could differentiate into osteoblastic cells in response to exogenous BMP-2 *in vitro* and *in vivo*. Our results suggest that oral mucosal fibroblast is a potential candidate for cell-mediated bone regeneration therapy. Currently, the most popular cell source for cell-mediated bone regeneration therapy is mesenchymal stem cells isolated from bone marrow; however, the application of these cells is inconvenient for dentists because the cells are usually isolated from iliac bones or vertebrae. We believe that the application of oral mucosal fibroblasts to cell-mediated bone regeneration therapy will provide a unique and convenient opportunity to develop new bone regeneration therapies in the field of dentistry.