国際シンポジウム
＝口腔顎顔面領域の発生と再生＝

Dental and Craniofacial Morphogenesis and Tissue Regeneration

主催：九州大学大学院　歯学研究院
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「口腔組織の再生・再建医療研究」
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１３：００～１８：００
場所：西鉄グランドホテル「鳳凰の間」

Special Lecture （13:00-16:00）

Chair: Kazuaki NONAKA,

Section of Pediatric Dentistry, Faculty of Dental Science, Kyushu University

Hiroshi NAKANISHI,

Laboratory of Oral Aging Science, Faculty of Dental Science, Kyushu University

Making new teeth from stem cells.

Paul Sharpe

Department of Craniofacial Development, Dental Institute, Kings College London

All our organs develop in the early embryo from simple origins, most often from interactions between just two types of cell, epithelium and mesenchyme. In order to devise methods of constructing organs and tissues for replacement or repair, these embryonic epithelial-mesenchymal cell interactions must be reproduced in the laboratory. Since the most important characteristic of embryonic cells is their plasticity, stem cells are the most likely source of cells to be engineered to produce organ primordia that can be used for transplantation. Teeth are organs that can be easily accessible and non-essential for life. Replacing missing or damaged teeth using stem cell-based tissue engineering is thus not only clinically and commercially valuable but can also provide a test case for using stem cells for replacement of an organ that is not life-threatening to the patient. Teratomas are stem cell tumours of the adult ovary that often include ectopic teeth. Thus in this pathological situation, stem cells, in this case germ cells, are capable of forming a complete organ. Using this and an understanding of the early developmental processes in the embryos, we are developing methods of producing human tooth primordia for transplantation into the adult mouth. The procedure involves reproducing the epithelium and mesenchymal cells that form embryonic tooth primordia from cultured stem cells. In animal studies we have shown that stem cells that are easily cultured such as embryonic stem cells, embryonic neural stem cells and adult bone marrow cells can all form tooth primordia that are able to develop into complete teeth in adults. Similarly studies are now ongoing with human stem cells.

The molecular regulatory mechanism of palatogenesis.

Yang Chai*, Xun Xu, Jun Han, Yoshihiro Ito, and Pablo Bringas, Jr.
Center for Craniofacial Molecular Biology, University of Southern California

Cleft palate represents one of the major groups of congenital birth defects in the human population. Despite recent advancements in medical intervention, babies born with cleft palate often suffer multiple handicaps that significantly compromise the quality of their lives. Multiple growth and transcription factors have been identified as critical regulators for palatogenesis that control the interaction and fate determination of the cranial neural crest (CNC) derived palatal mesenchyme and medial edge epithelium (MEE). Specifically, members of the transforming growth factor-β (TGF-β) superfamily mediate a wide range of biological activities, including cell proliferation, differentiation, extracellular matrix formation, and induction of homeobox genes, suggesting that TGF-β signaling is important in pattern formation and organogenesis.

During craniofacial development, TGF-β is expressed in the craniofacial epithelium and the CNC-derived mesenchyme, suggesting tissue-specific functions in regulating craniofacial development. To investigate the functional requirement for TGF-β signaling, we generated animal models with epithelim or CNC-derived mesenchyme specific inactivation of *Tgfbr2* gene and investigated the molecular mechanism of TGF-β signaling in regulating the fate of craniofacial epithelial or the CNC-derived mesenchyme cells, respectively. Using mice with conditional inactivation of *Tgfbr2* in the epithelium, we showed that TGF-β signaling was specifically required for regulating palate fusion. At the cellular level, TGF-β signaling was critical for mediating apoptosis of MEE cells during palatal fusion. On the other hand, conditional *Tgfbr2* gene ablation in the CNC resulted in complete cleft secondary palate, calvaria agenesis, and other skull defects with complete phenotype penetrance. Significantly, disruption of the TGF-β signaling did not adversely affect CNC migration. Cleft palate in *Tgfbr2* mutant mice resulted from a cell proliferation defect within the CNC-derived palatal mesenchyme. The midline epithelium of the mutant palatal shelf remained functionally competent to mediate palatal fusion once the palatal shelves were placed in close contact in vitro. Our data suggest that TGF-β IIR plays a critical, cell autonomous role in regulating the fate of MEE and CNC cells during palatogenesis. To explore the molecular mechanism of TGF-β signaling in regulating craniofacial development, we analyzed downstream target genes affected by altered TGF-β signaling in either the epithelium or the CNC-derived mesenchyme. Comprehensive analysis revealed specific TGF-β signaling network in regulating craniofacial development. Collectively, our study demonstrates that TGF-β

signaling plays an essential role in cell fate determination of the craniofacial epithelium and the CNC-derived mesenchyme and provides animal models for the study of abnormal craniofacial development.

Roles of Runx2 and BMP in osteoblast differentiation and bone regeneration.

Akira Yamaguchi
Section of Oral Pathology, Department of Oral Restitution, Graduate School of Tokyo Medical and Dental University

Recent advance in bone cell biology provides us a lot of important information for molecular mechanism underlining bone formation and bone resorption. To develop biology-based regenerative medicine/dentistry, we conduct BONE REGENERATION PROJECT by investigating molecular mechanism underlying osteoblast differentiation and bone regeneration. I will introduce you two topics in this project.

1) Transcription factors regulating osteoblast differentiation: Runx2 is an essential transcription factor that regulates osteoblast differentiation and bone formation. We established two clonal cell lines (C2 and C6) from calvarial region of Runx2-deficient mice. Both cell lines expressed extremely low levels of osteoblast phenotypes, but treatment with rhBMP-2 induced these cell lines to express substantial levels of osteoblast phenotypes including alkaline phosphatase and osteocalcin, suggesting that transcription factors other than Runx2 play some important roles in regulation of osteoblast differentiation. Identification of such transcription factors may provide important information to understand molecular mechanism in osteoblast differentiation and bone formation.

2) Extensive gene expression analyses during bone regeneration: Gene expression profile during bone regeneration has not been clarified well. We extensively analyzed genes expressed during bone regeneration using GeneChip (Affimetrix) in a mouse bone regeneration model. We selected 40 genes that were up-regulated at an early stage of bone regeneration. Among these genes, we demonstrated that the genes relating Notch signaling play important roles in osteoblast differentiation and bone regeneration. Our study indicated that Delta1/Jagged1-activated Notch signaling stimulated BMP2-induced osteoblast differentiation by interacting with BMP signals.

Finally, I would like to discuss roles of Runnx2 and BMP signals in osteoblast differentiation and bone regeneration.

Gap junctional communication regulates ameloblast differentiation.

Satoshi Fukumoto[1], Aya Yamada[1], Emiko Fukumoto[2], Kenji Yuasa[1], Kazuaki Nonaka[1]
[1]Division of Pediatric Dentistry, Kyushu University
[2]Division of Oral Hearth Services Research, Nagasaki University

Tooth development was regulated by their specific gene expression. To identify the molecules that express stage specifically in tooth germ, we performed the digital differential display (DDD) method using pooled molar UniGene library. The molecules that expressed in tooth germ stronger than other organs were checked their expressions in mouse incisor using the immunofluorescence. We found that Gja1 mRNA in tooth germ was expressed stronger than that of other 9 organs by DDD method. The tissue expression of Gja1 in mouse incisor was stage specific in amelogenesis, and in odontoblasts. It expressed strongly in secretary stage, and no expression in maturation stage of ameloblasts. Further, Gja1 disrupted mice showed inhibition of tooth germ, salivary gland, and lung morphogenesis, similar to occulodentdigital dysplasia (ODDD). These results suggest Gja1 expression may be associated with ODDD phenotype in teeth, especially anodontia, microdontia and amelogenesis imperfecta.

Oral Presentation by Graduate School Students (16:00-18:00)

Title	Chaired by:
1. Role of neurotrophic factor NT-4 in ameloblast differentiation. K. Yoshizaki, S. Fukumoto, Y. Kamasaki, K. Hirano, H. Harada, A. Nakasima, K. Nonaka	S. Fukumoto T. Kiyoshima
2. Impairment of Interaction between Microglia and Axotomized Facial Motoneurons and Decreased Neuronal Survival in Cathepsin S-deficient Mice. HP Hao, H. Nakanishi	S. Fukumoto T. Kiyoshima
3. L3/Lhx8, a LIM homeodomain encoding gene, is expressed in the developing maxilla of chick embryo. M. Inoue, M Kawakami, K Tatsumi, T Manabe, A Wanaka, T Kirita	T. Kiyoshima I. Kobayashi
4. Possible functions of Pgk1 in the tooth development. J Honda, I Kobayashi, T Kiyoshima, H Sakai	I. Kobayashi T. Kiyoshima
5. BMP-2 promotes osteoblast differentiation in Runx2-deficient calvarial cell lines. Tingjiao Liu, A Yamaguchi	I. Kobayashi M. A. Kido
6. Cystatin C Accelerates the Osteogenic Differentiation of Bone Marrow Mesenchymal Stem Cells and Increases Bone Formation in vivo. A Danjo, T Yamaza, D Shimohira, MA Kido, T Tanaka	M. A. Kido I, Kobayashi
7. Effects of enamel matrix derivative on the injured pulp tissues. H Kaida, H Anan, N Fujishiro, K Maeda	M. A. Kido H. Maeda
8. Expression and transcriptional regulation of Osterix transcription factor during odontoblast differentiation. M Ishikawa, K Iohara, A Akamine, M Nakashima	H. Maeda M. A. Kido
9. Characterization of clonal human periodontal ligament cell lines. S Fujii,, H Maeda, N Wada, A Tomokiyo, A Akamine	H. Maeda S. Matsuya
10. Topical application of statin affects the bone healing around implants. Y Moriyama, Y Ayukawa, K Koyano	S. Matsuya H. Maeda
11. Fabrication of Low-Crystalline Hydroxyapatite from Gypsum Based on Phosphate Treatment. Y Suzuki, S Matsuya, M Nakagawa, Y Ayukawa, K Koyano, K Ishikawa	S. Matsuya H. Maeda

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Hidefumi Maeda
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Sigeki Matsuya
Department of Biomaterials, Faculty of Dental Science, Kyushu University

Oral Presentation by Graduate School Students (16:00-18:00)

1. Role of neurotrophic factor NT-4 in ameloblast differentiation.

K. Yoshizaki[1,4], S. Fukumoto[1], Y. Kamasaki[2], K. Hirano[1], H. Harada[3], A. Nakasima[4], K. Nonaka[1]

[1]Section of Pediatric Dentistry, Faculty of Dental Science, Kyushu University, [2]Division of Pediatric Dentistry, Nagasaki University, [3]Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry, [4]Section of Orthodontics Dentistry, Faculty of Dental Science, Kyushu University

Objectives: Neurotrophic factors regulate survival, differentiation, and growth in the nervous system. In addition, they are expressed in tooth germ during their development. However, detail mechanism of tooth development regulated by neurotrophic factors has never been clearly understood. Here, we focused on the role of neurotrophin-4 (NT-4) in ameloblast differentiation, which is detected at high level in ameloblasts at early stage of amelogenesis.

Methods: To analyze the expression of enamel matrix in dental epithelial cell line, we performed RT-PCR after stimulated with or without NT-4. ERK1/2 phosphorylation by exogenous administration of NT-4 was analyzed by western immunoblotting. Further, to examine the in vivo function of NT-4, NT-4 disrupted mice were used for histological analysis of tooth development.

Results: In vitro assay, NT-4 induced the expression of ameloblastin and enamelin in dental epithelial cells. Further, enamel matrix thickness of mutant mice was changed at postnatal day 3 (P3) compared with wild type. The mRNA expression of ameloblastin and amelogenin in mutant mice was changed in P3.

Conclusion: NT-4 regulated the expression of enamel matrix proteins, ameloblastin and enamelin both in vitro and in vitro. This result suggests that neurotrophin, especially NT-4, may be important for regulation of ameloblast differentiation.

2. Impairment of interaction between microglia and axotomized facial motoneurons and decreased neuronal survival in cathepsin S-deficient mice.

Hai Peng Hao, Hiroshi Nakanishi
Laboratory of Oral Aging Science, Faculty of Dental Science, Kyushu University

Trauma to facial nerve axotomy occurs as a result of traffic accidents or due to damage during surgical manipulations. It has been reported that expression level of cathepsin S (CS), a lysosomal cysteine protease, and its endogenous inhibitor cystatin C (CC) were markedly up-regulated in microglia in response to facial nerve axotomy. Besides functions in the endosoma/lysosomal system, there is substantial evidence that CS is involved in extracellular proteolysis, because CS degrades extracellular matrix molecules even at neutral pH. However, the precise role of CS in activated microglia that is believed to be closely associated with axonal regeneration is poorly understood. Therefore, we have attempted to elucidate the precise role of CS in activated microglia after facial nerve axotomy by the use of CS-deficient mice.

In wild-type mice, microglia accumulated in the axotomized side and spread on the surface of axotomized motoneurons. Both CS and CC were markedly increased at mRNA and protein levels, whereas cathepsin B (CB) showed no significant change. In response to the axotomy, microglia were also activated and accumulated in the axotomized side of CS-deficient mice. However, the mean cell number of microglia accumulated in the axotomized side of CS-deficient mice was significantly smaller than that of wild-type mice. Furthermore, it was also noted that CS-deficient microglia abutted to axotomized motoneurons, but failed to spread on the neuronal surface. In contrast to the wild-type mice, both mRNA and protein levels of CB were significantly increased in the axotomized side of CS-/- mice. The intense immunoreactivity of CB was observed in both neurons and microglia abutted to the surface of facial motoneurons. Furthermore, primary cultured microglia prepared from CS-/- mice failed to adhere on the dishes coating with fibronectin, which is known to be increased in the surface of axotomized neurons. The mean number of facial motoneurons at 30 days after axotomy in CS-/- mice was significantly smaller than that of wild-type mice. On the basis of these observations, it is reasonable to consider that CS-deficiency impaired spreading of microglia on the surface of axotomized facial motoneurons. This impairment of cell-cell contact may be mainly responsible for the decrease in neuronal survival after axotomy in CS-deficient mice.

3. L3/Lhx8, a LIM homeodomain encoding gene, is expressed in the developing maxilla of chick embryo.

Masahide Inoue[1], Masayoshi Kawakami[1], Kouko Tatsumi[2], Takayuki Manabe[2],
Akio Wanaka[2], Tadaaki Kirita[1]
[1]Department of Oral and Maxillofacial Surgery and [2]Anatomy, Nara Medical University

LIM-homeodomain (Lhx) encoding gene plays critical roles in the control of pattern formation and cell type specification. Here we report a chick orthologues of L3/Lhx8 and the profiles of expression during the upper lip formation. The chick EST clone was highly homologous to the mouse Lhx8. Whole-mount in situ hybridization showed that dominant expression of L3/Lhx8 mRNA from stage 19-29 HH in the maxillary process as well as the lateral edge of the frontonasal mass. The signal was restricted to the mesenchyme underlying the oral ectoderm. To study the regulation of L3/Lhx8 gene expression, maxillary tissues of stage 23 HH chick embryos were transplanted into limb bud with or without the maxillary epithelium. The expression of L3/Lhx8 in the presence of the maxillary epithelium was at significant levels whereas no expression of L3/Lhx8 was detected in the epithelium-free mesenchyme. Beads soaked in recombinant Fgf-8b or Tgf-β3 induced expression of L3/Lhx8 in epithelium-free mesenchymal grafts. Our data suggest that the expression of L3/Lhx8 required epithelial mesenchymal interaction and Fgf-8b and Tgf-β3 may be responsible for Lhx gene expression in the maxilla.

4. Possible functions of Pgk1 in the tooth development.

Jun-ya Honda [1, 2], Ieyoshi Kobayashi [1], Tamotsu Kiyoshima [1], Hidetaka Sakai [1]
[1]Laboratory of Oral Pathology and Medicine, Division of Maxillofacial Diagnostic and Surgical Sciences, Faculty of Dental Science, Kyushu University
[2]Ddepartment of Orthodontics Division of Oral Health, Growth and Development, Faculty of Dental Science, Kyushu University

We previously performed cDNA subtraction between mouse embryonic day (E) 10.5 and E12.0 mandibles, and found that several genes were differentialy expressed. In this study, we examined the temporal and spacial expression pattern of *Phosphoglycerate kinase (Pgk) 1* which was one of differentially expressed genes, by means of *in situ* hybridization (ISH) method in the course of tooth germ formation. *Pgk 1* showed specific strong expression patterns in the mouse tooth germ at E10.5-E18.0. Strong expression was also found in the mouse brain, eye, tongue, heart, lung, liver, kidney and intestine at E15.0. We then studied Pgk1 protein by western blotting (WB) analysis using total protein isolated from tooth germ and tissues at E15.0. The results of WB showed distinct two bands, the one is original (49 kDa) and the other is high molecular protein. Interestingly, high molecular protein suggested more positive reaction in embryonic tissues and tooth germ than in adult tissues. We thought that high molecular band is a complex with GAPDH referring past literatures and based on molecular weight. We had the result of positive bands of Pgk1 and GAPDH with WB combined with immunoprecipitation (IP) using mouse liver at E15.0. In conclusion, our present results suggested that Pgk1 performed some functional roles in the development of the tooth germ and other embryonic organs by forming protein complex with GAPDH.

5. BMP-2 promotes osteoblast differentiation in Runx2-deficient calvarial cell lines.

Tingjiao Liu, Akira Yamaguchi
Section of Oral Pathology, Department of Oral Restitution, Graduate School of Tokyo Medical and Dental University

Although Runx2 is an essential transcription factor for osteoblast differentiation, we previously reported that primary cells isolated from calvariae of Runx2-deficient embryos expressed osteoblast markers such as alkaline phosphatase (ALP) activity and osteocalcin in response to rhBMP-2 (Cell 89:755-764,1997). These results suggest that BMP-2 stimulates osteoblast differentiation by Runx2-independent pathway. To investigate molecular mechanism of osteoblastic differentiation regulated by Runx2-independent pathway, we established two clonal cell lines, named C2 and C6, from calvariae of Runx2-deficient embryos. Both cell lines expressed extremely low or undetectable levels of mRNA relating to osteoblast differentiation at basal condition, whereas treatment with rhBMP-2 or transduction of *BMP-2* gene by adenovirus vector effectively induced both cells to express mRNA relating to osteoblast differentiation including ALP, osteocalcin and osterix. Induction of osteoblast differentiation makers by BMP-2 was more prominent in C6 cells than C2 cells. Transduction of *Runx2* gene by adenovirus vector also induced expression of mRNA relating osteoblast differentiation in both cell lines, but this effect was more prominent in C2 cells than C 6 cells. These results suggest that osteoblast differentiation in C2 cells is preferentially regulated by Runx2-dependent pathway, but that in C6 cells is preferentially regulated by Runx2-independent pathway using molecules induced by BMP-2. To explore such molecules, we conducted microarray analysis using C6 cells with or without rhBMP-2 treatment. RNA microarray analysis revealed that 72 genes were up regulated by rhBMP2 treatment in C6 cells including 14 transcription-relating molecules such as Msx2, Dlx2, Id1, Id2, Id3, Smad6 and Smad7. Genes involved in TGF-beta pathway, Wnt pathway and Notch signaling pathway were also up regulated in C6 cells by treatment with rhBMP-2. To confirm bone formation ability of C6 cells and calvarial cells isolated from wild type mice, we transplanted these cells into peritoneal cavity of athymic mice using diffusion chambers coated with rhBMP-2. Wild type calvarial cells generated mineralized bone and cartilage in the diffusion chambers, whereas C6 cells generated only cartilage but not mineralized bone. These results indicate that Runx2-deficient cells are capable of differentiating into certain stages of osteoblast lineage cells using transcription factors and signaling pathways other than Runx2, but incapable of generating mineralized bones. These results further confirmed that Runx2 is essential to bone formation.

6. Cystatin C accelerates the osteogenic differentiation of bone marrow mesenchymal stem cells and increases bone formation i*n vivo*.

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Introduction: Cystatin C (CysC) is well known as a natural cysteine proteinase inhibitor and present in several body fluids such as milk. Recently, a novel function of CysC was identified that CysC acts as an antagonist for transforming growth factor (TGF)-β receptor. TGF-β plays significant roles not only in the cell differentiation including mesenchymal stem cells (MSCs), but also in the development and regeneration of tissue and organ including bone. However, it has been not elucidated that CysC affects on the osteogenic differentiation of MSCs, still more on the bone formation and regeneration. In this study, we examined the effect of CysC on the osteogenic potentiality of human bone marrow MSCs (hBMMSCs) derived from bone marrow and *in vivo* bone formation.

Methods: hBMMSCs were cultured in a growth medium with or without CysC, and examined for immunofluorescence, cell proliferation and colony forming unit (CFU) assay. hMSCs were also cultured in a osteogenic medium in the presence of CysC and analyzed alkaline phosphatese (ALP) activity, mineralized nodule formation and bone sialoprotein (*BSP*) and *Runx2* gene expressions. CysC-stimulated hBMMSCs were transplanted to immunocompromised mice, and the transplants were analyzed. To evaluate an effect of systemic administration for bone formation, CysC was orally administrated in ovariectomized (OVX) mice. The analyses of X-ray, histology and RT-PCR were subjected for the osteogenic assay both in the transplants and in mice long bones (femur and tibiae).

Results: CysC treatment increased cell proliferation and colony formation of hBMMSCs. ALP activity and calcified nodule formation were also accelerated in CysC-stimulated group. CysC induced mRNA expression for BSP and *Runx2*. In transplant experiment, bone formation was increased in the group transplanted CysC-pretreated hBMMSCs. Gene expression for *BSP* and *Runx2* was increased in the CysC-pretreated group. In OVX mice administrated with CysC, bone density was increased by both X-ray and histological analyses. Osteocalcin (*OCN*) increasingly expressed in the CysC-treated mice.

Conclusion: Our results indicated that CysC accelerates not only proliferation and osteogenic potential of hBMMSCs, but also bone formation in vivo. Therefore, it is suggested that CysC is a new candidate for the treatment of osteolytic bone disease such as osteoporosis and periodontal disease in the field of hBMMSCs therapy.

7. Effects of enamel matrix derivative on the injured pulp tissues.

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Department of Periodontology, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University

Objectives: To investigate the pathobiological mechanisms of wound healing in the injured pulp tissues after the application of EMDOGAIN® gel containing enamel matrix derivative (EMD).

Methods: Pulpotomy was performed in the mandibular first molars of rats. EMD or Vitapex® (VIT) containing calcium hydroxide were applied to the exposed pulp tissues. We especially examined the relationship between the expression of IL-1β, TGF-β1, BMP-2, BMP-4 and DMP-1, and the behavior of reparative dentine formation showing alkaline phosphatase activity using immunohistochemical and enzyme-histochemical methods.

Results: In EMD-treated rats, a small number of IL-1β-expressing macrophages were observed around the microabscess during the early phase, and then cells expressing BMPs were rapidly increased in number. In contrast, cells expressing TGF-β1 were hardly seen throughout the experimental period. Newly formed reparative dentine was observed earlier in EMD-treated rats than that seen in VIT-treated rats. On the other hand, in VIT-treated rats, numerous macrophages expressing IL-1β were continuously observed around the abscess, and then cells expressing TGF-β1 were seen in the lesions. Subsequently, cells expressing BMPs were gradually increased in number with reparative dentine formation.

Conclusion: These results suggest that there is the difference in the time and extent of the expression of cytokine and growth factor between EMD and VIT-treated rats. It is speculated that EMD induced BMPs-expressing macrophages in the injured pulp tissues, and these cells created a favorable environment for promoting wound healing of the injured pulp tissues.

8. Expression and transcriptional regulation of Osterix transcription factor during odontoblast differentiation.

Masaki Ishikawa[1]*, Koichiro Iohara[2], Akifumi Akamine[1], Misako Nakashima[2]

[1]Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University

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Objectives: Osterix is a zinc finger-containing transcription factor, which is essential for bone formation. *Osterix* is also expressed in the mesenchyme of tooth germ. The precise function of Osterix in tooth development, however, is not clear. In this study, transcriptional regulation of *Osterix* by *Runt-related gene* (*Runx*) family and *bone morphogenetic protein 2* (*Bmp2*) during odontoblast differentiation was examined.

Methods: Localization of *Osterix* mRNA was compared with *Runx* family and *Bmp2* during tooth development by in situ hybridization. The expression of *Osterix* in tooth germ was compared in *Runx3* homozygotes with wild type. The expression of *Osterix* was also examined by real-time RT-PCR after transfection of *Bmp2* and co-transfection of *Runx2* and *Runx3*.

Results: *Osterix* mRNA was primarily expressed in the preodontoblastic layer at the late bell stage, and strongly expressed in the odontoblastic layer at the terminal differentiation stage. *Runx2, Runx3* were expressed in the dental papilla at the cap and the early bell stage. *Runx2* was no more detected at the late bell stage, while *Runx3* was restricted to the odontoblastic layer at the late bell stage, overlapping with *Osterix* and *Bmp2*. There was no conspicuous difference of expression of *Osterix*, *Runx2* and *Bmp2* in the tooth germ of *Runx3* homozygotes compared with wild type. Expression of *Osterix*, *Runx2* and *Runx3* was much increased 24 hrs after *Bmp2* gene transfection in primary pulp cells. *Runx3* gene transfection induced an increase of *Osterix* expression, and cotransfection of *Runx3* with *Runx2* resulted in inhibition of *Runx3*-stimulated *Osterix* expression.

Conclusion: These results suggest that Osterix might be implicated in odontoblast terminal differentiation, positively regulated by BMP2 and Runx3 and negatively by Runx2. No discernible change in expression of *Osterix* in *Runx3* homozygotes raising the possibility of overlapping functions of other signaling factors during odontoblast differentiation.

9. Characterization of clonal human periodontal ligament cell lines.

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Department of Endodontology and Operative Dentistry, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University

Objectives: The periodontal ligament (PDL) is a highly specialized tissue connecting the cementum with the tooth socket bone, and affects the life span of tooth. However, little is known about the precise characteristics and regenerative mechanism of human PDL, because of the absence of cell lines and specific makers, and the fact that the PDL tissue consists of heterogeneous cell populations. Therefore, to address these issues, we aimed to establish and characterize clonal human PDL cell lines immortalized by the transduction of *Simian Virus40 T-antigen (SV40T-Ag)* and *human telomerase reverse transcriptase (hTERT).*
Methods: Normal human PDL fibroblasts (HPLF) were obtained from a healthy first premolar of one volunteer. HPLF were transfected with *SV40T-Ag gene* and *hTERT gene*, named as STPLF. The expression of *SV40T-Ag* and hTERT in STPLF was ascertained by RT-PCR analysis and Western blotting analysis. Twenty clonal human PDL cell lines were obtained by limiting dilution of STPLF. Three of them, 1-4, 1-11, and 1-24, which were used in this study, were examined on the expression of STRO-1 and CD146 immunocytochemically, and gene expressions by RT-PCR technique. Moreover, 1-11 was examined on the potential to differentiate into osteoblastic or adipocytic cells, and transplanted into SCID mice.
Results: STPLF obviously expressed SV40T-Ag and hTERT, and showed stable proliferation at more than 120 population doublings (PD), while HPLF stopped at 20 PD. 1-4, 1-11, and 1-24 expressed *RUNX2, Col I, ALP, OPN, OCN, RANKL, OPG, Scx, Periostin, Col XII,* and *α-SMA* mRNA, comparable to HPLF. Immunocytochemical data showed that CD146 was expressed in 1-4 and 1-11, and STRO-1 was expressed in 1-11 and 1-24. 1-11 produced calcified matrices in the presence of ascorbic acid and β-glycerophosphate, and showed adipocytic differentiation in the adipogenic induction medium. When 1-11 was transplanted into SCID mice with β-TCP for four weeks, transplant produced bone-like structures around β-TCP.
Conclusion: The present results suggest that a clonal human PDL cell line, 1-11, would be derived from the immature cell present in PDL, and that STRO-1 and CD146 would be possible markers for such immature cells. This clone could be a very helpful model for the study of characteristics and regeneration of human PDL.

10. Topical application of statin affects the bone healing around implants.

Yasuko Moriyama, Yasunori Ayukawa, Kiyoshi Koyano
Department of Removable Prosthodontics, Faculty of Dental Science, Kyushu University

Objectives: HMG-CoA reductase inhibitors ("statin"), which inhibit a key enzyme in the mevalonate pathway, are widely used for the treatment of hyperlipidemia. Recent studies demonstrate that statins stimulate BMP-2 expression in osteoblasts, suggesting an anabolic effect of statins on the bone enhancement. The aim of the present study was to evaluate if the topical application of statin enhances the osteogenesis around the titanium implant, using a rat model.

Methods: Commercially-pure titanium rods were inserted in both tibiae of Wistar rats. Ten-week-old female Wistar rats were divided into 6 groups with 9 rats for each groups: (1) passive control (implant only); (2) active control (implant with 0μg fluvastatin-containing propylene glycol alginate (PGA)); (3) low-dose fluvastatin (implant with 3μg fluvastatin-containing PGA) treated; (4) medium-dose fluvastatin (implant with 15μg fluvastatin-containing PGA) treated; (5) high-dose fluvastatin (implant with 75μg fluvastatin-containing PGA) treated; and (6) ultra-high-dose fluvastatin (implant with 300μg fluvastatin-containing PGA) treated. The animals were sacrificed on first, second and fourth weeks of observation. Peri-implant bone formation was assessed using histomorphometric procedures by measuring bone-implant contact (BIC) and the volume density of the newly formed peri-implant osteoid density (OD) or mineralized bone density (MBD). Statistical differences among groups were determined by ANOVA and $P<0.05$ was considered significant.

Results: At week 1, there was no significant difference in BIC values among groups however, OD in 300μg group was significantly higher than other groups. At week 2, in the experimental groups, the medullary canal was filled with abundant bone trabeculae with mesh-like structure. Especially in 75μg group, this bone trabeculae seen in the medullary canal were somewhat thicker than those of control groups. BIC was significantly higher in both 15 and 75μg groups than in the passive control group. Peri-implant MBD was also significantly increased in 75 and 300μg groups in comparison with control groups. At 4 weeks, there was no significant difference in BIC values among the groups, but OD was significantly higher in passive control group than in the experimental groups.

Conclusion: Our histomorphometrical evaluations revealed the positive effect of topically-applied fluvastatin on the bone around the implant.

11. Fabrication of low-crystalline hydroxyapatite from gypsum based on phosphate treatment.

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Objectives: Hydroxyapatite (HAP) has been widely used as a bone substitute material. Most of the HAP products are prepared by sintering at high temperature. However, the original shape is difficult to retain due to shrinkage brought about by the sintering process. Furthermore, the sintered HAP cannot be resorbed by the osteoclasts, therefore the sintered HAP cannot be replaced with the newly-formed bone. In this study, we tried to fabricate a low-crystalline HAP with various shapes from gypsum block at lower temperature without sintering. Subsequently, we also investigated the biocompatibility of the fabricated material in vivo.

Materials and Methods: Calcium sulfate hemihydrate was mixed with distilled water and set gypsum was prepared. Gypsum blocks were immersed in phosphate solution at 100ºC for 24hours. The specimens were characterized by powder X-ray diffraction and FT-IR after the reaction. Biocompatibility of the fabricated material was investigated through an in vivo evaluation using rats. The material was filled into the artificial bone defect created in the rat tibia. As a control, sintered HAP was also filled in the same manner. After 2 and 4weeks, 1μm-thick sections of the implant sites were observed under light microscopy.

Results: By compositional analysis, we confirmed that low-crystalline HAP was fabricated with its original shape retained. Histologically, as early as 2weeks of implantation, the fabricated low-crystalline HAP was almost circumscribed by newly-formed bone. Resorption by the osteoclasts could also be observed on the fabricated HAP. On the other hand, sintered HAP was surrounded by connective tissue.

Conclusions: Low-crystalline HAP monolith was successfully prepared from gypsum through phosphate treatment at lower temperature and it showed much better biocompatibility and osteoconductivity than sintered HAP. Therefore it has a potential to be an ideal bone substitute material wherein new bone deposition and resorption took place.