

International Symposium 2015

Oral and Craniofacial Development and Diseases

December 10th, 11th, 2015

**Yumikura Hall
Osaka University**

Program

December 10th (Thu)

13:00 - Opening remarks **Yoshinobu Maeda** (Director, Osaka University Dental Hospital)

First session of Oral presentation for young investigators

Session Chair: **Makoto Abe** (Osaka Univ.)
Kyoko Oka (Fukuoka Dental College)

- 13:05 - Pleiotropic roles played by KLF4 during skeletal development
 Makoto Abe (Osaka Univ.)
- 13:22 - Tooth regeneration using iPS cell-derived neural crest cells
 Keishi Otsu (Iwate med Univ.)
- 13:39 - Augmented BMP signaling in the neural crest inhibits nasal cartilage morphogenesis by inducing p53-mediated apoptosis
 Satoru Hayano (Okayama Univ.)
- 13:56 - Recovery from age-related infertility under environmental light-dark cycles adjusted to the intrinsic circadian period
 Nana N. Takasu (Osaka Univ.)
- 14:13 - The function of H3K79 methylation of craniofacial development
 Daisuke Sakai (Doshisha Univ.)

Break (10min)

Second session of Oral presentation for young investigators

Session Chair: **Hiroshi Kurosaka** (Osaka Univ.)
Daisuke Sakai (Doshisha Univ.)

- 14:40 - Elucidating the role of TRPM7 in tooth development and its mineralization
 Kyoko Oka (Fukuoka Dental College)
- 14:57 - Roles of CD40 in immune response and periodontal inflammation
 Chiharu Fujiwara (Osaka Univ.)
- 15:14 - PKP1, a novel Wnt signaling regulator, is critical for tooth development and ameloblast differentiation
 Keigo Yoshizaki (Kyushu univ.)
- 15:31 - Nestin⁺ cells are pericytes in periodontal ligament
 Tomoaki Iwayama (Osaka Univ.)
- 15:48 - The role of Rdh10 in palatal development
 Hiroshi Kurosaka (Osaka Univ.)

16:05 Coffee break

16:30 – 17:30

Educational Lecture

Session Chair: **Hiroshi Kurosaka** (Osaka Univ.)

“Neural crest Cells: Evolution, Development and Disease”

Paul Trainor (Stowers Institute for Medical Research)

17:30 - 18:30 **Poster session**

18:30 - Party

December 11th (Fri)

13:00 - **Opening remarks** **Satoshi Wakisaka**, Project Leader (Osaka University)

First session of Invited lectures

Session Chair: **Satoshi Wakisaka** (Osaka Univ.)

Sachiko Iseki (Tokyo Medical and Dental Univ.)

13:05 - Teeth of the development, by the morphogenesis, for the future
Han-Sung Jung (Yonsei Univ.)

13:35 - The molecular mechanisms in tooth development
Atsushi Ohazama (Niigata Univ.)

14:05 - Regulation of endochondral ossification by transcription factors
Riko Nishimura (Osaka Univ.)

14:35 - 14:55 Coffee break

Second session of Invited lectures

Session Chair: **Han-Sung Jung** (Yonsei Univ.)

Riko Nishimura (Osaka Univ.)

14:55 - Exploration of Salivary Gland and Palatal Development Mechanisms using New Databases for Future Regenerative Medicine
Takayoshi Sakai (Osaka Univ.)

15:25 - Shh functions in oropharyngeal region
Sachiko Iseki (Tokyo Medical and Dental Univ.)

15:55 - Dentin Matrix Protein 1: bone biology and clinical application
Satoru Toyosawa (Osaka Univ.)

10min break

16:35 - 18:05

Plenary Lecture

Session Chair: **Takashi Yamashiro** (Osaka Univ.)

"Making Faces: The role of Cranial Neural Crest Cells in Neurocristopathies and Ribosomopathies"

Paul Trainor (Stowers Institute for Medical Research)

18:05 - 18:10

Closing remarks **Atsuo Amano** (Dean, Osaka University Graduate School of Dentistry)

December 10th (Thu)

First session of Oral presentation for young investigators

Session Chair: **Makoto Abe** (Osaka Univ.)

Kyoko Oka (Fukuoka Dental College)

13:05 - 13:22

Pleiotropic roles played by KLF4 during skeletal development

Makoto Abe

Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of dentistry

Normal modeling of skeleton occurs through cooperation between the neighboring bone forming osteoblasts. Osteoblasts secrete unique extracellular matrixes to form osteoid which functions as a template for future mineralized structure. Sufficient quality and quantity of the osteoid is not just important for matrix mineralization, but also crucial for normal recruitment and differentiation of osteoclasts. It is important to understand the signaling cascades used during bone formation, resorption, and also during the mutual interacting phase to control the bone-related disease.

Kruppel-Like Factor 4 (KLF4) is a transcription factor originally identified as a molecule predominantly expressed in epithelial tissue. KLF4 is also expressed transiently in immature osteoblasts, but the role played was completely unknown. Recent evidence suggests that osteoblastic-KLF4 plays numerous functions during skeletal development. These include the regulation of osteoblast differentiation, maintaining the precise cooperation between adjacent osteoblasts during the mineralization phase, and also non-cell-autonomously regulating osteoclast differentiation.

13:22 - 13:39

Tooth regeneration using iPS cell-derived neural crest cells

Keishi Otsu, Mika Kumakami-Sakano, Naoki Fujiwara, Hidemitsu Harada

Division of Developmental Biology & Regenerative Medicine, Department of Anatomy, Iwate Medical University

The discovery of induced pluripotent stem cells (iPS cells) allows us to obtain pluripotent stem cells without the use of embryonic cells and adult stem cells. Because they can differentiate into various cell types of the body, they are candidate to serve as a valuable source in regenerative medicine. We have recently developed an efficient culture protocol to induce neural crest like cells (NCLC) from mouse iPS cells. In recombination cultures between NCLC and mouse dental epithelium, NCLC exhibited a gene expression pattern involving dental mesenchymal cells. Further, after transplantation under kidney capsule, the recombinants formed the calcified tooth germ structure with periodontal tissue. In addition, NCLC differentiated into osteoblasts by the proper induction in vitro. After transplantation with unidirectional porous hydroxyapatite 3D scaffold (KURARIFE HA; Kuraray) to mouse calvarial defects, NCLC differentiated into osterix positive cells and formed osteoid at the bony defect edge. These results suggest that iPS cells will be promising cell sources for the tooth and periodontal tissue regeneration. In this presentation, we will introduce our recent attempt to investigate the potential of iPS cells for tooth regeneration and discuss the problems that must be overcome in order to achieve

whole tooth regeneration.

13:39 - 13:56

Augmented BMP signaling in the neural crest inhibits nasal cartilage morphogenesis by inducing p53-mediated apoptosis

Satoru Hayano, Yoshihiro Komatsu, Haichun Pan and Yuji Mishina

Department of Orthodontics, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences

BMP signaling plays broad roles in developmental patterning, including skull morphogenesis. We have previously reported that enhanced BMP signaling through BMPRII in neural crest cells causes craniosynostosis in the mouse. We have noticed that 55% of the mutants show neonatal lethality. These severely affected mutants exhibited nasal cartilage defects. We found increased apoptotic cells in mutant nasal cartilage primordia. Immunohistochemical staining revealed an increase of p53 in mutant nasal cartilage. To prove the idea that increased p53 causes excess apoptosis to lead the nasal cartilage defects, pifithrin- α , a chemical inhibitor of p53, was injected to pregnant mice. The treatment resulted in prevention of apoptosis in mesenchymal cells and neonatal lethality suggesting enhanced BMP signaling induces p53-mediated apoptosis. To dissect the molecular mechanism, we examined the expression level of p53 and its regulatory network for apoptosis. We found that expressions of Bax and Caspase3 were increased in the mutants; however, p53 expression level was unchanged. It is reported that Mdm2 ubiquitinates p53 to prompt its degradation and a BMP signaling component SMAD1 prevents from the MDM2-mediated p53 ubiquitination. We compared ubiquitinated p53 level between controls and the mutants by western blotting. We found that ubiquitination of p53 was decreased in the mutants. These results indicate that BMP signaling induces p53-mediated apoptosis by preventing p53 ubiquitination in early stage of chondrogenesis and thus appropriate level of BMP signaling is required for proper craniofacial morphogenesis.

13:56 - 14:13

Recovery from age-related infertility under environmental light-dark cycles adjusted to the intrinsic circadian period

Nana N. Takasu and Wataru Nakamura

Laboratory of Oral Chronobiology, Graduate School of Dentistry, Osaka University

Female reproductive function changes during aging with the estrous cycle becoming more irregular during the transition to menopause. We found that intermittent shifts of the light-dark cycle disrupted regularity of estrous cycles in middle-aged female mice, whose estrous cycles were regular under unperturbed 24-hr light-dark cycles. Although female mice deficient in Cry1 or Cry2, the core components of the molecular circadian clock, exhibited regular estrous cycles during youth, they showed accelerated senescence characterized by irregular and unstable estrous cycles and resultant infertility in middle age. Notably, tuning the period length of the environmental light-dark cycles closely to the endogenous one inherent in the Cry-deficient females

restored the regularity of the estrous cycles and, consequently, improved fertility in middle age. These results suggest that reproductive potential can be strongly influenced by age-related changes in the circadian system and normal reproductive functioning can be rescued by the manipulation of environmental timing signals.

Reference:

Takasu NN et al. *Cell Rep.* 12: 1407-13 (2015)

14:13 - 14:30

The function of H3K79 methylation of craniofacial development

Honami Ogo¹, Tomomi Nakano¹, Issay Kitabayashi², Jun Motoyama³, Toshio Watanabe¹ and **Daisuke Sakai**³

¹ Graduate School of Humanities and Science, Nara Women's University,

² National Cancer Center Research Institute,

³ Graduate School of Brain Science, Doshisha University

Epigenetic modifications such as methylation and acetylation of histones function as regulatory switches of gene expression through the altering of chromatin structure. Recent studies revealed that the expression of several leukemogenic and developmental genes depend on methylation of histone H3 on lysine 79 (H3K79me). However the mechanisms of cell- or tissue-specific H3K79me is poorly understood. Dot1L is a conserved methyltransferase mediating H3K79me, and its activity is thought to be dependent on the combination of cofactors in the complex. Thus we analyzed the role of Af10, one of the Dot1L cofactors for tissue-specific H3K79me and embryonic development. We generated *Af10* conventional knockout mouse (*Af10*-KO) and found that *Af10*-KO exhibited tissue-specific defects, such as clefting of the facial midline and hypertelorism. Consistent with this phenotype, *Af10* is strongly expressed in mesenchyme of nasal processes and maxillary prominences, and loss of *Af10* resulted in the decrease of mesenchymal cell number in nasal processes. Furthermore, H3K79me level was significantly diminished in nasal processes of *Af10*-KO. These results suggest that *Af10* is required for cranial-specific H3K79me and subsequent activation of craniofacial developmental gene expression. We are currently exploring the target genes of *Af10*-dependent H3K79me on craniofacial development.

Second session of Oral presentation for young investigators

Session Chair: **Hiroshi Kurosaka** (Osaka Univ.)

Daisuke Sakai (Doshisha Univ.)

14:40 - 14:57

Elucidating the role of TRPM7 in tooth development and its mineralization

Kyoko Oka¹, Kayoko Ogata^{1, 2}, Hidefumi Fukushima³, Fujio Okamoto², Hiroshi Kajiya², Masao Ozaki¹ and Koji Okabe².

¹ Section of Pediatric Dentistry, ² Section of Cellular Physiology, Fukuoka Dental College, ³ Tohoku University Graduate School of Dentistry

The function of ion channel has been unclear in enamel and dentin formation. TRPM7 is an unusual bi-functional protein containing an ion channel covalently linked to a protein kinase domain. Here, we report the TRPM7, predominantly expressed in ameloblast and odontoblast,

play the pivotal role in mineralization of dentin and enamel formation.

Screened the TRPM7 expression profile by in situ hybridization in mouse at E18.5, TRPM7 was strongly expressed in ameloblast and odontoblast. Tissue specific q-PCR analysis was also shown that the higher expression of TRPM7 in teeth to compare with other organs. Immunohistochemical analysis showed that TRPM7 was started to express in differentiated ameloblast and odontoblast from the bell stage. In lower incisor, TRPM7 expression of ameloblasts was seen throughout the entire process of amelogenesis except undifferentiated apical bud area. To exam the functional role of TRPM7 in mineralization, we performed knockdown TRPM7 in ameloblast (SF2) /odontoblast (mDP) cell lines using shRNA system. TRPM7 shRNA suppressed the expression of TRPM7 and TRPM7-like ionic current in SF2 and mDP. After cultured each cells with osteogenic differentiation medium, staining of von Kossa was clearly diminished, but ALP activity was not changed by TRPM7 depletion.

These results demonstrate the crucial importance of TRPM7 function during mineralization of both enamel and dentin formation.

14:57 - 15:14

Roles of CD40 in immune response and periodontal inflammation

Chiharu Fujihara¹, Richard J Hodes², Tomoaki Iwayama³, Satoru Yamada⁴, Shinya Murakami⁴

¹ Challenge to Intractable Oral Diseases, Center for Translational Dental Research, Osaka University Dental Hospital

² Experimental Immunology Branch, National Cancer Institute, National Institutes of Health

³ Department of Oral Anatomy and Developmental Biology, Graduate School of Dentistry, Osaka University

⁴ Department of Periodontology, Graduate School of Dentistry, Osaka University

The interaction between CD40 on antigen presenting cells and CD40 ligand (CD40L) on activated T cells has various functions in immune responses, such as antibody production and formation of germinal center (GC). However, it is still unknown the cell-type specific roles of CD40 in antibody responses and GC B cell formation. Thus, we generated B cell- and dendritic cell (DC)-specific CD40 conditional knockout mice and analyzed whose CD40 was important for antibody responses *in vivo*. We have revealed that CD40 on B cells, but not CD40 on DC, is essential for the induction of antibody responses *in vivo*.

CD40 is expressed on not only immune cells but also non-immune cells such as fibroblasts. Thus, beyond antibody formation, we next expanded the analysis of the possible roles of the CD40-CD40L axis between periodontal ligament (PDL) cells/infiltrated activated T cells. PDL cells expressed CD40 at mRNA and protein levels. Upon stimulation with soluble CD40L, PDL cells produced inflammatory cytokines. Interestingly, CD40 expression on PDL cells was dramatically inhibited by fibroblast growth factor-2 (FGF-2) that is used for periodontal regeneration to regulate PDL cellular functions. Our results suggest that the CD40-CD40L interaction between PDL cell/T cell plays a critical role in augmenting inflammatory responses during periodontal inflammation, and that suppressing CD40 by FGF-2 is a key to reduce excessive inflammation and accelerate the healing process which is essential for successful

periodontal regeneration.

15:14 - 15:31

PKP1, a novel Wnt signaling regulator, is critical for tooth development and ameloblast differentiation

Kanako Miyazaki, **Keigo Yoshizaki**, Chieko Arai, Han Xue, Keita Funada, Ichiro Takahashi
Section of Orthodontics and Dentofacial Orthopedics, Division of Oral Health, Growth and Development Faculty of Dental Science, Kyushu University

Wnt signaling pathway plays important roles for tooth development, especially during the tooth morphogenesis. We focused on Plakophilin 1 (PKP1), which is highly expressed in tooth on microarray data, and known as disease gene for ectodermal dysplasia/skin fragility syndrome. We hypothesized that PKP1 is a regulator of Wnt signaling pathway because of its armadillo repeat domain similar to β -catenin.

To identify the function of PKP1 during tooth development, we performed *ex vivo* organ culture experiments using PKP1 siRNA. The size of tooth germs was significantly smaller than that of the controls when siRNA was applied. Proliferation of dental epithelial cell was inhibited by PKP1 siRNA *in vitro*. Transfected PKP1-EmGFP was translocated from plasma membrane into nuclei by stimulation of Wnt5a, Wnt3a, and Lithium Chloride.

PKP1 is also known as an adhesion-related molecule of desmosome, and we found that PKP1 localization shifts to the plasma membrane during ameloblast differentiation. In addition, PKP1 siRNA disturbed localization of ZO-1 to tight junction, and inhibited the differentiation of ameloblast. Immunoprecipitation revealed that PKP1 bound to ZO-1.

These results suggest that PKP1 is involved in early tooth morphogenesis as a Wnt signal regulator, and affects to the differentiation of ameloblast via the cell adhesion molecules.

15:31 - 15:48

Nestin⁺ cells are pericytes in periodontal ligament

Tomoaki Iwayama¹, Satoshi Wakisaka¹ and Shinya Murakami²

¹ Department of Oral Anatomy and developmental biology, ² Department of Periodontology, Graduate School of Dentistry, Osaka University

Background:

The periodontal ligament contains a mesenchymal stem cell (MSC) /progenitor population to maintain tissue homeostasis. To date, only Nestin-positive cells in bone marrow were shown to be *bona fide* MSCs by rigorous *in vivo* characterization. It would be helpful for better understanding of tissue homeostasis to identify Nestin-positive population in periodontal ligament.

Materials and methods:

Periodontal ligament of Nestin-GFP transgenic mice was analyzed histologically. GFP-positive cells in the neonatal adipose tissue were FACS-sorted, and CFU-F assay was performed. Their differentiation capacities were characterized by inducing to fibroblasts, osteoblasts, or adipocytes *in vitro*. Furthermore, the Nestin-Cre/R26-tdTomato double transgenic mice were generated and their progeny was genetically traced *in vivo*.

Results:

Nestin-positive cells were located adjacent to capillaries

in periodontal ligament. FACS-isolated nestin-positive cells expressed MSC markers including CD44, CD90, CD105, Sca-1, PDGFRs, and formed CFU-F at high efficacy. When nestin-positive cells were cultured and induced, they differentiated into mineralized nodule forming osteoblasts, Col3a1 expressing fibroblasts and Oil Red O-positive adipocytes. In a lineage tracing experiment, nestin-lineage cells were also identified as a perivascular population.

Conclusion:

Nestin-positive cells are pericytes in periodontal ligament. They are MSC/progenitors, and maintain their perivascular localization during homeostasis, suggesting they are an MSC reservoir within periodontal ligament.

15:48 - 16:05

The role of Rdh10 in palatal development

Hiroshi Kurosaka, Lisa Sandell and Paul Trainor

Retinoic acid signaling is well known to be widely involved in craniofacial development. Either loss or gain of function of retinoic acid signaling during development could result in craniofacial abnormality such as cleft lip and/or palate. There are various genes involved in retinoic acid metabolism including Retinol dehydrogenase 10 (Rdh10) which catalyzes the first oxidative step in the metabolism of vitamin A to its active form retinoic acid. Previously we reported Rdh10^{trax} mutant mice which developed by our ENU mutagenesis screen and have loss of function mutation in Rdh10 gene together with various craniofacial defect and significant reduction of retinoic acid signaling. However, since the Rdh10^{trax} mutant mice were embryonic lethal around E12.0, we couldn't analyze the role of Rdh10 on later stage craniofacial development. In this study we used Rdh10^{fx/fx} mice crossed with a tamoxifen-inducible CreER^{T2} line in order to excise Rdh10 gene at different developmental stages in mouse to investigate temporal requirement of retinoic acid signaling in craniofacial development. By excising Rdh10 at E7.5 from developing mice lead to cleft lip, choanal atresia and secondary cleft palate at E15.5. Histological section revealed ectopic bone formation at anterior secondary palate which presumably part of the etiology on cleft lip or choanal atresia. Currently we are performing various gene expression analyses on those mutant mice and would like to discuss the significance of temporal requirement of retinoic acid signaling on mice palatal development.

“Neural crest Cells: Evolution, Development and Disease”

Paul Trainor

Stowers Institute for Medical Research

Neural crest cells comprise a migratory stem and progenitor cell population that gives rise to a diverse array of cells and tissues throughout the vertebrate body. Neural crest cells (NCC) are considered to be a vertebrate innovation that significantly contributed to their evolution, predation, radiation and adaptation to most niches of the planet. Neural crest cell formation encompasses several steps including induction and specification from a precursor neural stem cell pool, epithelial to mesenchymal transition, acquisition of polarity, delamination and migration from the neural tube. Work in aquatic and avian model systems has uncovered gene regulatory networks mediated by Wnt, BMP and FGF signaling that drive neural crest cell formation. However, knockout mouse models have failed to recapitulate a role for these pathways in mammalian neural crest cell induction. Thus the signals, switches and mechanisms governing neural crest cell formation in mammals remains poorly understood. We have identified novel roles for orphan nuclear receptors in mammalian neural crest induction. Loss-of-function mutants exhibit a maintenance of neural stem cell identity and an inability to differentiate and form neural crest cells. Global gene expression and protein interaction analyses, reveal that orphan nuclear receptors may act as bimodal switches in neural crest cell formation, firstly by repressing pluripotency genes while concomitantly activating neural crest specific genes, and secondly by regulating epithelial to mesenchymal transition. Our findings have identified a novel regulator of mammalian neural crest cell development and defined a temporal window for mammalian neural crest cell formation which is earlier than previously thought and raises important questions regarding the appropriateness of particular Cre mouse lines in studies of mammalian neural crest cell specification and induction.

December 11th (Fri)

First session of Invited lectures

Session Chair: **Dr. Satoshi Wakisaka** (Osaka Univ.)

Dr. Sachiko Iseki (Tokyo Medical and Dental Univ.)

13:05 - 13:35

Teeth of the development, by the morphogenesis, for the future

Dr. Han-Sung Jung

Yonsei University

Tooth development is regulated by progressive and mutual interactions between epithelium and mesenchyme. The molecular mechanisms underlying this instruction are well-preserved and most of the contributing molecules belong to several signalling families. Research focusing on mouse teeth has uncovered many aspects of tooth development, including molecular and evolutionary detailed and in addition offered a valuable system to analyse the regulation of epithelial stem cells. In mice, the spatial and temporal regulation of cell differentiation and the mechanisms of patterning during development can be analysed both in vivo and in vitro.

The mouse teeth that modulating the balance between inductive and inhibitory signals constitutes a key mechanism regulating the epithelial stem cells and cellular differentiation. I would share current ideas with additional maintenance for the location of the putative dental stem cells and for the stemness. Fine-tuning of the signalling in the regulation of the tooth morphogenesis, and that altering the levels of an inhibitor can cause variation in the tooth patterning. Furthermore, clinical implications including in the diagnosis, prevention and treatment of congenital defects as well as in the design of regenerative therapies would be of fundamental importance in Oral Biosciences.

13:35 - 14:05

The molecular mechanisms in tooth development

Dr. Atsushi Ohazama

Division of Oral Anatomy, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences

Teeth develop from a dynamic and complex reciprocal interaction between epithelium and cranial neural crest-derived mesenchyme. Tooth position, number, and shape are consistent in mammals. To determine the shape, position and numbers of teeth, tooth development is under strict genetic control. Many signaling pathways including Bmp, Shh, Fgf and Tgf are known to be involved in regulating tooth development. The nuclear Factor kappa B (NF-κB) pathway plays a major role in many physiological and pathological processes including organogenesis, immune response to infection, apoptosis, cell proliferation, cancer and stem cell regulations. I will introduce recent findings on molecular mechanisms of tooth development, especially, how NF-κB pathway is involved in tooth development.

14:05 - 14:35

Regulation of endochondral ossification by transcription factors

Dr. Riko Nishimura

Osaka University Graduate School of Dentistry, Department of Molecular and Cellular Biochemistry

Endochondral ossification plays critical roles in skeletal development, tissue patterning and craniofacial development. Endochondral ossification is a very unique and complex biological event formed by multiple steps. These multiple steps are sequentially harmoniously regulated by transcription factors, including Sox9, Runx2/3 and Osterix. We have identified several transcriptional partners of Sox9 including p54^{nrb}, Znfx219 and Arid5a, and showed their functional roles in endochondral ossification. We also demonstrated that Sox9 negatively regulates the late stage of endochondral ossification by interacting Ihh/PTHrP loop. In addition, we indicated that Osterix is essential for calcification and degradation of cartilage matrices during endochondral ossification. To further understand molecular basis of endochondral ossification, we attempted to identify novel transcription factors involved in endochondral ossification. To address this, we developed new molecular cloning approaches based on RNA-Seq and microarray analyses, and isolated Arid5b, Foxc1 and Zfhx4 as important transcription factors for endochondral ossification. In this symposium, I would like to introduce and discuss how these transcription factors regulate endochondral ossification and craniofacial development.

14:55 - 16:25

Second session of Invited lectures

Session Chair: **Dr. Han-Sung Jung** (Yonsei Univ.)
Dr. Riko Nishimura (Osaka Univ.)

14:55 - 15:25

Exploration of Salivary Gland and Palatal Development Mechanisms using New Databases for Future Regenerative Medicine

Dr. Takayoshi Sakai

Department of Oral-facial Disorders, Division of Functional Oral Neuroscience, Osaka University Graduate School of Dentistry

In the field of oral medicine, there are intractable diseases. However, for a majority of them, treatment methods have not yet been established. Salivary hypofunction is caused by therapeutic radiation for head and neck cancer as well as Sjogren's syndrome. Presently, there is no adequate treatment for patients with such irreversible gland damage, and offering better treatment options to these patients is the major impetus for re-engineering salivary glands. Cleft palate is among the most common craniofacial birth defects. Several genes have been identified that contribute to cleft palate, but the full spectrum of such genes and whether and how they interact is unknown. To elucidate the mechanisms of both salivary gland disease and cleft palate, five databases in oral and salivary gland development were recently created.

Laser microdissection in combination with T7-SAGE has been developed as a method for gene discovery of candidate molecules that may be essential for early organ morphogenesis (GSM555989 and 555990 in NCBI, *Nature* 423, 876-881, 2003, *Science* 329, 562-565, 2010). A combination of microdissection and microarray has been established as a method for gene discovery of candidate molecules that may be essential for palatal fusion (GSM1067620, 1067621 and 1067622 in NCBI, *PLoS One* 8, e61653, 2013). Using these databases, Fibronectin, Btbd7, and CEACAM1 were identified as important molecules in salivary gland or palatal development. Progress in understanding the mechanisms of salivary gland and palatal development may facilitate novel approaches to future tissue engineering and regeneration of organs.

15:25 - 15:55

Shh functions in oropharyngeal region

Dr. Sachiko Iseki

Section of Molecular Craniofacial Embryology, Tokyo Medical and Dental University Graduate School of Medical and Dental Sciences

Among *Sonic hedgehog* (*Shh*) enhancers, *MFCS4* is responsible for the expression in epithelium of oropharyngeal region in development. The compound heterozygous mutant of *Shh* and *MFCS4* (*Shh*^{+/-}; *MFCS4*^{+/-}) show cleft palate with full penetrance and hypoplasticity in tongue, epiglottis, arytenoid, thyroid cartilage, hyoid bone and cranial base.

Palatal shelves of *Shh*^{+/-}; *MFCS4*^{+/-} mice failed to elevate *in vivo* but successfully elevated in maxillary organ culture without tongue, which suggested that the tongue development was disturbed. Consistently, the arrangement and segmentation of intrinsic lingual muscles were impaired in the mutant.

In the primordia of pharyngeal cartilages and the cranial base that form directly under the pharyngeal epithelium, *Sox9* expression was down-regulated, resulting in hypoplastic cartilage in the mutant.

The severity of down-regulation of *Sox9* in affected tissues was related to the expression level of *Patch1*, *Shh* receptor.

These results suggest that *MFCS4* activation contributes to the development of the oro-pharyngeal supporting organs. We are currently investigating molecular mechanisms of the phenotypes.

15:55 - 16:25

Dentin Matrix Protein 1: bone biology and clinical application

Dr. Satoru Toyosawa

Department of Oral Pathology, Osaka University Graduate School of Dentistry

Dentin matrix protein 1 (DMP1) is a member of the SIBLING family of genetically related noncollagenous proteins in mineralized tissues. A unique feature of DMP1 is its unusually large number of acidic domains, which are negatively charged *in vivo*. DMP1 also contains a large number of phosphorylation motifs for Fam20C, and these regions become another negatively charged domain after phosphorylation. Because of its highly negative-charged nature, DMP1 can bind to calcium, thereby regulating matrix mineralization. *In vivo* analyses of DMP1-overexpressing transgenic mice showed increased bone mineral density in the cortical bone. These findings indicated DMP1 promoted the calcification process in the bone formation. To apply the capacity of DMP1 to bone regeneration, we developed the biomimetic DMP1/collagen composite which enhanced osteoconductive potency in rat calvarial defect.

DMP1 mRNA was expressed in osteocyte, but not in osteoblasts. Osteocyte has recently been found to play a significant role in the bone homeostasis. Given that DMP1 is produced in osteocytes, whereas other bone matrices are produced in osteoblasts, serum DMP1 represents a candidate biochemical marker for osteocyte activity. To assess the potential utility of DMP1, we developed new ELISAs for DMP1. Blood study by DMP1-ELISAs revealed that serum DMP1 levels decreased with age, and were most highly correlated with the level of mature-osteoblast markers.

Serum DMP1 may be a new biochemical marker for osteocyte-mediated bone turnover although further study is needed to evaluate its significance.

"Making Faces: The role of Cranial Neural Crest Cells in Neurocristopathies and Ribosomopathies"**Dr. Paul Trainor** (Stowers Institute for Medical Research)

Craniofacial anomalies account for approximately one-third of congenital defects. The majority of the bone, cartilage, connective and peripheral nerve tissues in the head and face are derived from a transient progenitor cell population called the neural crest. Consequently, defects in neural crest cell patterning are thought to underlie most congenital craniofacial anomalies. Understanding the etiology and pathogenesis of craniofacial malformations therefore is dependent upon a thorough knowledge of the mechanisms that govern neural crest cell formation, migration, survival and differentiation. Ribosome biogenesis is integral to cell growth and proliferation through its roles in translating mRNAs and building proteins. Disruption of any step in the process of ribosome biogenesis can lead to congenital disorders termed ribosomopathies. Given the ribosome's importance in all cell types, it is remarkable that disruptions in the global process of ribosome biogenesis leads to congenital anomalies with specific phenotypes including defects in the craniofacial, axial and limb skeleton. This is exemplified in conditions such as Treacher Collins syndrome and Acrofacial dysostosis, Cincinnati type, which are associated with mutations in TCOF1, POLR1C or POLR1D, and POLR1A respectively. Our research on the etiology and pathogenesis of Treacher Collins syndrome and Acrofacial dysostosis, Cincinnati type, has revealed the critical role of rDNA transcription, one of the rate-limiting steps of ribosome biogenesis, in neural crest cell development. Furthermore, we have determined that the spatiotemporal regulation of rRNA transcription can mechanistically underlie the phenotypic specificity of each ribosomopathy. Thus integrating ribosome biogenesis with regulators of neural crest cell, cartilage and bone development serves growth and differentiation during skeletal development, the perturbation of which results in congenital neurocristopathies and ribosomopathies. Furthermore, our work is facilitating the development of therapeutic approaches to prevent the pathogenesis of craniofacial malformation syndromes, which has broad implications for other congenital birth defects of similar etiology to Treacher Collins syndrome.

P-1

Identification of genetic risk factor for Japanese aggressive periodontitis by genome-wide association study

Jirouta Kitagaki, Shizuka Miyauchi, Motozo Yamashita, Satoru Yamada, Masahiro Kitamura, Shinya Murakami
Department of Periodontology, Osaka University Graduate School of Dentistry

Periodontitis is an inflammatory disease involving loss of gingival tissue and alveolar bone. Disease susceptibility to aggressive periodontitis (AgP) appears to be influenced by genetic risk factors. To identify these in a Japanese population, we conducted exome sequencing of 41 unrelated patients. We found that AgP is associated with SNP rs536714306 in the G-protein coupled receptor 126 gene, *GPR126* [c.3086 G>A (p.Arg1029Gln)], with *P*-value: 2.20×10^{-3} and odds ratio 9.09 (95% confidence interval: 1.64–50.36). cAMP ELISA analysis of cAMP concentrations suggested that rs536714306 impaired the signal transactivation of GPR126. Moreover, transfection of human periodontal ligament (HPDL) cells with wild-type or mutant GPR126 containing rs536714306 showed that wild-type GPR126 significantly increased the mRNA expression of bone sialoprotein (BSP) gene, while mutant GPR126 had no effect on BSP expression. The increase in expression of BSP was through the GPR126-induced increase of BMP-2 expression. These data indicate that GPR126 is important in maintaining the homeostasis of periodontal ligament tissues through regulating the cytodifferentiation of HPDL cells. The *GPR126* SNP rs536714306 negatively influences this homeostasis, leading to the development of AgP, suggesting that it is a genetic risk factor for AgP in the Japanese population.

P-2

KLF4 regulates cell-cell adhesion in osteoblasts

Yuto Takeuchi, Junji Fujikawa, Ahmed Nomir, Makoto Abe
Dept. of Oral Anatomy and Developmental Biology Osaka Univ. Graduate School of Dentistry

Matrix mineralization is achieved by cooperation of neighboring osteoblastic cells. Direct osteoblastic cell-cell adhesion mediated by adherens and gap junction is important for bone modeling and remodeling. We previously reported that Kruppel-Like Factor 4 (KLF4) controls the differentiation of osteoblasts. Here, we show that KLF4 regulates the expression of N-cadherin which is a dominant cadherin in osteoblasts. KLF4 reduced the expression of N-cadherin and also the formation of adherens junctions. In the transgenic mice expressing KLF4 in osteoblasts, the area of collagen distribution was abnormally reticular due to reduced cell-cell adhesion. Reduction of adherens junction neither increased the free cytoplasmic β -catenin nor activated canonical Wnt signaling. Instead, KLF4 upregulated Axin2 transcription without activating canonical Wnt signalling, and this induction was cancelled by Runx2.

P-3

The proteasome inhibitor bortezomib enhances cytodifferentiation and mineralization of periodontal ligament cells

Shizuka Miyauchi, Jirouta Kitagaki, Motozo Yamashita, Satoru Yamada, Masahiro Kitamura, Shinya Murakami
Department of Periodontology, Division of Oral Biology and Disease Control, Osaka University Graduate School of Dentistry

Objective: Ubiquitin proteasome pathway plays important roles in the regulation of proliferation and differentiation. Bortezomib (Velcade®), one of the proteasome inhibitors, is known to induce osteoblastic differentiation in a number of cell lines, including mesenchymal stem cells and osteoblastic precursor cells. Since periodontal ligament (PDL) contains multipotent mesenchymal stem cells, we examined the effects of bortezomib on osteoblastic differentiation of PDL cells.

Methods: A mouse PDL clone cell line, MPDL22, was cultured in

mineralization-inducing medium with or without bortezomib. Expression of calcification-related genes and calcified-nodule formation were assessed by real-time PCR and Alizarin Red staining, respectively.

Results: Bortezomib increased the expression of calcification-related mRNA, such as *ALPase*, *Bsp*, *Runx2* and *osteopontin*, and calcified-nodule formation in MPDL22 cells. These effects were induced by increasing the cytosolic accumulation and nuclear translocation of β -catenin, leading to an increase in expression of *Bmp-2*, *-4* and *-6* mRNA. Furthermore, bortezomib enhanced BMP-2-induced mRNA expression of *Bsp* and *osteopontin* and increased calcified-nodule formation in MPDL22 cells.

Conclusion: Bortezomib induced cytodifferentiation and mineralization of PDL cells by enhancing the accumulation of β -catenin within the cytosol and the nucleus and increasing the *Bmp-2*, *-4* and *-6* mRNA expression, suggesting that bortezomib may be a promising compound for use in periodontal regenerative therapy.

P-4

Investigation of medial edge epithelial cell behavior during secondary palate fusion

Aoyama Gozo, Kiyomi Mihara, Safiye Esra Sarper, Hiroshi Kurosaka, Takashi Yamashiro
Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Osaka University

Failure of secondary palate fusion will lead to cleft palate, the most frequent congenital craniofacial birth defects in humans. Palate fusion process involves the removal of medial edge epithelial (MEE) cells which lies on the edge of secondary palate process. It is known that apoptosis plays important role in the removal of MEE cells, and that another mechanisms, such as epithelial cell migration and epithelial-mesenchymal transition, may be involved in the removal of MEE cells. Whether apoptosis is solely responsible for the removal of MEE cells is inconclusive, however, because it is reported that palate fusion was caused under inhibition apoptosis.

In this study, paying attention to epithelial cell migration, we cultured unpaired palatal shelves of the mice that express Green Fluorescent Protein under the promoter of keratin 14 (K14-GFP) at embryonic day 14.5 (E14.5) and used live imaging technique in order to observe the behavior of MEE cells in vivo during secondary palate fusion. First, we confirmed that epithelial cells expressing GFP signaling disappeared from the edge of palatal shelves along anterior posterior axis. Secondary, we found that epithelial cells move dynamic before disappearing of GFP signaling. Our findings indicate epithelial cell migration could possibly relate to the removal of MEE cells.

P-5

Mutual Regulation between Hypoxic Responses and PLAP-1 in Periodontal Ligament Cells.

Satomi Yamamoto, Masahide Takedachi, Keigo Sawada, Chiaki Morimoto, Asae Hirai, Tomoaki Iwayama, Toshihito Awata, Satoko Yamaba, Satoru Yamada and Shinya Murakami
Department of Periodontology, Osaka University Graduate School of Dentistry

Introduction:

Cellular responses to hypoxia regulate various biological events via activation of hypoxia-inducible factor (HIF)-1 α . Interestingly, it has been reported that several hypoxic responses can be observed in periodontal ligament cells (PDLcs). PLAP-1 (Periodontal Ligament Associated Protein-1), an extracellular matrix specifically expressed in the periodontal ligament, plays important roles in cellular functions of PDLcs. However, the involvement of PLAP-1 in the hypoxic responses has not been examined yet.

Methods:

We cultured PDLcs in normoxic (20% O₂) or hypoxic (1% O₂) condition. In some experiments, PDLcs were cultured with or

without deferoxamine (DFO, inhibitor of HIF-1 α degradation), or chetomin (HIF inhibitor). PLAP-1 expression was examined by RT-qPCR and western blotting. We also examined whether PLAP-1 could modulate the HIF-1 α expression in PDLs by treating the PDLs with PLAP-1 siRNA.

Results:

PLAP-1 expression in PDLs was upregulated by hypoxic condition and DFO treatment, and was suppressed by chetomin treatment. Furthermore, suppressing PLAP-1 expression by siRNA transfection resulted in increase in the protein expression of HIF-1 α .

Conclusion:

This study demonstrated that the expression of PLAP-1 is upregulated in hypoxic condition through HIF-1 α activation and that the hypoxia-induced PLAP-1 modulates HIF-1 α signaling in PDLs.

P-6

Relationship between galectin-1 expression and tumor immunity at gingival squamous cell carcinoma

Yuri Noda, Mitsunobu Kishino, Sunao Sato, Katsutoshi Hirose, Satoru Toyosawa

Department of Oral Pathology, Osaka University Graduate School of Dentistry

Galectin-1(Gal1) is the beta-Galactoside-binding protein which regulates cell-cell adhesion, anti-inflammation response and angiogenesis in normal tissue. Gal1 overexpresses in cancer plays important roles in cancer progression for regulating cell cycle, migration and immune escape. About tumor immunity, it is considered that Gal1 suppresses the immune response by inducing apoptosis of activated T-cells in the tumor. However, there is not shown histological evidence of the T-cell apoptosis in human tumor specimens. In this study, we analyzed the relationship between Gal1 expression and apoptotic T-cells of gingival squamous cell carcinoma (GSCC) and other clinicopathological factors. Eighty specimens of GSCCs were stained by immunohistochemistry using the anti-Gal1, the anti-CD3/anti-Cleaved-caspase3 and the anti-CD8/anti-Cleaved-caspase3 antibodies. Gal1 expression and T-cell apoptosis were evaluated on 6 high-power fields. We showed that Gal1 expression of GSCC was significantly correlated with T-cell infiltration, and apoptosis of the T-cells. Moreover, it was significantly correlated with some clinicopathological features, such as lymph node metastasis, histological differentiation and overall survival. These findings suggest that the Gal1 may contribute to the immune escape of GSCC cells, and that the Gal1 expression may be a useful marker for GSCC.

P-7

Roles of FKBP12 and BMP Type II Receptors in Activation of Mutant Forms of BMP Type I Receptor, ALK2, which are Responsible for Heterotopic Ossification in Fibrodysplasia Ossificans Progressiva (FOP)

Aiko Machiya^{1,2}, Mai Fujimoto^{1,2}, Satoshi Ohte¹, Sho Tsukamoto¹, Mai Kuratani¹, Naoto Suda², Takenobu Katagiri¹

¹ Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University,

² Division of Orthodontics, Meikai University School of Dentistry

Fibrodysplasia ossificans progressiva (FOP) is a rare hereditary disorder characterized by progressive heterotopic ossification (HO) in soft tissues, such as skeletal muscle, tendon and ligament. In patients with FOP, the orthotopic ankylosis of temporomandibular joints causes progressive limitation of jaw opening. HO in FOP is caused by gain-of-function mutations of ALK2, a type I receptor for bone morphogenetic proteins (BMPs). FK506-binding protein-12(FKBP12) has been shown to repress an activity of the receptors. In the present study, we examined the role of FKBP12 in the activation of mutant ALK2 found in FOP.

Over-expression of FKBP12 in C2C12 cells reduced a BMP activity induced by 12 mutant ALK2 except ALK2 (PF197-8L), which carries a mutation in the binding domain with

FKBP12. Indeed, ALK2 (PF197-8L) did not interact with FKBP12 in CoIP experiments. However, HO was induced after a biopsy in the patient carrying ALK2 (PF197-8L) similar to other patients with FOP. We have reported that BMP type II receptors are involved in the activation of the mutant ALK2. In the presence of BMP type II receptors, FKBP12 did not inhibit the BMP signaling induced by any mutant ALK2, suggesting that the type II receptors cancel the suppression by FKBP12 and induce HO in FOP. Taken together, we conclude that the HO in FOP is induced by a co-operation with the mutant ALK2 and type II receptors.

P-8

Roles of CD40 in immune response and periodontal inflammation

Chiharu Fujihara¹, Richard J Hodes², Tomoaki Iwayama³, Satoru Yamada⁴, Shinya Murakami⁴

¹ Challenge to Intractable Oral Diseases, Center for Translational Dental Research, Osaka University Dental Hospital

² Experimental Immunology Branch, National Cancer Institute, National Institutes of Health

³ Department of Oral Anatomy and Developmental Biology, Graduate School of Dentistry, Osaka University

⁴ Department of Periodontology, Graduate School of Dentistry, Osaka University

The interaction between CD40 on antigen presenting cells and CD40 ligand (CD40L) on activated T cells has various functions in immune responses, such as antibody production and formation of germinal center (GC). However, it is still unknown the cell-type specific roles of CD40 in antibody responses and GC B cell formation. Thus, we generated B cell- and dendritic cell (DC)-specific CD40 conditional knockout mice and analyzed whose CD40 was important for antibody responses *in vivo*. We have revealed that CD40 on B cells, but not CD40 on DC, is essential for the induction of antibody responses *in vivo*.

CD40 is expressed on not only immune cells but also non-immune cells such as fibroblasts. Thus, beyond antibody formation, we next expanded the analysis of the possible roles of the CD40-CD40L axis between periodontal ligament (PDL) cells/infiltrated activated T cells. PDL cells expressed CD40 at mRNA and protein levels. Upon stimulation with soluble CD40L, PDL cells produced inflammatory cytokines. Interestingly, CD40 expression on PDL cells was dramatically inhibited by fibroblast growth factor-2 (FGF-2) that is used for periodontal regeneration to regulate PDL cellular functions. Our results suggest that the CD40-CD40L interaction between PDL cell/T cell plays a critical role in augmenting inflammatory responses during periodontal inflammation, and that suppressing CD40 by FGF-2 is a key to reduce excessive inflammation and accelerate the healing process which is essential for successful periodontal regeneration.

P-9

Establishment of in vitro amelogenesis imperfecta model

Keiko Miyoshi¹, Arya Adiningrat^{2,3}, Ayako Tanimura¹, Ryna Dwi Yanuarieska^{2,4}, Dian Yosi Arinawati², Taigo Horiguchi¹, Takafumi Noma¹

¹ Dept. Mol. Biol., Biomed. Sci., Tokushima Univ. Grad. Sch., Japan,

² Grad. Sch. Oral Sci., Tokushima Univ., Japan,

³ Dept. Biomed. Sci., Sch. Dentistry, Faculty of Med. and Health Sci., UMY, Indonesia,

⁴ Dept. Dentomaxillofacial Rad., Faculty of Dent., UGM, Indonesia

Amelogenesis is one of the essential events during tooth formation, and the understanding of its molecular mechanism is essential to regenerate tooth. Disorder of the process causes amelogenesis imperfecta (AI), a hereditary disease with defective enamel.

Recently, we found that mutation of the gene encoding Specificity Protein 6 (SP6, also known as Epipofin) causes AI in a subline of SHRSP rat (AMI). SP6 is a transcription factor with three zinc finger domains, and specifically expressed in the dental epithelium through amelogenesis. However, two-base-insertion in *Sp6* gene has been found in AMI (SP6-AMI), resulting in the missing of the third zinc finger domain. To elucidate the molecular mechanism of SP6-directed amelogenesis, we established the

dental epithelial cell clones, ARE cells, as an *in vitro* AI model. Two ARE clones were selected based on the expression of amelogenesis related genes. The subcellular localization, transcription activity, and DNA-binding ability of wild type SP6 (SP6-WT) and SP6-AMI were comparatively examined in control dental epithelial cells, G5, ARE cells, and non-dental epithelial COS-7 cells. The results suggested that the third zinc finger domain of SP6 might play an important role for SP6 stability, modification, or transcription activity, together with the cell-type specific co-activator(s). We propose that ARE cells are useful tool to find the crucial molecular mechanism of SP6-directed amelogenesis.

P-10

The functional elucidation of vesicles in tooth mineralization

Ikumi Michikami, Shinji Kawai, Satoshi Wakisaka

Challenge to Intractable Oral Diseases, Center for Frontier Oral Science, Osaka University Graduate School of Dentistry

Some patients show rapid progression of dental caries, significant attrition or a fracture of the tooth, even though there is no obvious family history and underlying diseases. From this clinical background, we speculated that the other factors, not simply malfunctional genetic factors, caused calcification disorders in teeth. In this study, we focused on membrane trafficking system as a factor that affects calcification of teeth, and the identification of genes related to the vesicular transport in the tooth-forming cells.

The SNARE (Soluble NSF Attachment protein Receptor) proteins are involved in membrane vesicle fusion. We identified Snap23, one of the SNARE family, expression in tooth by qRT-PCR. In order to confirm the expression and localization of Snap23, we performed sectional in situ hybridization on a murine tooth germ. In situ analysis showed that the specific expression of Snap23 in ameloblast. Therefore, we generated the conditional knock-out mice lacking the Snap23 specifically in ameloblast, S23^{fl/fl}; K14-Cre, and analyzed the phenotype of the mice for the purpose of elucidating the mechanism of calcification of teeth. As a result of the micro-CT analysis, the formation of enamel of S23^{fl/fl}; K14-Cre mice in the first molar was less than that of the control littermates. Our results suggest that Snap23 is involved in the calcification of enamel.

P-11

Nestin+ cells are pericytes in periodontal ligament

Tomoaki Iwayama¹, Satoshi Wakisaka¹ and Shinya Murakami²

¹ Department of Oral Anatomy and developmental biology,

² Department of Periodontology, Graduate School of Dentistry, Osaka University

Background:

The periodontal ligament contains a mesenchymal stem cell (MSC) /progenitor population to maintain tissue homeostasis. To date, only Nestin-positive cells in bone marrow were shown to be *bona fide* MSCs by rigorous *in vivo* characterization. It would be helpful for better understanding of tissue homeostasis to identify Nestin-positive population in periodontal ligament.

Materials and methods:

Periodontal ligament of Nestin-GFP transgenic mice was analyzed histologically. GFP-positive cells in the neonatal adipose tissue were FACS-sorted, and CFU-F assay was performed. Their differentiation capacities were characterized by inducing to fibroblasts, osteoblasts, or adipocytes *in vitro*. Furthermore, the Nestin-Cre/R26-tdTomato double transgenic mice were generated and their progeny was genetically traced *in vivo*.

Results:

Nestin-positive cells were located adjacent to capillaries in periodontal ligament. FACS-isolated nestin-positive cells expressed MSC markers including CD44, CD90, CD105, Sca-1, PDGFRs, and formed CFU-F at high efficacy. When nestin-positive cells were cultured and induced, they differentiated into mineralized nodule forming osteoblasts, Col3a1 expressing fibroblasts and Oil Red O-positive adipocytes. In a lineage tracing

experiment, nestin-lineage cells were also identified as a perivascular population.

Conclusion:

Nestin-positive cells are pericytes in periodontal ligament. They are MSC/progenitors, and maintain their perivascular localization during homeostasis, suggesting they are an MSC reservoir within periodontal ligament.

P-12

Epithelial specific Runx1 knock-out causes enamel hypoplasia in mouse

Safiye Esra Sarper, Kiyomi Mihara, Gozo Aoyama, Hiroshi

Kurosaka, Jirou Miura, Takashi Yamashiro

Osaka University – Graduate School of Dentistry - Department of Orthodontics and Dentofacial Orthopedics

Enamel is one of the main tissue for making up the tooth and has the hardest combination in human body. Many genes are involved in enamel formation. If enamel formation altered human disease named enamel hypoplasia may occur. We have already discovered that epithelial elimination of *Cbfb* which is one of the co-factor of *Runx* gene family result in enamel hypoplasia in mice. In this study we focused on the role of *Runx1* which is another representative *Runx* family transcription factor during tooth development. Since it has been known that *Runx1* expression was observed only at dental epithelium we hypothesize that *Runx1* is involved in enamel formation.

Objective: In order to investigate how *Runx1* gene in the dental epithelium effects enamel formation and ameloblast differentiation

Methods: We generated epithelial specific knock out mouse using Cre-loxp (K14 Cre *Runx1*^{fl/fl}) system. Mineralized tissue was examined through micro-CT observation. In order to reveal histological defect of *Runx1* cKo mice we performed hematoxylin-eosin staining on frozen sections from dissected maxilla and mandible. For observing detailed enamel prism structure, electron micrographs was used to analyze from the surface of epoxy resin embedded tooth of adult mice.

Results: From gross morphological analysis of incisor teeth we revealed excessive attrition at tips of *Runx1* cKo incisors. Through micro-CT analysis we discovered shorter incisor in *Runx1* cKo mice. Electron micrographs showed that increased abrasion on occlusal surface of molars as well as incisors in *Runx1* cKo mice than control mice. Additionally enamel prism structure is obviously disturbed in *Runx1* cKo mice. Moreover histological analysis uncovered that *Runx1* cKo mice have retarded enamel formation and marked defect of ameloblast differentiation.

Conclusion: *Runx1* gene in dental epithelium is critical for ameloblast differentiation and enamel formation.

P-13

PKP1, a novel Wnt signaling regulator, is critical for tooth development and ameloblast differentiation

Kanako Miyazaki, Keigo Yoshizaki, Chieko Arai, Han Xue, Keita

Funada, Ichiro Takahashi

Section of Orthodontics and Dentofacial Orthopedics, Division of Oral Health, Growth and Development Faculty of Dental Science, Kyushu University

Wnt signaling pathway plays important roles for tooth development, especially during the tooth morphogenesis. We focused on Plakophilin 1 (PKP1), which is highly expressed in tooth on microarray data, and known as disease gene for ectodermal dysplasia/skin fragility syndrome. We hypothesized that PKP1 is a regulator of Wnt signaling pathway because of its armadillo repeat domain similar to β -catenin.

To identify the function of PKP1 during tooth development, we performed *ex vivo* organ culture experiments using PKP1 siRNA. The size of tooth germs was significantly smaller than that of the controls when siRNA was applied. Proliferation of dental epithelial cell was inhibited by PKP1 siRNA *in vitro*. Transfected PKP1-EmGFP was translocated from plasma membrane into nuclei by stimulation of Wnt5a, Wnt3a, and Lithium Chloride.

PKP1 is also known as an adhesion-related molecule of

desmosome, and we found that PKP1 localization shifts to the plasma membrane during ameloblast differentiation. In addition, PKP1 siRNA disturbed localization of ZO-1 to tight junction, and inhibited the differentiation of ameloblast. Immunoprecipitation revealed that PKP1 bound to ZO-1.

These results suggest that PKP1 is involved in early tooth morphogenesis as a Wnt signal regulator, and affects to the differentiation of ameloblast via the cell adhesion molecules.

P-14

Physiological properties of repetitive masseter activity during NREM sleep in guinea pigs

Takafumi Kato, Risa Toyota, Hiroyuki Yano, Makoto Higashiyama, Fumihiko Sato, Atsushi Yoshida
Department of Oral Anatomy and Neurobiology, Osaka University, Graduate School of Dentistry

Patients with sleep bruxism (SB) present rhythmic masticatory muscle activities (RMMA) during NREM sleep. However, no animal model has not been developed for investigating neurobiological aspects of SB. Therefore, we have conducted a comparative study on RMMA during sleep between humans and animals. Polygraphic recordings simultaneously with EMG activity from masseter muscle were done in normal healthy subjects and the freely-moving guinea pigs. RMMA episodes were visually scored. The EMG variables were analyzed and compared with those during chewing. Cortical and cardiac activities were quantified before and after the onset of RMMA. RMMA in human subjects and animals had common EMG characteristics in comparison to those of chewing. In addition, humans and animals, the occurrence of RMMA was associated with the signs of transient arousals such as a changes in EEG activity and RR-intervals. These results suggest that the RMMAs during NREM sleep in experimental animals share common physiological properties to those occurring in humans. Therefore, RMMAs in experimental animals can be a physiological marker for investigating the pathophysiology of rhythmic activations of the masticatory muscles during NREM sleep in humans.

P-15

Recovery from age-related infertility under environmental light-dark cycles adjusted to the intrinsic circadian period.

Nana N. Takasu and Wataru Nakamura

Laboratory of Oral Chronobiology, Graduate School of Dentistry, Osaka University

Female reproductive function changes during aging with the estrous cycle becoming more irregular during the transition to menopause. We found that intermittent shifts of the light-dark cycle disrupted regularity of estrous cycles in middle-aged female mice, whose estrous cycles were regular under unperturbed 24-hr light-dark cycles. Although female mice deficient in *Cry1* or *Cry2*, the core components of the molecular circadian clock, exhibited regular estrous cycles during youth, they showed accelerated senescence characterized by irregular and unstable estrous cycles and resultant infertility in middle age. Notably, tuning the period length of the environmental light-dark cycles closely to the endogenous one inherent in the *Cry*-deficient females restored the regularity of the estrous cycles and, consequently, improved fertility in middle age. These results suggest that reproductive potential can be strongly influenced by age-related changes in the circadian system and normal reproductive functioning can be rescued by the manipulation of environmental timing signals.

Reference:

Takasu NN et al. *Cell Rep.* 12: 1407-13 (2015).

P-16

Periodontal tissue regeneration by transplantation of adipose tissue-derived multi-lineage progenitor cells.

Keigo Sawada, Masahide Takedachi, Satomi Yamamoto, Chiaki Morimoto, Asae Hirai, Masao Ozasa, Tomoaki Iwayama, Yuko

Sano, Masahiro Kitamura and Shinya Murakami
Department of Periodontology, Osaka University Graduate School of Dentistry

The reconstruction of periodontium destroyed by periodontal diseases is a major goal of periodontal regenerative therapy. Due to unsatisfied efficacy of current periodontal regenerative therapies, development of new cell-based therapies has been hoped for. In this study, we examined the efficacy and mechanism of periodontal tissue regeneration by transplantation of adipose tissue-derived multi-lineage progenitor cells (ADMPCs).

Preclinical study utilizing a canine periodontitis model demonstrated the efficacy and safety of transplantation of ADMPCs for periodontal tissue regeneration. Then we started the First-in-Man clinical study. Subcutaneous adipose tissue-derived ADMPCs were isolated and cultured in a cell processing isolator. Autologous transplantation of ADMPCs was accomplished in a patient. 36-week follow up indicated that ADMPCs transplantation resulted in reduction of probing pocket depth, clinical attachment gain and improvement of radiolucency. No transplantation-related adverse events were observed.

On the other hand, *in vitro* studies revealed that trophic factors released by ADMPCs stimulated differentiation of human periodontal ligament cells into mineralized tissue-forming cells. This effect was partly dependent on insulin-like growth factor binding protein 6 derived from ADMPCs.

These results clearly indicate that ADMPCs transplantation can induce significant periodontal regeneration. The efficacy and safety is now further investigated in the clinical study.

P-17

Foxc2-CreERT2 lineage tracing reveals cranial and cardiac neural crest cell function

Mohammed Badrul Amin, Mohammad Khaja Mafij Uddin, Mohammad Johirul Islam, Naoyuki Miura, **Kazushi Aoto**

Department of Biochemistry, Hamamatsu University School of Medicine

The forkhead transcriptional factors, *Foxc1* and *Foxc2* are important for craniofacial, eye, heart, vertebrate, and kidney development. Both genes are similarly expressed during embryogenesis. However, cell-type and time-specific difference and function of *Foxc1* and *Foxc2* are not yet fully understood. Here we performed *Foxc2* genetic lineage tracing analysis using Tamoxifen inducible Cre recombinase-based mice (*Foxc2-CreERT2; R26R*). We found *Foxc2* marked craniofacial and cardiac neural crest cells (NCC) population, and its derived periocular mesenchyme, nasal cartilage and cardiac mesenchyme including cardiac septum and aortic arch. Moreover, we confirmed NCC expression of *Foxc2* in double fluorescence immunohistochemistry using specific *Foxc1* and *Foxc2* antibodies. Interestingly, many *Foxc2* positive cells were observed in nasal cartilage and cardiac outflow tract region than *Foxc1* cells. *Foxc1* was also expressed in oral epithelium. These results suggested that *Foxc2* has new function in NCC. Now we are checking about NCC derivative tissues in *Wnt1-Cre; Foxc2-flox/flox* embryos and developmental-stage-specific function in *Foxc2-CreERT2/flox* embryos.

P-18

The function of H3K79 methylation on craniofacial development

Honami Ogo¹, Tomomi Nakano¹, Issay Kitabayashi², Jun Motoyama³, Toshio Watanabe¹ and Daisuke Sakai³

¹ Graduate School of Humanities and Science, Nara Women's University

² National Cancer Center Research Institute

³ Graduate School of Brain Science, Doshisha University

Epigenetic modifications such as methylation and acetylation of histones function as regulatory switches of gene expression through the altering of chromatin structure. Recent studies revealed that the expression of several leukemogenic and developmental genes depend on methylation of histone H3 on lysine 79 (H3K79me). However the mechanisms of cell- or

tissue-specific H3K79me is poorly understood. Dot1L is a conserved methyltransferase mediating H3K79me, and its activity is thought to be dependent on the combination of cofactors in the complex. Thus we analyzed the role of Af10, one of the Dot1L cofactors for tissue-specific H3K79me and embryonic development. We generated *Af10* conventional knockout mouse (*Af10*-KO) and found that *Af10*-KO exhibited tissue-specific defects, such as clefting of the facial midline and hypertelorism. Consistent with this phenotype, *Af10* is strongly expressed in mesenchyme of nasal processes and maxillary prominences, and loss of *Af10* resulted in the decrease of mesenchymal cell number in nasal processes. Furthermore, H3K79me level was significantly diminished in nasal processes of *Af10*-KO. These results suggest that *Af10* is required for cranial-specific H3K79me and subsequent activation of craniofacial developmental gene expression. We are currently exploring the target genes of *Af10*-dependent H3K79me on craniofacial development.

P-19

Retinoic-acid-induced cleft palate by regulating *Sim2* and sonic hedgehog signaling

Qi Wang, Hiroshi Kurosaka, Takashi Yamashiro

Department of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry, Osaka University

Cleft palate is a common congenital anomaly in humans and is thought to be caused by genetic and environmental factors. Intake of retinoic acid (RA) or its precursor, vitamin A, during early pregnancy is associated with increased incidence of craniofacial lesions including cleft palate. However, the pathogenetic mechanism of cleft palate caused by excess RA is not fully understood.

The aim of the present study is to investigate the effects of excess RA on early palatogenesis in mouse fetuses and analyze the teratogenic mechanism. We gave all-trans RA (25 mg/kg body weight) to ICR pregnant mice by gastric intubation at embryonic day 8.5, 9.5 and 10.5, and harvested embryos at E11.5. It was found that the distance between RA-treated embryonic palatal shelves was wider, and in those regions both of apoptotic cell death and cell proliferation increased. Moreover, we discovered that expression of Sonic hedgehog (*Shh*), and its downstream genes *Ptch1* and *Gli 1* were reduced in palatal region of RA-treated embryos. We also observed the downregulation of single-minded homolog 2 (*Sim 2*) expression which is known to be associated with cleft palate. Furthermore, the incidence of cleft palate due to overdose RA was reduced by administration of SAG (*Shh* signaling agonist). Thus, our results suggest that one of the pathogenesis of cleft palate after excessive RA exposure is likely to be associated with the downregulation of *Shh* signaling pathway and *Sim2*.

P-20

Fate of TRPS1-daughter cells during the joint and cardiac development.

Ahmed G. Nomir^{1,2}, Makoto Abe¹, Yuto Takeuchi¹, Junji Fujikawa¹, Satoshi Wakisaka¹

¹ Dept. of Oral Anatomy and Dev. Biol., Osaka Univ. Grad. School of Dentistry

² Dep. of Anatomy and Embryology, faculty of veterinary medicine, Damanhour university, Egypt

Tricho-rhino-phalangeal syndrome (TRPS) exhibits an autosomal dominantly-inherited craniofacial and skeletal abnormality. Joint problems are one of the diagnostic features of TRPS. Recent reports revealed that relatively high population of the TRPS patients exhibit congenital heart defects. To understand the etiology of these problems, we generated a novel transgenic mice strain which contain approximately 4kb murine *Trps1* proximal promoter sequence followed by Cre cDNA. We crossed these mice with Cre reporter strain mice to identify the daughter cells in the joints and cardiac regions. X-gal staining revealed a restricted area of staining in fore and hind limb articular structures in embryonic and postnatal pups. X-gal labeled cells were

observed in the endocardial cushion. Later, extensive staining was observed in valves, atrial and ventricular walls and also in the interventricular septum. *Trps1* mRNA expression was observed in the developing joints of the limbs and restricted in a region of the cardiac valves. These results indicate that the sequence examined indeed contain skeletal cell and endocardium enhancer of *Trps1*, and partially explain why TRPS patients exhibit these problems. Also this novel *Trps1*-Cre mouse will be a useful strain to achieve Cre-mediated recombination in *Trps1* expressing (or expressed) cells during cardiac and chondrocytic development.

P-21

Studies on mineralization system of skeletal tissues

Shinji Kawai, Ikumi Michikami, Atsuo Amano, and Satoshi Wakisaka

Challenge to Intractable Oral Diseases, Center for Frontier Oral Science, Osaka University Graduate School of Dentistry

Osteoblasts secrete collagens or hydroxyapatites to form a hard tissue such as bones. It is considered that several genes function during the bone matrix release in periodontal tissues. Here we hypothesize that SNARE gene function during the bone matrix secretion to form bones and teeth. In this study, we aimed to analyze the function of SNARE genes in hard tissue using genetically modified mice.

First, we analyzed the expression of SNARE genes by qRT-PCR and in situ hybridization, and identified high expression of a SNARE gene in bone tissue. Over-expression of the SNARE gene promoted mineralization, but the knockdown inhibited mineralization in osteoblasts. Next, we generated specifically deficient the SNARE gene in osteoblasts. The shorter body length and shorter limb was observed by a soft X-ray in the SNARE knockout mice. By pQCT, bone mineral density was reduced in the mice. The bone structure parameters such as bone volume/tissue volume bone, trabecular thickness, and trabecular number were decreased by a micro-CT, and in bone morphometry, bone formation parameters were decreased, meaning that mineralization of the bone was delayed in the SNARE conditional knockout mice.

Our results were suggested that the SNARE gene is involved in the secretion of the bone matrix by osteoblasts. It is expected that our findings help the understanding in pathogenesis of metabolic disorders such as osteoporosis and ectopic calcification of blood vessels and joints.

P-22

A novel transcription factor *Zfhx4* is critical for chondrogenesis and craniofacial development

Eriko Nakamura, Kenji Hata, Michiko Yoshida, Tomohiko Murakami, Yoshifumi Takahata, Makoto Abe, Satoshi Wakisaka, Toshiyuki Yoneda, Riko Nishimura

Challenge to Intractable Oral Diseases, Center for Translational Dental Research, Osaka University Dental Hospital

Endochondral ossification plays critical roles in skeletal development and craniofacial development. Endochondral ossification is regulated by transcription factors, including *Sox9*, *Runx2*, and *Osterix*; however, considering the sequential and harmonious regulation of endochondral ossification, we hypothesized that yet-unidentified transcription factors contribute to endochondral ossification. To address this, we performed microarray analyses using mouse limb bud cells and isolated a novel transcription factor *Zfhx4*, putatively proposed to be responsible for the 8q21.11 Microdeletion Syndrome. To examine the role of *Zfhx4*, we first generated the *Zfhx4*^{-/-} mice. *Zfhx4*^{-/-} mice died of respiratory failure within a day after birth. *Zfhx4*^{-/-} mice exhibited trivialized thoracic cavity, malformation of proximal skeletal elements, domed skull, and cleft palate. Histological analyses indicated that palatal shelves were normally formed but not elevated in the E14.0 *Zfhx4*^{-/-} mice. Moreover, calcification and degradation of cartilage matrices were markedly suppressed in the femur of *Zfhx4*^{-/-} mice. Furthermore, MMP13 expression was dramatically down-regulated in the *Zfhx4*^{-/-} mice as like

Osterix-deficient mice. Fluorescent imaging analyses showed co-localization of Zfhx4 with Osterix, but not with Runx2. Co-immunoprecipitation experiments also indicated the physical association between Zfhx4 and Osterix. To examine the interaction Zfhx4 and Osterix *in vivo*, we generated the Zfhx4^{-/-}; Osterix^{+/-} mice. Chondrogenesis is more severely impaired in Zfhx4^{-/-}; Osterix^{+/-} mice than in Zfhx4^{-/-} mice. These results suggest that Zfhx4 regulates the late stage of chondrogenesis and palate formation.

P-23

Generation of a Loeys-Dietz syndrome mouse model

Satoru Yamada, Kenichiro Tsushima, Toshihito Awata, Satoko Yamaba and Shinya Murakami
Department of Periodontology, Osaka University Graduate School of Dentistry

Objectives: Loeys-Dietz syndrome (LDS), which is caused by heterozygous missense mutation in either TGF- β receptor 1 or 2 gene, is a syndromic connective tissue disorder and phenotypically similar to Marfan syndrome. Through an epidemiological study in collaboration with National Cerebral and Cardiovascular Center in Japan, we found one LDS patient with aggressive periodontitis (AP) like-disease. Considering possible relationship between LDS and AP, we generated a knockin (KI) mouse strain with the LDS mutation and performed phenotype analyses. **Methods:** We constructed KI vector to introduce the LDS mutation and established a KI mouse strain by homologous recombination in ES cells. We performed genotyping and observed the longevity of KI mice until 180 days after birth. We carried out histological analysis of aorta by Elastica van Gieson (EVG) staining. We stimulated mouse embryonic fibroblasts (MEFs) isolated from KI mice with TGF- β and examined PAI-1 expression by real-time PCR. **Results:** Heterozygote of KI mouse was born normally according to Mendel's laws, but the homozygote was observed only in 0.5% after birth. Kaplan-Meier survival curve showed reduced longevity of KI hetero mice. By EVG staining we found the elastic fiber fragmentation in the 24-week-old KI mouse aorta. KI MEFs showed down-regulated response to TGF- β compared to wild-type MEFs, suggesting the malfunction of TGF- β signal in KI mouse. **Conclusions:** In this study, we established a LDS model mouse that recapitulated the LDS phenotype. We envisage this LDS model mouse will be useful for the analysis of pathomolecular relationship between LDS and AP.

P-24

Novel mutant mice exhibiting craniofacial and axial skeletal defects

Junji Fujikawa, Ahmed Nomir, Yuto Takeuchi, Makoto Abe
Department of Oral Anatomy and Developmental Biology, Osaka University Graduate School of Dentistry

Normal skeletal development is important for physical protection of the internal organs and provides the rigid space for hematopoiesis in the bone marrow. Also, it is crucial for our daily locomotion and expansion of the lung for breathing. Recent evidence has shown that bone functions as an endocrine organ by secreting factors such as FGF23 and DMP1. Appropriate animal models had contributed a lot to understand the mechanisms of bone development and bone-related disease. We have recently identified a spontaneous mouse strain which showed severe axial skeletal defects. The abnormal skeletal phenotype was observed in both male and female mice, and was considered to be inherited in an autosomal recessive manner. Precise analysis of the skeletal phenotype revealed that this mouse showed severe cleft palate and asymmetrically fused ribs. Although the rib phenotype somewhat resembled the condition observed in human genetic disorder, spondylothoracic dysostosis (STD), there was also many inconsistent bone defects not seen in STD. In order to identify the responsible gene, we performed whole exome sequencing followed by sequential filtering to narrow down the candidates. We have so far narrowed down to almost 500 genes.

P-25

Elucidation of anabolic effects in bone by intermittent PTH administration

Katsutoshi Hirose, Sunao Sato, Kaori Oya, Mitsunobu Kishino, Yuri Noda, Miki Sharyo, Satoru Toyosawa
Department of Oral Pathology, Osaka University Graduate School of Dentistry

[Background] Intermittent administration of parathyroid hormone (PTH) is known to have an anabolic effect on bone, hence, it has been used for clinical treatment of osteoporosis. However, PTH has diverse effects on bone metabolism depending on the mode of administration and the mechanism of PTH actions is not fully understood. To address this issue, we elucidated the effects of intermittent administration of PTH corresponding to the mode of administration for clinical treatment of osteoporosis. [Materials and methods] Male 13-week-old SD rats were treated with human PTH [1-34] at 30 (μ g/kg) or the vehicle, 3 times/week for 6 months. The experimental and the control rats at 1, 3 and 6 months after onset of treatment were studied. [Results and Discussion] DEXA analysis indicated PTH-treated group had increased bone mineral density in tibiae. In histomorphometrical analysis, an increase in trabecular thickness and new bone formation (as reflected by increases in MAR and BFR) was observed. Serum level of osteocalcin in PTH-treated group was enhanced relative to control, but no differences in serum TRAP5b, NTX, P1NP, Dmp1, and Sclerostin. These findings suggested that intermittent PTH treatment may enhance minimodeling bone formation, with mature osteoblast activity and without altering bone resorption rate.