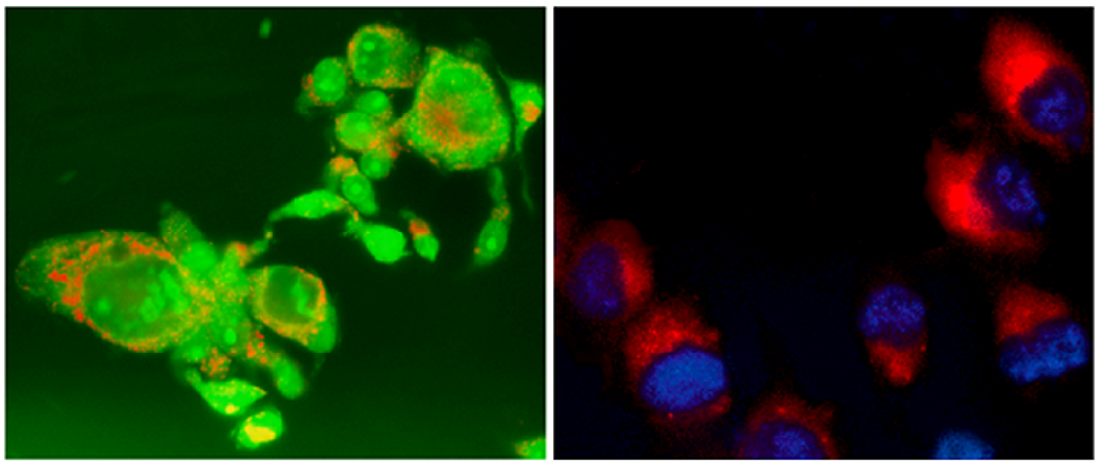
Kyudai Oral Bioscience 2013

—7th International Symposium —

March 8-9th, 2013

Fukuoka Recent Hotel, Fukuoka, Japan

PROGRAM & ABSTRACTS



Kyushu University Faculty of Dental Science

○会期：平成 24 年 3 月 8 日～9 日
3 月 8 日（金）13:00～18:30、懇親会 18:30～20:00
3 月 9 日（土）9:00～17:20

○会場：福岡リーセントホテル 舞鶴の間 A（２階）：下図参照
住所：福岡市東区箱崎 2-52-1
電話：092－641－7741

○主催：九州大学大学院　歯学研究院
歯学研究院研究プロジェクト
「口腔組織の再生・再建医療」、「口腔健康科学」
文部科学省　特別教育研究経費
「歯学連携ネットワークによる口腔からＱＯＬ向上を目指す研究」

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PROGRAM

March 8th (Fri)　　*MAIZURU* Room A（2nd Floor）

Opening remarks

13:00 - 13:05　Hiroshi Nakanishi (*vice Dean*)
13:05 - 13:10　Akifumi Akamine (*Dean, Faculty of Dental Science, Kyushu University*)

Session 1: Dental and Craniofacial Morphogenesis and Tissue Regeneration

Session 1A

■Chair person: Kazuaki Nonaka (*Department of Pediatric Dentistry*)

13:10-13:40　*S1A-1*　Yuji Mishina
Department of Biologic and Material Sciences, University of Michigan, Ann Arbor, MI, USA

13:40-14:10　*S1A-2*　Han-Sung Jung
Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Korea

14:10-14:40　*S1A-3*　Jonathan Schoenecker
Vanderbilt University Medical Center, Nashville, TN, USA

14:40-15:00　Coffee Break

Session 1B

■Chair person: Seiji Nakamura (*Section of Oral and Maxillofacial Oncology*)

15:00-15:30　*S1B-1*　Takayoshi Sakai
Department of Oral-Facial Disorders, Osaka University, Graduate School of Dentistry, Osaka, Japan

15:30-15:50　*S1B-2*　Haruyoshi Yamaza
Faculty of Dental Science, Kyusyu University, Fukuoka, Japan

15:50-16:10　Coffee Break

Session 1C

■Chair person: Toshio Kukita (*Dept of Molecular Cell Biology and Oral Anatomy*)

16:10-16:50　*S1C-1*　Agnès Vignery
Orthopedic and Rehabilitation and Cell Biology Yale University School of Medicine, New Haven, CT, USA

16:50-17:20　*S1C-2*　Kenichi Tezuka
Department of Oral Maxillofacial Science, Gifu University Graduate School of Medicine, Gifu, Japan

17:20-17:50　*S1C-3*　Akihito Yamamoto
Department of Oral and Maxillofacial Surgery, Nagoya University Graduate School of Medecine, Nagoya, Japan

18:00-20:00　Getting Together Party, MC: Ichiro Takahashi (*vice Dean*)
***CRYSTAL* Room（2nd Floor）**

March 9th (Sat) *MAIZURU* Room A（2nd Floor）

Session 2: Regulation of Chronic Inflammation and Diseases

Session 2A

■ **Chair person: Katsumasa Maeda (*Department of Periodontology*)**

9:00- 9:25 ***S2A-1*** **Terukazu Sanui**
Department of Periodontology, Faculty of Dental Science, Kyusyu University, Fukuoka, Japan

9:25-10:00 ***S2A-2*** **Thomas E. van Dyke**
Department of Periodontology, The Forsyth Institute Cambridge, MA, USA

10:00-10:25 ***S2A-3*** **Koichi Tabeta**
Center for Transdiciplinary Research, Niigata University, Niigata, Japan

10:25-10:50 Coffee Break

Session 2B

■ **Chair person: Hiroshi Nakanishi (*Department of Aging Science and Pharmacology*)**

10:50-11:15 ***S2B-1*** **Zhou Wu**
Department of Aging Science and Pharmacology. Faculty of Dental Science, Kyusyu University, Fukuoka, Japan

11:15-11:45 ***S2B-2*** **Jessica L Teeling**
Centre for Biological Science, University of Southampton, Southampton, UK

11:45-12:10 ***S2B-3*** **Sung Joong Lee**
Department of Neuroscience and Oral Physiology, and Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea

12:30-13:30 Lunch
For the Invited Speakers: Restaurant *SARAFURU* (1st Floor)

Session 3: Oral Health Science

■ **Chair person: Yoshihisa Yamashita (*Section of Preventive and Public Health Dentistry*)**

13:30-13:50 ***S3-1*** **Toru Takeshita**
Section of Preventive and Public Health Dentistry, Faculty of Dental Science, Kyusyu University, Fukuoka, Japan

13:50-14:20 ***S3-2*** **Todd D. Taylor**
Laboratory for MetaSystems Research, Computational Biology Research Core, RIKEN Quantitative Biology Center, Yokohama, Japan

14:20-14:50 ***S3-3*** **Liping Zhao**
Shanghai Center for System Biomedicine, Shanghai, China

14:50-15:10 Coffee Break

Session 4: PhD Student Session

■Chair person: Masato Hirata (*Laboratory of Molecular and Cellular Biochemistry*)

15:10-15:20 *S4-1* Lan Ma (*Department of Pediatric Dentistry*)
15:20-15:30 *S4-2* Kyosuke Toyoda (*Department of Periodontology*)
15:30-15:40 *S4-3* Urara Tanaka (*Department of Periodontology*)
15:40-15:50 *S4-4* Tomohiro Onimura (*Section of Oral and Maxillofacial Oncology*)
15:50-16:00 *S4-5* Xin-Wen Zhang (*Department Aging Science and Pharmacology*)

16:00-16:20 Coffee Break

■Chair person: Takeshi Yokoyama (*Department of Dental Anesthesiology*)

16:20-16:30 *S4-6* Yu Yasutake (*Laboratory of Molecular and Cellular Biochemistry*)
16:30-16:40 *S4-7* Daichi Muramatsu (*Section of Oral and Maxillofacial Oncology*)
16:40-16:50 *S4-8* Saori Sako (*Department of Dental Anesthesiology*)
16:50-17:00 *S4-9* Jane Harland (*Department of Biomaterials*)
17:00-17:10 *S4-10* XNT Tram (*Department of Biomaterials*)

Closing remarks

17:10-17:20 Seiji Nakamura (*vice Director, Kyushu University Hospital*)

ABSTRACTS

Session 1

Dental and Craniofacial Morphogenesis and Tissue Regeneration

S1A-1

Smad-dependent BMP Signaling through Type IA Receptor in Cranial Neural Crest Cells Directs Their Cell Fate towards Chondrocytes to Cause Craniosynostosis

Yuji Mishina

Department of Biologic and Materials Sciences,
University of Michigan, Ann Arbor, MI, USA

Dr. Mishina's laboratory is interested in functions of bone morphogenetic protein (BMP) signaling during bone development/remodeling and craniofacial development. We recently developed several mouse lines to conditionally decrease or increase levels of BMP signaling using a Cre-loxP system. Using these systems, we have found that BMP signaling in osteoblasts is critical for maintenance of bone mass and biomechanical properties, BMP signaling in early embryos is critical for ciliogenesis that is essential to establish a left-right asymmetry, and BMP signaling in cardiac neural crest cells is important for valve functions during heart development. In this seminar, I would like to talk about our recent findings how BMP signaling is critically involved in skull morphogenesis through a tight regulation of its signaling activity.

Craniosynostosis describes conditions in which one or more sutures of the infant skull are prematurely fused, resulting in facial deformity and delayed brain development. Approximately 20% of human craniosynostoses are thought to result from gene mutations altering growth factor signaling; however, the molecular mechanisms by which these mutations cause craniosynostosis are incompletely characterized, and the causative genes for diverse types of syndromic craniosynostosis have yet to be identified.

We recently found that enhanced BMP signaling through the BMP type IA receptor (BMPR1A) in cranial neural crest cells, but not in osteoblasts, causes premature suture fusion in mice. In support of a requirement for precisely regulated BMP signaling, this defect was rescued on a *Bmpr1a* haploinsufficient background, with corresponding normalization of Smad phosphorylation. Moreover, in vivo treatment with LDN-193189, a selective chemical inhibitor of BMP type I receptor kinases resulted in rescue of craniosynostosis. The finding that relatively modest augmentation of Smad-dependent BMP signaling leads to premature cranial suture fusion suggests an important contribution of dysregulated BMP signaling to syndromic craniosynostoses, and potential strategies for early intervention.

S1A-2

A Wnt/Shh/Sostdc1 Negative Feedback Loop Governs the Spatial Patterning of Teeth

Han-Sung Jung

Department of Oral Biology, College of Dentistry, Yonsei University, Seoul, Korea

Each vertebrate species displays specific tooth patterns in each quadrant of the jaw: the mouse has one incisor and three molars, which develop at precise locations and at different times. Sonic hedgehog (Shh), Wnt and Sostdc1 are key signalling molecules involved in the spatial patterning of teeth and other ectodermal organs such as hairs, vibrissae and feathers. For example, conditional Shh- and Smo-deficient mice such as *K14-Cre;Shh*$^{flox/flox}$ and *K14-Cre;Smo*$^{flox/flox}$ exhibit same morphological aberrations in tooth development: the dental lamina is absent, and the first (M1) and second molars (M2) are fused. Interestingly, M1/M2 fusion is also observed in *Sostdc1*$^{-/-}$ and *Lrp4*$^{-/-}$ mice, as have supernumerary molars and incisors. While it has been reported that a Wnt/Shh negative feedback loop exists in tongue papillae and that Sostdc1 (also known as USAG-1, Ectodin and Wise) is a secreted inhibitor of the Wnt pathway, the relationship between Shh and Sostdc1 is yet to be clarified. Here, by utilizing maternal transfer of 5E1 (an IgG1 monoclonal antibody against Shh protein) through the placenta, we show that blocking Shh signalling by 5E1 results in molar fusion and supernumerary tooth formation and that Sostdc1 is a downstream target of Shh, which suggests a Wnt/Shh/Sostdc1 negative feedback loop governing the spatial patterning in teeth. Furthermore, we propose a new reaction–diffusion model, in which Wnt, Shh and Sostdc1 act as the activator, mediator and inhibitor, respectively, confirming that such interactions do indeed lead to patterning consistent with tooth patterning of mouse.

S1A-3

Plasmin but not Fibrin is Essential for Fracture Healing

[1]Masato Yuasa, [1]Nicholas Mignemi, [1]Heather A Cole, [1]Lynda O' Rear 1,[1]Jeffry S Nyman, [2]Justin M Cates, [1,2]Herbert S Schwartz, [1,2,3]Jonathan G Schoenecker

[1]Department of Orthopaedic and Rehabilitation, [2]Pathology, [3]Pediatrics
Anderbilt University Medical Center, Nashville, TN, USA

INTRODUCTION: For a century the formation of a fibrin clot has been considered to be the initial, and essential, phase of fracture repair. In addition to providing hemostasis, the clot is thought to recruit stem cells and localize growth factors that are required for fracture healing. Hence, many principles of fracture care and pharmaceuticals have been developed to enhance a fibrin matrix in the fracture bed (**1**).

Despite its beneficial and essential role in the initial phases of healing, the persistent accumulation of fibrin has been implicated as a factor of delayed skin wound (**2**). In addition, recent evidence in chronic diseases such as Alzheimer's (**3**), multiple sclerosis (**4**) and muscular dystrophy (**5**) have also implicated the accumulation of fibrin as a key molecular component of the pathophysiology of disease. From these studies we formulated the hypothesis that accumulation of fibrin would impair fracture healing.

Materials and Methods: All mice were used for this study at 8 weeks of age. To avoid sex-related confounding effects on developmental bone growth and fracture, only male mice were used in this study (12-16g). Mice were placed under anesthesia using 60 mg/kg ketamine and 4 mg/kg xylazine. We created a mid-shaft femur fracture by open method on mice (Wild type (WT)); n= 15, plasminogen deficient (Plg-/-) which cannot remove fibrin; n= 14, and fibrinogen deficient (Fbg-/-) which cannot make a fibrin clot; n=6). 23 gauge needles (0.6414mm) were used for fixation with retrograde fashion. Fracture healing was followed radiographically using a Faxitron X-ray system (Lincolnshire, IL) weekly from 1 week to 6 weeks post-fracture (PF). We sacrificed the mice at 2 and 6 weeks PF. Fracture healing was analyzed by X-ray, micro Computed Tomography (uCT; uCT40 Scanco medical AG, Switzerland), Microfil (Flow Tech Inc., Carver, MA) injection and histology. We dissected the femur and fixed them in 4 % paraformaldehyde and embedded into paraffin. After sectioned, we stained the sections by H&E, Safranin-O, CD31 immunohistochemical (IHC) (anti mouse CD31, BD Pharmigen) and Fibrin IHC staining (given by Dr. J. Degen, Division of Developmental Biology, Children's Hospital Research Foundation, Cincinnati, Ohio). The extent of fracture healing in the mice was quantified by both X-ray and micro CT. We quantified the healing

by a grading scale, which accounts for development of ossified callus and mineral remodeling by x-rays and by measuring their structural and tissue level properties of bone by uCT. We injected antisense oligonucleotide of fibrinogen to Plg (-/-) mice (Plg-/- ASO treated; n=10) weekly to restrict fibrinogen level in blood 2 weeks prior to fracture. Fibrinogen levels in plasma were measured 6 weeks PF by fibrinogen ELISA (Immunology Consultants Laboratory, Inc, OR). **STATISTICS:** Comparisons among the groups were performed using the one-way analysis of variance with the least significant difference procedure was used for analyzing 2 or multiple groups, respectively. A level of $p< 0.05$ was considered significant for the differences between mean values (*).

RESULTS: Weekly X-rays revealed that WT and Fbg (-/-) mice formed ossified bridging callus by 3 weeks PF which was almost completely remodeled by 6 weeks PF. uCT and histological results revealed that callus formation in WT and Fbg (-/-) mice has completely bridged by 6 weeks PF. However, Plg (-/-) mice failed to develop an organized mineralized callus and failed to unite or remodel the disorganized matrix (**Figure.1**). uCT at 6 weeks PF in Plg (-/-) mice revealed that they still retained poor remodeled callus formation and disorganized mineralization at the fracture site and moreover 71% (ten out of fourteen mice) displayed non-bridging callus. Microfil injection demonstrated that, at 2 weeks PF, vascularity in the callus was significantly reduced in Plg (-/-) mice compared to WT and Fbg (-/-) mice (**Figure2-a**). Consistent with these findings, there remained abundant avascular cartilage in Plg (-/-) mice at 2 weeks PF in histological sections compared to WT mice. We confirmed the migrating neovessels into avascular cartilage by CD31 IHC staining. Fibrin IHC staining showed abundant fibrin interposed between the avascular cartilage and vascularized bone (**Figure2-b**). At 6 weeks, whereas in WT, vessels crossed over the fracture site, those in Plg (-/-) mice did not cross over the non bridging callus. Fibrinogen level in blood of plg (-/-) mice treated with ASO was significantly reduced although that of Plg (-/-) mice was 2-2.5 folds higher than WT mice. Fracture healing was impaired in Plg (-/-) mice and was partially rescued by ASO injection when we measure the fracture healing by grading scale. uCT revealed that structural properties of the fracture site in Plg (-/-) mice at 6 weeks PF were statistically lower than those of WT, Fbg (-/-) and Plg (-/-) ASO treated mice.

DISCUSSION: This is the first study to demonstrate that, as opposed to what has been accepted, fibrin is not essential for fracture healing and that accumulation of fibrin can result in delayed, disorganized non-unions. From our data, it is speculated that with insufficient fibrinolysis, fibrin can become a structural barrier to cell invasion, in particular endothelial cells and osteoblasts into avascular fracture callus. These results may provide valuable insight into novel means of improving fracture healing in these populations by targeting fibrin degradation.

SIGNIFICANCE: These results demonstrate that dysfunction of fibrinolysis leads disruption of neovascularization in the fracture healing. These findings have potential clinical implications in

disease conditions which are known to have alteration of fibrin clots rendering it more resistant to fibrinolysis as well as primary dysfunction of the fibrinolytic system such as diabetes, smoking (6) and aging.

1: Richard M Berg, Calcification in callus formation and fracture repair. ANNALS of Surgery Vol 93(5); 1931

2: Thomas H. Bugge, Loss of Fibrinogen Rescues Mice from the Pleiotropic Effects of Plasminogen Deficiency. Cell Vol. 87, 709–719, November 15, 1996

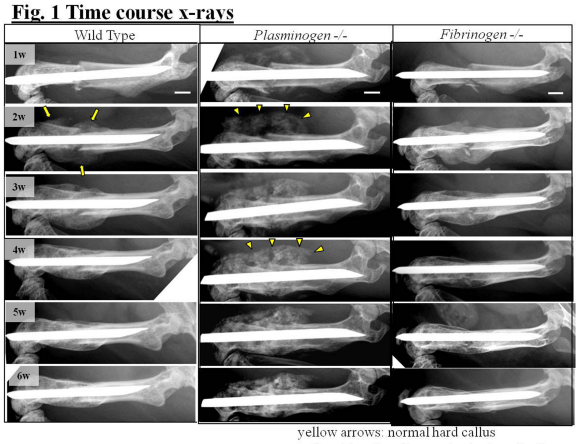
3: Marieke van Oijen, Fibrinogen Is Associated With an Increased Risk of Alzheimer Disease and Vascular Dementia. *Stroke 2005, 36:2637-2641*

4: Akassoglou K., Fibrin depletion decreases inflammation and delays the onset of demyelination in a tumor necrosis factor transgenic mouse model for multiple sclerosis. *Proc Natl Acad Sci U S A. 2004,101(17): 6698–6703*

5: Berta Vidal, Fibrinogen drives dystrophic muscle fibrosis via a TGFB /alternative macrophage activation pathway. *GENES & DEVELOPMENT 2008, 22:1747–1752*

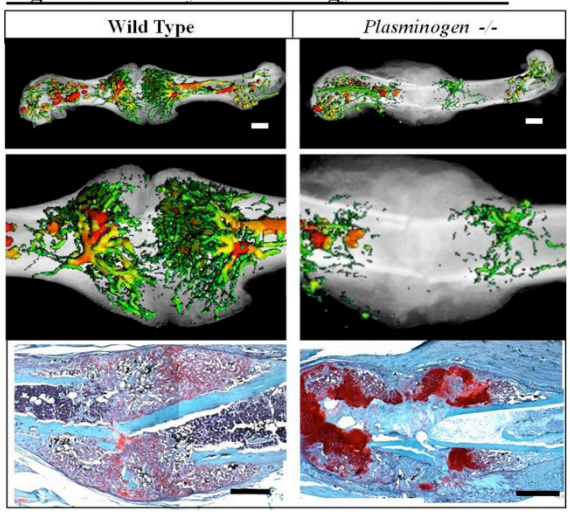
6: Rajat S. Barua, Effects of Cigarette Smoke Exposure on Clot Dynamics and Fibrin Structure : An Ex Vivo. *Arterioscler Thromb Vasc Biol. 2010;30:75-79*

Fig. 1 Time course x-rays



yellow arrows: normal hard callus
yellow triangles: disorganized mineralization

Fig. 2 Vascularity and Histology at 2 weeks PF



S1B-1

Exploration of Functional Regulators for Branching Morphogenesis of Salivary Gland by Microanalysis of Gene Expression

Takayoshi Sakai

Department of Oral-Facial Disorders, Graduate School of Dentistry, Osaka University, Osaka, Japan

Many organs such as salivary glands, kidneys and lungs form multiple epithelial clefts during the process of branching morphogenesis in embryonic development.

The developing submandibular salivary gland provides an excellent model system for clarifying the mechanisms comprising this phenomenon. In cleft formation, buds are delineated by the conversion of epithelial cell-cell adhesions to cell-matrix adhesions, but the mechanisms of cleft formation are not clear. Here we show a set of approaches being used to identify and characterize molecules necessary for branching morphogenesis. A combination of laser microdissection with T7-SAGE has been established as a method for gene discovery of candidate molecules that may be essential for early organ morphogenesis (Current Protocols in Cell Biology 19.3.1-19.3.30, 2002). We have identified *Fibronectin* and *Btbd7* at cleft-forming sites and provided insights into the mechanisms of dynamic cleft propagation in branching morphogenesis (GSE22374: T7-SAGE transcriptome of cleft versus end bud epithelial cells of embryonic mouse salivary glands). The matrix protein fibronectin is required for salivary, kidney, and lung branching. Wedges of fibronectin translocate inward as clefts form between randomly motile epithelial cells, accompanied by loss of the cell-cell adhesion molecule E-cadherin in cells adjacent to the fibronectin (Nature 423, 876-881, 2003). How a matrix molecule can drive cleft formation and branching is unknown. We have identified Btbd7 as a dynamic regulator of branching morphogenesis. Btbd7 provides a mechanistic link between the extracellular matrix and cleft propagation through its highly focal expression leading to local regulation of Snail2 (Slug), E-cadherin, and epithelial cell motility (Science 329, 562-565, 2010). Inhibition experiments show that Btbd7 is required for branching of embryonic mammalian salivary glands and lungs. Hence, *Btbd7* is a regulatory gene that promotes epithelial tissue remodeling and formation of branched organs. Progress in understanding the mechanisms of salivary branching morphogenesis should provide novel approaches to future tissue engineering or regeneration of damaged salivary glands.

S1B-2

Caloric Restriction in Health

Haruyoshi Yamaza

Department of Pediatric Dentistry, Graduate School of Dental Science, Kyushu University, Fukuoka, Japan

Caloric restriction (CR) retards aging processes and extends mean and maximum lifespan, and the effects of CR are demonstrated in a wide range of animals including C. elegans, Drosophila and rodents. Although the findings have been confirmed repeatedly in many laboratories, the mechanism of CR has not been elucidated. Recent discoveries of longevity assurance genes emphasize the importance of reduced growth hormone (GH)–insulin-like growth factor (IGF)-1 signaling with a decrease in serum insulin levels.

We previously reported a new long-lived transgenic (Tg) dwarf rat model, in which the GH–IGF-1 axis is selectively suppressed by overexpression of the antisense GH gene, that significantly lives 7%–10% longer than wild-type (WT) rats. Subsequent studies revealed that these Tg rats, which fed the foods ad libitum (AL), have similar phenotypes to WT-CR rats. The plasma IGF-1 levels are moderately reduced in both groups of rats as compared to WT-AL rats. In WT-CR and Tg-AL rats, glucose tolerance was normal or slightly improved, whereas the glucose-induced insulin response was lower. To investigate the potential role of the GH–IGF-1 axis in the CR effect, recombinant human (rh)IGF-1 was administered continuously to WT-CR, WT-AL, and Tg-AL rats for 14 days and their responses were analyzed. Infusion of a physiologic dose of rhIGF-1 elicited negative feedback regulation of rat IGF-1 production, thus the total IGF-1 concentrations did not increase compared with the infusion of the normal saline in all three rat groups. In addition, rhIGF-1 infusion in WT-CR and Tg-AL reduced the plasma concentration of adiponectin, which was increased in long-lived animals by CR and the reduction of GH-IGF-1 axis, on the level of control group.

The homolog of FoxO, which is the downstream of insulin/IGF-1 signal and transcription factor, is involved in the lifespan extension in C. elegans and Drosophila. We focused on FoxO1. Therefore, we investigated the roles of FoxO1 in the effects of CR using FoxO1 knockout (KO) mice. Although the lifespan did not differ significantly between the WT and KO mice in AL or CR conditions, the anti-neoplastic effect of CR, as indicated by reduced incidence of tumors at death in the WT-CR mice, was mostly abrogated in the KO-CR mice

In conclusion, adiponectin and FoxO1 could be the important factors through insulin/IGF-1 signaling pathway for CR effects such as lifespan extension and anti-neoplasm.

S1C-1

The Making of Multinucleate Macrophages: Osteoclasts and Giant cells

Agnès Vignery

Yale School of Medicine, Orthopaedics and Cell Biology, New Haven, CT, USA

Macrophages are innate immune cells that are omnipresent in tissues and play key roles in immunity, development, remodeling, and tissue repair. In addition, in rare instances, macrophages can fuse to form a multinucleate osteoclast, in bone, or a giant cell, in chronic inflammatory reactions. While our understanding of the fusion mechanism of viruses with host cells, to inject their DNA or RNA, has made major progress, the fusion mechanism of macrophages remains poorly understood. Indeed, the fusion of cells in general, remains obscure. Such cell-cell developmental fusion events include fertilization, which results from the fusion of a sperm cell with an oocyte; the formation of multinucleate skeletal muscle cells, which originate from the fusion of myoblasts; and the formation of the placenta, in which cytotrophoblasts fuse into syncytiotrophoblasts. The molecular mechanisms that mediate cell-cell fusion remain to be discovered.

Our hypothesis has been that viruses have stolen from macrophages their fusion machinery. Because macrophages are ubiquitously present in tissues and organs as mononucleate cells, we hypothesize that the fusion machinery must be transiently induced at the onset of fusion so as to limit fusion in time, hence in cell size. As a result of cDNA microarray and monoclonal antibody screens, we have identified SIRPa, its receptor CD47, CD200 and its receptor CD200R, CD44 and KCa3.1/SK4 as molecules transiently induced at the onset of fusion. Because SIRPa-CD47 and CD200-CD200R belong to the superfamily of immunoglobulins, we propose that they allow macrophages to recognize each other as self, hence adhere and fuse with one another to form a new multinucleate cell rather than to kill one another. Such a recognition mechanism is therefore essential and might have played a central role in the evolution of the innate immune system.

We therefore regard the fusion machinery of macrophages as a complex molecular assembly that has evolved to ensure the recognition of non-self by macrophages to protect organisms against pathogens and foreign bodies, which are bigger than pathogens, such as implants. In the case of osteoclasts, they can be regarded as a task force that shaped up in response to the formation of calcified bone, which appeared evolutionary as a new challenge.

S1C-2

Dental Pulp Cells as a Source for iPS Cell Banking

Ken-ichi Tezuka

Department of Tissue and Organ Development,
Gifu University Graduate School of Medicine, Gifu, Japan

Human dental pulp cells (DPCs) are present in the cell population isolated from dental pulp tissues (Gronthos *et al.*, PNAS 2000, JDR 2002; Takeda *et al*., JDR 2008; Iida *el al*. Arch. Oral Biol. 2010). We reported that viral introduction of four transcription factors (*OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*) can reprogram DPCs into induced pluripotent stem (iPS) cells, which closely resemble embryonic stem cells (Tamaoki *et al.*, JDR 2010, Okita *et al.* Nature Methods 2011). However, we also found that establishing quality-controlled iPS cell lines from a large number of individual patients is not easy and validation of them requires considerable time and cost.

Human leukocyte antigen (HLA) plays an important role in rejection of tissues and cells transplanted from allogenic donors. In recent days, tissue transplantation is conducted without complete matching of HLA because of shortage of the donors. Instead of matching HLA types, immnosuppressants are frequently used to prevent immune reaction both host to graft and graft to host directions; however, when the immune system function is suppressed, there is an increased susceptibility to infectious diseases and cancers.

To solve this problem, usage of HLA haplotype-homo donors has been considered in iPS cell therapy. HLA haplotype-homo donors have a couple of identical HLA gene sets, resulting in presentation of HLA molecules half in the variation. Therefore, iPS cells derived from HLA haplotype-homo donors are expected to be successfully transplanted to many patients with less possibility of rejection. Potential risk of GVHD will be omitted by making iPS cells to differentiate strictly into desired cell types without contamination of hematopoietic cells. We screened approximately 200 DPC lines to find three patients having only one genotype in each of three HLA loci, A, B, DRB1. If iPS cells will be established from these three patients, they are expected to show complete match with approximately 20% of the Japanese population.

We will discuss about the advantages and disadvantages of DPCs as one of the somatic cell sources for future iPS cell banking and regenerative medicine.

S1C-3

Multifaceted Neuro-Regenerative Activities of Human Dental Pulp Stem Cells Promote Locomotor Recovery after Spinal Cord Injury in Rats

Akihito Yamamoto

Department of Oral and Maxillofacial Surgery,
Nagoya University Graduate School of Medicine, Nagoya, Japan

Spinal cord injury (SCI) often leads to persistent functional deficits due to loss of neurons and glia and to limited axonal regeneration after injury. We have reported that transplantation of adult dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs) into the completely transected adult rat spinal cord resulted in marked recovery of hindlimb locomotor function. Our study revealed that engrafted DPSC and SHED exhibited three major therapeutic benefits for recovery after SCI, including (a) inhibition of SCI-induced multicellular apoptosis, which promoted the preservation of neural fibers and myelin sheaths; (b) regeneration of the transected axon through the direct inhibition of multiple axonal growth inhibitor signals derived from SCI-circumstance; (c) replacement of lost or damaged oligodendrocytes after SCI through specific differentiation into mature oligodendrocytes under the extreme conditions of SCI. Thus, the data indicate that SHED/DPSCs promote substantial functional recovery after SCI through both cell-autonomous/cell-replacement and paracrine/trophic effects.

In current study, we have examined the level of benefits of paracrine effects alone for the treatment of SCI. We found that the intrathecal administration of serum-free conditioned medium (CM) derived from SHED, but not bone marrow stromal cells or skin fibroblasts, promoted remarkable functional recovery. This recovery was supported by a strong immunoregulatory function of SHED-CM, which dramatically converted the pro-inflammatory SCI-circumstance into a tissue repair/regenerating platform by modulating the microglia/macrophage phenotype. SHED-CM induced robust differentiation of the anti-inflammatory M2 microglia/macrophages in the injured SC. Furthermore; we found that in vitro SHED-CM directly induced IL-10-producing M2 microglia in a manner synergistic with a major extracellular matrix component of glial scar. In addition, I will introduce you remarkable benefits of SHED-CM for the treatment of hypoxic-ischemic (HI) brain injury in neonatal infants. HI is a major cause of acute mortality and chronic neurologic morbidity for which there currently is no promising therapy. Taken altogether, our study demonstrates that SHED-CM may enable the establishment of new stem-cell-based neuroregenerative therapies that do not involve cell transplantation.

References

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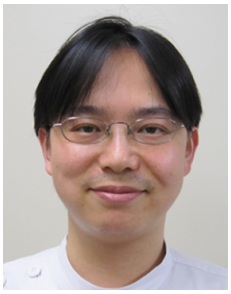
Session 2

Regulation of Chronic Inflammation and Diseases



S2A-1

Inhibition of Sprouty2 Polarizes Macrophages toward M2 Phenotype in Periodontitis

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Periodontal disease is a chronic inflammatory response to bacterial pathogens involving the supporting tissues of the teeth. The anerobic bacterium, *Porphyromonas gingivalis*, has been implicated as a major etiologic agent in the development and progression of periodontitis. Lipopolysaccharide (LPS) from *P. gingivalis* induces pro-inflammatory cytokines in host macrophages. Macrophages can acquire distinct functional phenotypes, referred to as classically activated, pro-inflammatory macrophages (M1) and alternatively activated, anti-inflammatory macrophages (M2). M2 macrophages are associated with homeostatic functions linked to wound healing and tissue repair. Therefore, the inhibition of pro-inflammatory cytokine production in macrophages is the important biological reaction for the homeostasis after elimination of the etiologic agent.

Sprouty proteins are identified as inhibitors of fibroblast growth factor (FGF) receptor. Specifically Sprouty2 (Spry2) functions as a negative regulator of receptor tyrosine kinases (RTKs) signaling, and is expressed in several developing organs including kidney, lung, brain, heart, skeletal muscle and craniofacial area. Furthermore, Spry2 has conserved function to modulate morphogenesis in several tissues. Recently, we demonstrated that inhibition of Spry2 induced cell proliferation and differentiation of MC3T3-E1osteoblastic cells, while it diminished cell proliferation of GE1gingival epithelial cells *in vitro*.

We report here that suppression of Spry2 polarizes J774 mouse macrophages toward alternative macrophage activation (M2) phenotype when J774 cells are stimulated with LPS from *P. gingivalis*. This polarization is defined by surface protein expression, cytokine production and arginase activity. These data provide evidence that Spry2 inhibitors affect the inflammatory process at the level of the macrophage and shifts macrophage polarization from pro-inflammatory properties toward anti-inflammatory ones in periodontitis.

Additionally, we are confirming how this response affects the periodontal tissue regeneration or the para-inflammation of periodontitis.

S2A-2

Resolution of Inflammation in Periodontal Regeneration

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Resolution of inflammation is an active; agonist mediated well-orchestrated return of tissue homeostasis. Lipoxins and Resolvins are endogenous lipid mediators (eicosanoids) that actively regulate the resolution of acute inflammation. Lipoxin A_4 and Resolvin E1, derived from arachidonic acid and eicosapentaenoic acid, respectively, are endogenous anti-inflammatory and pro-resolving mediators that regulate leukocyte migration and enhance macrophage phagocytosis of apoptotic neutrophils. These small lipid molecules act through specific G-protein linked receptors on inflammatory cells. Resolution receptor agonists have been shown to have significant impact in inflammatory bone diseases, such as periodontitis and arthritis. Periodontitis is an inflammatory disease that is initiated by oral microbial biofilm. It is the host response to the biofilm that destroys bone (osteoclastic resorption) in the pathogenesis of the disease. Resolution agonists have been demonstrated to promote regeneration of bone lost to disease and to prevent inflammatory bone loss in animals. Mechanistic studies have revealed that bone cells, particularly osteoclasts and osteoblasts, express functional resolution receptors on their surface that regulate RANKL:OPG ratios and the consequent osteogenic and osteolytic responses. The potential for therapeutic benefit of resolution agonists in clinical periodontal regeneration is being examined in large animal models.

S2A-3

Mechanisms Linking Periodontitis to Atherosclerotic Diseases - Oral Infection and Lipid Metabolism -

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Coronary heart disease (CHD) is the leading cause of death in Japan and other developed countries. The major pathway underlying CHD pathology is atherosclerosis. Several epidemiological studies have suggested a link between atherosclerosis and infection/inflammation such as *Chlamydia pneumoniae* infection, *Helicobacter pylori infection,* and periodontitis.

Our project adopted as Periodontal Medicine project in Niigata University Transdisciplinary Research aimed to reveal underlying mechanisms linking periodontitis to atherosclerotic diseases, which we believe gives us opportunity to develop novel clinical markers reflecting periodontal status in terms of increasing progression of atherosclerosis or considering risk of atherothrombotic vascular event. Using animal model has an advantage to address dissecting mechanisms of the pathogenesis linking two diseases. Several studies have suggested that certain periodontopathic bacteria accelerate atherogenesis in mouse model. However, the results varied except for accelerating atherogenesis due to the study design such as infection method, infection period and genetic background. In fact, the mechanisms linking cholesterol accumulation to periodontal infection-induced inflammation are largely unknown.

To address these issues, we orally infected both C57BL/6 and hyperlipidemic C57BL/6.KOR-*Apoe*^{shl} (B6.Apoeshl) mice with *Porphyromonas gingivalis* and evaluated atherogenesis, gene expression in the aorta and liver and systemic inflammatory and lipid profiles in the blood. The changes in the expression of key genes involved in cholesterol turnover, such as liver X receptor and ATP-binding cassette A1 were observed by *P. gingivalis* infection. *P. gingivalis* infection had a potential role of shifting genes targeting the expression of LDL receptor, which resulted in the disturbance of regulatory mechanisms of the cholesterol level *in vitro* study.

Our animal study indicated periodontal infection itself does not cause atherosclerosis, but it accelerates it by inducing deteriorating lipid metabolism in addition to systemic inflammation and, particularly when underlying hyperlidemia or susceptibility to hyperlipidemia exists, it may contribute to the development of coronary heart disease.

S2B-1

Cathepsin B-dependent Novel IL-1β Production Pathway in Microglia

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Interleukin-1β (IL-1β) promotes various chronic neurological disorders such as dementia and pain. Activated microglia are the main cellular source of IL-1β in the brain. However, it remains unclear why the IL-1β production during acute inflammatory reactions becomes chronic. Production of IL-1β is tightly regulated. Two signals mediated by Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are generally required for the production and secretion of IL-1β. TLRs lead to the transcriptional induction of pro-IL-1β though activation of nuclear factor (NF)-κB. The NLRP3 inflammasome contributes to the conversion of pro-caspase-1 to mature caspase-1.

Recently, we have found that chromogranin A (CGA), a neuroendocrine secretory proteins, can potently induce the production and secretion of IL-1β by microglia in a cathepsin B (CatB), a lysosomal cysteine protease, dependent manner. CGA activated NF-κB to drive pro-IL-1β transcription through the activation of TLR4 and its co-receptor CD14. CGA also bound on the scavenger receptor class A to form phagolysosomes and then increasingly induced expression of CatB. CatB in turn induced proteolytic maturation of pro-caspase-1 in phagolysosomes of microglia without activation of the NLRP3 inflammasome (*Terada et al., 2010; Sun et al., 2012*).

We further explored the roles of CatB in age-dependent impairment of hippocampal long-term potentiation (LTP), which is the cellular basis for learning and memory. Chronic systemic inflammation drives microglia to produce IL-1β (*Wu et al., 2005, 2007, 2008*) and impaired the formation of hippocampal LTP (*Liu et al., 2012*) even in the middle-aged animals. Minocycline, a known microglial activation inhibitor, significantly suppressed microglial production of IL-1β and restored the formation of LTP in the middle-aged animals subjected to systemic inflammation. The age-dependent impairment of LTP was not observed in the aged CatB-/- mice. Furthermore, CatB-/- mice was resistant to the chronic inflammatory pain. Interestingly, CGA could cause chronic pain through induction of CatB-dependent IL-1β production by spinal microglia (*Sun et al., 2012*).

We conclude that CGA is a CatB-dependent endogenous potent IL-1β inducer of microglia. Therefore, the CGA-CatB pathway can induce chronic inflammation, which is associated with age-dependent cognitive deficits and inflammatory pain.

S2B-2

Immune Adaptation in the Central Nervous System in Response to Systemic Infections

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Systemic bacterial infections are a common cause for morbidity and mortality in the elderly, and in particular those with a neurodegenerative disease, but the mechanism underlying these observations remain unclear. Experimental models of neurodegeneration have shown that LPS-induced systemic inflammation increases neuronal damage, a process that is believed to be mediated by activation of primed microglia. The effects of a real systemic bacterial infection on the innate immune cells of healthy, aged and diseased brain are less well described, and therefore, we have investigated the acute and long term effects on microglia and brain vascular endothelial cells in response to an experimentally induced bacterial infection. Young (3m), aged (18m) and mice with a pre-existing neurodegenerative disease, were given a single systemic injection of live *Salmonella typhimurium*. Inflammatory cytokines were measured in serum, spleen and brain, and microglia and endothelial cell phenotypes studied by immunohistochemistry. Serum cytokine levels (i.e. IFNγ, IL-1β, IL-6) peaked at day 7 after infection with *S. typhimurium* and were significantly reduced three weeks post-infection. In contrast, brain cytokine levels (IL-1β, IL-12) in *S. typhimurium* infected mice increased over three weeks, following high circulating IFN-γ levels. Furthermore, systemic bacterial infection was associated with a heightened response to a secondary challenge in the brain, suggesting priming of innate immune cells in the CNS in response to a systemic bacterial infection. Infection of aged mice and mice with a pre-existing neurodegenerative disorder showed increased phenotype changes of microglial cells, increased sickness behaviours, increased metabolic changes and a significant increase of infiltrating CD3+ T cells into the brain parenchyma.

These studies reveal that the innate immune cells in the brain are activated by systemic infections, which may lead to prolonged and damaging cytokine production. This exacerbation of the inflammation and the priming of the brain innate immune cells may have a profound effect on the progression of pre-existing neurodegenerative disease.

S2B-3

Preceding Role of Satellite Glia in Nerve Injury-Induced Microglia Activation and Pain Hypersensitivity

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Increasing evidence supports the notion that spinal cord microglia activation plays a causal role in the development of neuropathic pain after peripheral nerve injury; yet the mechanisms for microglia activation remain elusive. Previously, we have reported that nerve injury-induced microglia activation and subsequent pain hypersensitivity are attenuated in toll-like receptor 2 (TLR2) and Nox2 knockout mice, suggesting an important role of TLR2-Nox2-ROS signaling pathways in the nerve injury-induced spinal cord microglia activation and neuropathic pain. More recently, we found that proinflammatory gene expression and macrophages infiltration in the dorsal root ganglion (DRG) after nerve injury are also reduced in the TLR2 knockout mice. In these mice, the nerve injury-induced spontaneous pain behavior was also compromised. However, macrophages infiltration in the DRG *per se* has minimal effects on the spinal cord microglia activation in the nerve-injured mice suggesting putative role of satellite glial cells (SGC) but not macrophages in the development of neuropathic pain. To address the function SGC in the nerve injury-induced spinal cord microglia activation and pain hypersensitivity, we generated $IKK^{f/f}$/CNPase-Cre mice, in which IKK/NF-kB-dependent SGC activation is abrogated. In these mice, we found that nerve injury-induced spinal cord microglia activation and pain hypersensitivity was significantly attenuated compared to control mice. These data demonstrate that SGC activation is preceding to the spinal cord microglia activation and pain hypersensitivity due to peripheral nerve injury.

Session 3

Oral Health Science

S3-1

Dental Plaque Development in Adults with No Previous Experience of Dental Caries

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Dental caries is now recognized to be caused by dental plaque microbiota developed into a highly acidogenic community, rather than an emergence of a single or multiple pathogens. To explore a 'healthy' development pattern of plaque bacterial community, we compared an assembly of plaque bacterial community in young adults with and without caries experience. Plaque samples accumulated for 1, 2, 3, 4, 5 and 7 days were collected from 19 subjects (mean age, 23 ± 6 years) using a retrievable hydroxyapatite disk model. In both caries-free and -active subjects, total bacterial amount and microbial diversity steadily increased over time. On the other hand, the bacterial amount in caries-free subjects increased more slowly than caries-active subjects. The early plaque of caries-free subjects exhibited a more diverse community with higher proportions of *Neisseria, Gemella* and TM7 genera incertae sedis compared with caries-active subjects, and the relative abundances of *Granulicatella*, *Actinomyces* and *Veillonella* less markedly increased in the later stage. This study identified a succession pattern of plaque bacterial community in caries-free adults. Our results suggest that inter-individual difference of species arrangement within plaque is associated with susceptibility to dental caries.

S3-2

Metagenomics: An Introduction and Applications to Dentistry

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Metagenomics (sometimes referred to as environmental genomics, ecogenomics or community genomics) is the study of metagenomes, genetic material recovered directly from environmental samples as opposed to that grown from clonal cultures. Living organisms generally do not reside in isolation, but in most cases live in harmony with many other species. Human beings depend on several diverse microbial-rich communities, for example in the intestinal tract and oral cavity, and on the skin, for normal health maintenance. Just as importantly, in the environment, complex microbial communities in soil, water, air, and elsewhere are responsible for maintaining the health of our planet. Only a small fraction of these microbes can be cultivated in the laboratory, so currently very little is known about them. Without understanding this microbial compliment, we cannot truly understand the vast majority of life on earth, or the human genome itself. Identification of the microbial proteins, both known and novel, and hence their functions in these sometimes extreme environments will provide clues for understanding how they work, how the microbes cooperate and compete with one another, how the microbes interact with the host or other organisms, and how we may manipulate them to our advantage in order to improve human health and better manage our environment. The ultimate goal of metagenomic research is to be able to model and predict the behavior and interactions of whole environments of organisms. Eventually we would like to understand how the entire 'meta-system' (host genome, microbes, viruses, other eukaryotes, metabolites, environment, etc.) works and to be able to predict how perturbations in the system impact its various parts. By combining new high-throughput sequencing technologies and next-generation supercomputing capabilities we will finally able to study these complex environments on an enormous scale. I will present an introduction to metagenomics and give some examples of how it is being applied to the field of dentistry and oral health.

S3-3

Microbiome-wide Association Studies (MiWAS) for Hunting down the Obesity Bugs

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Accumulating evidence indicates that gut microbiota may play a pivotal role in onset and progression of obesity and insulin resistance, the core conditions of metabolic syndrome (MetS) via two different but complementary pathways, i.e. regulation of energy metabolism and provocation of chronic inflammation. However, these mechanistic findings are obtained almost exclusively with rodent models. Their relevance to humans still remains a question. It is also controversial whether the obesity-associated changes of gut microbiota happen at broad taxonomic-level or are more relevant with specific phylotypes. Microbiome-wide association studies, MiWAS, try to answer this question by correlating structural variations of gut microbiota with variations of host metabolic phenotypes. The structural variations can be characterized with next-generation sequencing of either the phylogenetic marker gene 16S rRNA or the whole microbiome. Host metabolic phenotypes can be captured as bodyweight, body fat content, insulin sensitivity, pro- or anti-inflammatory markers, and lipo-polysaccharide-binding protein (LBP), etc. Multi-variate statistics are used to identify associations between phylotypes/functional genes and host metabolic parameters. Due to the structural elasticity of gut microbiota, nutritional or drug interventions can be implemented to modulate gut microbiota of a diseased population to achieve improvement of host metabolic phenotypes. Samples collected from the same host before and after the intervention can yield much stronger associations than cross-sectional strategies such as those in classical genome-wide association studies (GWAS). We employed this MiWAS strategy in dietary therapy of morbid obesity/diabetes in humans to show that specific phylotypes which are more relevant with MetS can be identified, isolated and demonstrated to be causatively contributing to MetS development in humans.

Session 4

PhD Student Session

S4-1

Long Term Cryopreserved Dental Pulp Tissues of Exfoliated Deciduous Teeth is a Feasible Stem Cell Resource for Regenerative Medicine

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Human exfoliated deciduous teeth have been considered to be a promising source for stem cell-based regenerative therapy because they contain unique postnatal mesenchymal stem cells (MSCs) capable of multipotency and immunomodulatory function. However preservation technique of the teeth has not been developed for future therapy although it is impractical to isolate stem cells from human exfoliated deciduous teeth (SHED) immediately after exfoliated. In this study, we tested whether that cryopreserved dental pulp tissues of human exfoliated deciduous teeth contain retrievable SHED and compared the therapeutic efficacy of SHED isolated from the cryopreserved tissues (SHED-Cryo) with SHED isolated from the fresh tissues (SHED-Fresh). SHED-Cryo isolated from the cryopreserved dental pulp tissues for over 2 years (25-30 months) owned MSC properties including clonogenicity, self-renew, MSC marker expression, multipotency and *in vivo* tissue regenerative capacity and expressed similar MSC properties of SHED-Fresh. To examine the immunomodulatory functions of SHED-Cryo on human systemic lupus erythematosus (SLE), SHED-Cryo were intravenously transplanted into human SLE model MRL/*lpr* mice. Systemic SHED-Cryo-transplantation improved SLE-like syndrome including short lifespan, elevated autoantibody levels and nephritis-like renal dysfunction. SHED-Cryo amended elevated levels of IL-17-secreting helper T (Th17) cells in MRL/*lpr* mice and directly inhibited the differentiation of Th17 cells under *in vitro* co-culture system. SHED-Cryo expressed similar immunomodulatory efficacy to SHED-Fresh. Furthermore, we examined SHED-Cryo-mediated bone regeneration in calvarial bone-defect model mice. SHED-Cryo implantation was able to repair the defect with substantial bone formation resembled to SHED-Fresh implantation. These data suggest that cryopreservation of deciduous teeth provides a desirable clinical approach for potential therapeutic applications.

S4-2

Proteomic Approach to Understanding Endocytosis of Amelogenin

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Objectives: Emdogain (enamel matrix derivative, EMD) is used to stimulate regeneration of periodontal tissues. However, the exact molecular mechanisms of periodontal regeneration remain largely unknown. In this study, we investigated the proteins bound to amelogenin, the major (>95%) component of EMD, to elucidate the molecules involved in the periodontal tissue regeneration.

Methods: We firstly examined the pattern of cellular internalization of amelogenin by adding rAMEL exogenously to SaOS-2 osteoblastic cells and human PDL cells. Secondary, we screened the proteins bound to amelogenin by GST pull down assay followed by proteomic analysis. Purified GST fusion amelogenin immobilized on glutathione was incubated with the cell lysates. *After extensive washing, bound proteins ware resolved by* using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)*, and protein spots were visualized by CBB staining and silver staining*. To identify proteins, the major spots were excised from gel, and subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. The identified protein was examined by Western blot methods in the SaOS-2 osteoblastic cells.

Results: Confocal microscopy indicated that rAMEL attached to the plasma membrane and internalized in both cell lines. Our proteomic approach showed number of proteins bound to amelogenin. We identified 16 proteins in lysate of *SaOS-2 osteoblastic cells, and 13 proteins in the membrane fraction of human PDL cells.*

Conclusions: We identified new amelogenin binding proteins. Several membrane-associated proteins were identified as amelogenin binding partners. They include endoplasmic reticulum proteins, mitochondrial membrane proteins, and nuclear proteins. We identified Grp78/Bip protein both in cytosolic and membrane-enriched fractions.

S4-3

The Role of Sprouty2 Protein in Osteoblasts and Periodontal Ligament Cells

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Objectives: Sprouty proteins are identified as inhibitors of fibroblast growth factor (FGF) receptor. Specifically Sprouty2 functions as a negative regulator of receptor tyrosine kinases (RTKs) signaling. On the other hand, osteoblasts and PDL (periodontal ligament) cells are critical participants during the periodontal tissue regeneration process. However, the regenerative mechanisms induced by Sprouty2 in osteoblasts and PDL cells remain largely elusive. The purpose of this study was to investigate whether Sprouty2 could be new therapeutic targets for periodontal tissue regeneration.

Methods: MC3T3-E1 osteoblastic cells and human PDL cells were transfected with a control plasmid, or a dominant negative mutant plasmid of Sprouty2. For immunoblot analysis, cells were extracted after growth factor stimulation and proteins were detected by various antibodies. Using MTT assay, growth factor-induced cell proliferation was measured in transfected osteoblasts and PDL cells. A scratch wound healing assay was conducted with transfected PDL cells stimulated by growth factors. In order to evaluate osteoblast differentiation, ALP assay was performed using Sprouty2 dominant negative mutant MC3T3-E1 cells.

Results: Transduction of Sprouty2 mutation enhanced bFGF+EGF-induced ERK activation in MC3T3-E1 cells and PDL cells. In addition, when osteoblastic cells and PDL cells were stimulated with bFGF+EGF, cell proliferation assay revealed that Sprouty2 mutant cells proliferated faster than control cells. Migration activity of bFGF+EGF-stimulated Sprouty2 mutant PDL cells was slightly higher than control cells. ALP activity was enhanced in Sprouty2 mutant MC3T3-E1 cells.

Conclusions: We demonstrated that Sprouty2 dominant negative mutants activate cell proliferation of MC3T3-E1 osteoblastic cells and PDL cells stimulated by bFGF+EGF, and that suppression of Sprouty2 induces cell migration of PDL cells and ALP activity of osteoblasts. In other words, the use of Sprouty2 inhibitors and bFGF+EGF to bony defects may effectively allow alveolar bone to grow.

S4-4

Sprouty2 Controls Osteoblast Proliferation and Differentiation via MAPK and Smad Pathways

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Objectives: Fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) play essential roles for bone formation and osteoblast activity via mitogen-activated-protein-kinase (MAPK) and Smad pathways. Sprouty family is known as intracellular inhibitor of the FGF signaling, and four orthologs of Sprouty have been identified in mammals. Among them, Sprouty2 and Sprouty4 double knockout mice show the abnormal teeth, mandible, and limb. Furthermore, MAPK pathway has been also reported to participate in BMP signaling. In this study, we clarified the role of Sproutys for the proliferation and differentiation of osteoblast.

Materials and Methods: The expression of *Sproutys* was examined by RT-PCR, in human osteoblast like cells (SaOS-2). After Myc-tagged Sprouty2 plasmid was transfected into SaOS-2, the cell proliferation was determined by WST-8 assay. In SaOS-2, each phosphorylation of MAPK and Smad1/5/8 by FGF or BMP-2 stimulation was investigated by western blotting, and the expression of *RUNX2* and *ALP* known as transcription factor for osteoblast differentiation was analyzed by real-time PCR.

Results: mRNA of *Sprouty2* and *Sprouty4* was induced by FGF stimulation in SaOS-2. In terms of the degree of cell proliferation, the number of cells overexpressed with Sprouty2 decreased compared to that of controls. The phosphorylation of MAPK and Smad1/5/8 were downregulated in SaOS-2 transfected with Sprouty2. Furthermore, Sprouty2 suppressed the mRNA expression of *RUNX2* and *ALP*.

Conclusion: These results suggest that Sprouty2 can negatively control osteoblast proliferation and differentiation by downregulating MAPK and Smad pathways, and also suppress the induction of transcription factors for osteoblast differentiation.

S4-5

Cathepsin S Contributes to the Development of Chronic Pain

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Neuropathic pain is a significant clinical problem because two well-established classes of analgesics, opiates and the nonsteroidal anti-inflammatory drugs (NSAIDs), have limited efficacy. The lack of suitable therapy has motivated us to identify novel pharmacological targets for neuropathic pain. It is well known that spinal microglia play essential roles in the induction of neuropathic pain. Cathepsin S (CatS) is a member of cysteine protease family, which is preferentially expressed in cells of mononuclear-phagocytic origin. We may therefore speculate that CatS plays specific roles in the reaction of microglia and/or monocytic cells during the development of neuropathic pain. In the present study, we have thus examined the effects of CatS-deficiency and a specific CatS inhibitor on the development of neuropathic pain.

CatS-deficiency significantly attenuated the development of neuropathic pain and the mean number of Iba1-positive cells accumulated in the dorsal spinal cord after nerve injury. CatS significantly increased in spinal microglia from 1 day after nerve injury. It is generally believed that microglia are involved especially in the induction of neuropathic pain (up to 3 days after nerve injury). However, we found that increase in the cell number of Iba1-positive cells peaked at 14 days. Furthermore, Z-FL-COCHO, a specific inhibitor of CatS, significantly reversed already established neuropathic pain. Therefore, some populations of Iba1-positive cells in the dorsal spinal cord may be originated by infiltrated inflammatory monocytes because CatS is essential for the recruitment of inflammatory monocytes through proteolytic degradation of the basal membrane. To examine this, we conducted immunoblot analyses of cathepsin F (CatF), which is expressed in monocytes/macrophages, but not in microglia. We found the expression of CatF in the spinal cord of wild-type, but not CatS-deficient mice, after nerve injury. Finally, we examined the effect of splenectomy on the development of neuropathic pain, because spleen is a major source of monocyte development after injury. The splenectomy significantly inhibited the development of neuropathic pain.

Our results strongly suggest the prevention of monocyte recruitment by CatS inhibitors can be a useful therapeutic strategy for the pain relief.

S4-6

Zoledronic Acids Enhance Lipopolysaccharide-Stimulated Inflammatory Reaction in Macrophages

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Purpose: Critical side effects of Bisphosphonates (BP) are reported to occur bisphosphonate-related osteonecrosis of the jaw (BRONJ). Recent study reported that the initiation of BRONJ was involved in the innate immunity. The activation of immune cells is induced by inflammation, and excessive inflammation causes tissue destruction. In this study, to examine BRONJ correlate innate immunity, we used the macrophage which is one of the most important cells in the innate immunity, and analysis the effects of BPs on the macrophage.

Material, and Methods: A murine macrophage cell line, RAW264.7 was pretreated with Zoledronic acid (ZOL) which is a nitrogen-containing bisphosphonate, and analyzed the expression of Toll-like receptor 4 (TLR4). To determine the effects of inflammatory mediators, inflammatory cytokines expression was investigated by real-time PCR and nitric oxide (NO) production by Griess methods. The activation of IκB-α as an intracellular signaling molecule was examined by Western blot analysis. LPS-induced apoptosis was measured by flow cytometry.

Result: The TLR4 expression showed no significant difference by pretreated with ZOL compared with the control. On the other hand, cytokines and NO production were increased by pretreated with ZOL. Furthermore, ZOL induced the activation of NF-κB through the enhancement of IκB-α degradation. LPS-induced apoptosis was increased by pretreated with ZOL.

Conclusion: These results suggest that ZOL induce the activation of innate immunity, and the bone destruction found in BRONJ may be caused by the excessive production of inflammatory cytokines.

S4-7

Roles of the Bone-Derived Osteocalcin as a GLP-1 Secretagogue

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It has recently been reported that bone is an endocrine organ, since osteocalcin (OC) produced by osteoblasts is revealed to trigger the secretion of insulin; OC in uncarboxylated form (ucOC) but not carboxylated GlaOC acts as a potent insulin secretagogue, regulating energy metabolism by increasing energy expenditure and insulin sensitivity in the target organs. Incretin hormone glucagon-like peptide 1 (GLP-1) is a gastrointestinal hormone secreted by enteroendocrine L cells in the small intestine, and has a numerous physiological actions including potentiation of glucose stimulated insulin secretion, enhancement of β-cell growth and survival.

We here found that mouse small intestine and mouse enteroendocrine cell line STC-1 cells expressed Gprc6a, a putative ucOC receptor. Serum GLP-1 was increased by ucOC at both intraperitoneal and oral applications, but such an effect was not observed with GlaOC. STC-1 cells also responded to ucOC to secrete GLP-1 into the culture medium. GlaOC was effective only in the case of oral administration. Moreover, serum insulin level was increased in response to ucOC application, which was inhibited by GLP-1 receptor antagonist exendin(9-39), indicating that the insulin-releasing effect of ucOC is in part attributed to the action of GLP-1 released from the gut. These findings indicate that ucOC acts through its receptor in the small intestine, triggering secretions of GLP-1 and insulin.

S4-8

Swallowing Action May Attenuate Fentanyl-Induced Cough

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Aim: Intravenous fentanyl often induces cough during induction of anesthesia. This fentanyl-induced cough is not always benign, and sometimes spasmodic and/or explosive. But the mechanism is still not clear. We found swallowing action, when it was asked at induction of anesthesia to reduce pharyngeal discharge for facilitating laryngoscopy, attenuated that fentanyl-induced cough. In the current study, we investigated the relation between occurrence of fentanyl-induced cough and swallowing action retrospectively.

Method: We checked the anesthesia records of patients, 21-60 year-old, ASA-PS I or II, who underwent head and neck surgery from October 2010 to May 2012. Subjects were selected on condition that their anesthesia was induced with intravenous fentanyl first. Additionally, they were divided into two groups, swallowing action (S) when fentanyl was administered and no swallowing action (non-S). Patient's background, dose of fentanyl and occurrence of cough were investigated from their records. The data were compared between the groups using Mann-Whitney U-test or Chi square test. A p-value less than 0.05 were considered as the level of statistical significance.

Result: Forty-eight patients were suitable for this study. There were no significant differences in their background and dose/weight of fentanyl between S group (n=23) and non-S group (n=25). In S group, 4 of 23 patients (17.4%) had cough, while 13 of 25 patients (52.0%) had cough in non-S group. The occurrence of fentanyl-induced cough was significantly less in S group (p=0.027). This swallowing action was a simple and effective method to reduce fentanyl induced cough.

Conclusion: Swallowing action, when fentanyl is administered intravenously, may be useful to avoid cough during induction of anesthesia.

Key Words: fentanyl-induced cough, swallowing action

S4-9

Increasing Mechanical Strength of Carbonate Apatite Block by Ca Salt Introduction

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Carbonate apatite (CO_3Ap) is an ideal bone substitute as it has excellent osteoconductivity and bioresorbability. Research has shown that CO_3Ap block can be fabricated using calcium carbonate (calcite: $CaCO_3$) block as a precursor, based on the dissolution-precipitation reaction, and the mechanical strength of CO_3Ap block was increased by introduction of calcium lactate. However, the resultant CO_3Ap block still needs improvement, as higher mechanical strength is desirable for clinical use in load-bearing sites. The objectives of this study were to investigate the feasibility of using calcium acetate in the Ca salt introduction method and examine the effect of reducing porosity, by the addition of extra CO_3Ap crystals, on the mechanical strength of CO_3Ap block. Calcium acetate (1 M) was introduced into the micropores of CO_3Ap block under vacuum at room temperature. Next, the CO_3Ap block was exposed to carbon dioxide (CO_2) gas under atmospheric pressure for 14 days to carbonate the calcium salt to calcite. Finally, the CO_3Ap block was immersed in 1 M disodium hydrogen phosphate (Na_2HPO_4) aqueous solution at 60°C for 14 days to transform the calcite into CO_3Ap. Results suggest that the Ca salt introduction method enables fabrication of reinforced CO_3Ap block with greater mechanical strength than the original CO_3Ap block and sufficient strength to be a promising bone substitute in load-bearing areas.

S4-10

Hydrothermal Conversion of Calcite Foam to Carbonate Apatite

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We have previously reported an inverse ceramic foam method aiming to improve mechanical strength of calcite foam that has interconnected porous structure. The modified foam was obtained by multiple polyurethane coating. In previous study, calcium hydroxide ($Ca(OH)_2$) slurry was poured into the foam. After drying, the foam was burnt out to remove polyurethane and to carbonate $Ca(OH)_2$ under mixed O_2-CO_2 atmosphere. $Ca(OH)_2$ was completely converted to calcite. Those calcite foams are the useful precursors for the preparation of the three-dimensional (3D) interconnected porous carbonate apatite (CO_3Ap) foam. Therefore, we investigated the hydrothermal treatment of calcite foam to CO_3Ap foam in phosphate solution. The results indicated that the conversion ratio depends on the degree of porosity of calcite precursor. The CO_3Ap foam showed the increase in the compressive strength compared to conventional method. So, it is concluded that CO_3Ap foam fabricated in this study could be a good candidate for the bone replacement material in clinical application.