Abstracts of the 21st Nippon Medical School Foundation
Academic Meeting for Foreign Researchers

Date: 22nd January, 2011
Hours: 10:30 A.M.-4:00 P.M.
Place: Nippon Medical School Kitsuou Hall
Administration: Foreign Researchers enrolled in Nippon Medical School Foundation

1. Clinical experience and basic research in ophthalmology

You Zhou¹, Tsutomu Igarashi¹², Koichi Miyake², Nagisa Asakawa¹², Noriko Miyake², Takashi Shimada¹ and Hiroshi Takahashi¹

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I have been in Japan for more than 6 months, during which time I have gained clinical experience and carried out laboratory work in the Department of Ophthalmology. Clinical experience included training in the diagnosis and treatment of corneal diseases, cataracts, glaucoma, retinal diseases, and various other ophthalmic diseases. This experience will be invaluable when I return to China to work as an ophthalmologist. Our Department employs ocular gene therapy with the use of anti-neovascularization factors to target retinal and choroidal neovascular diseases, such as diabetic retinopathy and aged macular degeneration (AMD). Recently, we showed that adeno-associated virus (AAV) vector type 8 is useful in ocular gene therapy. AAV is capable of transferring genes to non-pathogenic, low-immunity dividing and non-dividing cells. I have tried to create AAV type 8 vectors encoding small interfering RNA (siRNA) of vascular endothelial growth factor (VEGF) to inhibit retinal neovascularization. VEGF levels are increased in retinal pigment epithelium (RPE) cells in patients with AMD. My aim is to inhibit the expression of VEGF in human RPE cell line by using AAV type 8 vectors encoding siRNA of VEGF.

2. An investigation into the mechanisms underlying the heterogeneity of secretinergic modulation of GABAergic synaptic transmission in the cerebellar cortex

Ying Zhang, Fumihito Saito and Hidenori Suzuki
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The polypeptide secretin was first identified as a chemical messenger in the pancreas in 1902, but there is now increasing interest in the role it plays in the central nervous system rather than the gastrointestinal system. It has been reported that secretin facilitates GABAergic transmission onto
Purkinje cells in the cerebellar cortex. However, using whole-cell patch-clamp recording, we observed heterogeneity of secretinergic modulation of stimulation-evoked inhibitory postsynaptic currents (eIPSCs). In fact, secretin did not induce any obvious potentiation of IPSC amplitude in approximately 24% of the cells. Therefore, we further explored the nature of the heterogeneity of secretinergic modulation using cerebellar slices prepared from young rats. First, we examined the effects of secretin under the blocking Gi-protein-coupled receptors (GABA_B, A_1 and CB_1 receptors), because secretin may induce activation of these receptors via direct or indirect pathways. Then we assessed the possibility of postsynaptic GABA_A receptor saturation by reducing the probability of GABA release or by blocking GABA binding with bicuculline. However, we still observed heterogeneity. These results suggest that the heterogeneity of secretinergic modulation is not due to the presynaptic activation of Gi-protein-coupled receptors or postsynaptic GABA_A receptor saturation.

3. **Bovine viral diarrhea virus quasispecies detected in an RK13 cell line originating in a rabbit kidney**

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   Bovine viral diarrhea virus (BVDV) is a species in the genus Pestivirus of the family Flavivirus. It is ubiquitous worldwide, and is one of the cattle industry’s most significant sources of financial loss. As BVDV infection generally causes viremia in cattle, most sera from infected animals are contaminated with BVDV. Therefore, researchers have studied BVDV contamination in various cell lines. This study concerns the characterization of BVDV detected adventitiously in an RK13 cell line (ATCC) originating in a rabbit kidney. Detection and titration of non-cytopathogenic BVDV are generally done by enzyme immunostaining, immunofluorescent staining, or the exaltation of Newcastle disease virus (END) method. We isolated the END-positive virus (END⁺ virus), the major viral population in the RK13 cell line; we then isolated the END-negative virus (END⁻ virus), the minor viral population, using the reverse plaque formation method, which depends on the complete resistance of END⁺ virus-infected cells to superinfection by vesicular stomatitis virus. We named these the RK13/END⁺ virus and the RK13/END⁻ virus, respectively. The RK13/END⁺ virus did not interfere against heterologous viruses such as vesicular stomatitis virus and Newcastle disease virus, whereas the RK13/END⁻ virus stably interfered against such heterologous viruses. The RK13/END⁺ and RK13/END⁻ viruses propagated in different cell cultures (MDBK-SY, BT, FS-L3, CPK-NS, and primary rabbit kidney). Phylogenetic trees based on 5'UTR sequences showed that both strains are located in the BVDV-1b branch, and are closely related to the CP7 strain. These results show that BVDV isolated from the RK13 cell line is a quasispecies consisting of at least two viruses showing both END-positive and END-negative characteristics. The RK13/END⁻ virus is a unique BVD virus which shows intrinsic interference. We intend to analyze interference between the RK13/END⁺ and RK13/END⁻ viruses.

4. **ACELLULAR DERMAL MATRIX SEEDED WITH ADIPOSE STEM CELLS AS A SOFT TISSUE FILLER**

   Hakan Orbay¹, Yoshihiro Takami¹, Kyoko Kobe¹, Hiroshi Mizuno² and
This study was designed to test the efficacy of acellular dermal matrix (ADM) seeded with adipose derived stem cells (ASCs) as a soft tissue filler. 20 male Fischer rats weighing 250-300 g were used for the study. Soft tissue augmentation in a left dorsal a rectangular area was attempted either with ADM (group I), ADM seeded with ASCs (group II), ASCs mixed with collagen gel (group III), collagen gel (group IV). ADM was obtained from the dorsal skin of rats by an acellularization process. ASCs were harvested from the inguinal fat pads of rats. Adhesion of ASCs onto the ADM scaffold was confirmed before implantation. Histological analysis was carried at post-operative 8 weeks. No cells could be detected in ADM before seeding but after seeding frozen sections and SEM revealed attached cells (average; 0.71x10^6 cells/cm^2) on the papillary dermis surface of ADM. Absorption rate was significantly higher in group I in comparison with group II. Thickness of the subpanniculus fibrotic tissue layer, where ADM were placed, and vascular density were both highest in group II. In conclusion, ADM seeded with ASCs might be a useful tool for soft tissue augmentation.

5. Biological Effects of Cellular Stretch on Human Dermal Fibroblasts

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**Purpose:** The development and progression of scars are determined by mechanical forces that are covered in current algorithms for the treatment and prevention of scars. However, the causes and effects of external mechanics in scar fibroblasts have not been clarified. The purpose of this study was to explore the biological effects of cellular stretch on human dermal fibroblasts and the mechanisms behind these effects.

**Methods:** Human dermal fibroblasts seeded onto a silicon chamber were subjected to periodic stretch-relaxation stimulation for 24 hours; control fibroblasts underwent identical procedures except for the stretching (n=3). Finite element analysis was performed to calculate the extension rate. Dynamic images were taken at 15-min intervals under a time-lapse microscope for 24 hours, after which morphological evaluations were carried out. Microarray analysis and real-time polymerase chain reaction evaluations were also performed to assess changes in related gene expression.

**Results:** Extension rate of the stretching in silicon chambers is 120%. Compared with the controls, the stress-loaded fibroblasts tended to align perpendicularly to the direction of stretch; cellular migration and intercellular contacts increased, and cellular proliferation decreased. Various gene expressions including TGF-β were changed by the cellular stretch.

**Conclusions:** Understanding of the responses of fibroblasts to mechanically induced morphological
changes and the resulting changes in gene expression provide new perspectives on the mechanisms of scar development. These perspectives may lead to new treatments.

6. Effects of the *Fusarium* mycotoxin deoxynivalenol on *in vitro* rumen fermentation

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The objective of this study was to determine the effects of deoxynivalenol (DON) on *in vitro* rumen fermentation in cattle by assessing pH, ammonia-N, total gas, volatile fatty acid (VFA) production, and DON degradation with two different carbon sources (corn starch or cellulose). Fifty milliliters of ruminal fluid:buffer (1:1) was incubated for 6 hours with each of the following: 1.5 g corn starch; 1.5 g corn starch + DON at 40 mg/kg dry matter (DM); 1.5 g cellulose; and 1.5 g cellulose + DON at 40 mg/kg DM. The different carbon sources markedly influenced all rumen fermentation parameters ($P<0.05$); DON had a negative impact on certain aspects of rumen fermentation capacity, such as ammonia-N and total gas production, with acetate and propionate production in particular tending to be reduced ($P<0.01$). It may be possible that some carbon sources can influence and limit the effects of DON. The DON degradation rate was clearly affected by the carbon source ($P=0.0105$), with cellulose leading to a higher rate of DON degradation than corn starch, indicating that the concentrate/forage ratio in the diet may have an effect on DON degradation in the rumen.

7. Laparoscopic and Thoracoscopic Surgery for Esophageal Diseases

Kyaw Htet, Tsutomu Nomura,
Hiroshi Makino, Nobutoshi Hagiwara,
Masao Miyashita and Eiji Uchida
Department of Surgery, Nippon Medical School

In Nippon Medical School’s Department of Gastrointestinal Surgery, patients with esophageal achalasia, hiatal hernias, and esophageal cancer are treated with laparoscopic and thoracoscopic surgery as follows:

1) In patients with esophageal achalasia, a laparoscopic procedure is used to cut the thickened esophageal muscles (Heller’s myotomy), and Dor fundoplication is performed to prevent reflux esophagitis after the operation.
2) A laparoscopic procedure is used in patients with hiatal hernias to return the stomach into the abdominal cavity; crural repair and Nissen or Toupet fundoplication are performed.
3) Patients with esophageal cancer undergo a thoracoscopic procedure in the prone position.
involving esophagectomy and lymph node dissection; a laparoscopic procedure is then used to perform gastric conduit formation and intra-abdominal lymph node dissection with the patients in the supine position.

In this presentation, the present status of laparoscopic and thoracoscopic surgery for esophageal diseases in Japan will be discussed, the clinical features of patients and the various laparoscopic and thoracoscopic procedures will be described, a video of operations by the staff of the Department of Gastrointestinal Surgery will be shown, and the clinical outcomes will be evaluated.

8. Endoscopic submucosal dissection for esophageal and gastric neoplasms

Zhang Li, Tsutomu Nomura, Nobuyuki Sakurazawa and Eiji Uchida
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Endoscopic submucosal dissection (ESD) is an advanced technique of therapeutic endoscopy for superficial gastrointestinal neoplasms. In Nippon Medical School’s Department of Surgery 1, ESD has been widely applied in the treatment of early-stage gastric and esophageal carcinomas. Chromoscopy and magnification endoscopy with narrow band imaging (NBI) are promising modalities to evaluate the stage of cancer and possibility of nodal metastases. ESD consists of three steps: 1) injecting fluid into the submucosa to elevate the lesion; 2) cutting the mucosa surrounding the lesion; 3) dissecting the submucosa beneath the lesion. In this presentation, the current status of ESD will be discussed, a novel technique invented by Dr. Sakurazawa and known as spring-assisted ESD will be introduced, and clinical data on patients who have undergone ESD over the last 6 months in the Department of Surgery 1, including demographic data, clinical features, and histopathological evaluations of the resected specimens, will be reviewed.

9. Apurinic apyrimidinic endonuclease-1 (APE-1) is regulated by NF-kB signaling pathway in esophageal cancer

Song Junmin1, Seiji Futagami1, Tetsuro Kawagoe1, Mayumi Shimpuku1, Hiroyuki Nagoya1, Akane Horie1, Tomotaka Shindo1, Nobue Ueki1, Masafumi Kusunoki1, Kazumasa Miyake1, Katsuhiko Iwakiri1, Hiroshi Makino1, Masao Miyashita2, Yoshio Hoshihara4, Shinichi Tsuchiya2 and Choitsu Sakamoto1

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4Ministry of Economy, Trade and Industry Clinic
**AIMS:** Our study was designed to investigate the functions of NF-kB in human esophageal cancer and to ascertain whether it interacts with apurinic apyrimidinic endonuclease-1 (APE-1) and cyclooxygenase-2 (COX-2).

**METHODS:** The expressions of APE-1, COX-2, P65, and monocyte chemoattractant protein-1 (MCP-1) were evaluated by immunohistochemistry in 74 esophageal cancer tissues. Western blotting and real-time PCR were performed to detect the protein and mRNA expressions of APE-1, COX-2 and P65 in MCP-1 stimulated with or without MG-132 pretreated esophageal squamous carcinoma cell lines (KYSE220).

**RESULTS:** In human esophageal cancer tissues, positive expression of APE-1 was localized in nuclear pattern, while positive expression of MCP-1, COX-2 and P65 was localized in cytoplasm pattern of cancer cells. In addition, APE-1 and COX-2 expression levels in the study group with high expression of MCP-1 protein were significantly increased as compared to that higher than those in the low expression group. In vitro study, MCP-1 stimulation significantly increased the protein and mRNA expressions of APE-1, which was in turn reduced by MG-132, a inhibitor of NF-kappa B.

**CONCLUSION:** APE-1 is regulated by the NF-kB pathway in human esophageal cancer tissues and cell lines. The clarification of the role of NF-kB in human esophageal cancer may lead to new treatment modalities for this cancer.

**10. Muscle-directed systemic gene therapy for pancreatic cancer using adeno-associated virus vector (type 8)-mediated MDA7/IL-24**

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Melanoma differentiation-associated gene-7/interleukin-24 (MDA7/IL-24) selectively induces apoptosis in cancer cells and exerts immunomodulatory and anti-angiogenic effects, as well as potent anti-tumor bystander effects, making it an ideal candidate as a new anticancer gene therapy. To examine the feasibility of adeno-associated virus (AAV) vectors expressing MDA7/IL-24 in systemic cancer gene therapy for pancreatic cancer, we generated an AAV type 8 vector expressing MDA7/IL-24 (AAV8-IL24). Previous *in vitro* studies showed that medium conditioned by AAV-IL24-transduced C2C12 cells induces tumor cell-specific apoptosis against the hamster pancreas cancer cell line (PGHAM-1). We are now conducting *in vitro* experiments to investigate whether gemcitabine induces tumor cell-specific apoptosis against PGHAM-1. To assess the *in vivo* effects of muscle-targeted AAV-mediated systemic delivery of MDA7/IL-24 in a normal hamster model, we injected AAV8-IL24 (1.4x10¹⁵ vg/body) into the quadriceps muscle. ELISA analysis showed increasing levels of IL-24 in plasma over a 4-week period, reaching a peak (739.1±244.7 ng/ml) 4 weeks after injection. To assess the *in vivo* effects of MDA7/IL-24 in a pancreatic cancer hamster model, we are establishing a model in which PGHAM-1 cells expressing the luciferase gene are inoculated into the hamster pancreas. After a single injection of AAV8-IL24 (1.4x10¹⁵ vg/body) into the quadriceps muscle, tumor cell growth will be monitored by IVIS. ELISA analysis will be done for the level of IL-24. We will investigate *in vivo* apoptosis with TUNEL assay and any anti-angiogenic effects with immunohistochemistry. Effects on survival will also be investigated.
11. Influence of FASN and SCD polymorphisms on fatty acid composition and melting point in various adipose tissues of Korean Native cattle (Hanwoo)

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The objective of this study was to determine whether polymorphisms of fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD) genes have any association with the fatty acid composition or fat-melting point (MP) of 4 different adipose tissues (coelomic [CL], perirenal [PR], intramuscular [IM] and subcutaneous [SC]) from Hanwoo steers (n=44). We applied a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method to determine the FASN and SCD genotypes based on their polymorphisms. No significant differences in FASN genotypes were observed in total saturated fatty acids (SFAs) or unsaturated fatty acids (UFAs) from any of the adipose tissues (P > 0.05). The UFA and monounsaturated fatty acid (MUFA) was trend to higher with SCD AA genotype in CL, PR, and IM except for SC (P > 0.05). MP varied significantly between genotypes, indicating an increase in the proportion of SFA and decrease in UFA or MUFA, especially in IM adipose tissue (P < 0.05). The allele frequencies were little biased the allele G or V, indicating that frequencies of genotype AA were as low as 0.20 and 0.11 in the FASN and SCD genes, respectively. Overall, although the genotypes of neither gene were significantly correlated to the fatty acid composition, they were related to the genetic advantage in MP. These results suggest that single nucleotide polymorphisms in the FASN and SCD genes may be useful markers to improve the beef quality of Hanwoo.

12. Study on the Genetic Diversity of Mongolian Native White Horses

The polymorphisms of white coat color genes and their effects on whitening or graying

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We have previously reported that the major coat color genes of the Mongolian native white horse are the melanocortin 1 receptor (MC1R) and agouti signaling protein (ASIP), but that the expression of these genes can be inhibited by the white coat color genes. In this report, we discuss the polymorphisms of the syntaxin 17 (STX17), membrane-associated transporter protein (MATP), tobiano (To), sabino (SB1) and c-KIT genes and their effects on the whitening or the graying of the Mongolian native white horse coat.

Genomic DNA samples from 50 Mongolian native white horses were used for polymerase chain reaction (PCR) of the STX17, MATP, To, SB1 and c-KIT genes.
Genotyping of five white coat color related-genes was performed by PCR-restriction fragment length polymorphism, PCR-amplified fragment length polymorphism and PCR-direct sequencing methods.

The genotype frequencies of GG and Gg were high in STX17 gene, which is related to graying; the G gene frequency was 0.69. C<sup>c</sup>C<sup>cr</sup> and C<sup>cr</sup>C<sup>cr</sup> mutant-types were detected in the MATP gene, which is associated with coat color dilution; the C<sup>cr</sup> gene frequency was 0.2. In the To gene, which is related to coat spot patterns, Toto mutant-type was detected in four horses. However, no genotype variation was recognized in SB1 gene.

In the c-KIT gene, which promotes the growth of melanocytes, a single nucleotide polymorphism was found in a non-synonymous substitution region in Exon 14. The Mongolian native white horses studied included a cremello, a palomino and a dun. Coat whitening in these horses was recognized as being influenced by MATP genes.

Analysis of the genetic variations in the white coat-related genes in Mongolian native white horses, confirmed that graying of the coat color is caused early in a horse’s life by the STX17 gene. Analysis also showed that the MATP and a c-KIT genes play a simultaneous role in whitening. We believe this causes increased inhibition of melanocytes or secretion of the enzyme for melanin synthesis, and that consequently the whitening process begins when the horses are still foals.


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Introduction: Personalized genetic medicine is the most effective strategy in anticancer chemotherapy. The effectiveness and toxicity of the drugs used differ from person to person according to their genotype or single nucleotide polymorphisms (SNPs). SNPs can help predict an individual’s response to drugs, a matter that has been studied and applied clinically in some countries such as Japan and the USA, but no studies on SNP frequency in the Mongolian population have been reported to date. Several SNPs in the dihydropyrimidine dehydrogenase (DPD), thymidylate synthase (TS) and methylenetetrahydrofolate reductase (MTHFR) genes are associated with 5-fluorouracil treatment outcome and toxicity in patients with esophageal or colorectal cancer. In the MTHFR gene, two common functional polymorphisms are known to be associated with decreased activity of MTHFR.

Purpose: To establish methods for genotyping SNPs, and to examine SNP frequencies in relation to anticancer chemotherapy in the Mongolian population.

Methods: We want to establish a method for genotyping SNPs in the MTHFR gene (rs1801133 and rs1801131) using high-resolution melting curve analysis (hrMCA) and small amplicon genotyping (SAG). We are awaiting ethical approval from the government of Mongolia, Nippon Medical School, and the Mongolian National Cancer Center.

Present results: We used hrMCA and SAG to genotype SNPs, successfully differentiating SNP haplotypes (wild homo, SNP hetero and SNP homo).

Further plan: After receiving ethical approval, we will expand our research to compare clinical outcomes and toxicities with SNP frequency in a large sample Mongolian population.

14. Use of High-Resolution Melting Curve Analysis of Genomic DNA to Screen for COL3A1 Mutations in Ehlers-Danlos Syndrome, Vascular Type

Banyar Than Naing, Atsushi Watanabe and Takashi Shimada
Ehlers-Danlos syndrome, vascular type (vEDS) (MIM #130050) is an autosomal dominant disorder caused by heterozygous mutations of the type III procollagen gene (*COL3A1*). Most *COL3A1* mutations are detected in total RNA from patient-derived fibroblasts, which requires invasive skin biopsy. High-resolution melting curve analysis (hrMCA) has recently been developed as a post-polymerase chain reaction (PCR) mutation scanning method that enables simple, rapid, cost-effective, and highly sensitive mutation screening of large genes. We applied hrMCA to screen the entire coding region of *COL3A1*, using genomic DNA extracted from peripheral blood. PCR primer pairs for *COL3A1* (52 amplicons) covered all coding regions of 52 exons, including splicing sites. Eight known *COL3A1* mutations for validation samples and other new mutations were successfully detected with hrMCA. In addition, two nonsense mutations of *COL3A1* that were not found using the total RNA method were identified. Furthermore, we established a small amplicon genotyping (SAG) method for detecting three high frequent coding-region single nucleotide polymorphisms (SNPs) (rs1800255:G>A, rs1801184:T>C, and rs2271683:A>G) in *COL3A1* mutation screening to differentiate mutations before sequencing. We were also able to predict nonsense mutations by checking the sequence discrepancy between genomic DNA and cDNA at these coding-region SNPs. Thus, our combined method of hrMCA and SAG using genomic DNA enables the detection of *COL3A1* mutations with high efficiency and specificity. We are now applying hrMCA to screen for *COL3A1* mutations in patients with suspected vEDS using genomic DNA, and we are looking forward to applying the method to screen for mutations of other genes.