【ENGLISH】

Summary of Research Project Results

(Projects launched in JFY2000)

Japan Society for the Promotion of Science (JSPS)

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Systematic Genetic Study of Asthma and Atopic Dermatitis Based on the Genome-wide SNP Informatics

Project Leader:
Ituro INOUE Visiting Associate Professor,
The Institute of Medical Science, The University of Tokyo

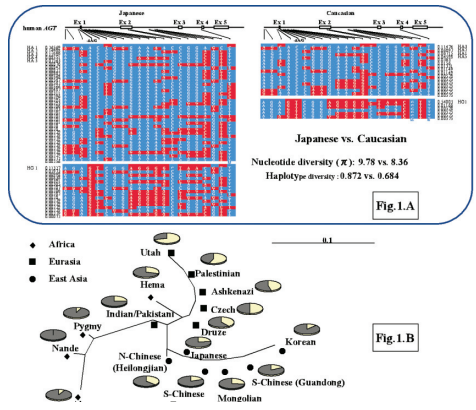
1. Objective

Asthma is a complex syndrome with many clinical phenotypes in both adults and children. Both genetic and environmental factors equally play important roles in the etiology of asthma. Identification of disease gene has been recognized as a core task to understand the etiology of the disease. However, genetic study of complex diseases has been hampered by a number of factors, including lack of knowledge on the appropriate candidate genes together with their sequences and insufficient information on sequence variations to evaluate allelic association with the disease. Over the past five years, human genome sequence is accomplished and a large number of SNPs in the genome is available. Also taken the advantage of high throughput genotyping technology, we screened asthmatic patients to identify genes and genetic variations related to asthma and related diseases.

2. Summary

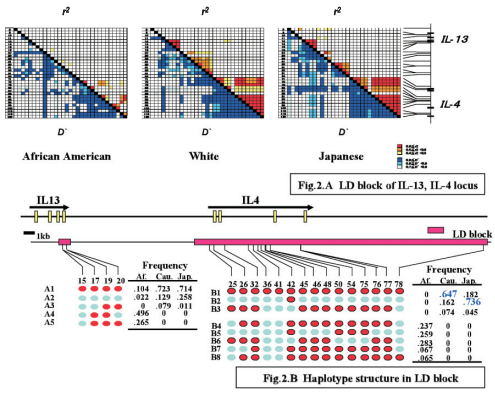
2-1. SNPs for linkage disequilibrium study and population history :

To better understand the genetic variation between populations, we investigated the roles of population history and natural selection in shaping patterns of genetic diversity in angiotensinogen (*AGT*), by sequencing the entire *AGT* gene (14,400bp) in 736 chromosomes from Africa, Asia and Europe. An analysis of allele frequencies confirmed that the A-6 and the associated T235 variant are present at substantially higher frequencies in African populations than in non-African populations, as suggested previously. In addition, haplotypes carrying the M235 variant showed elevated levels of linkage disequilibrium, suggesting that they have risen to high frequency recently. Departures from neutral expectation tested by Tajima's *D*, Fu and Li's *D** and *F**, and Fay and Wu's *H* tests in some, but not all, regions of the *AGT* gene indicate that patterns of diversity in the gene cannot be accounted for by human population history, which would affect all regions equally. Taken together, patterns of genetic diversity in *AGT* suggest that natural selection has favored the M235 variant over the T235 variant in some populations. (Fig.1A, B)



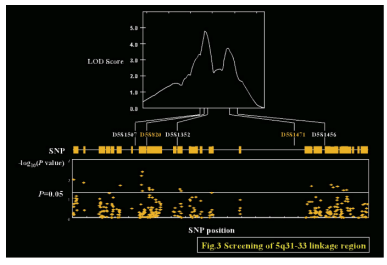
2-2. Haplotype structure of IL-4, IL-13 locus :

A 25.6 kb region at chromosomal location 5q31, covering the entire human interleukin 13 (*IL-13*) and interleukin 4 (*IL-4*) genes has been reported to be associated with bronchial asthma. We have examined nucleotide variation at this locus in African, European American, and Japanese populations, using 120 diallelic variants. A block of strong linkage disequilibrium ($|D'| > 0.7$) spans a 10 kb region containing *IL-4* in European American and Japanese populations, and is present but less clear in African samples. Two major haplotypes at *IL-4*, account for >80% of haplotypes in Europeans Americans and Japanese. These haplotypes are quite diverged from each other and the ancestral haplotype, and tests of neutrality show that these haplotypes appear to have been maintained by balancing selection. The most common haplotype in the European American population is much less common in the Japanese population, and vice versa. The evidence of balancing selection at *IL-4* suggests that *IL-4* may account for some genetic variance underlying susceptibility to asthma and other allergic diseases, while the strong LD observed in the *IL-4* region may allow more efficient disease-association studies using this locus. (Fig2A, B)



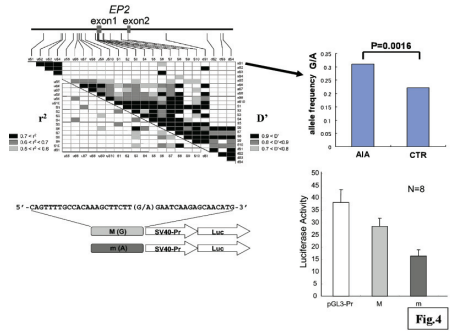
2-3. Positional cloning of gene responsible for atopic asthma :

In the genome-wide linkage search with 47 Japanese families with atopic asthma, evidence of linkage was detected on chromosome 5q31-33 (maximum lod score = 4.8). The linkage region spanning 20 cM was screened with 500 SNPs for susceptibility to asthma. For the first screening, the asthma patients with family history or strong mite-sensitive (RAST > 4). Several SNPs showed association with asthma ($P < 0.05$) and the SNPs were subjected to further study to identify the susceptibility. Genes identified are under investigation for functional properties. (Fig.3)



2-4. Genetic study of aspirin-intolerant asthma :

Aspirin-intolerant asthma (AIA) is a subtype of bronchial asthma characterized by development of bronchoconstriction evoked by nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the cyclooxygenase pathway, leading to enhancement of the lipoxygenase pathway. We evaluated allelic association of 370 single nucleotide polymorphisms (SNPs) of 63 candidate genes, mostly from the arachidonic acid metabolic cascade, with AIA. After two rounds of screening with 198 AIA patients, multiple SNPs in the prostaglandin E_2 receptor subtype 2 (EP2) gene were associated with AIA ($P < 0.05$). Among the 77 SNPs identified in the EP2 gene, we selected 17 SNPs based on linkage disequilibrium and allelic frequencies (minor allele frequency > 0.1) for further association study. SNPs in the promoter region of the EP2 gene, uS5, uS5b, and uS7, were significantly associated with AIA (permutation P = 0.039-0.001). The most significantly associated SNP, uS5, was located in the regulatory region of the EP2 gene (AIA 31.1% vs. control 22.1% (permutation P = 0.0016) or vs. ATA 22.2% (permutation P = 0.0017)). In *in vitro* reporter assay in HCT116 cells, the site containing the uS5 allele showed reduced transcription activity. Taken together, these results suggest that uS5 allele serves as a target of a transcription repressor protein. A functional SNP of the EP2 gene associated with risk of AIA should decrease the transcription level, resulting in reduction of the PGE_2 braking mechanism of inflammation and involvement in the molecular mechanism underlying AIA.(Fig.4)



3. Primary Publications

(1) Nakajima T, Wooding S, Satta Y, Jinnai N, Goto S, Hayasaka I, Saitou N, Guan-Jun J, Tokunaga K, Jorde LB, Emi M, Inoue I. Evidence for natural selection in the HAVCR1 gene: high degree of amino-acid variability in the mucin domain of human HAVCR1 protein. *Genes Immun.* 6, 398-406, 2005.

(2) Jinnai N, Sakagami T, Sekigawa T, Kakihara M, Nakajima T, Yoshida K, Goto S, Hasegawa T, Koshino T, Hasegawa Y, Inoue H, Suzuki N, Sano Y, Inoue I. Polymorphisms in the prostaglandin E2 receptor subtype 2 gene confer susceptibility to aspirin-intolerance asthma: a candidate gene approach. *Hum Mol Genet*, 13, 3203-3217, 2004.

(3) Sakagami T, Witherspoon DJ, Nakajima T, Jinnai N, Wooding S, Jorde LB, Hasegawa T, Suzuki E, Gejyo F, Inoue I. Local adaptation and population differentiation at the interleukin 13 and interleukin 4 loci. *Genes Immun*, 5, 389-397, 2004.

(4) Nakajima T, Wooding S, Sakagami T, Emi M, Tokunaga K, Tamiya G, Ishigami T, Umemura S, Munkhbat B, Jin F, Guan-jun J, Hayasaka I, Ishida T, Saito N, Pavelka K, Lalouel J-M, Jorde LB, Inoue I. Natural selection and population history in the human angiotensinogen gene (AGT): 736 AGT sequencing in worldwide chromosomes. *Am J Hum Genet*, 74, 898-916, 2004.

(5) Nakajima T, Jorde LB, Ishigami T, Umemura S, Emi M, Lalouel J-M, Inoue I. Nucleotide diversity and haplotype structure of the human angiotensinogen gene in two populations. *Am J Hum Genet* 70, 108-123, 2002.

Systematic Expression Profile Analysis and its Application to Personalized Medicine

Project Leader:
Yusuke NAKAMURA Professor, The Institute of Medical Science, The University of Tokyo

1. Objective

Through two types of genome-wide approach, expression profile analysis using cDNA microarray and association study using thousands of gene-based SNPs, we attempted to clarify detailed molecular mechanisms causing or susceptible to diseases. Identification of genes of medical and biological importance should impact on development of novel therapeutic drugs as well as diagnostic tools, and contribute to establishment of the personalized medicine.

2. Summary

2-1. Genome-wide expression profile analysis :

We have established our own cDNA microarray system consisting of 32,000 cDNA clones, coupled with laser-microbeam microdissection. Through expression analysis of nearly one thousand clinical cancer tissues as well as various cell lines that were exposed to certain chemicals or were transfected with p53 or beta-catenin gene, we have identified a number of genes of medical and biological importance.

(1) p53-target genes:

Mutations of the p53 gene are the most common genetic alterations found in human cancers, and are known to play crucial roles in tumor development and progression. The p53 gene encodes a protein functioning as a transcription factor. Identification of these downstream genes involved in the p53-signaling pathway should provide the deep insight into the molecular mechanisms that mediate tumor-suppressor activities as well as cell-survival or death signal against cellular stresses. We have been attempting to isolate p53-target genes through cDNA microarray analysis.

Our recent efforts for isolation of p53-target genes and their functional analysis identified genes that have various physiological functions such as apoptosis (p53AIP1 and STAG1), DNA repair (p53R2), inhibition of angiogenesis (BAI1), re-entry into cell cycle (p53RFP), positive regulation of p53 (p53DINP1), immune response (IRF5 and Fractalkine), cell survival (p53CSR) and determination of cell fate (p53RDL1). Our results should contribute to a better understanding of the mechanism in which p53 determine the fate of cells having various cellular stresses and also the process how p53 function as a tumor suppressor gene.

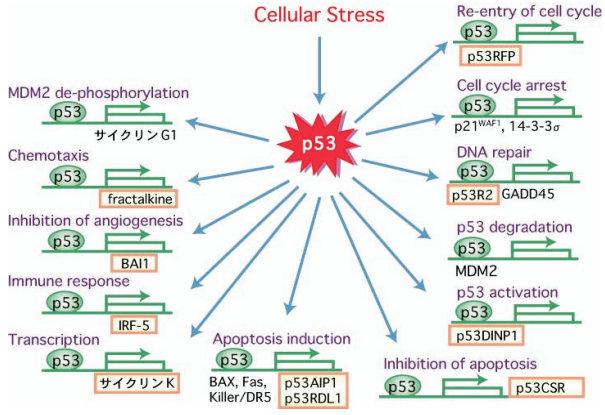


Fig.1. P53-target genes and their functional roles

(2) Screening of molecular targets for development of treatment and diagnosis of cancer.:

We have analyzed clinical cancer samples of the liver, pancreas, stomach, colon, esophagus, bile duct, uterus, lung, ovary, kidney, urinary bladder, testis, prostate, breast, and soft tissues as well as acute and chronic myeloid leukemias. We have selected hundreds of candidate genes by the criteria as follows; (a) gene expressions were transactivated in a large proportion of cancer tissues in comparison with their corresponding normal tissues and (b) expression was not observed or hardly detectable in any important vital organs. The further functional analysis of them identified dozens of genes that are likely to function as oncogenes in various cancers. The suppression of expression of such genes with small-interfering RNAs (siRNAs) induced cell cycle arrest, apoptosis, or suppression of anchoring-dependent cell growth.

For example, one of the good candidates that we identified as a molecular target for development of drugs is *SMYD3,* a gene that is over-expressed in the majority of colorectal and hepatocellular carcinomas. SMYD3 formed a complex with RNA polymerase II through an interaction with the RNA helicase HELZ and transactivated a set of genes that included oncogenes, homeobox genes and genes associated with cell-cycle regulation. It also functions as histone methyltransferase. We also developed antibodies against HIG2 (hypoxia-inducible protein-2) that showed growth-suppressive effect on renal cell carcinoma (RCC)

cells. The functional and clinical analysis indicated that HIG2 functions as oncogene in an autocrine manner and measurement of serum HIG2 is likely to be a good tumor marker of kidney cancer. In addition we developed monoclonal antibodies against FZD10 that was specifically expressed in synovial sarcomas. *In vivo* experiment using xenograft derived from human synovial sarcoma revealed a strong accumulation of the antibody injected intravenously into the tumor, indicating a possible application of antibody for treatment of this type of tumor.

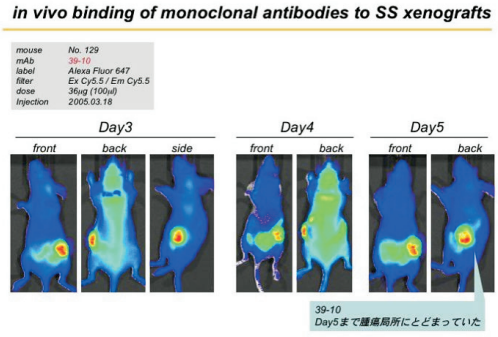


Fig.2. Accumulation of anti-FZD10 antibody in the xenograft derived from human synovial sarcoma (red spots indicate the accumulated antibody in the tumor)

(3) Prediction of effect of chemotherapy and radiation therapy.:

We applied a cDNA microarray system representing 27,648 genes to select a defined set of genes that could predict responsiveness of advanced NSCLCs to gefitinib. Statistical analysis of expression profiles in 17 clinical samples identified dozens of genes that were differentially expressed between gefitinib-responders and non-responders. A gefitinib-sensitivity assay *in vitro* brought to light at least one biological mechanism of Gefinitib-resistance of NSCLC cells, i.e. induction of resistance by amphiregulin (AREG). In addition, we established prediction method for a certain protocol for CML, uterine cancer, and bladder cancer.

(4) Genes responsible for deafness:

Through cDNA microarray analysis of gene expression in human cochlea and vestibule, we detected specific expression of several genes in these tissues and analyzed their mutations in DNAs isolated from patients with deafness. So far we have identified mutations in three genes, μ-crystallin (*CRYM*; also known as NADP-regulated thyroid hormone-binding protein), FBOX2, and KIAA1199. Mutations in CRYM implied the possible involvement in the potassium-ion recycling system for development of deafness.

(5) Genes related to arrhythmia and osteoporosis:

We isolated a mammalian gene, *ZNT5*, whose expression transiently increased in response to intimal denudation of rabbit aorta. Mice deficient for this gene showed poor growth and a decrease in bone density due to impairment of osteoblast maturation to osteocyte. More than 60% of male null-mice died suddenly because of the bradyarrhythmias.

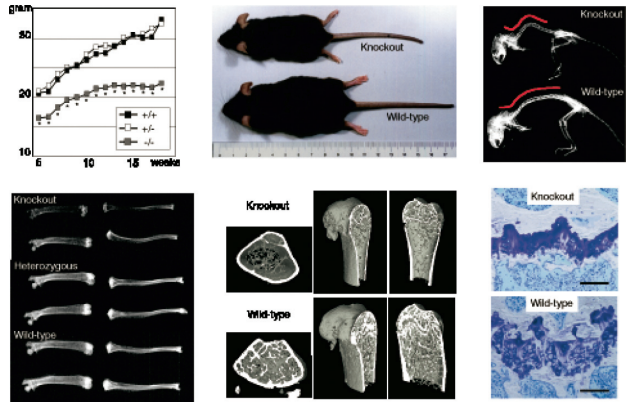


Fig.3. Phenotypic changes (scoliosis and osteoporosis) in mice lacking ZNT 5 gene

2-2. Genome-wide association analysis using gene-based SNPs:

We performed the genome-wide association study using 465 patients with IgA nephropathy and 483 patients with inflammatory bowel disease by genotyping nearly 90,000 gene-based SNPs selected from our database, by means of the high-throughput multiplex PCR-Invader assay method. So far we have identified five candidate genes for IgA nephropathy, L-selectin, E-selectin, HLA-DRA, polymeric immunoglobulin receptor (*PIGR*), and immunoglobulin μ-binding protein 2 (*IGHMBP2*). We have also identified one cytokine as a strong candidate susceptible to inflammatory bowel disease.

3. Concluding Remarks

These results indicated that systematic analysis of expression profiles and genome-wide SNP analysis should be a very effective approach for identification of molecules that are susceptible or responsible for diseases and could be potential targets for development of novel therapeutic drugs and diagnostic tools.

4. Primary Publications

(1) S. Okamura et al.: p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis. Molecular Cell, 8:85-94, 2001.

(2) T. Takei et al.: Association between single-nucleotide polymorphisms in selectin genes and IgA nephropathy. Am. J. Human Genetics, 70:781-786, 2002.

(3) C. Tanikawa et al.: p53RD L1 regulates p53-dependent apoptosis. Nature C ell Biology, 5:216-223, 2003.

(4) T. Kimura et al.: Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. Nature Genetics, 34:440-445, 2003.

(5) R. Hamamoto et al.: SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nature Cel l Biology, 6:731-740, 2004.

(6) M. Tsuge et al.: VNTR Polymorphism of E2F-1 binding element in the 5' flanking region of SMYD3 is a risk factor for human cancers. Nature Genetics, in press, 2005.

Identification of Genes Involved in Brain Diseases

Project Leader:
Shoji TSUJI Professor, Graduate School of Medicine,
The University of Tokyo

1. Objective

1-1. The objective of this project is to identify genes involved in the pathogenesis of neurologic diseases based on genome analyses.

1-2. The single gene diseases to be focused on include autosomal dominant spinocerebellar ataxia, autosomal recessive spinocerebellar ataxia, and cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL).

1-3. The neurologic diseases with complex trait to be focused on include multisystem atrophy (MSA) and ALS/PDC in Kii Peninsula.

1-4. For diseases with identified causative genes, effort will be made to elucidate molecular mechanisms of the neurologic diseases.

2. Summary

2-1. We identified the causative gene, APTX, as the causative gene for early-onset ataxia with hypoalbuminemia and ocular motor apraxia (EAOH) and that APTX functions as an enzyme involved in the single stranded DNA break-repair system.

2-2. For autosomal dominant ataxia, we have mapped a locus for an autosomal dominant ataxia to chromosome 3p26.1-25.3.

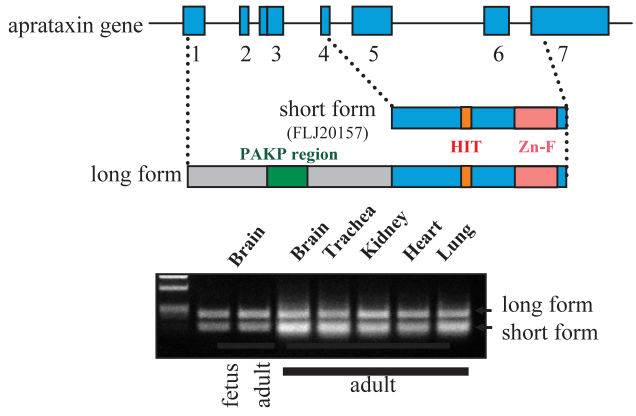
2-3. We have identified rhe causative gene for an axonal form of Charcot-Marie-Tooth disease.

2-4. Genome-wide linkage analysis of CARASIL has revealed a candidate locus. Positional cloning approach is in progress.

2-5. As the approach toward neurodegenerative diseases with complex trait, we have recently identified familial cases of multiple system atrophy (MSA). Non-parametric linkage analysis of familial MSA cases has revealed several candidate loci.

2-6. Based on studies on cell culture systems and transgenic mice, the molecular pathogenesis of polyglutamine diseases has been shown to be caused by suppression of transcriptional activation as a result of intranuclear accumulation of mutant proteins with expanded polyglutamine stretches.

Genomic organization of aprataxin gene and the two major mRNA forms.



3. Concluding Remarks

Based on intensive genome-wide analysis, causative genes have been identified for EAOH and an axonal form of Charcot-Marie-Tooth disease. For neurologic diseases with complex trait, integratted analyses for familial as well as sporadic cases of MSA

are in progress.

We have identified the roles of single-strand DNA break repair systems in the pathogenesis of spinocerebellar degeneration. In polyglutamine diseases, we have identified the role of intranuclear accumulation of mutant proteins with expanded polyglutamine stretches and leading to transcriptional sysregulkations.

4. Primary Publications

(1) Shimohata, T, Nakajima, T, Yamada, M, Uchida, C, Onodera, O, Naruse, S, Kimura, T, Koide, R, Nozaki, K, Sano, Y, Ishiguro, H, Sakoe, K, Ooshima, T, Sato, A, Ikeuchi, T, Oyake, M, Sato, T, Aoyagi, Y, Hozumi, I, Nagatsu, T, Takiyama, Y, Nishizawa, M, Goto, J, Kanazawa, I, Davidson, I, Tanese, N, Takahashi, H and Tsuji, S.: Expanded polyglutamine stretches associated with CAG repeat diseases interact with TAFII130, interfering with CREB-dependent transcription. Nature Genet. 26:29-35, 2000

(2) Date, H., Onodera, O., Tanaka, H., Iwabuchi, K., Uekawa, K., Igarashi, S., Koike, R., Hiroi, T., Yuasa, T., Awaya, Y., Sakai, T., Takahashi,T., Nagatomo, H., Sekijima, Y., Kawachi,I., Takiyama, Y., Nishizawa, M., Fukuhara, N., Saito,K., Sugano, S. and Tsuji, S.: Early-onset ataxia with ocular motor apraxia and hypoalbuminemia is caused by mutations in a new HIT superfamily gene. Nature Genet. 29: 184-188, 2001

(3) Hara, K, Fukushima, T, Suzuki, T, Shimohata, T, Oyake, M, Ishiguro, H, Hirota, K, Miyashita, A, Kuwano, R, Kurisaki, H, Yomono, H, Goto, J, Kanazawa, I and Tsuji, S. Japanese SCA families with a distinct phenotype linked to a locus overlapping with SCA15 locus Neurol. 62:648-651, 2004

(4) Züchner S, Mersiyanova, I V. Muglia, M. Bissar-Tadmouri, N. Rochelle, J. Dadali, E L. Zappia, M. Nelis, E. Patitucci, A. Senderek, J. Parman, Y. Evgrafov, O. Jonghe, P D. Takahashi, Y. Tsuji, S. Pericak-Vance, M A. Quattrone, A. Battaloglu, E. Polyakov, A V. Timmerman, V. Schröder, J M. Vance, J M. Battologlu, E. Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nature Genet. 36:449-51, 2004.

(5) Shimohata, M, Shimohata, T, Igarashi, S, Naruse, S, and Tsuji1, S. Interference of CREB-dependent transcriptional activation by expanded polyglutamine stretches - Augmentation of transcriptional activation as a potential therapeutic strategy for polyglutamine diseases. J. Neurochem. 93:654-663, 2005

Whole Body Functional Genomics Directing toward the Understanding of Disease-related Genes

Project Leader:
Mitsuo ITAKURA Professor, Institute for Genome Research, The University of Tokushima

1. Objective

To understand the mechanism of disease processes in the whole body, the following studies have been done.

1-1. Identification of disease-causing genes of 3 congenital metabolic diseases by linkage analysis of the pedigree followed by positional cloning.

1-2. Quantitative trait loci (QTL) analysis of F2 progenies generated between spontaneously diabetic db mouse and non-diabetic D2 or C3H mouse to find the disease-susceptibility genes for diabetes.

1-3. A large scale association study on type 2 diabetes and rheumatoid arthritis using region-wide 2-stage association approach using evenly-spaced common single nucleotide polymorphism (SNP) markers.

1-4. Analysis on the whole body effect of gene modifications in laboratory animals including mouse, drosophila, and medaka.

2. Summary

2-1. We discovered the uromodulin gene as an etiology for Familial Juvenile Hyperuricemic Nephropathy (FJHN). We discovered GDD1 as the etiological gene for autosomal dominant gnathodiaphyseal dysplasia (GDD). Locus for GDD was determined by the linkage analysis of a large Japanese family to be 11p14.3 to 15.1 on chromosome 11. The new gene GDD1 encodes protein of 913 amino acids of which amino acid at position 356 was found to be mutated in patients with GDD not only in Japanese family, but also in African American family. We discovered autosomal dominant CSPG2 (Chondroitin Sulphate Proteo-Glycan 2) as the etiological gene for Wagner syndrome (WS). WS is characterized by visual acuity loss due to progressive chorioretinitis. Locus for WS was determined by the linkage study of a three-generation large Japanese family to be 5q13-14 on chromosome 5. The gene CSPG2 is mutated at the splice acceptor site in intron 7, i.e. from AG to GG causing the splicing out of 39 bases with the loss of 13 amino acids.

2-2. We generated the F2 progenies by inter-crossing F1 progenies heterozygous for db between obese diabetic db mice and non-diabetic non-obese D2. We performed Quantitative Trait Loci (QTL) analysis on F2 progenies. By applying the expression QTL, i.e. regarding the mRNA level as one of the quantitative traits, we detected 17 genes as the candidate to modify the diabetic traits as the modifier out of 76 genes in this region. By generating F2 progenies between F1 progenies heterozygous for db and non-diabetic non-obese C3H, we detected 6 modifier loci.

2-3. We identified SEC8L1 as the disease susceptibility gene for rheumatoid arthritis. We previously detected the locus of 7q31-34 spanning 25.3 Mb by the affected sib pair analysis in 53 Japanese pedigrees. We selected 728 SNPs with the minor allele frequency of more than 15% covering 107 genes in 179 genes in this candidate region. We performed 2-stage association study using 760 patients with rheumatoid arthritis and 760 healthy normal control subjects. We detected the SNP showing significant association with the disease status with $P = 5.9 \times 10^{-6}$ in 2 x 2 allele type contingency table of chi-square test.

We identified SOCS2 as the disease susceptibility gene for type 2 diabetes. In the region of 12q15 to 12, we selected 536 gene-centric SNPs and 49 intergenic SNPs to cover 92 genes out of 128 genes in this target region. These SNPs were gene-centric evenly-spaced (one in every 10 kb) and common SNPs with the minor allele frequency larger than 10%. We detected disease-susceptibility haplotype block including the SNP showing significant association with the disease status with $P = 1.3 \times 10^{-4}$ in 2 x 2 allele type contingency table of chi-square test. Adenoviral transfer of SOCS2 to cultured islet beta cell line of MIN6 or isolated rat islets showed 25% reduction in the insulin release.

2-4. We produced transgenic mice (Tg) expressing Pax6 in pancreatic islet beta cells (beta cells), which showed islet tumors. We produced Tg expressing regI in beta cells exhibiting diabetes and pancreatic tumors. Tg expressing FGF8 or FGF 10 in beta cells showd hepatocyte or exocrine pancreatic differentiation. Tg expressing constitutive active form of CDK4 in beta cells showed the increased

islet area from 2.0% of the whole pancreas in the littermates to 20.0% in Tg. We produced homozygous state of Tg for the porcine active form of TGFb1 driven by the glucagon promoter. Due to the paracrine action of TGFb1, the growth of beta cells was arrested. TGFb1 homozygous mice showed decreased glucose tolerance, decreased plasma insulin concentrations, reduction in size of whole pancreas to one third, and that of endocrine islets to one fifth in comparison with those in the littermates. The size of the endocrine islets is determined in vivo by the rate of G1 to S progression.

2-5. We analyzed the function of FMR1, the etiologic gene for fragile X syndrome in drosophila (fruit fry). We generated the knockout for dFMR1, the homologue of human FMR1. They showed the disturbed diurnal variation, disturbed memory, and morphological abnormality of the neuron.

Based on the biochemical analysis, we discovered that dFMR1 is involved in the RNAi molecular pathway. By the genetic analysis using the mating of various mutants of drosophila, we discovered that dFMR1 is involved in the RNAi molecular pathway from the genetic basis. These observations opened up the new research field of new disease entity due to the disturbance in RNAi molecular pathway.

2-6. We analyzed the recognition of self and non-self by T-cells in the whole body approach. We used the antigen receptor transgenic mice showing positive and negative selection in vivo. Based on the microarray analysis of immature thymocytes, we detected Immune-associated nucleotide-1 (IAN1) and IAN4 as the up-regulated genes.

We used ethyl nitrosourea-induced mutagenesis in medaka and generated the homozygotes for the mutation to understand the organogenesis of the thymus. We used ragI as the probe to visualize the developing thymus. We have screened 538 F2 progenies covering about 60% of the whole genome of medaka. We discovered 22 mutant medaka showing the abnormal thymus organogenesis. We analyzed the stage at which abnormality starts to occur by the gene expression analysis representing the cell-specific expression in the cell lineage.

3. Concluding Remarks

3-1. Identification of etiologic gene mutations in 3 congenital metabolic diseases enables not only the genetic diagnosis, but also helps to understand the gene function in the whole body.

3-2. As the efficient method for the association study, we proposed the followings: selection of candidate regions based on linkage analysis, association study using evenly-spaced (1 every 10 kb) common SNP markers (minor allele frequency larger than 15%), and the relatively large scale of 760 to 950 cases and controls, respectively.

3-3. We have examined the minor allele frequency of more than 90,000 SNPs in 46 Japanese and showed that about 70% of them have minor allele frequency larger than 15% (ttp://www.genome.tokushima-u.ac.jp/dgi/JAPDGI/ASNPs/index_English.html)

3-4. We discovered that dFMR1 protein, etiological gene product of fragile X syndrome, interacts with Argonaute (AGO) protein. Because AGO protein is the essential component of RISC (RNA-induced silencing complex), and because RNAi (RNA interference) pathway is regulated by RISC, our discovery opened the new disease entity relating with RNAi pathway. We further discovered that AGO1 is involved in miRNA pathway and AGO2 in RNAi pathway.

3-5. In the process of T-cell differentiation, specific T-cells that are advantageous for survival are selected. One mechanism relating with IAN1 and 4, and another mechanism relating with migration into and out of thymus are playing the important roles. Together with the discovery of genes essential for thymus development, our observation should open the new regulatory system for immune differentiation.

Based on the whole body functional analysis of various genes both in human and experimental animals, the further development of functional genomics directing toward the understanding of the disease and metabolic regulation is expected to proceed.

4. Primary Publications

(1) Inoue S, Shimoda M, Nishinokubi I, et al. A role for the Drosophila fragile X-related gene circadian output.Curr Biol. 2002 Aug 6;12(15):1331-5. PMID: 12176363

(2) Kudo E, Kamatani N, Tezuka O, et al. Familial juvenile hyperuricemic nephropathy: detection of mutations in the uromodulin gene in five Japanese families.Kidney Int. 2004 May;65(5):1589-97. PMID: 15086896

(3) Tsutsumi S, Kamata N, Vokes TJ, et al. The novel gene encoding a putative transmembrane protein is mutated in gnathodiaphyseal dysplasia (GDD).Am J Hum Genet. 2004 Jun;74(6):1255-61. PMID: 15124103

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(5) Ueno T, Saito F, Gray DH, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes.J Exp Med. 2004 Aug 16;200(4):493-505. Erratum in: J Exp Med. 2004 Oct 4;200(7):following 946. PMID: 15302902

(6) Hamada D, Takata Y, Osabe D, et al. Association between single-nucleotide polymorphisms in the SEC8L1 gene, which encodes a subunit of the exocyst complex, and rheumatoid arthritis in a Japanese population.Arthritis Rheum. 2005 May;52(5):1371-80. PMID: 15880602

Human Molecular Biology and Medicine Based on Genome Analysis

Project Leader:
Nobuyoshi SHIMIZU Professor,Keio University, School of Medicine

1. Objective

We attempt to perform comprehensive genome analysis of human chromosomes 22, 21, and 8. We try to identify all the genes in the analyzed sequences and elucidate their functions. We intend to develop human molecular biology and medicine by integrating novel findings on these genes in terms of genomic structures, tissue expression profiles, protein functions, and mechanisms of gene regulation. Using Keio BAC library and our ability of efficient genome analysis, we also perform hunting for disease genes including those caused by gene-dosage effects. We also investigate the physiological significance of the proteins and the pathogenesis of disease-associated genes which we previously found. These genes include *PARKIN* for autosomal recessive juvenile parkinsonism (ARJP), *AIRE* for autoimmune disease APECED, and *MYOCILIN* for a glaucoma GLC1A.

2. Summary

2-1. Human genome analysis (mapping, sequencing, and gene analysis) :

We have contributed to the mapping, sequencing and analysis of the particular parts of five chromosomes 22, 21, 8 (8q22-q24.1 region: 14 Mb), 6 (*PARKIN* gene: 1.4 Mb) and 2 (Immunoglobulin κ Locus). Comprehensive analysis of the sequence data with computer-aided annotation and experimental inspection enabled us to find genes that are associated with important biological functions as described below.

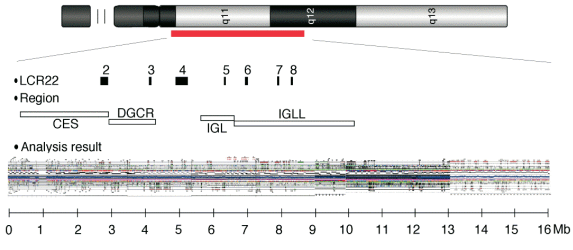

Fig.1. Gene map of human chromosome 22

The Argonaute family consists of 4 *PIWI* genes and 4 *EIF2C* genes and all the protein products interact with a DICER protein to participate in the new regulatory mechanism called RNA interference. DGCR8 is also a component of molecular apparatus for generation of siRNA and miRNA. The YPEL family consists of 5 similar but distinct genes whose protein products appear to be involved in the centrosome function.

For chromosome 21 genes, we investigated structure and function of many candidate genes (*DYRK1A*, *SIM2*, *UMODL1*, *TRPM2*, *ZNF295*, *ZNF298*, etc.) for Down syndrome. We also found 48 *KAP* (keratin-associated protein) genes on chromosome 21, and 93 genes in total human genome. The KAPs are essential for hair formation in association with the keratin intermediate filaments. Interestingly, 16 *KAP* genes on 21q22.3 are located within introns of the recently identified gene *TSPEAR*. Surprisingly, transcriptional direction of 8 out of 16 *KAP* genes is the same as that of *TSPEAR* gene. This finding suggests "a novel transcription mechanism".

2-2. Identification of the genes responsible for diseases caused by gene-dosage effects:

We are developing a DNA microarray using the Keio BAC library. We have so far made a 7.8-K BAC microarray on which 7,809 BAC clones are spotted to cover one third of the human genome. The CGH (comparative genomic hybridization) analysis by BAC microarray is being used for detecting chromosomal anomalies associated with human tumors and various congenital diseases such as Down syndrome and DiGeorge syndrome.

We proved that TBX1 gene in the DiGeorge syndrome (DGS) critical region (DGCR) of 22q11.2 is the main causative gene for DGS and conotruncal anomaly face syndrome (CAFS). We discovered another candidate gene *DGCR8* for those syndromes. The expression profile of *DGCR8* in developing mouse embryos is consistent with the clinical phenotypes associated with DGS/CAFS.

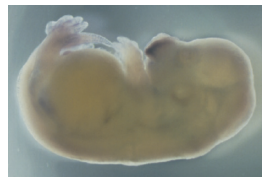

Fig.2. Expression of *DGCR8* gene in mouse embryo

2-3. Positional cloning of the causative gene *TMPRSS3* for autosomal recessive non-syndromic deafness DFNB8/10:

We identified *TMPRSS3* gene as a pathogenic gene for familial deafness DFNB8/10. We showed that the product of *TMPRSS3* gene is a trans-membrane serine protease that is localized in the endoplasmic reticulum within cells. We suggested that the epithelial amiloride-sensitive sodium channel (ENaC), which is expressed in the inner ear, could be a substrate of TMPRSS3. We are now investigating the role of *TMPRSS3* gene mutation in hearing loss using mouse models.

2-4. Functional analysis of previously found disease genes:

We determined the complete 1.4-Mb sequence of the gigantic gene *PARKIN* responsible for autosomal recessive juvenile parkinsonism (ARJP), and determined the precise breakpoints of the large deletion mutations frequently found in the ARJP patients. Furthermore, we proved that the gene product Parkin is a ubiquitin ligase (E3) which has an ability to transfer ubiquitin to target proteins. This discovery supported the idea that parkinsonism is caused by abnormality in the protein degradation system in neuronal cells.

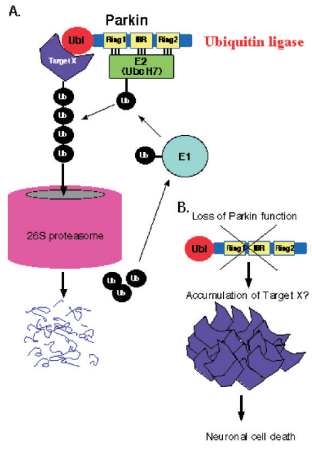

Fig.3. Molecular pathogenic model of parkinsonism

We found that AIRE protein has both transcriptional activator and E3 ubiquitin ligase activities. We have recently established permanent lines ($AIRE^+$ cells) derived from thymic epithelial cells which express *AIRE* gene. $AIRE^+$ cells are invaluable for the biochemical analysis of *AIRE* function in relation to the promiscuous gene expression in thymic epithelial cells.

We analyzed the expression of *myocilin* (*MYOC*) and *optineurin* (*OPTN*) genes both associated with glaucoma. The alteration in expression of *MYOC* but not of *OPTN* under stress suggests that different mechanisms are involved in development of glaucoma associated with the two genes.

2-5. Construction of an integrated knowledge base for mutations and polymorphisms in human disease genes *MutationView*:

MutationView has now collected 17,119 entries of mutations/ polymorphisms from 2,147 literatures, dealing with 292 genes involved in 500 diseases. We have also developed highly intelligent information retrieval system utilizing descriptions in OMIM.

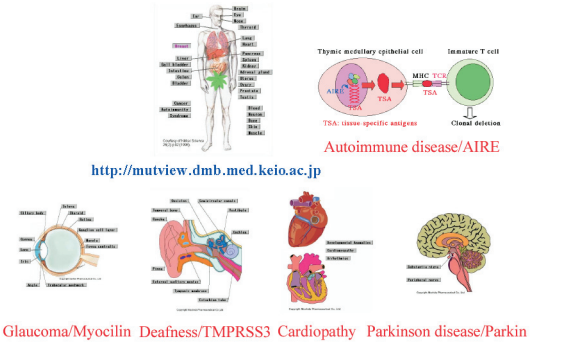

Fig.4. *An integrated knowledge database-MutationView*

3. Concluding Remarks

We identified many novel genes by meticulous analysis of the highly accurate DNA sequence of the human genome. These should serve as a firm foundation for biomedical research in the future. Our research on the disease genes impacted on the development of diagnostic methods and new drugs for Parkinson disease, autoimmune disease, glaucoma and so on. Furthermore, novel tools including genome-wide BAC microarray will be invaluable for comprehensive analysis of the human genome.

4. Primary Publications

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(5) Sasaki, T., Shiohama, A., Minoshima, S. and Shimizu, N.; Identification of Eight Members of the Argonaute Family in the Human Genome, *Genomics*, 82(3): 323-330 (2003)

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Identification of Disease-related Genes Using Microsatellite Polymorphisms

Project Leader:
Hidetoshi INOKO Professor, School of Medicine, Tokai University

1. Objective

We have focused on microsatellite markers with a higher degree of polymorphism than SNP (single nuclelotide polymorphism), enabling to narrow down the candidate susceptible region to 100 kb efficiently in association mapping. The aim of this project was to discover disease-related genes using 27,000 microsatellite followed by SNP based association analysis on the 100 kb candidate region at the genome-wide level.

2. Summary

2-1. Collection of polymorphic microsatellite markers :

We optimized pooled-DNA genotyping method which utilizes DNA mixture consisting of aliquot DNA from each sample to identify polymorphic microsatellite markers efficiently and to save costs. Polymorphism of all the microsatellite markers which were designed across the whole human genome were tested using pooled-DNAs from 100 individuals of normal-healthy Japanese. At first, we collected 9,880 known microsatellite markers, which were polymorphic in Caucasian populations, and tested for their polymorphism in Japanese population: 9,099 of these known microsatellite were found to be polymorphic in Japanese. Then we designed 56,207 of new microsatellite markers across the whole human genome and screened: 30,950 markers displayed a high degree of genetic polymorphism. As a result, we obtained a total of 40,049 microsatellite markers which showed polymorphism in the Japanese population.

The average resolution of these markers was one microsatellite every 83.2 kb, and the average heterozygosity and allele frequency were 0.69 and 7.0, respectively. Therefore, we have succeeded in collection of microsatellite markers which show polymorphism in the Japanese population across the entire human genome with high density enough to be utilized for genome-wide case-control study.

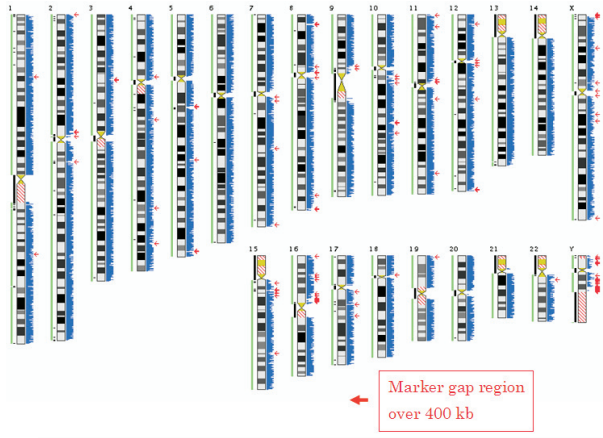


Fig.1. Summary of polymorphic microsatellite markers

2-2. Genetic homogeneity of Japanese population :

We investigated allelic lineages of 116 Japanese samples using 36 microsatellites on Y chromosome and on autosomal chromosomes. Any markers did not show any significant difference in allele frequencies among these groups, suggesting that there are no hierarchical or stratified population structure in our Japanese samples. This result supports the possibility of application for genome-wide association study using polymorphic microsatellite markers.

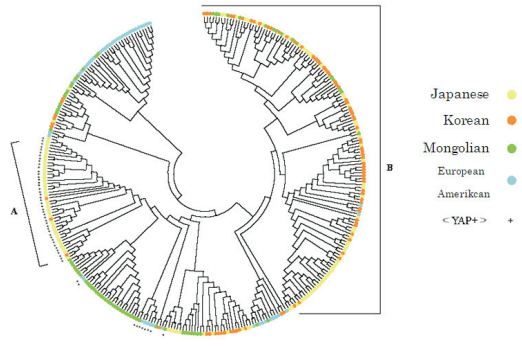


Fig.2. Haplotype dendrogram based on microsatellite markers in Y chromosome

2-3. Survey of genes associated with complex disorder in HLA region :

We performed case-control analyses on rheumatoid arthritis and other HLA-associated diseases as a target in the human leukocyte antigen (HLA) region by precise mapping of their candidate gene regions using microsatellite markers. We found the candidate gene segment for rheumatoid arthritis which was found to be located telomeric region of the HLA-class III region by microsatellite-based association mapping, then a susceptibility gene of this complex disorder being successfully identified by following SNP-based association analysis. The ranges of the LD region observed in SNPs (30 kb) were shorter than those of microsatellites (100 kb). These results demonstrated that our approach of microsatellite-based genome-wide study is quite expecting to survey susceptible genes complex disorders: namely, mapping of candidate regions (100 kb) by genome-wide association analysis using polymorphic microsatellite markers at first, then followed by identification of disease responsible genes by association analysis with SNPs within thus narrowed down candidate regions.

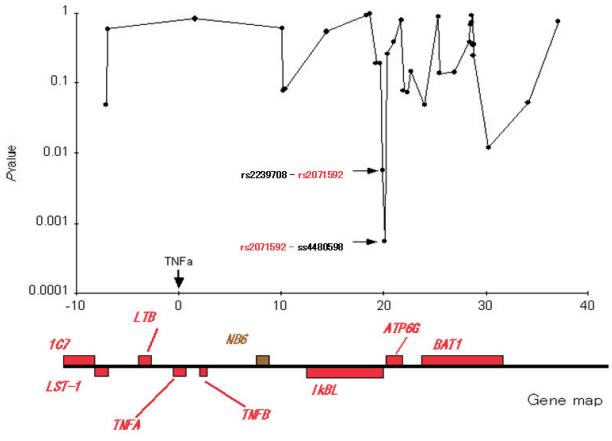


Fig.3. Association study of pairwise haplotypes.

2-4. Genome-wide surveys of genes associated with complex disorders :

We proceed to mapping of susceptibility regions by genome-wide case-control studies for rheumatoid arthritis, psoriasis, hypertension, and strong myopia. Forty-seven candidate regions which were associated with rheumatoid arthritis (RA) were identified: we conduced case-control pooled-DNA genotyping method for the first, second, and third screening to RA, and then markers which showed statistically significant difference of allele frequencies between normal and patient populations throughout these three independent screenings were confirmed by individual genotyping analysis. Seven candidate regions which showed the top 7 strongest disease-association among 47 candidate regions by genome-wide microsatellite-based association mapping were analyzed for identification of RA susceptible genes with SNP-based association study, and seven susceptibility genes associated with rheumatoid arthritis were thus identified from each of seven candidate regions. Two among seven susceptible RA genes were known susceptibility genes and five represent new rheumatoid arthritis susceptibility genes.

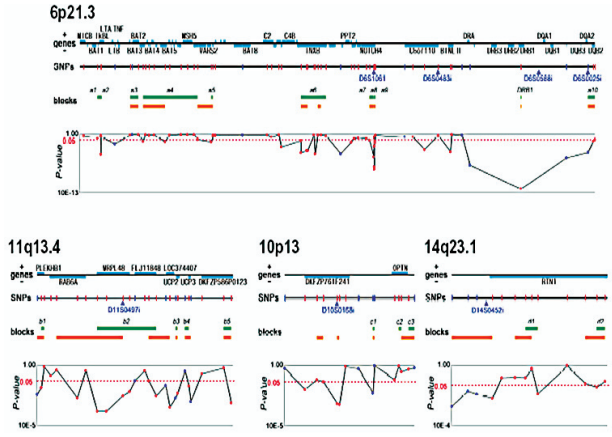


Fig.4. Association study using SNP markers around RA-Susceptibility regions

3. Concluding Remarks

In this way, our microsatellite-based genome-wide association technique was established as a new and powerful method to survey disease-associated genes across the whole human genome to achieve our final goal of this project. We have been undertaking case-control analyses for other 20 common or complex diseases, such as psoriasis, hypertension, strong myopia etc using our method, and could identify approximately 30 candidate regions (100 kb) for each disease. Although time-consuming and cost problems do not enable us to identify susceptibility genes of these diseases by SNPs genotyping of a lot of candidate regions thus narrowed down by genome-wide microsatellite-based association studies, we are planning to continue to and put considerable effort into these study for identification of as many as susceptible loci of common or complex diseases.

4. Primary Publications

(1) Tamiya G et.al: “Whole genome association study of rheumatoid arthritis using 27,039 microsatellites.” *Hum Mol Genet*, (2005) *in press.*

(2) Okamoto K et.al:” Identification of IkBL as the second Major Histocompatibility Complex-Linked susceptibility locus for rheumatoid arthritis.” *Am J Hum Gene*, 72, 303-312, (2003).

(3) Katoh T et.al: “Genetic Isolates in East Asia:A study of Linkage Disequilibrium” *Am J Hum Gene*, 71 395-400, (2002).

Genetic Analysis of Osteoporosis by Systematic SNP Typing

Project Leader:
Mitsuru EMI Professor, Institute of Gerontology, Nippon Medical School

1. Objective

Osteoporosis is a major cause of fragility fractures that usually happen to aged individuals, and thus the increased prevalence of osteoporotic fractures are regarded as serious socioeconomic problem in advanced nations like Japan. By clarifying its etiological basis, establishment of a practical method for pre-symptomatic diagnosis and the development of novel preventive therapy are expected for realizing healthier future life.

Several candidate polymorphisms have been tested as possible genetic risks for osteoporosis, however none of them were proved to be commonly involved.

Here in this study, we aimed to clarify genetic risks of osteoporosis, as a part of mechanisms causing osteoporosis, expecting to provide a basis for development of preventive diagnostics and therapies.

2. Summary

The project was carried out, pursuing the following working-aims.

2-1. Establishment of multi-step screening for the osteoporosis susceptibility genes :

To identify reliable genetic factors of osteoporosis susceptibility, a method of multi-step screening was pursued. Completion of this aim required high quality evaluation of reliable phenotypes, reasonable numbers of study subjects from general population, high quality selection of nucleotide variations (SNPs) to be analyzed, high through-put analysis of SNP genotypes, and determination of reasonable method for statistical analysis.

About 2,000 of study subjects from general population in Eastern Japan were collected through health check program conducted by Tokyo Metropolitan Institute of Gerontology, one of the leading research groups of epidemiological study on common diseases in Japan. 1,500 healthy subjects were recruited by hospital based method conducted by three leading hospitals in Japan, i.e., University hospital of The Tokyo University (Tokyo), Tokyo Metropolitan Hospital of Geriatric Medicine (Tokyo), and Research Institute and Practice for Involutional Diseases (Nagano). Bone quality assessed by bone mineral density of wrist, spine and hip joint by DXA equipment were evaluated in combination with the effect of age and body mass index of the individuals as an adjustment procedure. Possibly influential SNPs were selected from public database, on the hypothesis that those were in the coding or regulatory regions of annotated genes.

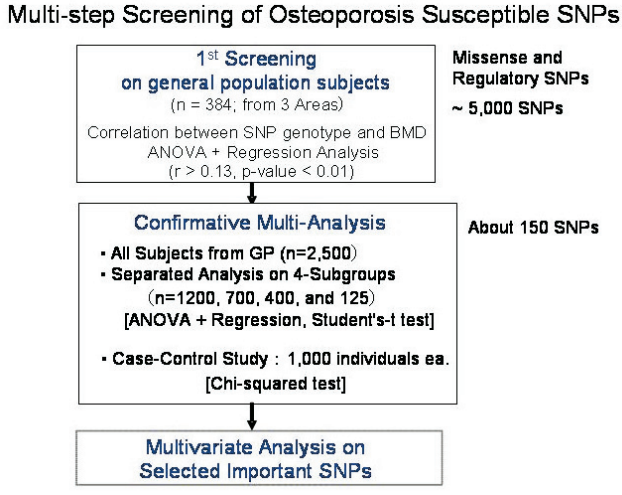


Fig.1. Outline of the multi-step screening

2-2. Identification of strong candidate for the susceptibility SNPs, and analysis of linkage disequilibrium :

As a result of the screening analysis on 5,000 SNPs, possible 64 SNPs were identified localized in the 52 gene loci. Linkage disequilibrium of these loci was analyzed. By confirmative multi-analysis using entire subject groups that includes 1,000 individuals from general population and 400 healthy subjects from a hospital, significance and meanings of these SNPs were judged and estimated.

2.3 Analysis of molecular function of the nucleotide variations, and estimation of interactive effects of these SNPs :

Since we identified several important candidate SNPs both statistically and mechanistically, we

further pursued biological and molecular mechanism of the effect of nucleotide variations. Examples were low density lipoprotein receptor related protein 5 (LRP5), gonadotropin-releasing hormone (GnRH), pituitary glutaminyl cyclase (QPCT), interleukin 1 receptor associated kinase 1 (IRAK1), etc. We also examined combined effects of these polymorphisms on bone mass regulation by multiple regression analysis (Fig. 2; Conceptual diagram).

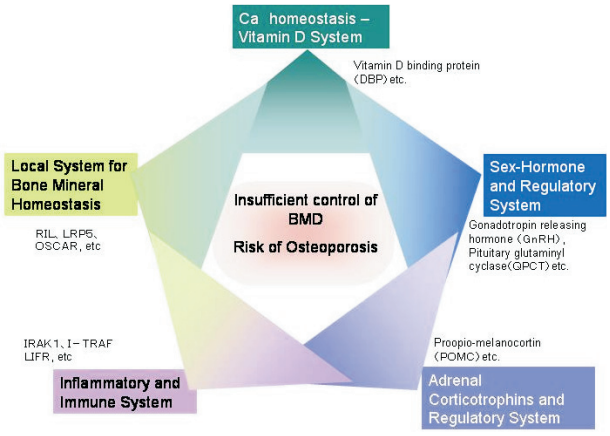


Fig.2. Multiple components of regulatory systems or susceptible genes for bone mass regulation and osteoporosis risks were tested.

Serum cholesterol level is one of the confounding factors influencing bone metabolism. Thus we examined if the same set of polymorphisms (5,000 SNPs) were correlated with the serum cholesterol levels. The results were encouraging because the strongest correlation was detected reproducibly in every subset of our study subjects between HDL cholesterol and a well known mutation that is found in Japanese population relatively often. We are continuing investigate on this aspect of interaction between homeostatic controls of bone and lipid metabolism by genetic factors.

2.4 Development of the predictive diagnostic markers and estimation of clinical outcomes :

Since we identified several important candidate SNPs, a set of polymorphisms were utilized for developing a prototype of the predictive diagnostic marker set, and a glass slide assay plate was made by which multiple nano scale invader assay could be performed all at once. This part of study was assisted by BML (BioMedical Laboratories Inc.) as a collaborator. Two types of patent application were made on 2003.

3. Concluding Remarks

As the conclusive outcome of this project, we identified a great many numbers of reasonable candidate polymorphisms as osteoporosis susceptibility SNPs; the identified number was grater than that proposed in the past decade. And at least, some of them were shown to be functionally important. We are expecting that identification of one real susceptibility-SNP would improve the detection sensitivity for other ones. By promoting those efforts of investigations, our study would result in development of polymorphic markers for drug-sensitivity, and development of therapeutics for novel molecular targets.

We believe that our study on this project was carried out properly on the outlined objectives of this program, “furnish solutions for global issues in the 21st century, while promoting further socio-economic development and creating a richer living standard for the Japanese people”.

4. Primary Publications

(1) Shimizu, M., Kosaka, N., Shimada, T., Nagahata, T., Iwasaki, H., Nagai, H., Shiba, T., Emi, M. : Universal Fluorescent Labeling (UFL) Method for Automated Microsatellite Analysis. DNA Res. 9(5) 173-178, 2002.

(2) Iwasaki, H., Emi, M., Ezura, Y., Ishida, R.,Kajita, M., Kodaira, M., Yoshida, H.,Suzuki, T., Hosoi, T., Inoue, S., Shiraki,M., Swensen, J., Orimo, H.: Association of a Trp16Ser variation in the Gonadotropin Releasing Hormone (GnRH) Signal Peptide with Bone Mineral Density, revealed by SNP-dependent PCR (Sd-PCR) Typing. Bone. 32(2) 185-190, 2003.

(3) Ishida, R., Emi, M., Ezura, Y., Iwasaki, H.,Yoshida, H., Suzuki, T., Hosoi, T., Inoue,S., Shiraki, M., Ito, H., Orimo, H.: Association of a haplotype (196Phe/532Ser) of variations in the Interleukin-1-Receptor-Associated Kinase (IRAK1) Gene with Low Radial Bone Mineral Density inTwo Independent Poulations. J. Bone. Miner. Res. 18(3) 419-423, 2003.

(4) Ishida, R., Ezura, Y., Emi, M., Kajita, M.,Yoshida, H., Suzuki, T., Hosoi, T., Inoue,S., Shiraki, M., Ito, H., Orimo, H.: Association of a promoter haplotype (-1542G/-525C) in the Tumor Necrosis Factor Receptor Associated Factor-Interacting Protein (I-TRAF) Gene with Low Bone Mineral Density in Japanese Postmenopausal Women.: Bone. 33(2) 237-241, 2003.

(5) Ezura, Y., Kajita, K., Ishida, R., Yoshida,S., Yoshida, H., Suzuki, T., Hosoi, T., Inoue, S., Shiraki, M., Orimo, H., Emi, M.: Association of Multiple Nucleotide Variations in The Pituitary Glutaminyl Cyclase Gene (QPCT) with Low Radial-Bone Mineral Density in Adult Women. J. Bone. Miner. Res. 19(8) 1296-1301, 2004.

Biological Systems Database and Genome Information Science

Project Leader:
Minoru KANEHISA Professor, Institute for Chemical Research, Kyoto University

1. Objective

The increasing amount of genomic information is the basis for understanding principles of how higher-order biological systems, such as the cell, the organism, and the biosphere, are formed, as well as for medical, industrial, and other practical applications. However, current informatics technologies cannot readily uncover higher-level complexity of such biological systems, although they are quite effective to find and characterize building blocks of genes and proteins. Here we develop knowledge-based methods for uncovering higher-order systemic behaviors of the cell and the organism from genomic information. The reference knowledge is stored in KEGG, Kyoto Encyclopedia of Genes and Genomes, and associated bioinformatics technologies are developed both for basic research and practical applications.

2. Summary

2-1. New concept of the database :

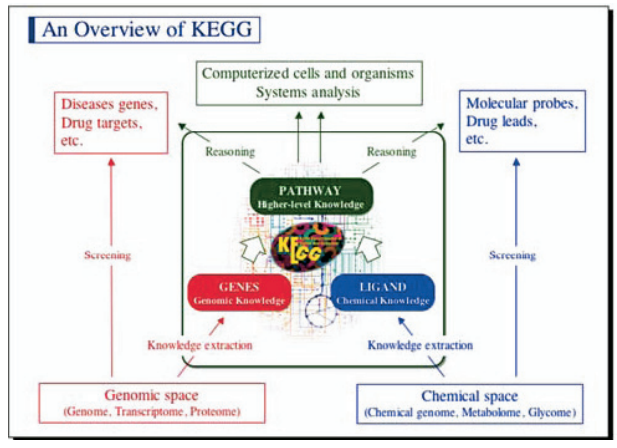
An ultimate goal of bioinformatics is a complete computer representation of the cell and the organism, which will enable computational prediction of higher-level complexity, such as molecular interaction networks involving various cellular processes and phenotypes of entire organisms, from genomic information. From this perspective, a new concept of the biological database has been developed. As shown below, the database is a computer representation of the biological system, and it has been successfully implemented as KEGG.

What is Database?

	NCBI	Kyoto
Database	Repository / Infrastructure	Computer representation of biological systems
Collection	All available data in given domains	Building blocks and wiring-diagrams
Integration	Linking	Reconstruction
Implementation	Entrez	KEGG
Retrieval	Individual data (eg., BLAST)	Graph features (eg., cliques in SSDB)

2-2. The KEGG database :

The KEGG database project was initiated in our laboratory in 1995, the last year of the first five-year phase of the Japanese Human Genome Program, and then continued in the second five-year phase, with supports from the Ministry of Education as grants-in-aid for scientific research on priority areas. With a new funding under the Millennium Project the current research-for-the-future program was launched in 2000, and KEGG was significantly expanded. As shown below, KEGG is a biological systems database, integrating genomic information (GENES database) and chemical information (LIGAND database) in terms of network information (PATHWAY database).



Traditional bioinformatics technologies have focused on finding useful genes and molecules, such as disease genes and drug targets, by screening of large-scale data. In contrast, our approach is first to understand wiring diagrams (molecular interaction networks) of building blocks and then to find functions and utilities of biological systems as a whole. KEGG is a reference knowledge base containing current knowledge on such wiring diagrams, and it is used worldwide as a unique resource for reconstructing metabolism and other cellular processes from genomic information and for understanding systemic functional meanings and utilities.

During the five years of this project we have developed various new features in KEGG, which are summarized below.

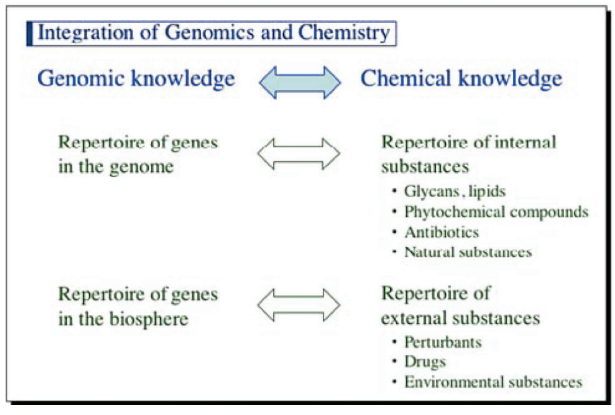
(1) For network information, we expanded the PATHWAY database from a collection of metabolic pathways to a more comprehensive collection containing signaling and various other

regulatory pathways as well as human disease pathways. In addition, an XML version of pathway maps was made available to facilitate computational analysis of KEGG pathways. KEGG has become the international standard of pathway information.

(2) For genomic information in the GENES database, we introduced the KO (KEGG Orthology) system, and developed an automatic method of KO assignment and KEGG pathway mapping, enabling rapid analysis of genomic sequences and EST sequences. By improving the KO system and the automatic assignment program, we hope to make KEGG as the international standard for genome annotations.

(3) For chemical information in the LIGAND database, we introduced GLYCAN [4] in addition to COMPOUND and REACTION. We also developed graph-based algorithms for chemical compound structure comparison and glycan structure comparison, as well as the RC (Reaction Classification) system, which can be used for automatic assignment of EC numbers. These advanced activities resulted in the international collaborations with the NCBI, the EBI, and the Consortium for Functional Glycomics.

(4) The entire KEGG resource is made available at the enhanced KEGG website for general use, as well as through the newly developed KEGG API (application programming interface) for custom use to meet specific needs.



2-3. Integration of Genomics and Chemistry :

The representation of the biological system (ontology) in KEGG is based on the concept of the graph, especially the nested graph and the line graph. The nested graph is a graph whose nodes can themselves be graphs. It is used for representing KEGG network hierarchy and for pathway reconstruction and functional inference.

The line graph is a graph derived by interchanging nodes and edges. The metabolic pathway can be viewed either as a network of genes (enzymes) or as a network of compounds, meaning that one can be generated from the other by the line graph transformation. With this concept, we have undertaken new research on integrated analysis of genomic and chemical information. The gene repertoire in the genome would tell us about all substances that should be produced by an organism, and conversely chemical structures of natural substances would tell us about the genes that should be present in the genome. The integration of genomics and chemistry is becoming more important now that chemical genomics initiatives generate large amounts of experimental data.

3. Concluding Remarks

The number of accesses to the KEGG/GenomeNet website has increased four fold during the five-year period of this project. It is by far the best-used database site in Japan, despite the fact that it has been developed and maintained by a single laboratory. In fact, it is one of the major sites in the world as is apparent by the Google links search showing the number of links made from other sites. We acknowledge the Millennium funding for this achievement.

Number of links by Google

Database	Address	Links
NCBI	www.ncbi.nlm.nih.gov	29,800
ExPASy (SwissProt)	www.expasy.org	18,300
EBI	www.ebi.ac.uk	13,200
GenomeNet (KEGG)	www.genome.jp	9,430
DDBJ	www.ddbj.nig.ac.jp	620
JSNP	snp.ims.u-tokyo.ac.jp	55
PDBj	www.pdbj.org	23
H-invitational	www.h-invitational.jp	19

As of 16 July 2005.

4. Primary Publications

(1) Kanehisa, M. and Bork, P. (2003) Bioinformatics in the post-sequence era. *Nat. Genet.* 33, 305-310.

(2) Kanehisa, M., Goto, S., Kawashima, S., and Nakaya, A. (2002) The KEGG databases at GenomeNet. *Nucleic Acids Res.* 30, 42-46.

(3) Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004) The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277-D280.

(4) Hashimoto, K., Goto, S., Kawano, S., Aoki-Kinoshita, K.F., Ueda, N., Hamajima, M., Kawasaki, T., and Kanehisa, M. (2005) KEGG as a glycome informatics resource. *Glycobiology*, in press.

(5) Hattori, M., Okuno, Y., Goto, S., and Kanehisa, M. (2003) Development of a chemical structure comparison method for integrated analysis of chemical and genomic information in the metabolic pathways. *J. Am. Chem. Soc.* 125, 11853-11865.

(6) Kotera, M., Okuno, Y., Hattori, M., Goto, S., and Kanehisa, M. (2004) Computational assignment of the EC numbers for genomic-scale analysis of enzymatic reactions. *J. Am. Chem. Soc.* 126, 16487-16498.

Development and Application of Computer Programs for Mapping Disease-related Genes Using Polymorphic Markers

Project Leader:
Naoyuki KAMATANI Professor, Institute of Rheumatology,
Tokyo Women's Medical University

1. Objective

We make algorithms and computer software based on the algorithms for the trait mapping (localization of the genome sequences associated with diseases and drug reactions) using genetic polymorphisms. We make a home page to explain various technologies for the trait mapping and to educate researchers. We assist various researchers who perform studies based on genetic polymorphisms concerning the field of statistical genetics.

2. Summary

2-1. Construction of algorithms and application of computer programs to the real data. :

We developed 8 algorithms and implemented them in computer programs. Among them, the following 6 were published in papers.

(1) Checkfam (1: The number here corresponds to the number in the reference list): This program detects incompetence in the genotypic data in pedigrees. We developed a method and implemented it in the computer program that can be used on the Web (http://www.genstat.net).

(2) SimPack (2): This program performs various simulations for linkage analyses and linkage disequilibrium-based analyses. By the simulation, the program evaluates the study design. Especially, we developed an algorithm for genome-wide association studies based on linkage disequlibrium. Step-wise focusing method turned out to be the most efficient strategy. Recent studies by other researchers support this notion.

(3) Ldsupport (3): A haplotype is a list of alleles at linked loci derived from a single parent. This program infers by the maximum likelihood method population haplotype frequencies as well as individual diplotype configurations. In the genomic level, the complete information is the diplotype configuration which is a combination of 2 haplotypes. Now it is difficult to determine individual diplotype configurations and the diplotype configurations inferred statistically is used.

(4) Penhaplo (4): This program performs both testing and inference based on haplotype and diplotype configurations using cohort, clinical trial and case-control samples. It is generally shown that multiple linked loci show statistical significance in the association studies using linkage disequlibrium, and the association of the phenotypes not to SNPs but to haplotypes are in question. This program tests the association between the phenotypes and haplotypes. In addition, the maximum likelihood estimated penetrances are also calculated.

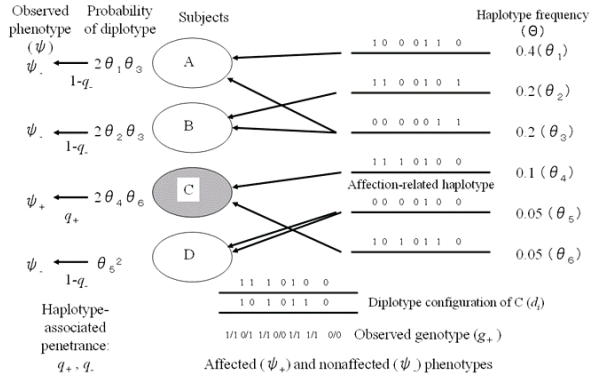


Fig.1. Sample space for PenHaplo (for alternative hypothesis)

(5) AssignHaplo (5): This program assigns minor allele of an uncommon SNP within a haplotype block to a major haplotype. Majority of the uncommon SNPs within haplotype blocks are assigned to single major haplotypes.

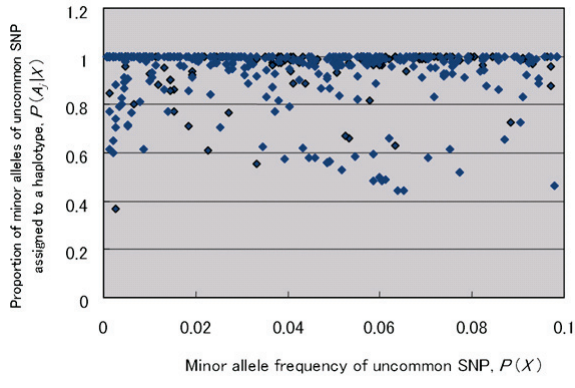


Fig.2. Proportion of minor alleles of intra-block uncommon SNP assigned to a haplotype, P (Aj |X)

(6) Popstruct (6): This program detects the structuring of the population, and performs the test of association in the presence of the structure. Since population structure is a major bias for association studies, the present program is very useful.

2-2. Explanation and education of methods :

We opened a home page (http://www.genstat.net) to explain various methods of statistical genetics. We also published a book for the same purpose (Statistical Genetics in the Postgenome era, Yodo-sha, Tokyo). We explained and educated many researchers in this field.

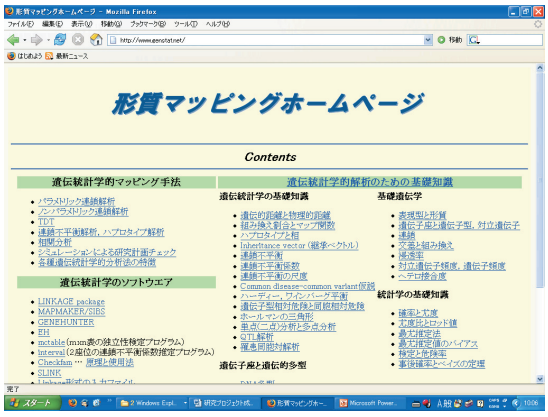


Fig.3. Trait mapping home page

2-3. Assistance in the polymorphic data analyses :

We delivered the above programs to other researchers and assisted them in the data analyses. We have been collaborators of many other groups and become co-authors in 34 papers as a result of such collaborations. The contents of those papers include causes of diabetes mellitus, complications of diabetes, drug reactions, APRT deficiency, AMP deaminase deficiency, metabolism of anti-cancer drug (Irinotecan), prion gene of cattles, ca uses of schizophrenia, complication of rheumatoid arthritis and drug-adverse reactions.

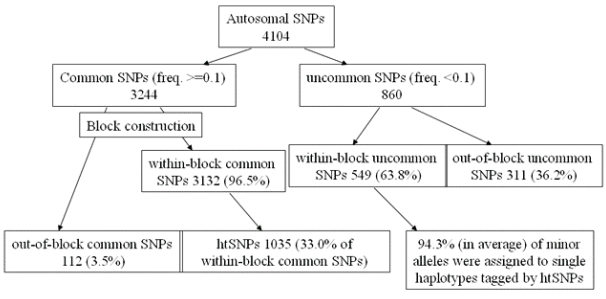


Fig.4. Statistics of SNPs of drug-related genes.

We constructed haplotypes using over 5,000 SNPs based on the data from 752 subjects. Then we selected several hundres htSNPs after constructing haplotypes. The data of this size do not exist elsewhere in the world scientific society.

Thus, in collaboration with Pharma SNP Consorsium (PSC) and RIKEN (SNP Research Center), we obtained blood samples from over 1,000 subjects. We genotyped all of them concerning over 5,000 SNPs in over 200 drug-related genes. The data of SNP allele frequencies are now open in the following cite.
(http://www.jpma.or.jp/psc/11data/index.html)

3. Concluding Remarks

If large-size genome data are obtained from individuals, they are expected to be useful for the maintenance of health and treatment of diseases. In the present project, we developed 8 algorithms including Penhaplo for the trait mapping (localization of the genomic sequences associated with diseases and drug responses) using genetic polymorphisms, and implemented them in computer programs. We found many genes associated with various diseases or drug reactions by either the delivery of the above programs to other researchers or by the collaborations. We have thereby been able to establish the basis of statistical genetics in Japanese scientific community which had been very week in this country but is expected to be quite important in science and medicine.

4. Primary Publications

(1) Saito M, Saito A, Kamatani N. Web-based detection of genotype errors in pedigree data. *J Hum Genet.*, 47, 377-379, (2002).

(2) Saito A, Kamatani N. Strategies for genome-wide association studies: optimization of study designs by the stepwise focusing method. *J Hum Genet.*, 47, 360-365, (2002).

(3) Kitamura Y, Moriguchi M, Kaneko H, Morisaki H, Morisaki T, Toyama K, Kamatani N. Determination of probability distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. *Ann Hum Genet.*, 66, 183-93, (2002).

(4) Kamatani N, Sekine A, Kitamoto T et al. Large-Scale Single-Nucleotide Polymorphism (SNP) and Haplotype Analyses, Using Dense SNP Maps, of 199 Drug-Related Genes in 752 Subjects: the Analysis of the Association between Uncommon SNPs within Haplotype Blocks and the Haplotypes Constructed with Haplotype-Tagging SNPs. *Am J Hum Genet.*, 75, 190-203, (2004).

(5) Ito T, Inoue E, Kamatani N. Association Test Algorithm Between a Qualitative Phenotype and a Haplotype or Haplotype Set Using Simultaneous Estimation of Haplotype Frequencies, Diplotype Configurations and Diplotype-Based Penetrances. *Genetics.*, 168, 2339-2348,(2004).

(6) Nakamura T, Shoji A, Fujisawa H, Kamatani N. Cluster analysis and association study of structured multilocus genotype data. *J Hum Genet.*, 50, 53-61, (2005).

Virulent/Valuable Genome Systems in Microorganisms

Project Leader:

Tetsuya HAYASHI Professor, Frontier Science Research Center,
University of Miyazaki

1. Objective

We live in the environments where numerous microorganisms exist. Many of them create symbiotic relationships with human beings by forming microflora on skin surfaces and mucous membranes of our body. They also contribute in building up and maintaining natural ecosystems. On the other hand, many microorganisms are recognized as pathogens. They include various causative agents of infectious diseases that have been emerged or re-emerged during the past decades. In order to develop new effective strategies against these pathogenic microorganisms, it is essential to understand their biological features as pathogens and the molecular mechanisms of their virulence. This would be possible by first determining the genome information of each pathogenic microorganism.

The main aims of this project are to determine complete genome sequences of various pathogenic bacteria and to construct genome databases of virulent and applicative genome systems for each bacterium. We also performed a wide range of post genomic researches towards the development of new effective anti-infection strategies based on these bacterial genome information.

2. Summary

2-1. During the five-years project period, we determined complete genome sequences of nine bacteria. Results of their genome analyses were published in journals (Table 1) and the constructed genome databases are now available through the websites of each sequencing team. Genome sequencing and sequence analyses have been completed for additional seven bacteria, which include emetic type *Bacillus cereus*, *Chlamydophila felis*, and *Serratia marcescence*. The results are submitted for or in preparation for publication. We also obtained 5-8x draft sequences for seven bacteria, and gap filling processes are under way. They include enterohemorrhagic *E. coli* O26, O111, and O103. Many plasmids and bacteriophages encoding important virulence factors have also been sequenced, and various programs useful for genome analyses have been developed.

Bacterium	genome size
Chlamydophlia pneumoniae	1.2 Mb
Escherichia coli O157 (Sakai strain)	5.6 Mb
methicillin-resistant *Staphylococcus aureus* (MRSA)	3.0 Mb
Clostridium perfringens	2.9 Mb
Vibrio parahaemolyticus	5.1 Mb
Streptococcus pyogenes (Group A *Streptococcus*)	1.9 Mb
Bacteroides fragilis	5.3 Mb
Nocardia farcinica	6.0 Mb
Staphylococcus saprophyticus	2.6 Mb

1 Mb; one milion bas pairs

Table 1. Published bacterial genome sequences

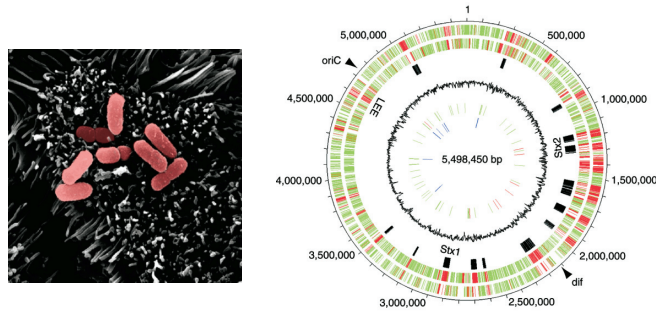


Fig.1. *Escherichia coli* O157 attached to the intestinal cells (left panel) and the genome structure (right panel).

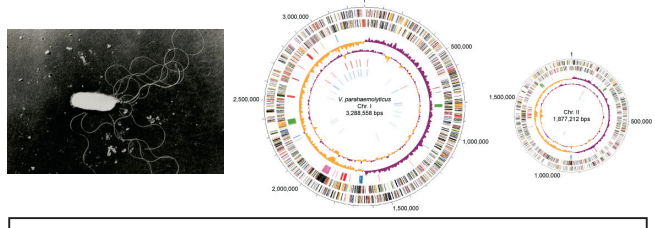


Fig.2. *Vibrio parahemolyticus* (left panel) and the genome structure. This bacterium possesses two chromosomes.

2-2. For each sequenced pathogen, varying types of post-sequencing studies have been conducted to obtain more details on their virulence-related genome systems and to seek for effective application. We gave a particular focus on the construction of microarray, which have been completed for the following nine bacteria: *C. pneumoniae*, *E. coli* O157, *C. perfringens*, *V. parahaemolyiticus*, *S. pyogenes*, *S. aureus*, *N. farcinica, Bacillus cereus*, and *S. marcescence*. In *E. coli* O157 and *C. perfringens*, we obtained many important findings on the genomic diversity and regulatory mechanisms of virulence gene expression by microarray analyses. Another important achievement was the development of the Whole Genome PCR Scanning method. It was originally developed for comparative analyses of O157 strains, but was successfully applied to comparative genomics of other bacteria. Futhermore, functions and regulations of many newly identified potential virulence genes were assessed in each sequenced bacteria.

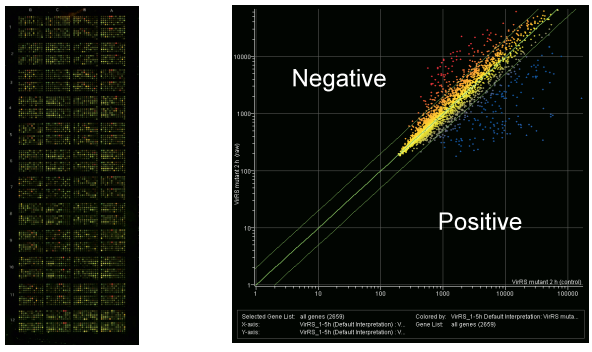


Fig.3. DNA microarray of *Clostridium perfringens* (left panel) and the result of gene expression profiling analysis of a mutant strain harboring a mutation in a virulence regulator gene (right panel).

3. Concluding Remarks

We have determined 16 bacterial genome sequences. This achievement was far more than we have expected at the beginning of the project. This owes mainly to the great improvement in the sequencing ability of the sequencing center leaded by Dr. Hattori, Kitazato University. We will continue the sequencing efforts for the seven unfinished genome sequences. Their complete genome sequences are to be published in the near future.

Data obtained by the genome sequence analyses and various post-sequencing studies provided many important findings. Of particular interests are the following four points.

(1) Most bacteria contain a large amount of foreign DNA, and lateral gene transfer has played important roles in the evolution of many pathogenic bacteria.
(2) Each pathogen contains a larger number of virulence-related genes than previously identified.
(3) There exits a high level of strain-to-strain genomic diversity in each bacterial species.
(4) A wide range of genes, including various metabolic genes, are required for the pathogens to propagate in human body. In order to obtain the overall understanding of the bacterial pathogenicity, analyses on functional and regulatory roles of these genes are also required.

We surely need to make more efforts to fully understand the pathogenicity of each pathogenic microorganism, but the genome information obtained in this project would configure valuable genomic databases for future studies and for future establishment of new effective anti-infection strategies.

4. Primary Publications

< Only genome sequence papers are listed here.>

(1) M. Shirai, *et al.*: Comparison of whole genome sequences of *Chlamydia pneumoniae* J138 from Japan and CWL029 from USA. Nucleic Acids Res., 28: 2311-2314, 2000.
(2) T. Hayashi, *et al.*: Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res., 8: 11-22, 2001 (supplement 8: 47-52).
(3) M. Kuroda, *et al.*: Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. Lancet, 357: 1225-1240, 2001.
(4) T. Shimizu, *et al.*: Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA., 99: 996-1001, 2002.
(5) K. Makino, *et al.*: Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. Lancet, 361: 743-749, 2003.
(6) I. Nakagawa, *et al.*: Genome sequence of an M3 strain of *Streptococcus pyogenes* reveals a large-scale genomic rearrangement in invasive strains and new insights into phage evolution. Genome Res., 13: 1042-1055, 2003.
(7) T. Kuwahara, *et al.: Geno*mic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. Proc. Natl. Acad. Sci. USA, 101: 14919-14924, 2004.
(8) J. Ishikawa, *et al.*: The complete genomic sequence of *Nocardia farcinica* IFM 10152. Proc. Natl. Acad. Sci. USA, 101: 14925-14930, 2004.
(9) M. Kuroda, *et al.*: Whole genome sequence of *Staphylococcus saprophyticus* reveals the pathogenesis of uncomplicated urinary tract infection. Proc. Natl. Acad. Sci. USA, 202: 13272-13277,2005.

Computational Biology on Genome Function Based on Expression and Phenotype Data

Project Leader:
Satoru KUHARA Professor, Faculty of Agriculture, Kyushu University

1. Objective

The genomic sequences of many important model organisms are on the verge of being elucidated. Our research goal is to develop technologies critical to post genomic analysis such as transcriptome analysis, proteome analysis and network based functional analysis to take advantage of this wealth of recently acquired biological information. Furthermore, using information science approaches, we will investigate methods leveraging genome structure to advance from single gene functional analysis to inference of system wide network based functional analysis.

2. Summary

2-1. Genome analysis support system :

We developed a semi-automated genome analysis support system called GAMBLER to greatly increase the speed of processes from sequencing through annotation(Fig.1). This system was designed to reduce the researchers intervention required and to reduce the complication in annotating thousands of ORFs in the microbial genome.

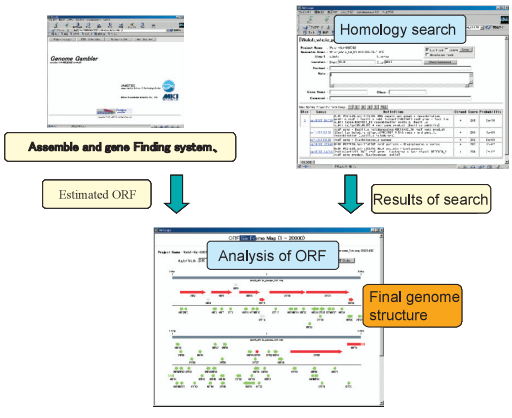


Fig.1. General flow in GAMBLER system

Using this system, we were able to rapidly determine the genomic sequence of almost of microbial genome projects in Japan. In *Bacteroides fragilis*, we found the DNA inversions regulating cell surface adaptation. (Satoru Kuhara).

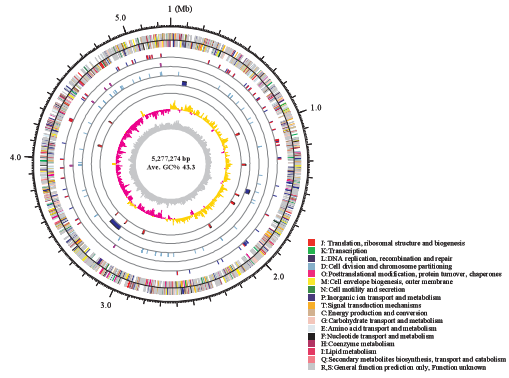


Fig.2. Genome structures in *Bacteroides fragilis*.

2-2. DNA chip of microbial genome :

In our project, we supply enough over 10000 chips for microbial research groups starting post genome research project, including *Chlamydia pneumoniae, E. coli O-157, Clostridium perfringens, Staphlococcus aureus, Vibrio parahemolyticus, Streptococcus pyogenes Saccharomyces cerevisiae and Chlamydia felis.* (Kosuke Tashiro)

2-3. Expression data analysis tools :

We found a new type of bias, called "print-order-bias". And we propose that the application of an appropriate combination of normalization methods to a microarray dataset not only removes biases effectively but also avoids additional biases to be added onto the expression profile data that may obscure the true expression levels of genes under study.

We introduce functional logistic discriminant analysis (FLDA) which is an extension of the classical method of logistic discriminant analysis to data where predictor variables are functions or curves. FLDA approach can effectively classify functions into two distinct classes by imposing smoothness constraint on the predictor functions and coefficient function by radial basis function expansion and regularization. The proposed method is illustrated through the analysis of yeast cell cycle(Fig.3). microarray data. (Sadanori Konishi, Kenji Sato)

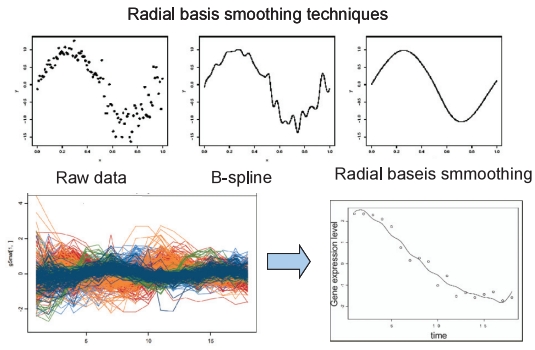


Fig.3. Smoothed gene expression patterns by Gaussian radial basis function networks

2-4. Gene Expression control network :

Boolean network approach. We have developed multilevel digraph approaches to analysis of Boolean network models. In this method, we first determine according to expression data whether a given gene has influenced the expression of another gene. By systematically altering the alignment, we are able to typify the topology of the vast majority of existing control relationships. Then, by eliminating the effects of indirect gene control influences, we are able to accurately and completely model gene control networks(Fig.4).

Graphical Gaussian Modeling approach. We also have developed a novel approach to infer the genetic networks from expression profiles. In our approach, the networks are inferred by a combination of cluster analysis and a method called 'Graphical Gaussian Modeling.

Application to Saccharom*yces cerevisiae*. We developed an extensive yeast gene expression library consisting of full-genome cDNA array data for over 500 yeast strains, each with a single gene disruption.

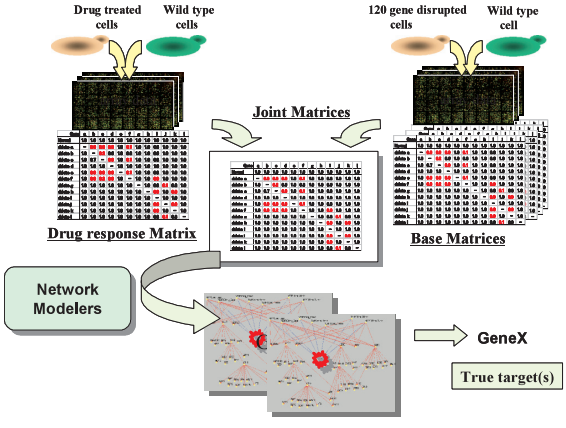


Fig.4. Drug targeting method based on gene networks

Using this data, combined with dose and time course expression experiments with the oral antifungal agent Griseofulvin, we identified CIK1 as an important affected target gene based on Boolean and Bayesian network analysis(Fig.5).

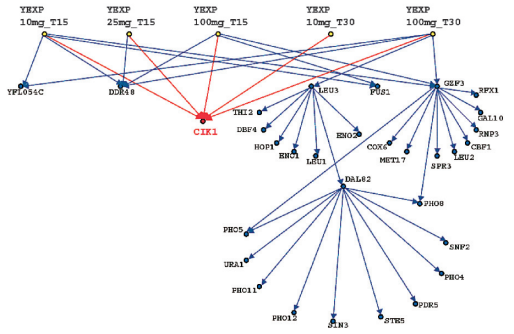


Fig.5. Target genes of Griseofulvin

3. Concluding Remarks

In genome research fields, we have developed the technologies critical to post genomic analysis such as transcriptome analysis and network based functional analysis to take advantage of this wealth of recently acquired biological information. We also investigate methods leveraging genome structure to advance from single gene functional analysis to inference of system wide network based functional analysis.

4. Primary Publications

(1) Savoie, C.J., et.al., Use of gene networks from full genome microarray libraries to identify functionally relevant drug-affected genes and gene regulation cascades. *DNA Research*, 10, 19-25, 2003.

(2) Aburatani, S., et.al., Discovery of novel transcription control relationships with gene regulatory networks generated from multiple-disruption full genome expression libraries. *DNA Research*, 10, 1-8, 2003.

(3) Araki, Y., Konishi, S. and Imoto, S. 2004. Functional discriminant analysis for microarray gene expression data via radial basis function networks, Proceedings of COMPSTAT'2004 Symposium, Physica-Verlag/Springer, pp. 613- 620.

(4) Uchida, S., et.al., Detection and Normalization of Biases Present in Spotted cDNA Microarray Data : a composite method addressing dye, intensity-dependent, spatially-dependent, and print-order biases. *DNA Research*. 12, 1, 2005.

(5) Aburatani, S., et.al., Deduction of a Gene Regulatory Relationship Framework from Gene Expression Data by the Application of Graphical Gaussian Modeling. *Signal Processing*, 83, 777-788, 2003.

(6) T. Kuwahara, T., et. al., Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. *Proc. Natl. Acad. Sci. U.S.A.,* 101, 14919-14924, 2004.

Analysis of Alzheimer Disease-Related Genes

Project Leader:
Masatoshi TAKEDA Professor, Graduate School of Medicine, Osaka University

1. Objective

The purpose of this project is to elucidate mechanism of the development of Alzheimer disease (AD) through searching for AD-related genes. AD is the first cause of dementia in the elderly, and its therapy and prevention are socially a strong matter of concern, provided by the estimate that the number of the patients could be more than 2,000,000 in 2025. Based on a neuropathological study, the major causative diseases of dementia are AD (52%), cerebrovascular dementia (28%), and Lewy body disease (18%).

AD is a neurodegenerative disorder, pathologically characterized by neuritic plaques and neurofibrillary tangles in the brain, and is thought as not monogenic but polygenic, highly heterogeneous disorders. Genetic analyses of early-onset autosomal dominant AD supported the amyloid cascade theory that AD is caused by abnormal production of beta amyloid. However, more than 90% of AD is a late-onset AD (LOAD), thought as one of polygenic diseases of which occurrence is promoted by environmental factors based on neuronal aging and genetic propensity. Mechanism to develop LOAD is complicated, and inversely the therapy and prevention of LOAD could be possible. Therefore, we searched for AD-related genes through genome analysis, and examined the risk effect or age-at-onset modification.

2. Summary

2-1. Correction of LOAD and control samples :

Screening of genetic risk effect on LOAD was examined using 300 patients evaluated by neuroimaging studies and 80 patients pathologically diagnosed, and 380 non-demented community-dwelled controls. Genetic risks were further evaluated using about 1000 patients and 1000 controls.

2-2. Risk genes reported previously :

We examined the risk effect of more than 60 genes reported previously, utilizing positional effects of SNPs closely located to the risk alleles of SNPs. However, we replicated the risk effect in only MME (3q21-q27), IDE (10q23) and APOC1 gene (19q13.2). On the other hand, among genes related to hypertension, we detected the risk effect in IL1RAP (3q28), COL9A1 (6q23) and ASE-1 gene (19q13).

2-3. β amyloid / cognition-related genes :

PS1 gene (14q24.3) is a causative gene of AD, and a SNP located in its promoter region indicated a genetic risk for LOAD. BDNF gene (11q13) is related to neuronal survival and the development of cognitive function, and an allele linked to decreased survival of neuronal cells in vitro also showed a risk for LOAD.

2-4. Genes related to lipid metabolism :

The APOE (19q13.2) gene, the sole risk gene generally accepted, is encoded in a gene cluster of apolipoprotein gene family. The APOC2 gene is located in this cluster, encoding an intragenic microsatellite, (GT)n(GA)m. We found that this repetitive sequence showed a positive correlation with age-at-onset of LOAD, and also an inverse correlation with serum apoC-II level, indicating that increased plasma apoC-II level, genetically modified by the APOC2 gene, promotes the age-at-onset of LOAD.

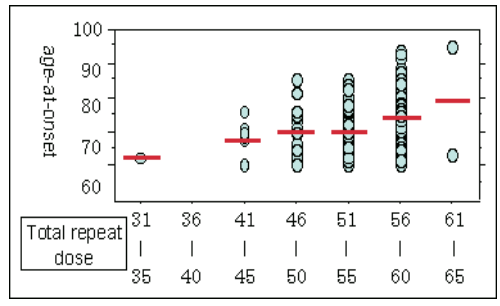


Fig.1. A correlation of the repeat dose of APOC2 microsatellite with age-at-onset of LOAD

To further elucidate the alteration of lipid metabolism on the occurrence of LOAD, we compared plasma lipid and apolipoprotein profiles between 26 LOAD and 26 sex- and age-matched non-demented subjects. We found that the decrease in plasma apoA-II, a component of HDL cholesterol, was the most prominent ($p<0.001$), suggesting that the APOA2 gene (1q21-q23), encoding apoA-II, could be a candidate for genetic risks. The APOA2 gene also harbors a microsatellite (TG)n, located only 6 bp upstream of exon 3, that could be related to gene expression.

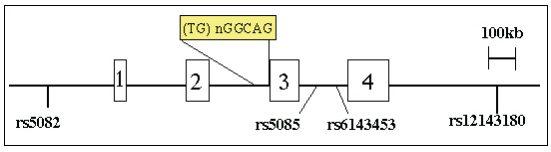


Fig.2. APOA2 gene structure

We found that in patients with APOE-ε4 positive LOAD, (TG) dose of the microsatellite was inversely correlated with the age-at-onset. Using a pSPL3 minigene expression system, we found that the length of the microsatellite (TG)n promoted the splicing out of exon 3, leading to deletion of amino acids encoded in exon 3. Thus, decreased plasma apoA-II level is affected by genetic variation of the length of the microsatellite (TG)n.

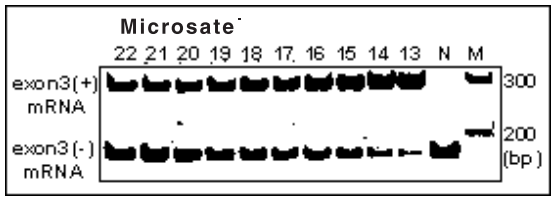


Fig.3.

It was concluded that these repeat dose of the microsatellite in APOA2 and APOC2 genes are the genetic modifiers of the age-at-onset of LOAD, provided by the evidence that control of plasma lipid could retard the occurrence of LOAD.

2-5. Genome scan of candidate risk loci :

Genome scanning of AD in Caucasian population noted many risk loci of AD, among which 10 risk loci are supported by more than two independent genome scan; 1q21-q23、4q32、5p12-p14、6q26-q27、9p23-p22、10p11-p14、10q24、12p13-p11、19q13 (APOE)、21q21-q22. Therefore, we targeted the short arm of chromosome 12 (12p) and chromosome 21, to perform linkage disequilibrium mapping of risk genes of LOAD. SNPs with a minor allele frequency at more than 5% were selected at 100-kb interval, and more than one SNP per gene were also chosen.

Marker	Odds ratio (95% C.I.)	p value
F12D18	5.66 (1.72 - 18.7)	0.00500
12p124H	3.84 (1.95 - 7.55)	0.00000
12p317	1.75 (1.19 - 2.63)	0.00300
12p316	1.56 (1.28 - 1.92)	0.00001
12p043	1.45 (1.14 - 1.85)	0.00087
12p161	1.37 (1.10 - 1.70)	0.00300
12p075	1.35 (1.10 - 1.64)	0.00227
12p212	1.26 (1.04 - 1.53)	0.02000

Table.1. LOAD-risk gene mapping at 12p.

Eight genes at 12p locus significantly showed linkage disequilibrium with LOAD. As for 21 locus, linkage disequilibrium was identified at 9 loci and 6 genes, among which one gene is located within Down syndrome critical region. We are studying function analysis of this gene in relation to β amyloid interaction. We also performed linkage disequilibrium mapping at 10q24 locus, and IDE and KNSL1 gene significantly showed linkage disequilibrium. Both genes are located oppositely, and we are currently examining detailed analysis of this promoter region.

2-6. Genes showing altered expression in AD hippocampus :

We also examined linkage disequilibrium with genes showing altered expression in hippocampus of AD. Five genes among those decreased expression in AD (27.8%), and 3 genes among those increased expression in AD showed significant associations. Notable association was found in POU2F1 gene (1q22-q23) ($p<0.0007$).

3. Concluding Remarks

We identified 27 novel risk genes and 14 risk loci of LOAD, in total. These genes and loci should be evaluated by detailed genome analyses, and these genetic factors could be a clue providing a novel approach in treating and preventing LOAD.

4. Primary Publications

(1) Kamino K, Kida T, Tanaka T, Tanii H, Okochi M, Kudo T, Kobayashi T, Takeda M: Apolipoprotein and β amyloid transport pathway. *PSYCHOGERIATRICS*, 2, 149-155 (2002).

(2) Nishimura M, Sakamoto T, Kaji R, Kawakami H: Influence of polymorphisms in the genes for cytokines and glutathione s-transferase omega on sporadic Alzheimer's disease. *Neurosci. Lett.*, 368, 140–143 (2004).

(3) Kida T, Kamino K, Yamamoto M, Kanayama D, Tanaka T, Kudo T, Takeda M: C677T polymorphism of methylenetetrahydrofolate reductase (MTHFR) gene affects plasma homocysteine level and is a genetic factor of late-onset Alzheimer's disease. *PSYCHOGERIATRICS*, 3, 4-10 (2004).

(4) Matsubara-Tsutsui M, Yasuda M, Yamagata H, Nomura T, Taguchi K, Kohara K, Miyoshi K, Miki T: Molecular evidence of presenilin mutation in familial early onset dementia. *Am. J. Med. Genet. (Neuropsychiatric Genetics)*, 114, 292-298 (2002).

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(6) Taguchi K, Yamagata H, Zhong W, Kamino K, Akatsu H, Hata R, Yamamoto T, Kosaka K, Takeda M, Kondo I, Miki T: Identification of hippocampus-related candidate genes for Alzheimer's disease. *Ann. Neurol.*, 57, 585–588 (2005).

2. Development / Differentiation / Regeneration

(1) Research Promotion Committee Members

○	Masatoshi TAKEICHI	(RIKEN)
	Shinichi AIZAWA	(RIKEN)
	Masuo OBINATA	(Tohoku University)
	Motoya KATSUKI	(National Institutes for Basic Biology)
	Shinichi NISHIKAWA	(RIKEN)
	Hiroshi HAMADA	(Osaka University)
	Hajime FUJISAWA	(Nagoya University)

○ : Committee Chairperson

(2) List of Research Projects

No.	Research Project	Project Leader
1	Establishment of Cell and Organ Transplantation Therapy Using Embryonic and Somatic Stem Cells	Nobuaki YOSHIDA (The University of Tokyo)
2	Regulation of Immune Response Based on Immuno-cytology and its Clinical Application to Cell Transplantation Medicine	Kenzaburo TANI (Kyushu University)
3	Tissue Regeneration and Construction by Control of Differentiation Programs	Norio NAKATSUJI (Kyoto University)
4	Characterization of Somatic Stem Cells and Tissue Reconstruction	Toshio SUDA (Keio University)
5	Studies on the Organogenesis and Disease Control in the Hepatobiliary and Panreatic System	Fumio ENDO (Kumamoto University)
6	Elucidation of the Principles of Development and Regeneration by Systematic Analysis of Genes	Naoto UENO (National Institute for Basic Biology)
7	Studies on Molecular Mechanisms Underlying Development, Differentiation and Regeneration of Neural Cells	Kazuhiro IKENAKA (National Institute for Physiological Sciences)

Establishment of Cell and Organ Transplantation Therapy Using Embryonic and Somatic Stem Cells

Project Leader:
Nobuaki YOSHIDA Professor, The Institute of Medical Science, The University of Tokyo

1. Objective

For the maintenance of self-renewal of embryonic stem (ES) cells, 2 transcriptional factors, Nanog and Oct-3/4, play essential roles. The expression of these factors is restricted to undifferentiated ES cells and is downregulated when the cells are induced to differentiate. However, the molecular basis for the ES cell self-renewal is still unclear. Our main purpose is to know what makes ES cells remain in the pluripotent state. On the other hand, It is essential to prospectively identify tissue stem cells to understand the mechanisms that regulate their self-renewal and multipotency. Furthermore, for the establishment of cell and organ transplantation therapy, we are analyzing the molecular mechanisms of kidney development. Our goal is to elucidate molecular mechanisms in kidney development. We also aim at derivation of kidney progenitors from stem cells, by utilizing knowledge obtained from molecular genetics.

2. Summary

2-1. Functional analysis of PTB in undifferentiated ES cells :

Rex-1 is one of the marker genes for undifferentiated ES cells whose expression is controlled by Oct-3/4 and unknown transcription factor named Rox-1. We identified PTB (polypyrimidine tract binding protein) as a candidate of Rox-1 and provide evidence that PTB is involved in the expression of Rex-*1* and *Nanog* genes. PTB null mice die around the stage of implantation and ES cells are not recovered from the blastocyats of these mice so far. The attempt to establish PTB null ES cells using Cre/loxP system in vitro has revealed the reduced growth rate in these cells when compared to wild-type ES cells. (Fig.1)

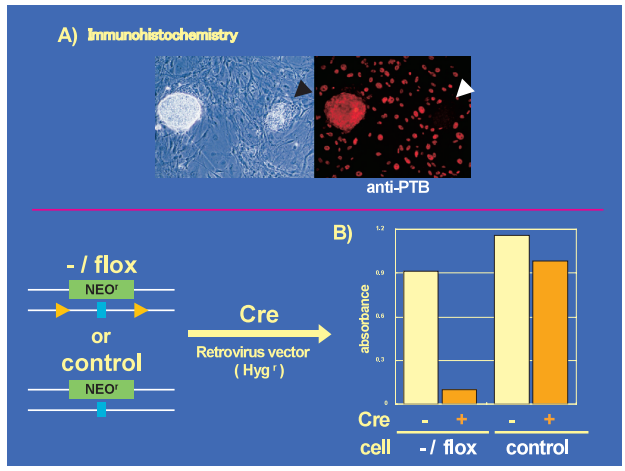

Fig.1. Reduced Growth Rate of PTB Null ES Cells

These result indicates the important roles of PTB both in ES cell growth/self-renewal and in early mouse development. We also found Sall4 has crucial role in undifferentiated ES cells through the analysis of knockout experiment *in vivo and in vitro*.
(Nobuaki Yoshida)

2-2. The role of Bmi-1 in hematopoietic stem cells :

Self-renewal and multi-lineage differentiation capabilities are two unique features of stem cells. Elucidation of the mechanisms by which stem cells self-renew or differentiate is crucial to developmental

biology and regenerative medicine. Using purified hematopoietic stem cells (HSCs) as a model, we have studied their self-renewal and commitment process. Paired-daughter cell analysis revealed that lineage commitment takes place asymmetrically at the level of HSCs under the influence of external factors. In addition, we have clarified that expression level of Bmi-1, but not other members of Polycomb group proteins is a critical determinant of HSC self-renewal. (Fig.2) (Hiromitsu Nakauchi)

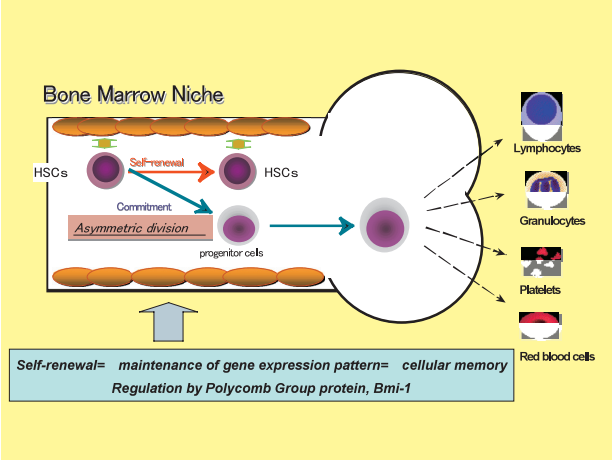

Fig.2. Expression level of Bmi-1 is a critical determinant of HSC self-renewal

2-3. The role of Sall1 in early kidney development :

Sall1 is a mammalian homolog of the *Drosophila* region-specific homeotic gene *spalt* (*sal*). We found that mice deficient in *Sall1* die in the perinatal period and that kidney agenesis or severe dysgenesis are present. *Sall1* is essential for ureteric bud invasion, the initial key step for metanephros development. Using *Sall1-GFP* knockin mice, we also set up an *in vitro* culture system, in which a single renal progenitor in the metanephric mesenchyme forms colonies consisting of several types of epithelial cells. Thus our colony-forming assay, which identifies multipotent progenitors in the embryonic mouse kidney, can be used for determining mechanisms of renal progenitor differentiation. (Fig.3)

(Ryuichi Nishinakamura)

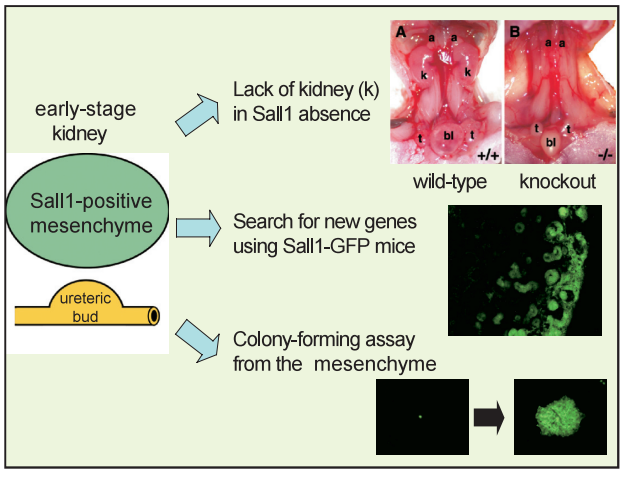

Fig.3. Sall1 has crucial role in early kidney development

3. Concluding Remarks

We have found two independent genes which are important for the maintenance of ES cells. Through the analysis of the function of these gene, the common mechanism for the maintenance of embryonic and somatic stem cells might be clarified. We also hope that the culture of human ES cells might become easier.

Lineage commitment takes place asymmetrically at the level of HSCs under the influence of external factors. Expression level of Bmi-1, but not other members of Polycomb group proteins is a critical determinant of HSC self-renewal.

We have found an essential gene for kidney development and are currently examining molecular functions of *Sall1*. We also plan to derive kidney progenitors from a variety of cell sources including embryonic stem cells, utilizing the colony-forming assay described above.

4. Primary Publications

(1) Takano H, Ema H, Nakauchi H. Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs. J Exp Med. 2004 199:295-302, 2004.

(2) Iwama A, Oguro H, Negishi M, Kato Y, Morita Y, Tsukui H, Ema H, Kamijo T, Yuko Kato-Fukui, Koseki H, van Lohuizen M, and Nakauchi H. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product, Bmi-1. Immunity. 21: 843-51, 2004.

(3) Takasato, M., Osafune, K., Matsumoto, Y., Yoshida, N., Meguro H., Aburatani, H., Asashijma, M., and Nishinakamura, R. Identification of kidney mesenchymal genes by a combination of microarray analysis and *Sall1- GFP* knockin mice. Mech. Dev, 121: 547-557. 2004.

(4) Sato, A., Matsumoto, Y., Koide, U., Kataoka, Y., Yoshida, N,. Yokota, T., Asashima, M., and Nishinakamura, R. Zinc finger protein Sall2 is not essential for embryonic and kidney development. Mol. Cell. Biol. 23: 62-69, 2003.

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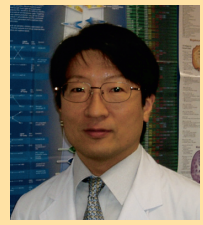
Regulation of Immune Response Based on Immuno-cytology and its Clinical Application to Cell Transplantation Medicine



Project Leader:
Kenzaburo TANI Professor, Medical Institute of Bioregulation, Kyushu University

1. Objective

As human embryonic stem (ES) cells have been established, the development of new transplantation medicine using cells or organs cultured from ES cells has become realistic. Such clinical research using human ES cells should be carefully done from ethical standpoint of view and should be preceded by preclinical research using animals more close to human. Common marmoset, small new world monkey, has several benefits as experimental animals including the hematological and immunological homology with human, being easy to handle with, and the existence of several experimental colonies in Japan. In this study, we tried to develop new cell therapies using marmoset ES cells and tested their usefulness and safety in vitro and in vivo.

Fig.1. Common marmosets

2. Summary

2-1. Establishment of CM ES cells :

First of all, we have established artificial ovulation methods in marmosets, surgical collection methods of fertilized eggs and isolation techniques of inner cell mass. We could successfully establish 3 new CM-ES cell lines and demonstrated their pluripotencies in vitro and in vivo, which were not demonstrated in CM-ES cell lines established by Thomson et al., in 1996. Our lines will be very helpful to develop human ES cell therapies as well as human disease models in monkeys.

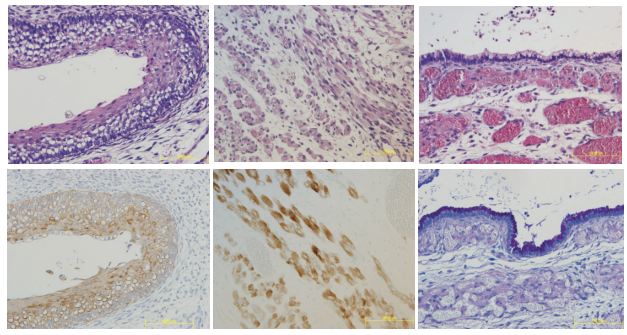


Fig.2. Teratoma formation of CMES cells was confirmed in immune deficient mice. :
Ectodermal (upper left), mesodermal (upper middle) and endodermal (upper right) characters are observed after HE staining. These cells are stained with cytokeratin (lower left), desmin (lower middle) and alucian blue-PAS (lower right).

2-2. Efficient hematopoietic cells induction from common marmoset (CM) embryonic stem(ES) cells :

We have tried various in vitro conditions including coculturing with OP9 stromal cells to induce hematopoietic stem cells from CM ES cells. The efficiency, however, was so poor. So we lentivirally transduced 6 known hematopoiesis-related genes, HoxB4, Bmi-1, Lh2, Tal1/scl, gata1, gata2 into CM-ES cells. We found tal1/scl gene efficiently induced CM CD34 positive cells in vitro and the cells were differentiated to three lineage blood cells. Currently, we are now trying to transplant these cells to common marmosets to determine the possibilities of using these Tal1/scl transduced cells as the hematopoietic stem cell source in vivo.

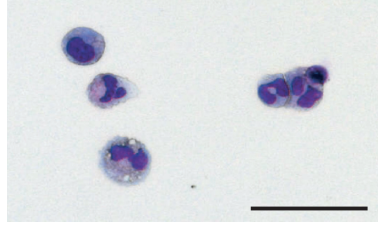


Fig.3. Granulocytes differentiated from Tal1/scl gene transduced common marmoset ES cells in vitro.

2-3. Identification and cloning of new stem cell expansion factors and the development of tissue homing method :

It is very important to develop stem cell expansion methods as well as efficient tissue homing method for the purpose of developing new cell therapies. Based on this idea, we have done the following research.

(1) Identification of hematopoietic stem cell or progenitors (HSC/Ps) expansion factor:

The ability of HSC/Ps to reconstitute the hematopoietic system of irradiated hosts is significantly enhanced in the absence of an intracellular adaptor protein, Lnk. We have developed a Lnk mutant that dominant-negatively inhibits the functions of Lnk endogenously expressed in the HSC/Ps and potentiate them for engraftment. Inhibition of Lnk-mediated pathways could be a potent approach to augment HSC/P engraftment without obvious side effects.

(2) Identification of stromal cell derived HSC/Ps expansion factor and ES cell maintenance factor:

We have cloned ISF (immune suppressor factor) gene using retroviral expression cloning methods and demonstrated its supporting ability of long-term culture-initiating cells when ISF was expressed in bone marrow stromal cells. The HSC/Ps proliferative activities were induced by Wint3a and Tie2, respectively through the suppression of TIMP3 and SFRP-1. Also, our results suggested Wint3a played important roles in the maintenance of ES cells.

(3) Identification of tissue homing factor:

Our results suggested that the CXCR4-SDF-1 system might be useful to localize CXCR4-expressing cells to bone surface.

3. Concluding Remarks

The regenerative medical research using common marmosets and the ES cells are considered to cast very important message in the field of clinical medicine, because the finding observed using these preclinical animal models will certainly suggest many important direct message to human medicine. As the Tal1/scl gene transduction facilitated the differentiation of marmoset ES cells to hematopoietic cells, this methods would open new stem cell transplantation method as well as transfusion method if our findings can also be applied to human ES cells. As the new stem cell expansion factors including Link dominant negative mutant and ISF, and tissue specific homing system of CXCR4-SDF-1 are considered to be promising method to develop novel cell therapy methods, further basic and clinical investigations are required.

4. Primary Publications

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(2) Soda Y, Tani K, Bai Y, Saiki M, Chen M, Izawa K, Kobayashi S, Takahashi S, Uchimaru K, Kuwabara T, Warashina M, Tanabe T, Miyoshi H, Sugita K, Nakazawa S, Tojo A, Taira K, Asano S : “A novel Maxizyme vector targeting a bcr-abl fusion gene induced specific cell death in Philadelphia chromosome-positive lymphoblastic acute leukemia” Blood, 104(2), 356-363(2004).

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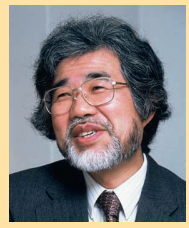
(5) Ema H, Sudo K, Seita J, Maeda A, Morita Y, Osawa M Takatsu K, Takaki S, Nakauchi H. : “Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice” Dev Cell., 2005 (in press).

(6) Sasaki E, Hanazawa K, Kurita R, Akatsuka A, Yoshizaki T, Ishii H, Tanioka Y, Ohnish Y, Suemizu H, Sugawara A, Tamaoki N, Izawa K, Nakazaki Y, Hamada H, Suemori H, Nakatsuj N, Okano H, and Tani K : “Establishment of Novel Embryonic Stem Cell Lines Derived from the Common Marmoset (Callithrix jacchus)” Stem Cells 2005 (in press).

Tissue Regeneration and Construction by Control of Differentiation Programs



Project Leader:
Norio NAKATSUJI Professor, Institute for Frontier Medical Sciences, Kyoto University

1. Objective

There are clear limits of the organ transplantation therapy, such as limited number of donors and immunological rejection. This research project is a basic research aimed at development of new therapies of regenerative medicine. It includes the following research lines in Institute for Frontier Medical Sciences.

(1) Establishment, characterization, culture and manipulation methods improvement of primate embryonic stem (ES) cell lines.
(2) Investigation of regulative mechanisms of the stem cell differentiation.
(3) Investigation of reprogramming mechanisms in dedifferentiation and transdifferentiation.

2. Summary

2-1. Establishment and characterization of cynomolgus monkey ES cell lines. Monkey ES cell lines are important for preclinical investigation. We have established several ES cell lines and distributed to other scientists in Japan after characterization of cell lines.

2-2. Improvement of culture methods for primate ES cells. Primate ES cells are much more difficult to maintain compared to mouse ES cells. We have succeeded to maintain for long periods by improvement of the serum-free medium and subculture method. As the results, we can now maintain monkey and human ES cells stably as the undifferentiated stem cells more than one-year periods. Also, we can now maintain primate ES cells without feeder cells for a limited period.

2-3. Development of gene transfection methods for primate ES cells. Primate ES cells are very sensitive to various stresses. We have developed reliable methods of electroporation and drug selection to produce ES cell lines with integrated transgenes (Fig.1).

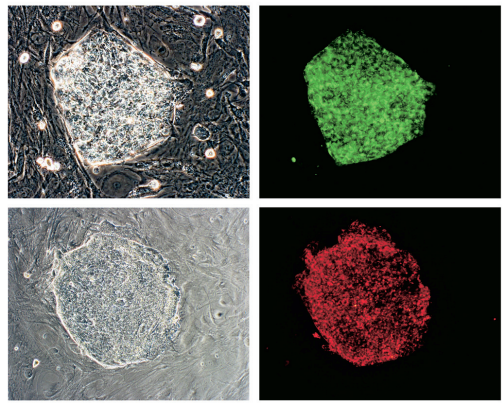


Fig.1. Cynomolgus monkey ES cell colonies after transfection of green or red fluorescent protein genes.

2-4. Analysis of maintenance mechanisms of undifferentiated status in primate ES cells, focused on LIF/STAT3 signaling. We have made clear that such signaling pathways are very different between primate and mouse ES cells.

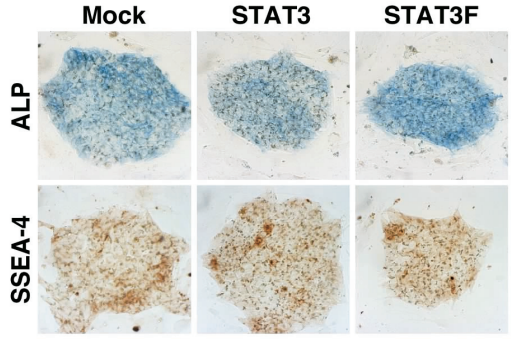


Fig.2. There is no effect when LIF/STAT3 signaling was inhibited by dominant negative vectors in monkey ES cells.

2-5. Preclinical investigation using monkey ES cell. We have carried out collaboration research with neurosurgeon group of Kyoto University Hospital. We have produced dopaminergic neurons from cynomolgus monkey ES cells and transplanted into brains of Parkinson disease model monkeys. They showed improvement of symptoms. This was recognized worldwide as the first success in treating Parkinson disease of monkeys using ES cells.

3. Concluding Remarks

We have succeeded in improvement of culture methods, cryopreservation methods, and gene transfection techniques in primate ES cells, thus enabling their stable maintenance and experimental manipulation. These methods are important basis for utilization of human ES cells in regenerative medicine. We also made clear that molecular mechanisms involved in maintenance of undifferentiated status are significantly different between primate and mouse ES cells. We can now maintain primate ES cells in the feeder-free condition for a limited period.

For the cell therapy using human ES cells, preclinical model system using monkey ES cells and monkey disease models is crucial for evaluation of effectiveness and safety. In such aspects, this research project is contributing greatly by providing basic technology and monkey ES cells for many medical researchers around Japan.

Also, our Institute for Frontier Medical Sciences is the national center of human ES cell research in Japan, because we are the only research group in Japan now establishing and distributing human ES cells to other biomedical research institutes throughout Japan. This research project has made solid basis of such important activity of our institute.

4. Primary Publications

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Characterization of Somatic Stem Cells and Tissue Reconstruction

Project Leader:
Toshio SUDA Professor, Keio University, School of Medicine

1. Objective

Stem cells are cells with self-renewal capacity as well as the capacity for differentiating into single or multiple lineages. Cell production is maintained throughout the lifetime of an individual by stem cells. Stem cell function is defined by the ability to reconstitute and maintain the tissues for long-term. The balance between self-renewal and commitment of stem cells is controlled by a combination of cell-intrinsic and external regulatory mechanism. The intrinsic cellular and molecular properties of stem cells have been extensively characterized. And recently, the niches or the specific microenvironment where stem cells reside in situ are studied at the molecular level. The concept of the stem cell niche was first proposed for the human hematopoietic system in the 1970's. A similar concept has also been postulated for the stem cells of epidermis, intestinal epithelium, nervous system and gonads.

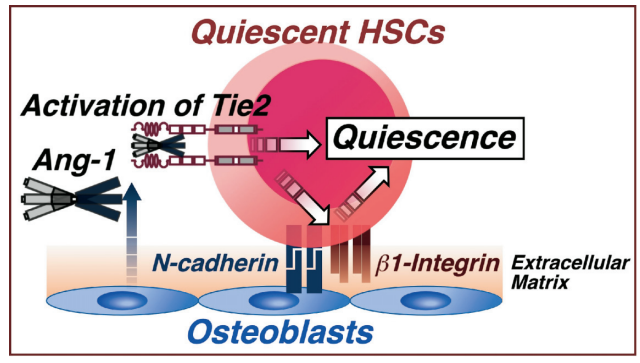
In this project, we try to characterize the somatic stem cells and tissue. In Keio University, Suda et al. attempted to identify the stem cell niche and show whether the self-renewal capacity of hematopoietic stem cells (HSCs) out of niches are sensitive to reactive oxygen species (ROS). In Kumamoto University, Taga et al. try to clarify whether that DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the brain. Moreover, Saya et al. investigate the mechanism for cell division and analyze the role of Aurora-A kinase in mitosis, which might be involved in the asymmetric cell division of stem cells.

2. Summary

2-1. Keio University research group :

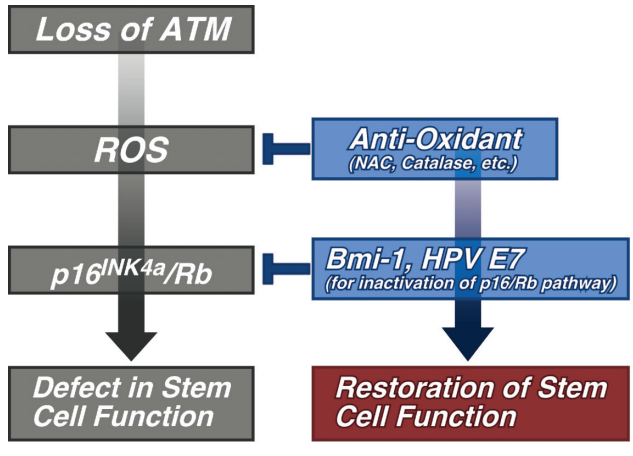
Interaction of HSCs with their particular microenvironments, known as the stem cell niches, is critical for cell cycle regulation of HSCs. We have revealed a molecular mechanism by which the cell cycle of HSCs is regulated by the niche. HSCs expressing the receptor tyrosine kinase Tie2 are adhere to osteoblasts in the BM niche. The interaction of Tie2 and its ligand Angiopoietin-1 (Ang-1) leads to tight adhesion of HSCs to osteoblasts through N-cadherin, resulting in maintenance of the long-term repopulating activity of HSCs.

Fig.1.



To analyze the self-renewal capacity of HSCs in the niche, we investigated the role of "Ataxia telangiectasia mutated" (ATM) protein, a key molecule of cell cycle checkpoint. ATM regulates the reconstitution capacity of the HSC, which was associated with the elevation of the ROS. Treatment of anti-oxidative reagents restored the reconstitution capacity of $ATM^{-/-}$ HSCs. From these findings, the involvement of oxidative stress is shown in interaction between HSCs and the niche.

Fig.2.



2-2. Kumamoto University research group :

We identified new molecular mechanisms regulating stem cell maintenance, differentiation, mitosis, cell motility and adhesion, which are essential events for tissue construction.

(1) We found that implantation of neural stem cells expressing BMP antagonist to spinal cord-injured mice contributes to aster recovery.
(2) We have identified a subpopulation of C6 glioma cells, based on the Hoechst33342-dye staining profile, which has cancer stem characteristics. This subpopulation shows malignant phenotype as well as multipotency.
(3) During embryogenesis, definitive hematopoiesis arises in the AGM region. We found that a Ras/MAP kinse pathway inhibitor Spred-2, an adaptor molecule Lnk, and STAT3 serine727 kinase HIPK2 inhibits AGM hematopoiesis.
(4) We clarified that Aurora-A kinase plays an important role in various mitotic events such as mitotic entry, centrosome separation and chromosome alignment and demonstrated its significance in tissue construction and tumorigenesis.
(5) We also investigated how adhesion molecule CD44 regulates cell motility and found that interalization of CD44 with hyarulonic acids and cleavage of CD44 by metalloproteases are critical steps for cells to move in the matrix.

3. Concluding Remarks

Stem cells are cells with self-renewal capacity as well as the capacity for differentiating into single or multiple lineages. Blood cell production is maintained throughout the lifetime of an individual by HSCs. The quiescent state of stem cells is thought to be an important mechanism for protecting cells from the stress. It begins to be elucidated that osteoblasts are critical components of the particular microenvironments, or niches for HSCs in adult bone marrow, and how stem cells persist in an immature state there. We have shown that the quiescence of HSCs is dynamically controlled by the signalings of receptors/ligands (Tie2/angiopoietins) and cell adhesion molecules such as N-cadherin.

On the other hand, glioma stem cells were identified as side population cells, which were shown to have the capacity to self-renew and to differentiate into multi-lineage pathway. The understanding of the relationship of normal and cancer stem cells to their niches should lead to development of new strategies directed toward regeneration medicine and cancer therapeutics.

4. Primary Publications

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(2) Marumoto T, Zhang D, Saya H: Aurora-A - a guardian of poles. Nat Rev Cancer. 5: 42-50, 2005, Review.
(3) Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T: Tie2/Angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell, 118: 149-161, 2004.
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(5) Setoguchi T, Nakashima K, Takizawa T, Yanagisawa M, Ochiai W, Okabe M, Yone K, Komiya S, Taga T: Treatment of spinal cord injury by transplantation of fetal neural precursor cells engineered to express BMP inhibitor. Exp Neurol, 189: 33-44, 2004.
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(7) Arai F, Ohneda O, Miyamoto T, Zhang X-Q, Suda T: Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. J Exp Med, 195: 1549-1563, 2002.
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Studies on the Organogenesis and Disease Control in the Hepatobiliary and Pancreatic System

Project Leader:
Fumio ENDO Professor, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University

1. Objective

We propose a transplantation therapy for 21st century ,which allow us to regenerate many organs, instead of organ transplantation between human to human that are restricted because of both ethical and economical problems. The hepatobiliary and pancreatic system are originated from endoderm. They are committed by many transcription factors. Their proliferation and differentiation are controled by a lot of known or unknown secreted factors. We tried to identify these factors and revealed molecular mechanisms of the development of the hepatobiliary and pancreatic system. Furthermore we challenged to reorganize hepatobiliary system by means of transplantation of progenitor cells derived from salivary gland. Our achievement should be useful for therapy not only hepatic but also for biliary and pancreatic diseases.

2. Summary

2-1. Achievements :

(1) We transplanted gene modified hepatic cells into model mice for hereditary hepatic disease. Using the cell transplantation, we identified new endodermal stem cells (Fig.1). We established technique to replace damaged hepatocyte of the model mice with gene modified hepatocyte (Fig.2). Furthermore, we found method for separating hepatic progenitor cells from adult. Next, we established method for separating stem cells of liver and pancreas. We can produce hepatic and pancreatic cells in vivo and in vitro.

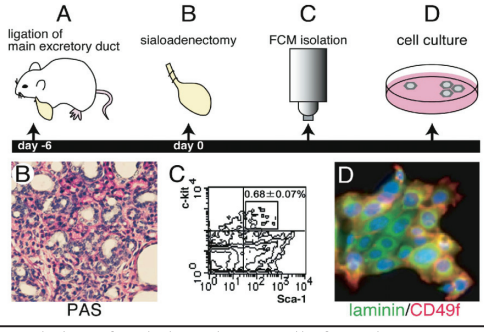

Fig.1. Isolation of endodermal stem cells from the mouse salivary gland(A). After 6 days of duct ligation, acinar cell depletion and ductal cell proliferation occurred in salivary gland(B). Single cell suspension were prepared form salivary gland. Labeled cells were collected by flow cytometry(C). Cultured stem cells were positive for CD49f and intracellular laminin(D).

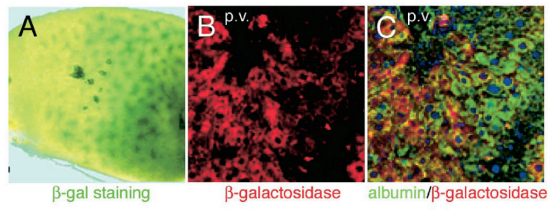

Fig.2. The liver from lethal mice with hepatic injury after transplantation of ubiquitous β-gal expressing (ROSA26) mouse salivary gland-derived endodermal stem cell (Hisatomi et al.). Transplanted donor cells were distributed in the whole recipient liver (A), and produced albumin (B,C). This stem cell transplantation therapy rescued lethal hepatic injury. (p.v. portal vein)

(2) We established method to isolate hepatic and pancreatic cells derived from pluripotent stem cells. In this method, GFP labeled cells were recognized as pancreatic progenitor cells. We investigated gene expressions of progenitor cells isolated by this method. We have got new founding after investigation of gene expression of translation factors.

(3) We have identified 4 new genes involved in morphogenesis of hepatobiliary and pancreatic development derived from chicken gut system. We have got new founding of spatiotemporal pattern of these genes.

(4) We achieved our initial plans to investigate endodermal stem cells. We established basic technologies of endodermal stem cell to apply regenerative medicine.

2-2. Biproducts from our study. :

(1) We elucidated a system for endodermal stem cells in the adult tissue. We established new methods for isolation of the endodermal stem cells using the system. The stem cell system required supporting cells to assist stem cell survival and proliferation. The finding of supporting cells is one of the breakthrough of adult stem cell study. We cloned the supporting cells from salivary gland derived cells. Humoral factors derived from the supporting cells stimulated very immature endodermal stem cells and let them survive and proliferate. The finding of supporting cells was very important for application of regenerative

medicine.

(2) Immature stem cells derived from the salivary gland were suit for donor cells to generate clone animals. We established cloned pig colony to use them for medical resource.

3. Concluding Remarks

Endo found endodermal stem cells in the salivary glands. We isolated the cells and analyzed the cells to established isolation method using cell sorting (Fig.1). The method enables us to isolate and detect endodermal stem cells in the rodent, pig and humans (Fig.3). Further study enables us to detect more immature cells. The new progenitor cells were cultured as floating cells. The cells were negative for VLA6 and extracellular laminin for long time in the culture. However, attachment of the floating cells to extracellular matrix enables us to developed VLA6 and intracellular laminin positive matured endodermal cells. During the process of this maturation, cells to support survival of immature cells were essential. We cloned the supporting cells to identify and elucidate the mechanisms of these cells. We found new evidences for existence, proliferation and differentiation of adult tissue endodermal stem cells. We convinced that regenerative medicine to regulate hepatobiliary and pancreatic diseases proceeds with these evidences. These outcomes of our project must be applied not only to fields of regenerative medicine as already shown in our study but also to many fields such as toxicology and drug delivery.

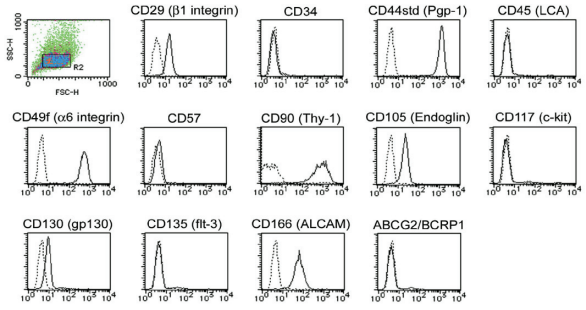

Fig.3. Forward scatter and side scatter pattern of salivary gland-derived endodermal stem cells can be seen. Cells were labeled with the following antibodies: CD29/integrin β1, CD34, CD44s/Pgp-1, CD45/LCA, CD49f/integrin α6, CD57, CD90/Thy-1, CD105/Endoglin, CD117/c-kit, CD130/gp130 common β-chain, CD135, CD166 and ABCG2/BCRP1. Continuous lines represent histograms using antibodies of each marker and dotted lines represent isotype control antibody treatment. 7-aminoactinomycin D was used to discriminate living cells from dead cells, which were excluded by flow-cytometric analysis.

Kume et al. created assay systems to evaluate development of pancreatic tissue with mouse embryonic stem cells. Using the system, process to differentiate endodermal stem cells from embryonic stem cells and endoderm specific tissue from endoderm were elucidated. In consequence of these study, efficient differentiation of endodermal stem cells from embryonic stem cells were generated. The process of differentiation can apply to human stem cells and contribute to human regenerative medicine.

Yokouchi et al. elucidated a process of liver development of chicken embryo, established differentiation markers, method for endoderm specific gene transfer and tissue culture for hepatic development of chicken. Using these chicken systems, fast and economical study for liver specific study for development were achieved compare to the mice system. On the other hand, expression pattern screening was completed to identify gene to contribute liver development. From these screening, 16 genes were identified to express interesting patterns. These genes contains 3 liver endoderm specific genes, 9 septum transversum paraxial mesoderm specific genes and 4 endothelial cell layer specific genes. Function of these genes will be proved to add new insight of mechanisms for liver development.

4. Primary Publications

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(3) Nakamura K., Mitsubuchi H., Miyayama H., Yatsunami K., Ishimatsu J., Yamamoto T., Endo F.: Complete absence of bile and pancreatic ducts in a newborn: a new entity of congenital anomaly in hepato-pancreatic development. J Human Genetics 48 (2003) 380-384.

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Elucidation of the Principles of Development and Regeneration by Systematic Analysis of Genes

Project Leader:
Naoto UENO
Professor, National Institute for Basic Biology,
National Institutes of Natural Sciences

1. Objective

Early development, initiated from fertilization of egg, involves dynamic morphological change through cleavage, diffrentiation and migrations of embryonic cells. These processes in early development appear to be controlled by a precisely programmed mechanism. In addition, we can perceive the presence of such a program in regeneration of tissues and organs of certain animals. The process of regeneration looks similar if it is not identical to that of early development. Mechanism underlying these phenomena "development & regeneration" can simply be attributed to the function of genes coded by the genome and their products namely proteins. Therefore, it is essential for us to understand how genes and proteins, which are predicted to be over 20 thousands of kinds in human, are regulating these complex biological phenomena and how genes themselves are regulated during embryogenesis. In this research project using an amphibian *Xenopus laevis* and a planarian *Dugesia japonica*, we purposed to establish a platform by which molecular mechanism of development and regeneration is systematically and comprehensively addressed based on information of genes.

2. Summary

2-1. Establishment of the platform for systematic and comprehensive analysis of genes :

Although Xenopus has been a useful model animal not only in developmental biology but also in cell biology, it was left behind of some model animals because of the lack of genome-wide information of expressed genes. Therefore, we first attempted to collect cDNAs by preparing high-quality and normalized cDNA libraries from embryo of different developmental stages and performed EST (expression sequence tag) sequencing. We acquired over 200 thousands of ESTs' information and then annotated them to nearly 20 thousands of unique genes (all information is disclosed on our we site XDB; http://xenopus.nibb.ac.jp/). Based on this resource, we fabricated a high-density cDNA microarray (Fig.1) for the systematic analysis of gene expression during early development. In addition, we have developed a database of spatial and temporal gene expression pattern examined by whole-mount in situ hybridization (Fig.2). These allow us to extend our studies to generate useful transgenic frogs (Fig.3) by introducing a variety of functional genes that are now available from our cDNA resource.

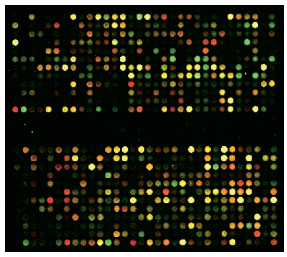

Fig.1 DNA microarray for the systematic analysis of gene expression.

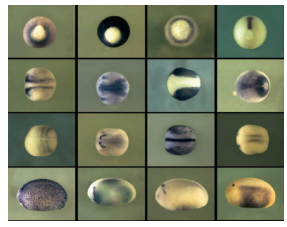

Fig.2 Gene expression database of *Xenopus laevis*

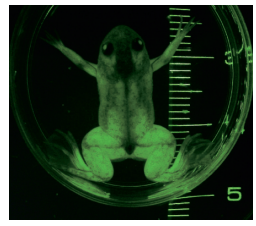

Fig.3 Transgenic *Xenopus* frog expressing GFP

2-2. Application to the study on Xenopus tail regeneration :

A comprehensive gene expression analysis using a cDNA array was performed to characterize a tail regeneration process of *Xenopus laevis* larval. It resulted in the identification of over a hundred of up- or down-regulated genes during the regeneration. The 48 up-regulated genes were categorized based on their putative functions, and the categories reflected the successive events during the regeneration process, such as inflammation response, wound healing, cell proliferation, and cell differentiation. A role of Wnt signaling in the regeneration was suggested by the array analysis and in situ hybridization. *xWnt-5a* was highly expressed in the distal portion of the regenerating tail, while *xSfrp-2*, a Wnt antagonist, was in a complementally manner. In order to revel the role of *xWnt-5a* in the regeneration process, an animal cap injected with *xWnt-5a* mRNA was grafted into an incision made at the dorsal portion of the proximal tail,

Fig.4 Ectopic tail induced by the transplantation of xWnt-5a expressing cells.

where *xWnt-5a* was never expressed and regeneration beyond a wound healing never occurred. The grafting experiment resulted into a formation of a complete secondary tail containing a notochord, spinal cord, muscle mass and fin (Fig.4). The host cells constituted a major portion of the secondary tail, and animal cap-derived cells were never found in the differentiated tissues.

2-3. Characterization of stem cells in regenerating planarian :

Planarians have a remarkable regenerative ability where a complete individual can regenerate and develop from small amputated pieces. This ability relies on pluripotent stem cells distribute throughout the entire body, originally called the neoblast. Planarians carry out regeneration by maintenance and regulation of these stem cells. To understand the mechanisms of planarian regeneration, we have focused on, and investigated the stem cells extensively.

We compared gene expression between normal planarians, stem cell eliminated-planarians by X-ray irradiation, and purified stem cells to identify stem cell specific genes. An extensive analysis (more than 16,000 genes) allowed us to identify at least 40 stem cell specific genes from planarians (Fig.5). These genes overall could be classified into two groups based on their *in vivo* expression patterns. Subsequent detailed analysis of cells expressing these gene groups, led us to find heterogeneous populations of planarian stem cells. The type 1 group of genes was expressed in the resting non-dividing stem cells, which were localized in specific regions in the body from head tip to tail tip. On the other hand, the type 2 group of genes was expressed in actively dividing stem cells present throughout the entire body. The stem cells expressing type 1 genes, resting stem cells, were smaller than stem cells expressing type 2 genes, the dividing stem cells.

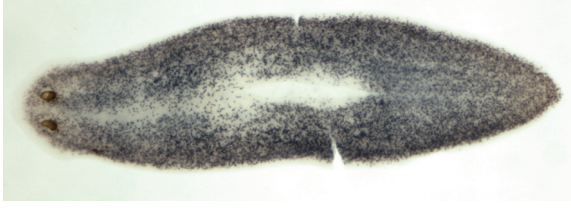

Fig.5 Genes that are specifically expressed in the stem cells .Discovery of such stem cell-specific genes helps to identify stem cells in the planarian.

3. Concluding Remarks

We have confirmed that the platform we have established in this project is very useful to identify genes that have essential function during early development. Structural information as well as spatial and temporal expression patterns facilitate prediction and experimental confirmation of gene function in vivo. In fact, our DNA microarray analysis identified an FGF target NRH and demonstrated that NRH has an essential function in regulating cell movements during *Xenopus* gastrulation. Likewise, the systematic and comprehensive approaches expanded our capability of investigating gene function in general.

In regeneration research, a similar approach using the DNA array was resulted in the identification of xWnt-5a as an important factor for regeneration recapturing normal development. Our result further supports an idea that xWnt-5a has an instructive role during the regeneration to organize proliferating cells in the wound to make a complete tail.

Regarding to the characterization of stem cells in planarian, we found that the resting stem cells can produce dividing stem cells. In planarians, the existence of stem cells in this resting-state is a new finding in 100 years of research. These results give a new insight into stem cell systems during planarian regeneration. Moreover our results show that planarians are a revived model animal for general stem cell research.

4. Primary Publications

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(4) Iioka, H., Ueno, N. and Kinoshita, N. (2004). Essential role of MARCKS in cortical actin dynamics during gastrulation movements. *J. Cell Biol.* 164, 169-74.

(5) Chung, H. A., Hyodo-Miura, J., Kitayama, A., Terasaka, C., Nagamune, T. and Ueno, N. (2004). Screening of FGF target genes in Xenopus by microarray: temporal dissection of the signalling pathway using a chemical inhibitor. *Genes Cells* 9, 749-61.

(6) Sugiura, T., Taniguchi, Y., Tazaki, A., Ueno, N., Watanabe, K. and Mochii, M. (2004). Differential gene expression between the embryonic tail bud and regenerating larval tail in Xenopus laevis. *Dev. Growth Differ* .46, 97-105.

(7) Tazaki, A., Kitayama, A., Terasaka, C., Watanabe, K., Ueno, N. and Mochii, M. (2005). Microarray-based analaysis of tail regeneration in Xenopus laevis larvae. *Dev. Dyn.* 233, 1394-404.

(8) Chung, H.A., Hyodo-Miura, J. Nagamune, T. and Ueno, N. (2005). FGF signal regulates gastrulation cell movements and morphology through its target NRH. *Dev. Biol.* 282, 95-110.

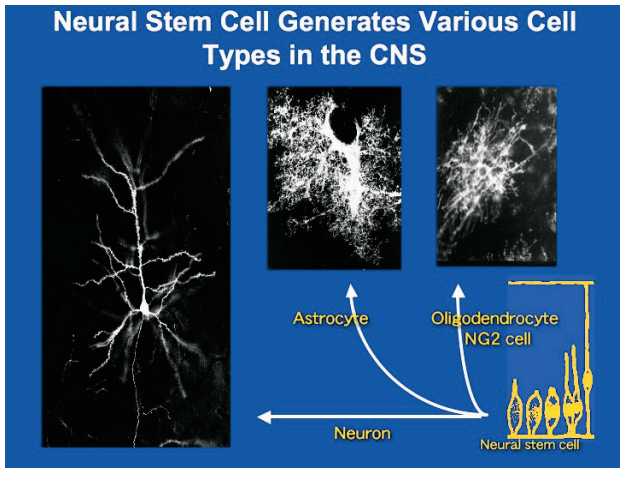
Studies on Molecular Mechanisms Underlying Development, Differentiation and Regeneration of Neural Cells

Project Leader:
Kazuhiro IKENAKA Professor, National Institute for Physiological Sciences, National Institutes of Natural Sciences

1. Objective

Brain cells were thought to have poor regenerating capacity and therefore it has been believed difficult to obtain an efficient therapeutic effect after brain injury or degenerating diseases. However, from the recent studies it became evident that adult brains still possess a few numbers of neural stem cells, which can expand themselves and generate various cell types in the central nervous system (Fig.1) Thus it is now important to understand how we can make the neural stem cells differentiate into various brain cell types that had been lost by injury or degeneration. This is the objective of our project.

Fig.1.



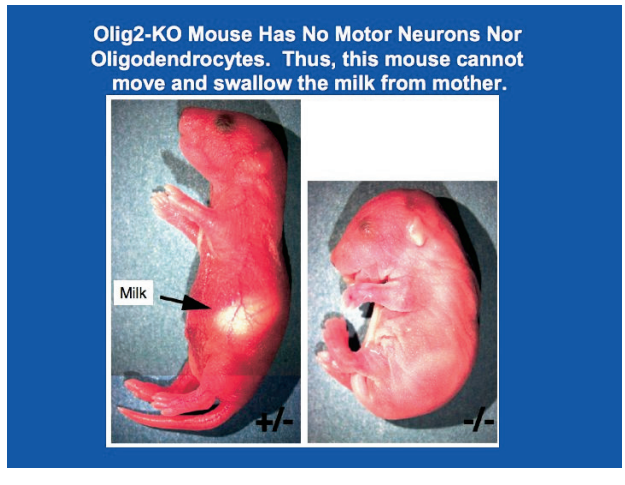
2. Summary

In this project we focused on the demyelinating diseases and Parkinson's disease, thus the brain cell types we aimed to generate from the neural stem cells were oligodendrocytes (OLs) and dopaminergic neurons.

2-1. The olig2 gene is essential for the generation of OLs. :

OLs are the cells responsible for the formation of myelin in the central nervous system and are generated from the neural stem cells. However, in contrast to the generation of neurons and astrocytes in the spinal cord, OL is generated from a restricted ventral region of the spinal cord. At the time when OLs are produced in this restricted region, neurons are produced from all other regions. Thus to know how this fine spatial restriction is acheived has been one of the major interests of the developmental biologists. The region from which OLs are generated is called the pMN domain and motor neurons are generated prior to OL generation from the same region. The olig2 gene has been isolated and found to be expressed solely in the pMN domain, and thus was expected to be deeply related to the OL generation. When we knocked out this gene, there were no OLs in the spinal cord as expected. Interestingly, the generation of motor neurons was also impaired (Fig.2). These observations demonstrated that the olig2 gene is essential for the generation of both oligodendrocytes and motor neurons.

Fig.2.



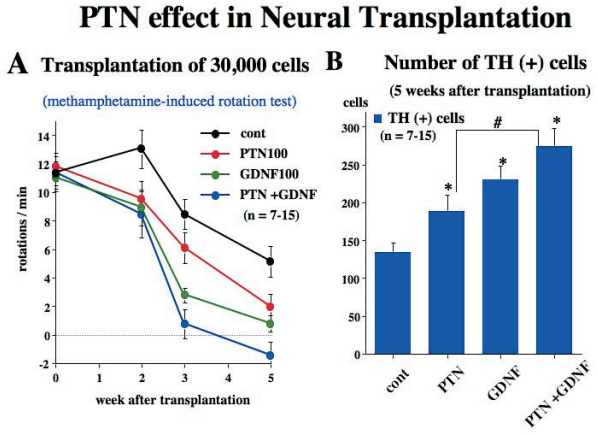
2-2. The differentiation of OLs is inhibited by the Wnt proteins. :

We demonstrated that the differentiation of OL is inhibited by the factor(s) present in the dorsal spinal cord. In this project we identified one the components of this factor(s) as the Wnt protein. It has been suggested that Wnt binds to the cell surface of OL precursor cells and controls the timing of OL development in the spinal cord. This allows OL to spread evenly in the entire region of the spinal cord. This is an important finding for the development of a new treatment for the demyelinating diseases such as multiple sclerosis.

2-3. Pleiotropin (PTN) has neurotrophic activity and enhances the differentiation of dopamine neurons. :

Differentiation factors related to doperminergic differentiation from the neural stem cells were investigated. Expression of pleiotrophin (PTN) was enhanced in dopamine-depleted striatum, and frequently found in ventral mesencephalon-derived neurospheres. Treatment of ES-derived neural stem cells with PTN increased the number of TH-positive neurons after doperminergic differentiation (Fig.3B). Treatment of cultured doperminergic neurons promoted the survival and PTN treatment of the donor cells during cell preparation induced the recovery of disturbed motor function after transplantation to hemi-parkinson model rats (Fig.3A).

Fig.3.



3. Concluding Remarks

We have identified PTN as a stimulator of dopaminergic neuron differentiation and Wnt as an inhibitor of OL differentiation. These findings are important for the development of new therapies.

4. Primary Publications

(1) Shimizu T., Kagawa T., Wada T., Muroyama Y., Takada S., Ikenaka K. Wnt Signaling Controls the Timing of Oligodendrocyte Development in the Spinal Cord. Dev Biol. 282(2) : 397-410, (2005).

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(3) Iwasaki Y, Hosoya T, Takebayashi H, Ogawa Y, Hotta Y, Ikenaka K : The potential to induce glial differentiation is conserved between drosophila and mammalian glial cell missing genens : Developmental 130 :6027-6035, (2003).

(4) Takebayashi H, Nabeshima Y, Yoshida S, Chisaka O, Ikenaka K, Nabeshima Y : The Basic Helix- Loop- Helix Factor Olig2 Is Essential for the Development of Motoneuron and Oligodendrosyte Lineages : Current Biology 12 : 1157-1163, (2002).

(5) Yamazaki Y, Makino H, Hamaguchi-Hamada K, Hamada S, Sigino H, Kawase E, Miyata T, Ogawa M, Yanagimachi R, Yagi T : Assessment of the developmental totipotency of neural cells in the cerebral cortex of mouse embryo by nuclear transfer : Proc. Natl. Acad. Sci. USA 98:14022-14026, (2001).

3. Plant Genetics

(1) Research Promotion Committee Members

○	Yasuyuki YAMADA	(Nara Institute of Science and Technology)
	Kunio IWATSUKI	(The University of the Air)
	Masaki IWABUCHI	(National Institute of Agrobiological Sciences)
	Naoki KATSURA	(National Agriculture and Bio-oriented Research Organization)
	Tatsuo SUGIYAMA	(RIKEN)
	Kokichi HINATA	(Iwate Biotechnology Research Center)
	Hiroo FUKUDA	(The University of Tokyo)

○ : Committee Chairperson

(2) List of Research Projects

No.	Research Project	Project Leader
1	Analyses of Molecular Mechanisms Controlling Plant Development and Production and Evaluation of Useful Transgenic Plants	Hiroshi KAMADA (University of Tsukuba)
2	Gene Analysis of Plant Biodiversity and the Effects of Transgenic Plants on the Environment	Isao INOUYE (University of Tsukuba)
3	Novel Gene Function Involved in Higher-Order Regulation of Nutrition-Storage in Plants	Kenzo NAKAMURA (Nagoya University)
4	Plant Molecular Breeding for High Assimilation Potential and Stress Tolerance Towards Food Security and Environmental Preservation	Hiroshi SANO (Nara Institute of Science and Technology)
5	Molecular Mechanisms on Regulation of Morphogenesis and Metabolism Leading to Increased Plant Productivity	Takashi HASHIMOTO (Nara Institute of Science and Technology)
6	RNA Silencing in Higher Plants and Functional Genomics	Fumihiko SATO (Kyoto University)
7	Studies of Intricate Mechanisms of the Regulatory Factors in Plant Cell Death and Proliferation	Hirofumi UCHIMIYA (The University of Tokyo)

Analyses of Molecular Mechanisms Controlling Plant Development and Production and Evaluation of Useful Transgenic Plants

Project Leader:
Hiroshi KAMADA Professor, Graduate School of Life and Environmental Sciences, University of Tsukuba

1. Objective:

We investigate some aspects of molecular mechanisms controlling plant development (embryogenesis, flower induction, fruit formation, etc) and growth (interaction between upper- and under-ground plant parts, synthesis of sulfur-containing amino acid, etc) and produce useful transgenic plants. Furthermore, we evaluate the characteristics of the transgenic plants, and establish the methods for the assessment of environmental effects in transgenic plants according to the Cartagena law.

2. Summary

2-1. Mechanism controlling morphogenesis :

On somatic embryogenesis that is an efficient method for plant regeneration, a new stress-induction method without phytohormone treatment was established. Modifying the method, we developed the efficient method for induction of somatic embryos in a model plant *Arabidopsis* (Fig.1).

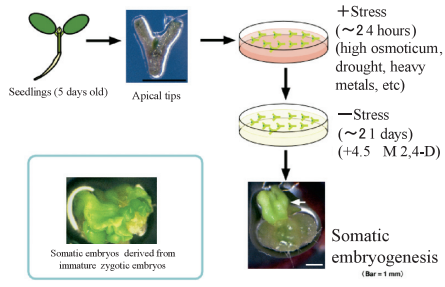


Fig.1. Stress-induced somatic embryogenesis in *Arabidopsis*

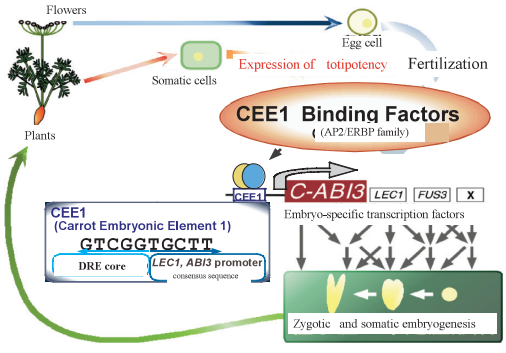


Fig.2. A model of molecular mechanisms controlling expression of embryo-specific genes

Using these methods, we had isolated and characterized a lot of embryo-specific genes, and then found a new DNA sequence (CEE1) determining embryo-specific expression of the genes as a *cis*-regulatory element and some transcription enhancing factors (AP2/EREBP family gene) that bind to CEE1 as *trans*-acting factors (Fig.2).

2-2. Control of methionine biosynthesis :

Methionine, an important essential amino acid containing sulfur, is used in protein synthesis and also involved in many cellular reactions after it is converted to *S*-adenosylmethionine (AdoMet). In many of biosynthetic pathways, the key-step enzyme is regulated by an allosteric mechanism. On the other hand, cystathionine gamma-synthase (CGS), the key-step enzyme of methionine biosynthesis in higher plants, is regulated at the step of mRNA degradation. A short stretch of amino acid sequence termed MTO1 region that is located within CGS itself is necessary for this regulation. It was first found that the degradation of *CGS* mRNA is induced by translation of the mRNA in the presence of AdoMet (Fig.3).

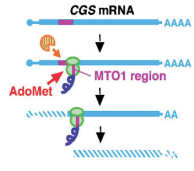


Fig.3. *CGS* mRNA degradation

2-3. Development of a method to prevent pollen dispersal from transgenic plants :

Ethylene is known as a phytohormone regulating plant development from seed germination to fruit formation and ethylene receptor has a role of sensing ethylene in plants. We introduced miss-sense mutation in this gene and expressed it in transgenic tobacco. The transgenic tobacco plants showed reduced sensitivity to ethylene. In addition, the transgenic plants showed lower seed production or sterility. Further detailed analysis showed that 1) pollen development was inhibited by abnormal development of tapetum tissue in anther (Fig.5), and 2) floral

architecture was altered into a heterostyly structure (Fig.4). The altered traits related to sterility were stable when the transgenic plants were grown in semi-containment greenhouse. The modified ethylene receptor genes could induce sterility in transgenic lettuce.

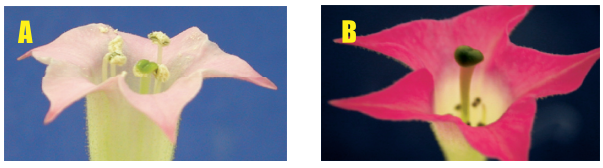


Fig.4. Floral architecture of transgenic plant with modified ethylene receptor.
A: Non-transgenic plant, B: Transgenic plant (Heterostyly)

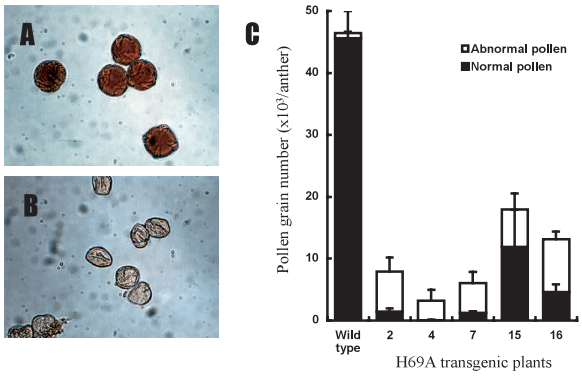


Fig.5. Pollen production of transgenic tobacco plants with modified ethylene receptor gene.
A: Pollen grain of non-transgenic plant,
B: Pollen grain of transgenic plant, C: Pollen production.

2-4. Analysis of gene diversity and development of the assessment method for gene flow :

To develop the assessment methods for environmental effects of transgenic plants, using wild carrot as a model plant, we examined ecological features, estimation methods of gene flow through pollen dispersion by pollinators, analytical methods of gene diversity in wild population, possibility of cross pollination between wild and domestic plants, and others.

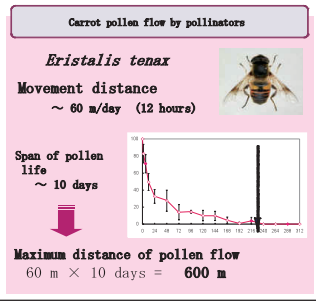


Fig.6. Estimation of pollen flow in wild carrot

It was found that the amplified fragment length polymorphism (AFLP) method is a most suitable method for estimation of gene diversity in wild carrot. According to the analyses on movement of pollinator insects and span of pollen life, it was estimated that the distance of pollen dispersal was 600 m (Fig.6).

3. Concluding Remarks

Some aspects of molecular mechanisms controlling plant morphogenesis (embryogenesis, flower induction, etc) were clarified and efficient methods for the production of transgenic plants were developed. Feedback regulation mechanism of CGS for biosynthesis of methionine (an important sulfur-containing amino acid) was elucidated. Moreover, using a modified ethylene receptor gene, a potential useful tool applicable in preventing pollen dispersal from transgenic plants was developed. On the other hand, we developed some assessment methods for environmental effects of transgenic plants.

4. Primary Publications

(1) Miho Ikeda-Iwai, Mikihisa Umehara, Shinobu Satoh and Hiroshi Kamada : Stress-induced somatic embryogenesis in vegetative tissues of *Arabidopsis thaliana*. Plant J., 34; 107-114 (2003).

(2) Yoshikatsu Matsubayashi, Mari Ogawa, Akiko Morita and Youji Sakagami : An LRR receptor kinase involved in perception of a peptide plant hormone. Science, 296; 1470-1472 (2002)

(3) Yukako Chiba, Ryoko Sakurai, Michiko Yoshino, Kimihiro Ominato, Mari Ishikawa, Hitoshi Onouchi and Satoshi Naito : S-adenosyl methionine is an effector in the posttranscriptional autoregulation of the cystathionine γ-synthase gene in *Arabidopsis*. Proc. Natl. Acad. Sci. USA, 100; 10225-10230 (2003).

(4) Masashi Asahina, Hiroaki Iwai, Akira Kikuchi, Shinjiro Yamaguchi, Yuji Kamiya, Hiroshi Kamada and Shinobu Satoh : Gibberellin produced in the cotyledon is required for cell division during tissue-reunion in the cortex of cut cucumber and tomato hypocotyls. Plant Physiol., 129; 201-210 (2002).

(5) Taichi Oguchi, Kimiyo Sage-Ono, Hiroshi Kamada and Michiyuki Ono : Characterization of transcriptional oscillation of an *Arabidopsis* homolog of *PnC401* related to photoperiodic induction of flowering in *Pharbitis nil*. Plant Cell Physiol., 45; 232-235 (2004).

(6) Min-Long Cui, Keita Takada, Biao Ma and Hiroshi Ezura : Over expression of mutated melon ethylene receptor gene *Cm-ETR1/H69A* confers reduced ethylene sensitivity to heterologous plant *Nemesia strumosa*. Plant Sci., 167; 253-258 (2004).

Gene Analysis of Plant Biodiversity and the Effects of Transgenic Plants on the Environment

Project Leader:
Isao INOUYE Professor, Graduate School of Life and Environmental Sciences, University of Tsukuba

1. Objective:

Plants biodiversity is a huge target of biological sciences, because its hierarchy covers from genes and proteins to populations and communities. To establish the guideline of conservation of genetic diversity and bio-safety, accumulation of broad range of basic information of biodiversity are indispensable. In this project, we investigated biodiversity of plants from three different points of view. 1. Phylogenetic study of plants and their character evolution: In contrast to animals, plants are comprised of 10 different lineages. Three major primary producers, Chlorophyta (green algae), Heterokontophyta (brown algae and their relatives) and Haptophyta (one major groups of marine phytoplankton) were studied. 2. Population-level diversity: genetic variations and its role on the maintenance of individual populations of *Primula sieboldii* as a model of endangered plants. 3. Development of risk assessment and tools for measuring genetic biodiversity: To establish basic methods for estimating genetic diversity and gene flow into the environment, methods to measure genomic diversity were studied using polyploidy plants. This study also aimed at establishing simple and inexpensive methods to estimate biodiversity of microorganisms (including those which are impossible to culture at present) in natural environment such as pond water and soil. To test above-mentioned methods and tools, bio-safety experiments of transgenic eucalyptus tree are planned using non-closed green house. In related to the aim of the project, problems in risk communication and related matters were analyzed and reviewed.

2. Summary

2-1. Major lineages of eukaryotic algae were investigated from phylogenetic point of view, and the following results were obtained. Two new classes of the stramenopiles (an assemblage including heterokont algae such as brown algae and diatoms), the Pinguiophyceae and Placidiophyceae (Placidia), were described. Monophyly of the coccolithophorids (Haptophyta with calcium cabonate scales) was confirmed and its probable ancient branch was discovered. Charalean algae were the closest relatives of the land plants. In addition to these primary goals, a novel group of eukaryotes was discovered and described as the phylum Kathableda and division Katablepharidophyta. A flagellate that would switches animal like and plant like stages in its life cycle was discovered (Fig.1). This deduced life form would show an early stage of the plastid acquisition.

Fig.1. Undescribed flagellate that is estimated at an early stage of plastid acquisition via. Endosymbiosis.

2-2. Development of a large number of microsatellite markers enabled us to analyze detailed gene flow and genetic structure within a number of populations of *Primula sieboldii* and the hierarchy of genetic variation throughout Japan.

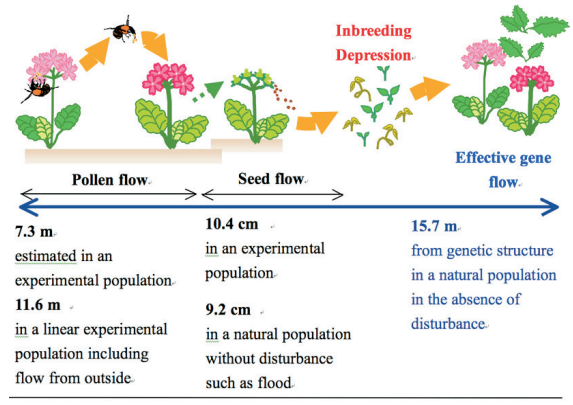


Fig.2. Estimation of gene flow distance using pollen and seeds.

2-3. These results helped to determine the genetic or evolutional conservation units of P. sieboldii. The multipurpose IBM (individual based model) was constructed based on the quantitative data on the pollen flow depending on the behavior of the pollinators and the seed flow by the seed dispersal

(Fig.2). These are useful to evaluate the extinction risk of fragmented populations especially in relation to the inbreeding depression and kinship structure formed by the effective gene flow. The strong inbreeding depression that was shown to appear at each life stage such as seed production, germination, seedling establishment, clonal growth or flowering suggests the accumulation of many lethal and deleterious genes are accumulated in the genome of the species. We should consider these genetic features in conservation practices. We obtained a partial linkage map through QTL mapping which might be useful in a more detailed genetic consideration in the conservation of the population of the species.

2-4. Risk assessment methods were examined and new tools and approaches for the measurement of genetic diversity were developed:

(1) plants: functional genomic marker based on cytochrome P450 for intraspecific genetic diversity,

(2) plants: holistic gene expression evaluation to compare homoeologous genomes for genomic diversity,

(3) microorganisms: nucleic acid extraction method and sequencing-gel based assessment on soil microbial species diversity by RISA and T-RFLP (Fig.3).

(4) Expressed Sequence Tags (EST) of wheat (*Triticum aestivum*), an allopolyploid plant, was analyzed, and changes of gene expression pattern in relation to genomic fusion (allopolyploidy). Also risk communication approach and methods are analyzed and some recommendation was made on public acceptance on the transgenic research.

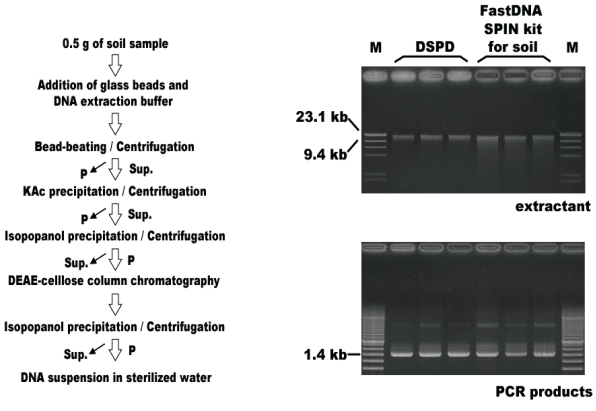


Fig.3. Simple DNA extraction from various types of soil

3. Concluding Remarks

There are various aspects in plant biodiversity, from gene to community-level. Single scientific project cannot cover all aspects of diversity, but our project successfully pointed the ways of future researches on plant biodiversity.

Discoveries of new class and phylum-level taxa imply that we still poorly understand diversity of plants, so that extensive taxonomic and phylogenetic studies are needed for comprehensive understanding of plant diversity.

Understanding of the hierarchy of genetic variation from gene level to ecosystem level, which revealed in this study, should greatly contribute to not only the basic study of conservation ecology, but also variation for the environmental assessment of genetically modified plants.

For assessment of bio-safety of environment, simple and inexpensive but accurate methods are indispensable. We have established basis of such methods that enable to evaluate the effect of transgenic plants to the environment and genetic stability of plants.

4. Primary Publications

(1) Okamoto, N. and Inouye, I. (2005) Is the secondary symbiosis in progress? Science 310:287.

(2) Okamoto, N. and Inouye, I. (2005) The Katablepharids are a distant sister group of the Cryptophyta: A proposal for Katablepharidophyta *divisio nova*/Kathablepharida *phylum novum* based on SSU rDNA and beta-tubulin phylogeny. Protist 156,163-179.

(3) Washitani, I., Ishihama, F., Matsumura, C., Nagai, M., Nishihiro, J. & Nishihiro, M.A. (2004) Conservation ecology of *Primula sieboldii*: synthesis of information toward the prediction of genetic/demographic fate of a population. Plant Species Biology, 20: 3-16.

(4) Ishihama, F., Ueno, S., Tsumura, Y., & Washitani, I. (2005) Gene flow and inbreeding depression inferred from fine-scale genetic structure in an endangered heterostylous perennial, *Primula sieboldii*. Molecular Ecology, 14: 983-990.

(5) Watanabe, K. N., T. Fujimura, K. Shimamoto, T. Hashimoto, N. Koizumi, H. Fukuda, S. Naito, K. Nakamura, T. Mimura, Y. Ohashi, K. Shimazaki, I. Terashima, H. Uchimiya and T. Yamaya. (2004) Negative fallout from public sentiment in Japan. Nature Biotech. 22, 904.

(6) Watanabe, K. N., M. Taeb and H. Okusu. (2004) Putting Cartagena into Practice. Nature Biotech. 22, 1207-1208.

Novel Gene Function Involved in Higher-Order Regulation of Nutrition-Storage in Plants

Project Leader:
Kenzo NAKAMURA Professor, Graduate School of Bioagricultural Sciences, Nagoya University

1. Objective:

Accumulation of storage macromolecules in the sink sites of plants after relocation and transport of nutrients is fundamental for crop productivity. In this project, we will use genome information and resources of model plants such as Arabidopsis and rice to identify novel gene functions involved in nutrient relocation, regulation of storage function in response to nutritional status, signal integration for the high-level accumulation of nutritional compounds, and coordinate regulation of relocation and storage by biological clock.

2. Summary

2-1. Roles of sugar and nitrogen transporters :

OsSUT1 is expressed in phloem sieve element and suggested to be involved in the loading of sucrose into phloem. We found that OsSUT1 is the major sucrose transporter responsible for the transport of sucrose to developing seeds and re-incorporation of sucrose into endosperm. We also identified roles of each members of ammonium transporters of rice in the uptake of nitrogen. (J. Yamaguchi)

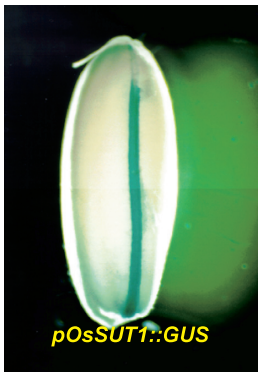


Fig.1. Expression of OsSUT1 in phloem of seed coat.

2-2. New insights into sugar signaling :

In general, icreased level of sugars in the source repress expression of photosynthetic genes while high-level of sugars promote expression of genes for the synthesis of storage macromolecules. Isolation and characterization of many mutants with altered response to sugars indicated the role of ABA in some of the sugar-inducible gene expression and the involvement of intact chloroplast function and a subunit of proteasome in some of the sugar-response. (J. Yamaguchi, A. Morikami, K. Nakamura)

2-3. Novel transcription factors involved in the sugar-inducible genes expression. :

Although the sugar-dependent modulation of gene expression in plants is mediated by multiple signal transduction pathways, only few transcription factors involved in sugar-regulated gene expression have been identified. We searched for transcription factors involved in sugar-inducible expression of sink-related genes by using various approaches, and identified 1) a novel transcription repressor HSI2 with B3 DNA binding domain, 2) AP2-tpe transcription activator ASML1/WRI1 (Fig.2), 3) ASML2 in a novel class of CCT domain proteins, and 4) HD-Zip-type transcription activator TB10 that binds to a *cis* element conserved among several sugar-inducible promoters. These factors affected expression of at least a subset of sugar-regulated genes, and transient expression of these factors in protoplasts showed either transactivation or transrepression of sugar-inducible reporter gene. Among these, ASML1/WRI1 is a regulator of seed oil accumulation and is expected to be useful in the control of seed oil. (K. Nakamura, A. Morikami)

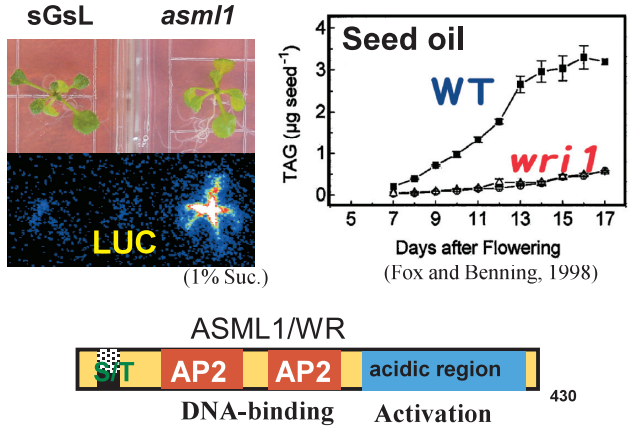


Fig.2. Activation of sugar-inducible genes by atransciption factor involved in seed oil accumulation

2-4. Gene cascades regulating expression of seed storage proteins :

Transcription factors that regulate expression of

seed storage protein genes under the control of seed-specific transcription factors LEC1, ABI3, FUS3 in the presence of ABA were identified. We found that ABA-enhancement of storage protein genes is mediated by ABA-dependent phosphorylation of specific Ser residue in TRAB1 by SAPK8~10. These and other results suggested transcription cascades that bring about high-level expression of seed storage protein genes during seed maturation. We also found that FUS3 and ABI3 regulates expression of not only structural genes for storage proteins but also genes involved in the processing and transport of storage protein precursors to the protein body, suggesting that gene expression and intracellular transport of storage proteins are under co-ordinate regulation. (T. Hattori)

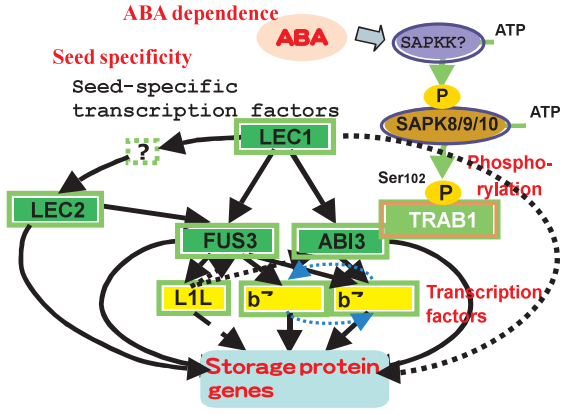


Fig.3. Regulatory networks regulating seed-specific and ABA-dependent high-level expression of seed storage proteins

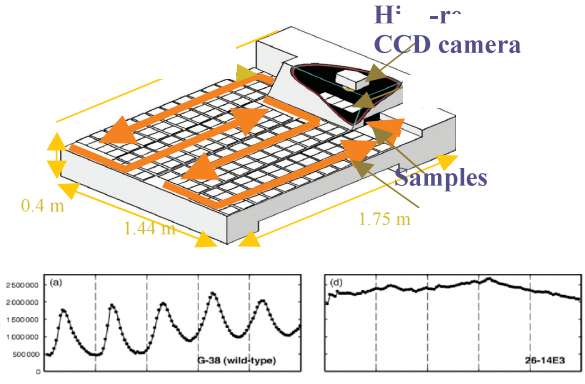


Fig.4. Automated, high-throughput, bioluminescence-monitoring apparatus (upper) used to screen for the arrhythmic mutant of *Arabidopsis thaliana* (lower).

2-5. Regulation by biological clock :

The 3-dimensional structure of the clock protein KaiC of cyanobacteria was elucidated. An automated, high-throughput, bioluminescence-monitoring apparatus that can monitor ~2000 individual plant seedlings was developed, and used for extensive screening of *Arabidopsis* mutants with altered circadian rhythms. Many mutants isolated included novel mutants such as six arrhythmic (AR) mutants. The AR mutants belonged to three complementation groups, and *AR1* gene coding for a novel central clock or clock-related protein was identified. (M. Ishiura)

2-6. Organ and vascular development :

We found that Asymmetric Leaves2 (AS2) is a member of a novel family of proteins and is essential for vein formation in leaves by repressing expression of class I knox homoeobox genes. The analysis of high-coverage gene expression profiling of genes for starch synthesis and storage proteins during the development of rice seeds was performed and a novel gene which might have a role in endosperm development was identified. (C. Machida, T. Masumura)

3. Concluding Remarks

Many novel genes such as sugar transporter important in relocation of sugars to seeds, transcription factors involved in the regulation of partitioning of carbohydrates in the sink and the expression of genes involved in sink functions were identified. Analysis of transgenic plants of these genes are in progress. In addition, novel high-throughput apparatus that is widely useful for post-genome research was developed.

4. Primary Publications

(1) Tsukagoshi, H., Saijo, T., Shibata, D., Morikami, A. and Nakamura, K. (2005) Analysis of sugar response mutant of *Arabidopsis thaliana* identified a novel B3 domain protein with the EAR motif that functions as an active transcriptional repressor. *Plant Physiol.* 138: 675-685.

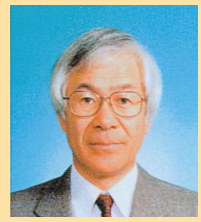
(2) Masaki, T., Mitsui, N., Tsukagoshi, H., Nishii, T., Morikami, A. and Nakamura, K. (2005) ACTIVATOR OF *Spo*^{min}*::LUC1/WRINKLED1* of *Arabidopsis thaliana* transactivates sugar-inducible promoters (Rapid Paper). *Plant Cell Physiol.* 46:. 547-556.

(3) Sonoda, Y., Ikeda, A., Saiki, S., Von Wiren, N., Yamaya, T. and Yamaguchi, J. (2003) Distinct expression and function of three ammonium transporter genes (*OsAMT1;1 -1;3*) in rice. *Plant Cell Physiol.* 44: 726-734.

(4) Kagaya,Y., Hobo, T., Murata, M. Ban, A. and Hattori, T. (2002) Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor TRAB1. *Plant Cell* 14: 3177-3189.

(5) Onai, K., Okamoto, K., Nishimoto, H., Morioka, C., Hirano, M., Kami-ike, N., and Ishiura, M. (2004) Large-scale screening of *Arabidopsis* circadian clock mutants by a high-throughput real-time bioluminescence monitoring system. *Plant J.* 40: 1-11.

Plant Molecular Breeding for High Assimilation Potential and Stress Tolerance towards Food Security and Environmental Preservation



Project Leader:
Hiroshi SANO Professor, Research and Education Center for Genetic Information, Nara Institute of Science and Technology

1. Objective:

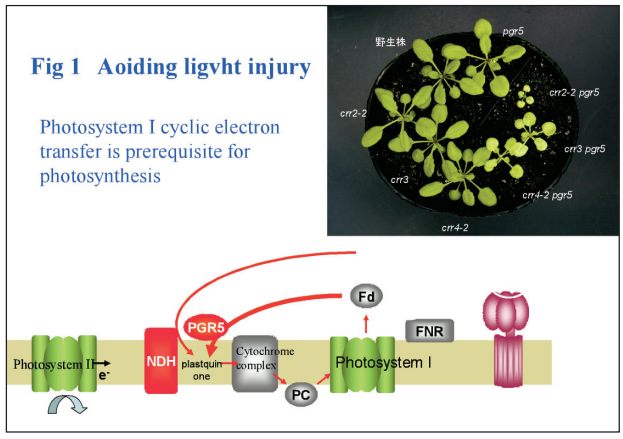
The most serious problem in the 21st century is food and environmental crises. The cause is clearly attributable to excess social activities of mankind, which exceeded the capacity of cycling system of earth. One of powerful methods to solve this is improvement of plant quality by adaptation of plant molecular breeding technology. The long-range aim of this project is to construct useful transgenic plants with high potentials in assimilation ability and in stresses tolerance. To this end, two experimental procedures are undertaken: increasing photosynthesis capacity and molecular breeding with stress resistant genes. During past five years, following results were obtained.

2. Summary

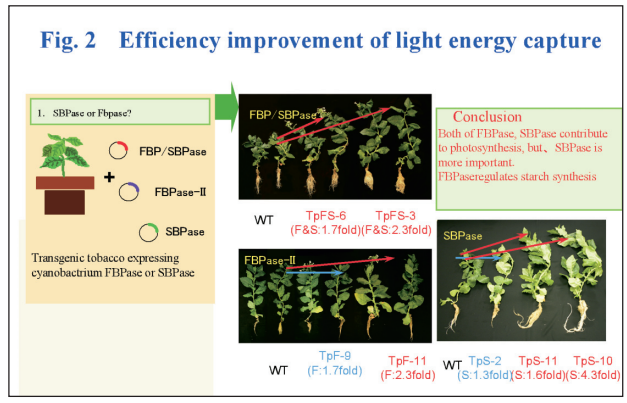
2-1. Improvement of assimilation capacity :

Assimilation of carbon, nitrogen and other compounds depends on plant capacity of energy production through photosynthesis. The improvement of carbon assimilation is the base for all of these, and therefore we focused on identification and strengthening of the limiting factor(s) of the system.

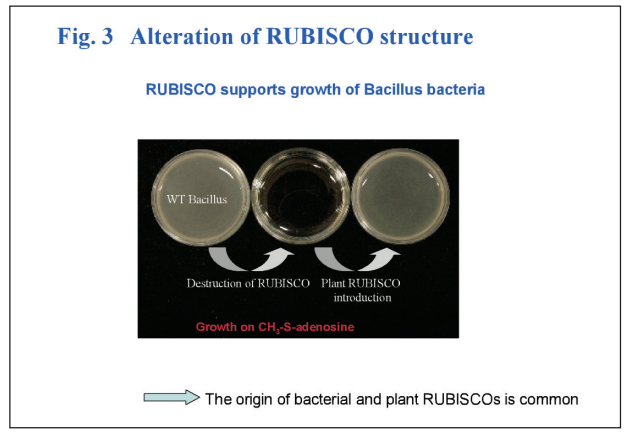
(1) The mechanism of photoprotection. The molecular mechanism to tolerate strong light intensity was elucidated (Fig.1).



(2) Improvement of chloroplast function. FBPase and SBPase were found to be essential for regulation of carbon flow. SBPase is the initial rate limiting factor in RuBP reproduction, and FBPase was essential for distribution of assimilated carbons. Plant growth rate and biomass were greatly increased by introducing FBP/SBP genes, which constitute the rate limiting step in Calvin cycle (Fig.2).



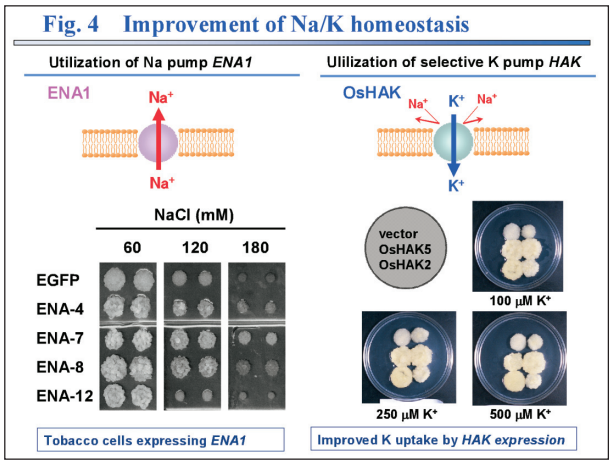
(3) Functional analyses of RUBISCO. The origin of RUBISCO was identified, and its evolution mechanism was proposed (Fig.3).



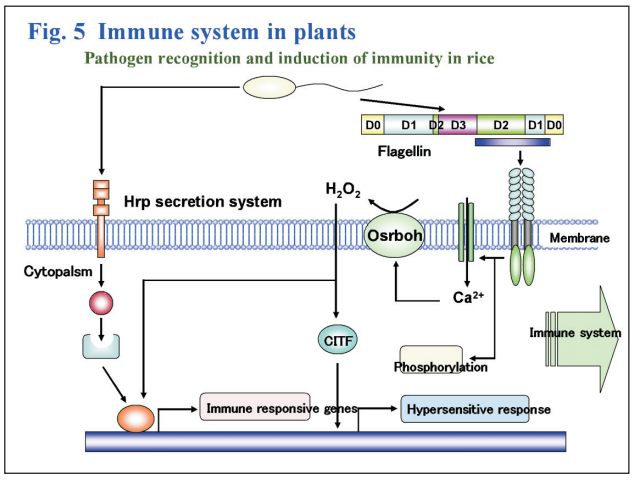
2-2. Molecular breeding

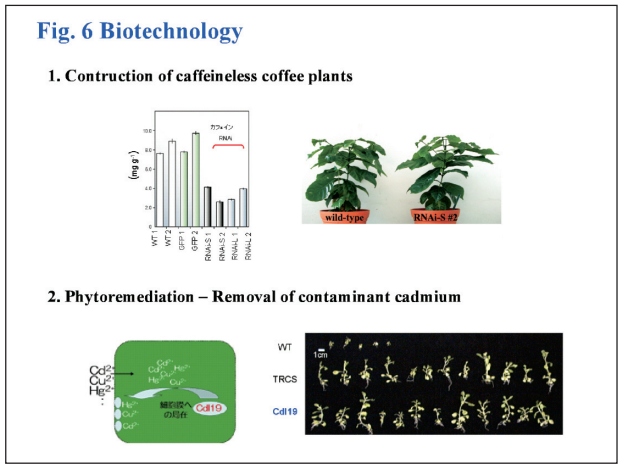
Environmental stresses including pathogen, wounding, salinity and drought suppress plant productive ability up to 40 to 80%. Transgenic technology is useful to confer crops tolerant ability against these stresses. Resulting plants can be used not only for improved food production but also for environment protection by phytoremediation.

(1) Molecular breeding of salt tolerant crops. Salt gives two serious damages to plants: osmotic imbalance and sodium ion toxicity. Focusing on the latter problem, we constructed transgenic tobacco cellsexpressing a gene for Na/K antiporter pump protein, and found them to be highly resistant to high level of sodium chloride (Fig.4).



(2) Immune system in plants. The molecular mechanism by which rice plants recognize bacterial pathogens was identified. Rice receptor protein recognizes bacterial fragellin proteins anot by the amino acid sequence but by the sugar modification (Fig.5). The resistance against a fungal pathogen, rice blight, was shown to be mediated through G-proteins, which are essential for pathogen recognition and signaling.





(3) Biotechnology. Caffeine biosynthetic pathway was clarified, and decaffeinated coffee plants were constructed. Similarly, transgenic tobacco in which caffeine was synthesized showed a strong repellant activity towards tobacco cutworms. A phytoremediation method for heavy metal contaminants was developed using genes for sulfur assimilating and heavy metal binding proteins (Fig.6). Epigenetic mechanism for gene silencing and activation was studied through analyses of DNA methyaltion.

3. Concluding Remarks

To ate, practical transgenic plants are few, such as soja beans, rice, maize and oil seed rapes. Research on crops that are tolerant against environmental stresses are far behind those commercial ones, and techniques to introduce multiple genes are still under development. Although improvement of assimilation capacity is urgent for global crop production, researches are only at laboratory scale, not offering even prototype transgenic samples. The present project has attempted to obtain fundamental knowledge of plant life and to develop techniques to construct transgenic plants that are possibly practically utilized.

4. Primary Publications

(1) Munekage Y, Hojo M, Meure J, Endo T, Tasaka M, Shikanai T (2002) *PGR5* is involved in cyclic electron flow around photosystem I and is essential for photoprotection in *Arabidopsis*. *Cell* 110: 361-371

(2) Munekage Y, Hashimoto M, Miyake C, Tomizawa K, Endo T, Tasaka T, Shikanai T (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. *Nature* 429: 579-582

(3) Ashida H, Saito Y, Kojima C, Kobayashi K, Ogasawara N, Yokota A (2003) A functional link between RuBisCO-like protein of *Bacillus* and photosyntheic RuBisCO. *Science* 302: 286-290

(4) Horie T, Yoshida K, Nakayama H, Yamada K, Oiki S, Shinmyo A (2001) Two types of HKT transporters with different properties of Na^+ and K^+ transport in *Oryza sativa*. *Plant J.* 27: 129-138

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(6) Fujiwara S, Tanaka, N, Takayama S, Isogai A, Che FS (2004) Rice cDNA microarray based gene expression profiling of the response to flagellin perception in cultured rice cells. *Mol Plant Microb Interact* 17: 986-998.

(7) Suzuki N, Yamaguchi Y, Koizumi N, Sano H (2002) Functional characterization of a heavy metal binding protein CdI19 from *Arabidopsis*. *Plant J.* 32: 165-173

(8) Ogita S, Uefuji H, Yamaguchi Y, Koizumi N, Sano H (2003) Production of decaffeinated coffee plants by genetic engineering. *Nature* 423:823

Molecular Mechanisms on Regulation of Morphogenesis and Metabolism Leading to Increased Plant Productivity

Project Leader:
Takashi HASHIMOTO Professor, Graduate School of Biological Sciences, Nara Institute of Science and Technology

1. Objective:

Various species-specific low-molecular weight organic compounds found in plants (secondary products) have long been utilized by us as medicines, dyes, spices, perfumes, and food additives. Since these useful plant metabolites are often difficult to synthesize chemically or to produce by bacterial fermentation, their efficient production systems in plants are desirable. To improve productivity of plant metabolites by genetic engineering, it is important to understand structural genes for biosynthesis enzymes and molecular mechanisms that regulate their expression.

When we view plant structures as the sites of metabolite production, we need to understand how developmental programs to shape organs and tissues are genetically connected to regulation of secondary metabolisms. In the future, genetic engineering approaches may be used to optimize plant architectures to increase production sites.

This project utilizes molecular biological and molecular genetic approaches to reveal biosynthesis and regulation of plant metabolites and to understand molecular mechanisms of root development. We hope to establish molecular foundation for increased production of useful plant metabolites and for optimizing plant architectures.

2. Summary

2-1. Metabolic regulation :

(1) Biosynthesis enzymes and transporters of nicotine were cloned from tobacco. Nicotine biosynthesis is regulated in response to wounding, which involves jasmonate signaling and a regulatory locus specific to nicotine accumulation.

(2) Transport mechanisms of berbeine and shikonin were studied in detail.

(3) Molecular networks between gene expression and metabolite profiles were revealed for flavonoids and campthothesin.

(4) Biosynthesis enzymes for the phytochrome chromophore were cloned, and used to engineer Arabidopsis plants with a novel phytochrome activity and photomorphological responses.

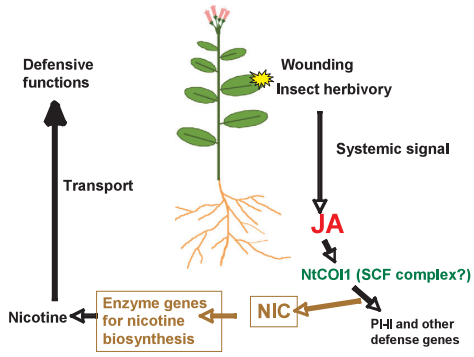

Fig.1. Regulation of Nicotine Biosynthesis

Insect herbivory and wounding to leaves initiate a signal transduction pathway involving jasmonic acid (JA) and possibly a SCF ubiquitin-dependent protein degradation complex, and activate general defense responses. In tobacco roots, a specific JA-dependent signaling pathway involving the regulatory gene NIC activates transcription of nicotine biosynthesis enzyme genes and transporters. Nicotine synthesized in roots is transported to leaves where it functions as a chemical defense against insects.

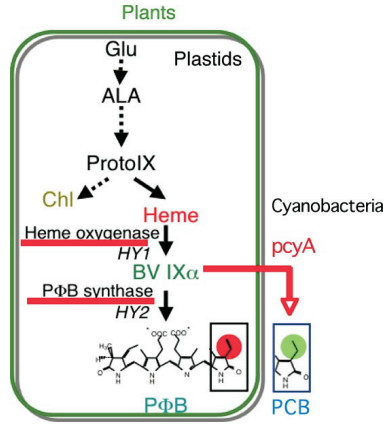

Fig.2. Metabolic Engineering of Chromophore

Plants synthesize the chromophore of phytochromes with the final step catalyzed by PϕB synthase (HY2) and respond to particular light wavelengths. Transgenic plants were engineered to produce the cyanobacteria-type chromophore

PCB (instead of PϕB) and were found to exhibit cyanabacteria-type photoresponses.

2-2. Root morphogenesis :

(1) Arabidopsis mutants with defective lateral root development were used to study molecular networks of root primordia formation.

(2) Several novel Arabidopsis mutants with defective adventitious roots in tissue culture system were obtained and their molecular characterization indicated RNA metabolism in adventitious root formation.

(3) Molecular determinants of pollen and stigma, and their downstream kinase, which function in *Brassica* self-incompatibility signaling, were identified. These studies opened an attractive strategy to regulate seed production in crops.

(4) Molecular mechanisms underlying how active oxygen species promote plant growth were studied.

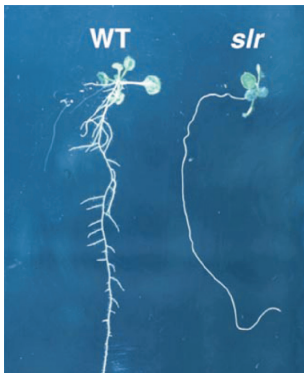

Fig.3. Lateral Root Development

Arabidopsis solitary-root (slr) mutant has a mutation in auxin-inducible protein IAA14, which is not effectively degraded in response to auxin, thus impairs lateral root development. Suppressors of slr revealed novel insights into lateral root formation.

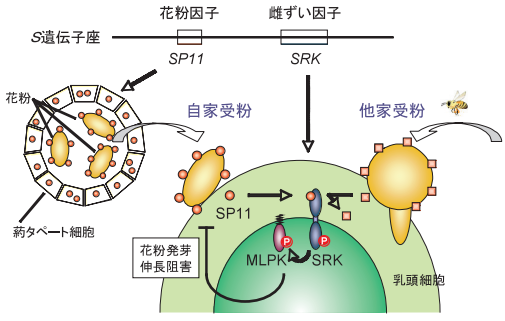

Fig.4. Self-incompatibility in Brassica

S loci determine self-incompatibility specificity and contain pollen ligand SP11 and stigma receptor SRK. Another kinase MLPK is required for the SP11/SRK signaling pathway, which ultimately inhibits water uptake and germination of self pollen.

3. Concluding Remarks

Biosynthesis of alkaloids and other plant-derived secondary products is actively studied world-wide. Although many genes encoding biosynthesis enzymes have been cloned, pathway-specific regulation and accumulation of metabolites have not well been characterized at the molecular level. Pathway regulators and novel transporters found in this project should be the first step to understand how environmental and developmental signals induce secondary metabolites, and how these molecules move from the sites of synthesis to the cells where they accumulate. Dynamic regulation of secondary metabolism will be explored in the future.

Signaling pathways of auxin and other plant hormones are steadily characterized. Present studies first identified molecular components of lateral/adventitious root formation in the general auxin pathway. Further studies will reveal many downstream players for root primordia formation and should lead a way to optimize root architecture for metabolite production.

4. Primary Publications

(1) T. Shoji, R. Winz, T. Iwase, K. Nakajima, Y. Yamada, and T. Hashimoto, Expression patterns of two tobacco isoflavone reductase-like genes and their possible roles in secondary metabolism in tobacco. Plant Mol. Biol. 50: 427-440 (2002).

(2) C. Kami, K. Mukougawa, T. Muramoto, A. Yokota, T. Shinomura, J. C. Lagarias and T. Kohchi Complementation of phytochrome chromophore-deficient Arabidopsis by expression of phycocyanobilin:ferredoxin oxidoreductase. *Proc. Natl. Acad. Sci. USA* 101: 1099-1104. (2004)

(3) N. Shitan, I. Bazin, K. Dan, K. Obata, K. Kigawa, K. Ueda, F. Sato, C. Forestier and K. Yazaki Involvement of CjMDR1, a plant MDR-type ABC protein, in alkaloid transport in *Coptis japonica*.. *Proc. Natl. Acad. Sci. USA* 100: 751-756. (2003)

(4) H. Fukaki, Y. Nakao, Y. Okushima, A. Theologis, and M. Tasaka, Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in Arabidopsis. Plant J. 44: 382-395, (2005).

(5) K. Murase, H. Shiba, M. Iwano, F.-S. Che, M. Watanabe, A. Isogai and S. Takayama. A membrane-anchored protein kinase involved in *Brassica* self-incompatibility signaling. *Science* 303: 1516-1519. (2004)

RNA Silencing in Higher Plants and Functional Genomics

Project Leader:
Fumihiko SATO Professor, Graduate School of Biostudies, Kyoto University

1. Objective:

The development of practical systems to identify gene functions based on RNA silencing (RNA interference; RNAi) as well as its application to the characterization of genome networks in higher plant systems have been investigated. The molecular bases of RNAi in plant cells were also investigated to contribute to the establishment of the next generation of genetic engineering with more controlled gene expression.

2. Summary

2-1. Development of simple transient RNAi system for the functional genomics :

An efficient transient RNAi method with artificially synthesized dsRNA has been developed. (Fumihiko Sato, Ei-ichiro Fukusaki)

This transient RNAi system has been successfully used to identify several trans-factors and previously unidentified biosynthetic genes in medicinally important isoquinoline alkaloid biosynthesis in *Coptis japonica* cells. (Fumihiko Sato)

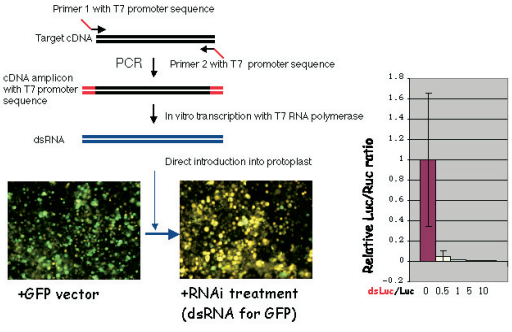

Fig.1. Development of transient RNAi system in *Coptis japonica* protoplasts.

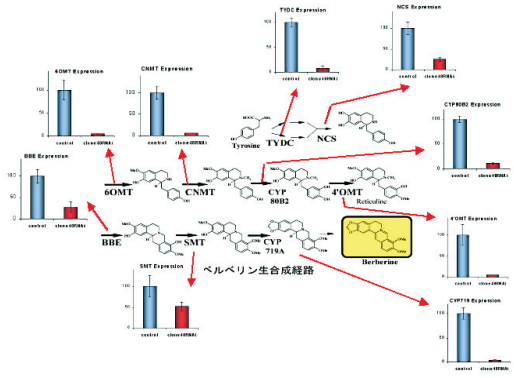

Fig.2. Application of transient RNAi has revealed a unique transcriptional network in isoquinoline alkaloid biosynthesis.

2-2. Development of differential RNAi (dRNAi), comprehensive RNAi for gene families, and a novel gene-substitution method based on dRNAi for functional genomics :

The differential RNAi (dRNAi) method for the gene-specific silencing of gene-families has been established using specific double-stranded (ds)RNA designed for a gene-specific region such as the 3' untranslated region (UTR) of transcript. Furthermore, a unique 37 bp-RNAi system for the comprehensive silencing of whole gene families in stable transformants has been established. Using dRNAi, the physiological functions of important photosynthetic PsbP in tobacco, chalcone synthase in anthocyanin synthesis in petunia, and Rac genes in signal transduction of pathogenesis in rice have been successfully characterized. (Fumihiko Sato, Ei-ichiro Fukusaki, Kazuyuki Isshiki, Ko Shimamoto)

A novel gene-substitution method by the ectopic expression of a complementary (or substitutional) expression vector in dRNAi host plants has been developed to substitute the gene function in transgenic plants. (Fumihiko Sato)

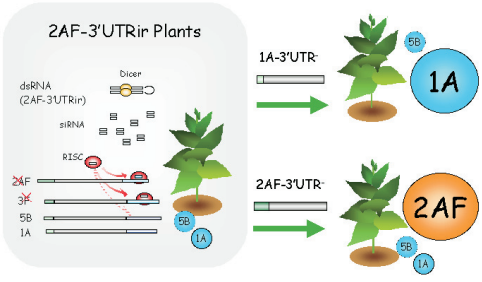

Fig.3. Effective gene-substitution method based on differential RNAi has been developed.

2-3. Development of efficient RNAi vectors for rice functional genomics :

Several RNAi vectors, such as practical RNAi vector (pANDA) based on the Gateway system, those for the quantitative control of RNAi using splicing mutation, and those for cell-specific silencing in endosperm have been developed. Using these vectors, *Wx* mRNA function was efficiently and quantitatively disrupted to modify the food quality of rice grain. pANDA vector was also successfully used for the characterization of a flowering-time control gene and disease-resistance genes in rice. (Kimiko Ito, Kazuyuki Isshiki, Ko Shimamoto)

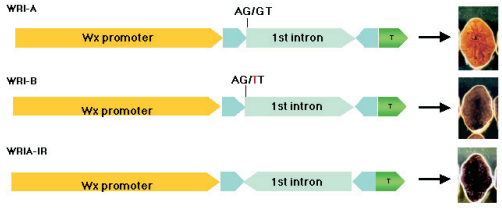

Fig.4. Cell-specific and qualitatively controlled RNAi vector has been developed to modify the amylose content in rice grain

2-4. RNAi and metabolic engineering :

RNAi has been successfully used for metabolic engineering in an isoquinoline alkaloid biosynthetic pathway to produce an important intermediate, reticuline, which is accumulated in transgenic cells. These novel cell lines have provided insight into understanding the generation of new biosynthetic pathways by metabolic engineering. (Fumihiko Sato)

2-5. Molecular characterization of DNA methylation :

The characterization of the low DNA-methylation mutant *ddm1* in Arabidopsis has revealed that several endogeneous genes as well as transposons can be de-suppressed by the reduction of DNA-methylation. Further analysis in rice also indicated the existence of a novel system of gene regulation/recognition in plant cells. (Tetsuji Kakutani)

2-6. RNAi and virus suppressors :

RNAi in plants is known to help defend against virus infection. On the other hand, viruses have RNAi suppressors to counteract RNAi. The NSs protein of *Tomato spotted wilt virus* has been characterized as the first RNAi suppressor in negative-strand RNA viruses, and it has been shown that NSs could interfere with multiple steps in the RNAi pathway. Also, a novel mechanism of RNAi suppression by *Red clover necrotic mosaic virus*, which requires multiple viral components, has been identified. (Kazuyuki Mise, Tetsuro Okuno)

3. Concluding Remarks

Novel RNAi methods (dRNAi, comprehensive RNAi, transient RNAi etc.) have been developed for functional genomics in plant. The application of these methods has revealed functional networks of genes. Further development of the gene-substitution method based on dRNAi could provide new tools for the development of plant functions using a more rational approach. Detailed characterization of the RNAi mechanism could also provide the molecular basis for the next generation of gene engineering.

4. Primary Publications

(1) Joseph G. Dubouzet, Takashi Morishige, Nanae Fujii, Chuang-Il An, Ei-ichiro Fukusaki, Kentaro Ifuku and Fumihiko Sato: "Transient RNA Silencing of Scoulerine 9-*O*-methyltransferase Expression by Double Stranded RNA in *Coptis japonica* Protoplasts", *Biosci. Biotech. Biochem.*, 69, 63-70 (2005).

(2) Kentaro Ifuku, Yumiko Yamamoto, Taka-aki Ono, Seiko Ishihara, and Fumihiko Sato: "PsbP Protein, but not PsdQ Protein, is Essential for the Regulation and Stabilization of Photosystem II in Higher Plants", *Plant Physiol.* 139, 1175-1184 (2005).

(3) Ryosuke Hayama, Shuji Yokoi, Shojiro Tamaki, Masahiro Yano and Ko Shimamoto: "Adaptation of Photoperiodic Control Pathways Produces Short-day Flowering in Rice", *Nature* 422, 719-722 (2003).

(4) Tetsu Kinoshita, Asuka Miura, Yeonhee Choi, Yuki Kinoshita, Xiaofeng Cao, Steven E. Jacobsen, Robert L. Fischer and Tetsuji Kakutani: "One-way Control of *FWA* Imprinting in *Arabidopsis* Endosperm by DNA Methylation", *Science*, 303, 521-523 (2004).

(5) Masahiro Tatsuta, Hiroyuki Mizumoto, Masanori Kaido, Kazuyuki Mise and Tetsuro Okuno: "The *Red clover necrotic mosaic virus* RNA2 *trans*-activator is also a *cis*-acting RNA2 Replication Element", *J. Virol.* 79, 978-986 (2005).

Studies of Intricate Mechanisms of the Regulatory Factors in Plant Cell Death and Proliferation

Project Leader:
Hirofumi UCHIMIYA Professor, Institute of Molecular and Cellular Biosciences, The University of Tokyo

1. Objective

Plants are thought to have unique systems, which support their life. Plants respond various environmental stresses, adapt and sustain their life. Factors responsible for abiotic and biotic stresses can trigger cell cycle arrest and cell death. The regulation of cell death and life is essential for certain aspects of plant development. A unique feature of plant cell cycle is the fact that the meristematic and differentiated cells are capable of re-entering the cell division. These mechanisms are a highly regulated and complex processes. The timing and efficiency of death and life have important roles in determining the yield of many crops. The major objective of this project is to identify and analyze factors which regulate plant cell proliferation and cell death. Increased knowledge of the regulation and processes of cell death and cell proliferation will provide information for plant breeders to generate crops with improved yield and stress-resistance with benefits for future society.

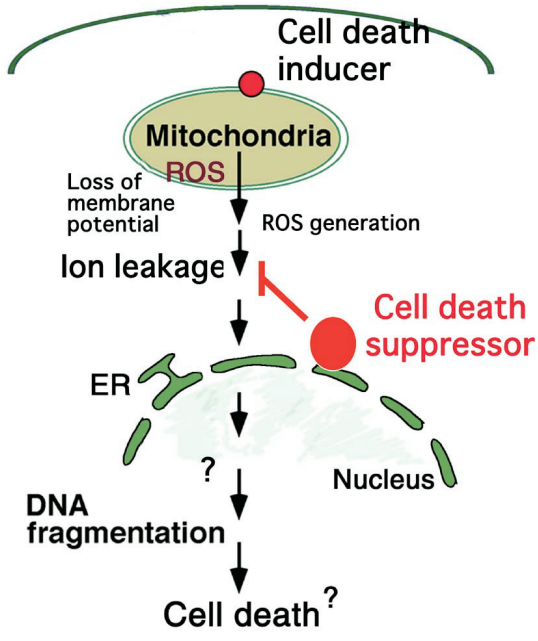

Fig.1. Schematic diagram of ROS-induced plant cell death. The expression of cell death inducer caused loss of membrane potential and ion leakage, and leaded cell death. Over expression of plant cell death suppressor inhibited such death phenomena.

2. Summary

In this project, we focused on molecular mechanisms, which are involved in regulation of plant cell life and death. Also, efforts were being made to find out fundamental mechanisms during plant growth and environmental adaptation. The results are summarized as followed.

(a) *Arabidopsis* BI-1 is a widely conserved endoplasmic reticulum membrane protein known as a cell-death regulator. We demonstrated that over-expression of BI-1 protein suppressed cell death induced by various stimuli such as hydrogen peroxide, salicylic acid and elicitor. Furthermore, the functional relationship between AtBI-1 and calcium signaling was also demonstrated.

(b) Cell division in plant is controlled by the CDKs. We found four CDK-activating kinases (CAKs) of *Arabidopsis* and proposed a CDK-phosphorylation cascade.

(c) The tapetum degradation is an event of the programmed cell death (PCD). We employed Bax and BI-1 genes to regulate PCD in the tapetum. It was presented that a certain signal for PCD was present in tapetum of anthers at the tetrad stage, and proper timing of PCD was essential for normal micro-sporogenesis.

(d) Trienoic fatty acids (TAs) are the major polyunsaturated fatty acid species in the membrane lipids in plant cells. We showed that TAs in chloroplast membrane lipids are involved in defense responses of plants against avirulent bacterial pathogens. Namely, linolenic acid, the most abundant TA, activated the NADPH oxidase that is responsible for oxidative burst.

(e) We demonstrated that plants have Na^+/K^+ transport proteins, which played important role in adaptation of cells to hyperosmotic conditions.

(f) The plant hormone ethylene regulates a wide range of developmental processes and the response of plants to stress and pathogens. Cross-talk between ethylene and sugar signaling was found. This indicates that a pathway controlling degradation of transcription factors responsible for ethylene signaling is a key for controlling plant growth and stress response.

3. Concluding Remarks

The objective of this project was to understand how plants live and die. We focused on the molecular mechanisms, which are involved in the regulation of cell proliferation and cell death. A major challenge to the world community in coming years is keeping food production in pace with the increasing world population. Our findings obtained from this project supplied novel materials to understand the biomass production and stress response to biotic and abiotic constrains. This study will provide a new knowledge of senescence and regulation of life, a fundamental aspect of plant biology as well as new approaches to improve the stress tolerance for important crops. A number of patent applications were filed for technologies obtained from this project.

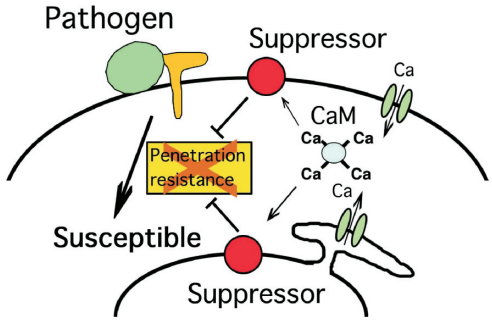

Fig.2. Schematic presentation of plant-pathogen interaction. Plants respond to pathogen through calcium signaling cascade. Plant cell death factors are involved in the regulation of plant-pathogen interaction. Further analysis will provide new approach to molecular breeding of crops.

4. Primary Publications

(1) Kawai - Yamada, M., Jin, L., Yoshinaga, K., Hirata, A. and Uchimiya, H.: Mammalian Bax-induced plant cell death can be downregulated by over-expression of *Arabidopsis Bax Inhibitor -1* (*AtBI-1*). Proc. Natl. Acad. Sci. USA., 98, 12295-12300 (2001).

(2) Yamaguchi, M., Kato, H., Yoshida, Y., Yamamura, S., Uchimiya, H. and Umeda, M.: "Control of *in vitro* organogenesis by cyclin-dependent kinase activities in plants", *Proc. Natl. Acad. Sci., USA* 100, 13, 8019-8023 (2003).

(3) Yanagisawa, S., Yoo, S.D., Sheen, J.: "Differential regulation of EIN3 stability by glucose and ethylene signaling in plants." *Nature* 425, 521-525 (2003).

(4) Kawai-Yamada, M., Ohori, Y. and Uchimiya, H.: "Dissection of Arabidopsis Bax inhibitor-1 suppressing Bax, hydrogen peroxide and salicylic acid-induced cell death" *Plant Cell* 16, 21-32 (2004).

(5) Takahara, T., Tasic, B., Maniatis, T., Akanuma, H., Yanagisawa, S.: "A delay in the synthesis of the 3' splice site promotes trans-splicing of the preceding 5' splice site." *Mol. Cell.* 18, 245-251 (2005).

(6) Hayashi, M., Takahashi, H., Tamura, K., Huang, J., Yu, L.H., Kawai-Yamada, M., Tezuka, T., and Uchimiya, H. "Enhanced dihydroflavonol-4-reductase activity and NAD homeostasis leading to cell death tolerance in transgenic rice." *Proc. Natl. Acad. Sci., USA* 102, 7020-7025 (2005).

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