Donatinon Laboratories

Department of Molecular and Developmental Biology
再生基礎医科学寄付研究部門(SBI, トミー, ロート製薬, 慈照会)

Visiting Professor	Sumiko Watanabe, Ph.D.	特任教授 医学博士	渡 辺 すみ子
Visiting Research Associate	Shinya Satoh, Ph.D.	特任助教 工学博士	佐 藤 伸 哉
Visiting Research Associate	Hideto Koso, MD, Ph.D	特任助教 医学博士	高 祖 秀 登

Our long-term goal is to understand the molecular mechanisms which coordinately regulate growth and differentiation of stem cells as well as differentiated cells with emphasis on intracellular signal transduction. For this purpose we are using models ranging from iPS and various culture cells, zebrafish, mouse, to monkey. Based on our research background on the area of cytokine signals, we now focus on the analysis of development and regeneration of neural retina.

The neural retina is a part of the central nervous system (CNS), and regeneration of the retina from retinal stem cells or other sources by transplantation is a critical issue from both clinical and neurobiological points of view. Although reports of successful regeneration of the CNS from neural stem cells (NSC) have appeared in the literature, such has not been the case for the vertebrate neural retina. Furthermore, the nature of retinal stem cells has not been clarified, making it difficult to attempt regeneration of the retina. Based on the techniquears and knowledge that have been accumulated through work on haematopoietic systems in our laboratory, we attempt to identify mammalian retinal stem cells and following developmental processes by revealing the expression pattern of cell surface proteins. We found that various CD antigens mark spatiotemporally distinct populations of retinal cells, and genes specifically expressed in such populations have been revealed by microarray analyses. Various signaling molecules and transcriptional factors are under investigation for their roles on retinal development. For developmental biological analyses, we use zebrafish in addition to mouse as model animals. We also continue to work on haematological projects, and bidirectional cooperative progress between neurological and haematological works is one of unique features of our laboratory. Projects, which gave major findings during 2011 are as follows.

1. Serine/Threonine kinase Melk regulates proliferation and glial differentiation of retinal progenitor cells

Rika Saito, Sumiko Watanabe

Serine/threonine kinase, Melk, was initially cloned in oocytes, but it is expressed in normal tissues and especially in cancer cells. We previously identified Melk as a gene that is highly expressed in immature mouse retinal progenitors. To analyze the function of Melk in embryogenesis, we cloned zebrafish Melk and reported that morpholino-based downregulation of Melk in zebrafish resulted in severe anemia (Saito et al. MCB, 2005). Melk-morpholino-treated zebrafish also showed microphthalmia, suggesting the participation of Melk in retinal development. In Melk-depleted retinas, differentiation of retinal neurons took place but was delayed, and the proliferative period of retinal progenitor cells

was prolonged, suggesting that Melk may regulate the timing of the transition from proliferation to differentiation. For more detailed examination, we performed gain- and loss-of-function analyses of Melk in mouse retinas. Knockdown of Melk by shRNA in mouse embryonic retinal explant culture resulted in decreased proliferative activity of retinal progenitors, and accordingly, overexpression of Melk slightly enhanced proliferation. Differentiation of retinal progenitor into subtypes of retinal neurons was not significantly affected, but Müller glia differentiation was perturbed by the level of Melk. Furthermore, process extension of glial cells was enhanced in the absence of Melk, suggesting that Melk is involved in the morphological differentiation of retinal cells. Taken together, our results suggest that Melk is primarily required for proper proliferation, and may play multiple roles in retinal development in vertebrates.

2. Identification of CD44 as a cell surface marker for Müller glia precursor cells

Toru Shinoe, Hiroshi Kuribayashi, Motoharu Seiki[1], Hiroyuki Aburatani[2], Sumiko Watanabe: [1]Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, [2]Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo.

In the retina, both neurons and glias differentiate from a common progenitor population. CD 44 cell surface antigen is a hyaluronic acid receptor expressed on mature Müller glial cells in the retina. We found that in the developing mouse retina, expression of CD44 was transiently observed at or around birth in a subpopulation of c-kit-positive retinal progenitor cells. The level of CD44 expression is probably regulated primarily by transcriptional level, but our results also suggested the involvement of posttranslational regulation. During in vitro culture, purified CD44/c-kit-positive retinal progenitor cells exclusively differentiated into Müller glial cells and not into neurons, suggesting that CD44 marks a subpopulation of retinal progenitor cells that are fated to become glias. Over-expression of CD44 inhibited the extension of processes by Müller glial cells and neurons, and involvement of hyaluronic acid in this process was suggested. Notch signaling is known to be involved in the specification of retinal progenitors into a glial fate. Activation of Notch signaling increased the number of CD44-positive cells in early development of the retina, and treatment with the Notch signal inhibitor, DAPT, at early, but not later, stages of retinal development abolished both CD44-positive cells and Müller glial cells. Together, CD44 was identified as an early cell surface marker of the Müller glia lineage, and Notch signalling was shown to be involved in commitment of retinal progenitor cells to CD44 positive Müller glial precursor cells.

3. The early retinal progenitor-expressing gene Sox11 regulates the timing of the differentiation of retinal cells

Ayumi Usui[3], Yujin Mochizuki[3], Atsumi Iida, Hiroyuki Aburatani[2], Sumiko Watanabe: [3]Department of Ophthalmology, Juntendo University School of Medicine.

Sry-related HMG box (Sox) proteins play diverse and critical roles in a variety of morphogenetic processes during embryonic development. Sox11 and Sox4 are members of the SoxC subtype. We found that Sox11 was strongly expressed in early retinal progenitor cells and that when expression of Sox11 subsided around birth, Sox4 expression began. To analyze the role of Sox11 and Sox4 in retinal development, we perturbed their expression pattern by expressing them ectopically in retinal explant culture. Overexpression of Sox11 or Sox4 in retinal progenitors resulted in similar phenotypes, that is, increased cone cells and decreased Müller glia. Birth-date analysis showed that cone cells were produced at a later developmental stage than that for normal cone genesis. Sox11-knockout retinas showed delayed onset and progress of differentiation of subsets of retinal cells during the embryonic period. After birth, retinal differentiation took place relatively normally, probably because of the redundant activity of Sox4, which starts to express around birth. Neither overexpression nor loss-of-function analysis gave any evidence that Sox11and Sox4 directly regulate transcription of genes critical to differentiation of subsets of retinal cells. However, histone H3 acetylation status of the early proneural genes was lowered in knockout retinas, suggesting that Sox11 regulates the timing of differentiation in retinal cells by creating an epigenetic state that helps to establish the competency to differentiate. Taking our findings together, we propose that the sequential expression of Sox11 and Sox4 during retinogenesis leads to the fine adjustment of retinal differentiation by contributing to preparing of competency of retinal progenitors.

4. Epigenetic regulation of retinal gene expression during retinal development

Shinya Satoh, Sumiko Watanabe

Trimethylation at Lys27 of Histone H3 (H3K 27me3) is mediated by the polycomb repressive complex 2 (PRC2) which contains the catalytic enzyme, Ezh1- or Ezh2-methyltransferase. This modification provides the platform to recruit PRC1 and form the polycomb group (PcG) complex, which works as the transcriptional repressor. On the other hand, H3K27me3 is demethylated by Jmjd3, Utx, or Uty demethylase, resulting in the activation of gene expression. To clarify the role of this modification in retinal gene expression during retinal development, we performed loss of function analysis of genes that catalyze this modification. When Jmjd3 expression was knocked down by the shRNA system in a retinal explant culture, expression of genes that are important for differentiation into bipolar cells was decreased. To reveal the role of Ezh 2 in retinal development, we analyzed the development of retinas that express the catalytically inactive Ezh2. Ezh2 expressing mutant retinas showed reduction in the thickness of the ONL and INL. RT-qPCR analysis showed strong induction of Ink4a/Arf genes in Ezh2 expressing mutant retinas, suggesting that Ink4a/Arf gene expression is regulated by H3K27me3 modification in retinal development. This data indicates that regulation of H3K27me3 modification is important for development of certain subsets of retinal cells.

5. Role of thyroid hormone receptor β2 in human red opsin gene induction

Yo Tanaka, Shinya Satoh, Sumiko Watanabe

Cone photopigments, known as opsins, are pivotal elements and the first detection module in color vision. There are several different opsins, and their expression pattern in cone is precisely regulated in transcription level. Red opsin is long wavelength opsin, and role of thyroid hormone and TRβ2 for transcriptional regulation has been suggested by several ways, but detailed mechanisms including target sequence in the enhancer of red opsin had not been revealed. We analyzed the requirement of the enhancer region of red opsin by analyzing a large different construct of red opsin enhancer/ promoter-luciferase plasmids. We found that TRβ2 affected 5′ and exon/intron region, but effects of T3 was only observed with exon/intron region. Furthermore, COUP-TFII suppressed the enhance/promoter activity of red opsin gene.

6. Role of Sall family transcription factors in eye development

Yukihiro Baba, Yui Watabe, Sumiko Watanabe

The vertebrate homolog of spalt, sall, includes four members, and Sall1 and Sall4 are involved in normal development, particularly of limbs and the nervous system, as well as several organs including the kidney and heart. The spalt genes are mutated in several human congenital syndromes and expressed in the developing embryo in discrete but overlapping patterns. We focus on Sall1 and Sall3 to reveal their roles in retinal development.

6.1 Induction of horizontal cell differentiation in mouse embryonic retinal cells by combinatorial expression of Foxn4, Prox1 and Sall3

Several transcription factors such as Foxn4, Ptf 1a, and Prox1 have been shown to be essential for horizontal cell fate determination. However, none of these factors were sufficient to induce horizontal cells from retinal progenitor cells in mouse retina. In addition, we found that the spalt-like zinc finger protein family Sall3 was essential for maturation of horizontal cells, but again it could not induce horizontal cell fate by itself. Therefore, a combinational function of these transcription factors might be required for horizontal cell differentiation. To verify this hypothesis, gain-of-function analysis was performed with transcription factors in combination. Various combinations of pCAG plasmids encoding Foxn4, Ptf1a, Prox1, Lim1, and Sall3 were introduced into mouse retinal explant culture, and retinal differentiation and proliferation were examined by immunostaining of frozen sections. Single overexpression of any candidate genes failed to enhance the expression of horizontal cell markers, in agreement with earlier studies. On the other hand, the combination of Foxn4, Prox1, and Sall3 induced the expression of horizontal cell markers, NF160, NFL, and Calbindin-D 28k. In addition, these cells localized at the appropriate position of mature horizontal cells. Monolayer culture confirmed horizontal cell-like morphology, such as long neurites. We concluded that horizontal cell differentiation is achieved by combinatorial funciton of Foxn4, Prox1, and Sall3.

6.2 Involvement of the transcriptional factor Sal-like 1 in the lens and retinal development

The transcription factor Sal-like 1 is widely expressed in the kidney, CNS, heart, limb bud and anus. Homozygous Sall1 knockout is lethal, resulting in fatality 24 hours after birth from absence or sever abnormalities of the kidney. In the eye, strong expression of Sall1 in the embryonic lens has been reported, suggesting a potential role played by Sall1 in the lens. We investigate the function of Sall1 in the eye, primarily

focusing on the lens. Endogenous expression of Sall1 in the developing eye was examined using heterozygous Sall1-EGFP mice. We found strong expression of Sall1 in the lens. Sall1 expression in the lens was already present at embryonic day11.5, and its expression was maintained after birth through to the adulthood. We also identified Sall1 expression in subset of cells of retina and the optic nerve. Morphological observation of eye revealed that many bubble-like structure in the Sall1-KO lens at embryonic day15.5. Examination by electron microscopy revealed that these bubbles were vacuoles in the primary fiber cells of the lens, and that these vacuoles appeared to be located between the fiber cell membranes. Taken together, an important role of Sall 1 in the development of the lens was revealed.

7. Molecular mechanisms regulating differentiation and proliferation of retinal stem/progenitor cells

Haruna Suzuki-Kerr, Yui Watabe, Sumiko Watanabe

Cell surface antigens are powerful tools for isolating specific subsets of retinal cells during development from cell mixtures without damaging the cells, which makes it possible to characterize their properties and identify genes that regulate their proliferation and differentiation. By screening retinal cells from mice at various developmental stages for their reactivity with over 150 different antibodies against various cell surface antigens, we identified SSEA-1 and c-kit as early and late progenitor markers, respectively. SSEA-1 marks retinal progenitor cells in the peripheral region of the retina at around E14 -E16. In the later stage of embryogenesis, SSEA-1 disappears and c-kit expression is observed in the retinal progenitor cells in the central region of the retina. We compared the gene expression patterns of regionally and temporally different subsets of retinal progenitor cells, SSEA-1-positive cells at E14, c-kit positive cells at P1, and differentiated c-kit negative cells at P1 using a microarray. We found that several genes are specifically expressed in SSEA-1 positive early retinal progenitor cells.

7.1 Requirement of Fezf2 in Differentiation of Cone OFF bipolar cells

We found the Fezf2, a transcriptional repressor of the Fez zinc finger family, to be strongly expressed in SSEA-1-positive cells. Fezf 2 expression pattern was characterized in the mature Fezf2$^{+/-}$ retinas by making use of the β-galactosidase reporter gene expressed under the Fezf2 promoter. Between postnatal day 14 and adult, Fezf2 expression was localized to a subset of cells in the inner nuclear layer (INL). These cells all expressed Chx10 but were mostly negative for islet1, suggesting that Fezf2 is expressed in a subset of Chx10＋/islet1-, cone OFF bipolar population. However, there was no aberrant morphological defect in the Fezf2 deficient retinas at P14 or P28, albeit the slight thinning of the INL was observed. Further examinations revealed that the thinning of INL was attributed to a selective reduction in Chx10＋/islet1- cone OFF bipolar cell number. Interestingly, many of cells expressing β-gal still persisted in Fezf2 deficient retinas at P14 and P28, implying that the observed loss of OFF bipolar cells was not a simple result of Fezf2-expressing cell death. Similar analysis was then repeated with P5¯P7 retinas, however, neither morphology nor marker (Chx10, islet1, active caspase-3) expressions differed between Fezf2 deficient retinas and equivalent controls, suggesting that the observed phenotype may start sometime after P7. In conclusion, Fezf2 is expressed in a subset of cone OFF bipolar cells in the mature retina, and its deficiency leads to a reduction in the number of cone OFF bipolar cells. These results imply roles played by Fezf2 in subtype specification and/or maturation of these bipolar cells.

7.2 The role of Zic family zinc finger transcription factors in the proliferation and differentiation of retinal progenitor cells

Members of the Zic family of zinc finger transcription factors play critical roles in a variety of developmental processes. Using DNA microarray analysis, we found that Zics are strongly expressed in SSEA-1-positive early retinal progenitors in the peripheral region of the mouse retina. Reverse-transcription polymerase chain reaction using mRNA from the retina at various developmental stages showed that Zic1 and Zic2 are expressed in the embryonic retina and then gradually disappear during retinal development. Zic3 is also expressed in the embryonic retina; its expression level slightly decreases but it is expressed until adulthood. We overexpressed Zic1, Zic2, or Zic3 in retinal progenitors at embryonic day 17.5 and cultured the retina as explants for 2 weeks. The number of rod photoreceptors was fewer than in the control, but no other cell types showed significant differences between control and Zic overexpressing cells. The proliferation activity of normal retinal progenitors decreased after 5 days in culture, as observed in normal *in vivo* developmental processes. However, Zic expressing retinal cells continued to proliferate at days 5 and 7, suggesting that Zics sustain the proliferation activities of retinal progenitor cells. Since the effects of Zic1, 2, and 3 are indistinguishable in terms of differentiation and proliferation of retinal progenitors,

the redundant function of Zics in retinal development is suggested.

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Donation Laboratory

Division of Antibody, Vaccine & Experimental Therapy
抗体・ワクチン治療寄付研究部門

Project Professor	Motoharu Seiki, D.M.Sc.	特任教授(兼)	医学博士	清 木 元 治
Project Professor	Kohzoh Imai, M.D., Ph.D.	特任教授(兼)	医学博士	今 井 浩 三
Project Professor	Kouhei Tsumoto, Ph.D.	特任教授(兼)	工学博士	津 本 浩 平
Project Assistant Professor	Taniguchi Hiroaki, M.D., Ph.D.	特任助教	医学博士	谷 口 博 昭

Tumors contain a small population of putative cancer stem cells (CSC), which possess unique self-renewal properties, and survive in a quiescent state for many years after remission and result in later relapse and metastasis. Therefore, it is conceivable that targeting CSCs will eradicate tumor-initiating cells, whereas conventional chemotherapies will only eradicate the bulk of a tumor.
Cancer stem cells and normal tissue stem cells utilize the same self-renewal pathway. However, researchers characterize some of changes, which occur in cancer stem cells, not in normal tissue stem cells. The design of new therapeutic agents should be aimed at targeting these unique molecular changes.
We have currently focused on studying these unique molecular changes, which occur in cancer stem cells, not in normal tissue stem cells. This could be a new therapeutic target against solid tumors.

(1) IGF1-R Kinase Inhibitors

Signaling through IGF1-R in normal cells leads to the activation of multiple intracellular pathways, mediated by the receptor-associated tyrosine kinase domain, by PI-3 kinase and Akt, yielding growth and enhanced survival. In cancer cells, IGF1-R plays an even more critical role because it contributes to the promotion of tumor growth by inhibition of the apoptosis, transformation, metastasis, and induction of angiogenesis via VEGF.

Chemosurviving cancer cells are enriched in CSCs and express increased levels of IGF-1R. Depletion of IGF-1R results in reduction of CSC phenotype in gastrointestinal cancer cells.

We have shown that a number of strategies against the IGF system in cancer have beneficial antitumor effects. Dominant negative mutants, siRNA, and antagonistic antibodies and small-molecule tyrosine kinase inhibitors are being evaluated for their ability to block signaling and, hence, the survival and growth of cancer cells. Similar inhibitory effects on tumor cell growth and metastasis are seen in vivo, in experimental animal models.

(2) Zinc-finger-containing transcriptional factor, Kruppel-like factor 2 (KLF2)

The Kruppel-like factor (KLF) proteins are multitasked transcriptional regulators with an expanding tumor suppressor function. KLF2 is a member of the KLF family of zinc-finger transcription factors and is involved in maintaining T-cell quiescence, regulating preadipocyte differentiation, endothelial cell function, lung development and the self-renewal of ES cells. Furthermore, KLF2 is one of the prominent members of the family because of its diminished expression in malignancies and its growth-inhibitory, pro-apoptotic and anti-angiogenic

roles.

We indicate that epigenetic silencing of KLF2 occurs in cancer cells through direct transcriptional repression mediated by the Polycomb group protein Enhancer of Zeste Homolog 2 (EZH2). Binding of EZH2 to the 5′-end of KLF2 is also associated with a gain of trimethylated lysine 27 histone H3 and a depletion of phosphorylated serine 2 of RNA polymerase.

Upon depletion of EZH2 by RNA interference, short hairpin RNA or use of the small molecule 3-Deazaneplanocin A, the expression of KLF2 is restored. The transfection of KLF2 in cells with EZH2-associated silencing showed a significant anti-tumoral effect, both in culture and in xenografted nude mice.

In this last setting, KLF2 transfection was also associated with decreased dissemination and lower mortality rate. In EZH2-depleted cells, which characteristically have lower tumorigenicity, the induction of KLF2 depletion 'rescued' partially the oncogenic phenotype, suggesting that KLF2 repression has an important role in EZH2 oncogenesis.

Most importantly, the translation of the described results to human primary samples demonstrated that patients with prostate or breast tumors with low levels of KLF2 and high expression of EZH2 had a shorter overall survival.

(3) PR domain-containing protein, PRDM14

PRDM have been linked to human cancers. To explore the role of the PR domain family genes in breast carcinogenesis, we examined the expression profiles of 16 members of the PRDM gene family in a panel of breast cancer cell lines and primary breast cancer specimens using semiquantitative real-time PCR.

We found that PRDM14 mRNA is overexpressed in about two thirds of breast cancers. Moreover, immunohistochemical analysis showed that expression of PRDM14 protein is also up-regulated. PRDM14 are known as a key transcription factor required for the maintenance of hESC identity and the reacquisition of pluripotency in human somatic cells.

Introduction of PRDM14 into cancer cells enhanced cell growth and reduced their sensitivity to chemotherapeutic drugs. Conversely, knockdown of PRDM14 by siRNA induced apoptosis in breast cancer cells and increased their sensitivity to chemotherapeutic drugs, suggesting that up-regulated expression of PRDM14 may play an important role in the proliferation of breast cancer cells.

That little or no expression of PRDM14 is seen in noncancerous tissues suggests that PRDM14 could be an ideal therapeutic target for the treatment of breast cancer.

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Division of Social Communication System for Advanced Clinical Research

先端医療社会コミュニケーションシステム社会連携研究部門

Project Professor Masahiro Kami, M.D., Ph.D.
Project Assistant Professor Tomoko Matsumura, M.D., Ph.D.

特任教授 医学博士 上 昌広
特任助教 医学博士 松村有子

The aim of our division is to establish and popularize state-of-art medicine and to promote translational research (TR). We investigate a methodology to develop national consensus in health care by using media.
We also perform individual case studies on H1N1 pandemic, economic burden of health care on patients, medical support for disaster-stricken area by the Great East Japan Earthquake on March 11, 2011 and clinical internship system. In each case, we also study the system of management, information circulation, and network.

【Medical Governance】

Tomoko Matsumura, Naoko Murashige, Yuko Kodama, Haruka Nakada, Koichiro Yuji[1], Masaharu Tsubokura, Masahiro Kami: [1]Department of Medicine (Department of Hematology/Oncology), Institute of Medical Science, The University of Tokyo.

We perform individual case studies on H1N1 pandemic, economic burden of health care on patients, and clinical internship system. In addition, we also study the system of management, information circulation, and network in each case and publish the results in scientific journals (Ohara M, et al., Yuji K, et al., Murashige N, et al. Yuji K, et al.). These results are widely published in news papers and popular magazines.

【Medical support for disaster-stricken area】

Masaharu Tsubokura, Yukio Kanazawa[2], Tomoyoshi Oikawa[2], Kyohei Takahahi[3], Akemi Takada[2], Tomoko Matsumura, Morihito Takita, Tamae Hamaki[4], Kazuhiko Kobayashi[5], Syuichi Iwamoto[4], Jinichi Mori[6], Yukie Takahashi[6], Masaki Miyasaka[4], Hideki Komatsu[7], Makoto Suzuki[7], Mamiko Ohara[7], Tsunehiko Komatsu[8], Tetsuya Miyashita[9], Tetsuya Tanimoto[10], Yuko Kodama, Masahiro Kami: [2]Minamisoma Municipal General Hospital, [3]Haramachi Central Maternity Clinic, [4]Tokyo Metropolitan Bokutoh Hospital, [5]JR Tokyo General Hospital, [6]Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital, [7]Kameda Medical Center, [8]Yokohama City University Hospital, [9]Teikyo University Chiba Medical Center, [10]Pharmaceuticals and Medical Devices Agency, JAPAN.

Collaborating with physicians who work in disaster-stricken area and many support physicians, we conducted rescue and evacuation activities for patients with hemodialysis and publish the results in scientific journals (Tsubokura M, et al.).

【Information Circulation on the Advanced Medicine】

Yukiko Sakamoto-Kishi, Naoko Murashige, Masahiro Kami, Tomoko Matsumura,

We investigate the role of the manga in the health care (Kishi Y, et al.). Through internet searches using Google, we investigated the characteristics of medical manga published in Japan, defined as those in which the main character is a medical professional and that occur in a medical setting. In our survey, it was suggested that Medical manga would be a powerful tool for increasing the awareness of the public regarding medicine.

【Economic Burden of Health Care on Patients】

Yuko Kodama, Ryoko Morozumi[7], Akihiko Matsui[8], Masahiro Kami, Tomoko Matsumura: [7]Faculty of Economics, University of Toyama, Toyama, Japan, [8]Faculty of Economics, the University of Tokyo, Tokyo, Japan.

Imatinib (Glivec), which is the first-line drug for chronic myelogenous leukemia (CML), is highly efficient. We clarified that the cost of Glivec in Japan was higher than the other countries by international research. We conduct a collaborate study with Professor Matsui at Faculty of Economics, the University of Tokyo, to review the utilization of Glivec and its cost in Japan. Because the economic burden on patients or the government with prevailing advanced medical care including anticancer drugs is an important issue, we continue further investigation.

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Division of Interactome Medical Sciences
インタラクトーム医科学社会連携研究部門

Project Associate Professor Etsuko Miyamoto-Sato, Ph.D. | 特任准教授 工学博士 宮 本 悦 子

In the division of "Interactome Medical Sciences", our developed technology in Japan, called as "puromycin technology", is used as analysis tools of interactome (i.e. comprehensive biomolecular interactions). Toward order-made medicine era, we will apply these tools to medical science. Interdisciplinary approach is required to accomplish our research. To develop the "interactome analysis pipeline", we collaborate with human genome center, where large sequence data is yielded from next generation sequencer and analyzed with supercomputer. Especially, we focus on "cancer" because of its diversity and individuality, and cancer researchers are also collaborated in our research. For cooperation study with the society and academia, industries are involved in our research.

Understanding and Control of Cancer Stem Cells for Realizing Personalized Genomic Medicine

Shigeo Fujimori[1], Takatsune Smimizu[2], Seiya Imoto[3], Masao Nagasaki[3], Rui Yamaguchi[3], Naoya Hirai[1], Kazuyo Masuoka[1], Taisuke Mori[4], Hideyuki Saya[2], Satoru Miyano[3], Etsuko Miyamoto-Sato[1,3]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. Tokyo, [2]Div. Gene Reg., IAMR, Keio Univ. Sch. Med, [3]Human Genome Ctr., Inst. Med. Sci., Univ. Tokyo, [4]Mol. Pathol. Div., Natl. Cancer. Ctr. Res. Inst.

On the verge of gene therapy, the interest of basic research in the medical care has transited from the conventional post-genomic to the personal genomic research. Especially, cancer is one of the diseases requiring personal genome medicine. To understand the individuality of cancer, we have to conduct various sorts of omics analyses and accurately comprehend the individual state of complicated biomolecular interaction networks by cell types. Our research project focuses on diversity of cancer stem cells (CSCs), and is aimed at discerning and regulation of CSCs by the collaboration of researchers having the model experiment system for studying CSCs, having technology for the omics data analysis and having network analysis technique bases on mathematical models. Different types of CSC model cells (Shimizu et al., *Oncogene*. 2010) are used in our research. Those CSC model cells that are derived from bone-marrow stromal cells of Ink4a KO mice and can be induced *in vitro* by the over expression of c-Myc. Although all of those CSCs cause osteosarcoma to develop after graft to the mouse, differ in malignancy. In order to compare those CSCs having different characteristics using omics data and systems biology techniques, we plan to conduct an analysis of large scale biomolecular interaction (interactome) in different CSC models using the *in vitro* virus method (IVV method; Miyamoto-Sato E. et al., *PLoS ONE*. 2010) coupled with next generation sequencer. The IVV method is our originally developed technology for protein analysis and is able to determine not only 'interacting partners' of the protein, but also 'interacting region (domain)' simultaneously, allowing us to analyze relationship between interactome and aberrance (e.g., splicing

abnormality and DNA mutation), which are frequently observed in cancer cells. Further, we have developed the database to investigate such relationship between them.

Highly efficient analysis of interactome using the *in vitro* virus method combined with high-throughput sequencing

Shigeo Fujimori[1], Naoya Hirai[1], Kazuyo Masuoka[1], Takanori Washio[1,2], Tomohiro Oshikubo[1,3], Tatsuhiro Yamashita[1,4], Etsuko Miyamoto-Sato[1,5]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. Tokyo, [2]RIKEN GENESIS Co., Ltd., [3]Fujitsu Adv. Eng. Ltd., [4]BioIT Biz. Dev. Unit, Fujitsu Ltd., [5]Human Genome Ctr., Inst. Med. Sci., Univ. Tokyo.

Current high-throughput 'interactome' datasets have not only low coverage but also low reliability yet. Although next generation sequencing (NGS) has been applied in various researches of molecular biology, it is not easy to utilize for analyzing interactome. In this research, to address these issues, we developed an IVV-HiTSeq method as an *in vitro* virus (IVV) method combined with NGS for the interactome analysis. The IVV method is one of the mRNA display methods and has often been utilized for the selection of prey proteins with bait from the large cDNA library. This technology is based on the technique of the formation of linkage between mRNA as genotype and their coding protein as phenotype, thereby an iteration of selection and amplification processes using mRNA tags for the enrichment is realized. Prey molecules enriched through iteration of several rounds of selection can easily be detected by sequencing of RNA moiety. In the IVV-HiTSeq method, prey DNA samples amplified with barcoded primers representing the round of selection are sequenced simultaneously. Obtained read data can be used as substitute for quantitative real-time PCR assays and has an ability to evaluate the statistical significance of interactions. We confirmed that our method can improve both the coverage and reliability of interactome datasets. Furthermore, our method has a potential to be applied to not only *in vitro* selections of PPI but also to detections of proteins that interact with nucleic acids or chemicals, suggesting that our method should be useful for exploring large space of protein sequences.

IRView: a database and viewer for protein interacting regions

Shigeo Fujimori[1], Naoya Hirai[1], Kazuyo Masuoka[1], Tomohiro Oshikubo[1,2], Tatsuhiro Yamashita[1,3], Takanori Washio[1,4], Ayumu Saito[5], Masao Nagasaki[5], Satoru Miyano[5], Etsuko Miyamoto-Sato[1,5]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. Tokyo, [2]Fujitsu Adv. Eng. Ltd., [3]BioIT Biz. Dev. Unit, Fujitsu Ltd., [4]RIKEN GENESIS Co., Ltd., [5]Human Genome Ctr., Inst. Med. Sci., Univ. Tokyo.

Protein-protein interactions (PPIs) are mediated through specific regions (sites/domains) on proteins. Some proteins have two or more protein interacting regions (IRs) and some of those IRs are competitively used for interactions with different proteins. To facilitate comprehension of such complicated interaction patterns at the region level, we developed the IRView, an online database that allows viewing of IRs in protein sequences. Currently, the IRView contains 3,417 IRs of human and mouse proteins obtained from several different sources, together with other annotated region data, such as InterPro domain/motif regions, non-synonymous single nucleotide polymorphism (nsSNP) sites and variable regions owing to alternative mRNA splicing. Since all region data in the IRView are stored and displayed as standardized positions on reference sequences, users can intuitively comprehend positional relations of each region to others via the user-friendly web interface. All records can be easily browsed and queried to search proteins of interest. The content of the database will be updated regularly. The IRView is publicly available at http://ir.hgc.jp/

PRD: A protein-RNA interaction database

Shigeo Fujimori[1], Katsuya Hino[2], Ayumu Saito[3], Masao Nagasaki[3], Satoru Miyano[3], Etsuko Miyamoto-Sato[1,2,3]: [1]Div. Interactome Med. Sci., Inst. Med. Sci., Univ. Tokyo, [2]Dept. Biosci. & Info., Keio Univ., [3]Human Genome Ctr., Inst. Med. Sci., Univ. Tokyo.

Although protein-RNA interactions (PRIs) have essential roles in a variety of cellular processes, compiled data on PRIs at the gene level are scarce compared with protein-protein interactions, which have been intensively surveyed and accumulated in public databases. Here, we present a PRI database (PRD) that provides information on PRIs described in the scientific literature. Currently the database contains $>$10,000 interactions imported from public databases and $>$400 originally curated interactions. Each interaction is linked to genes, articles, and taxonomy names, with identifiers used in the NCBI database. Furthermore, each record contains detailed information regarding protein

binding regions, conserved RNA elements, and detection methods (when available). Interaction data in our database were curated and stored according to the PSI-MI standard. Users can browse all recorded interactions and execute flexible keyword searches against the database via a web interface. Our database is not only a reference of PRIs, but will also be a valuable resource for studying characteristics of PRI networks. PRD can be freely accessed at http://pri.hgc.jp/. The content of the database will be continually updated.

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藤森茂雄、森泰昌、佐谷秀行、宮本悦子：タンパク質間相互作用ネットワーク解析によるがん個性の理解．システムがんNewsletter ２：４（2011）

Hiroyuki Ohashi, Etsuko Miyamoto-Sato: A next generation evolutionary molecular engineering system and the future prospects. Viva origino 40 suppl (2012)

Collaborative Research Unit

Division of Systems Biomedical Technology (Konika Minolta Technology Center)

システム生命医科学技術開発共同研究ユニット (コニカミノルタテクノロジーセンター)

Project Associate Professor Noriko Gotoh, M.D., Ph.D.
Project Assistant Professor Kunihiko Hinohara, Ph.D.

特任准教授 医学博士 後藤典子
特任助教 医学博士 日野原邦彦

Our major research interest is to elucidate the molecular mechanisms regulating cancer cells, stem cells, cancer stem cells and development. Our team has two important research directions: One is to clarify the basic principles underlying biology and the other is to apply the knowledge extracted from the basic principles to translational medicine. In particular, we are focusing on growth factor signaling, such as fibroblast growth factor (FGF) and epidermal growth factor (EGF). In order to achieve the goal, we take a challenging combinatorial approaches of molecular biology and systems biology, in addition to conventional methods of molecular biology.

1. Translational medicine by using systems biology approach

Asuka Nakata, Anna Mizutani, Tasunori Nishimura, Rui Yamaguchi[1], Masao Nagasaki[1], Teppei Shimamura[1], Seiya Imoto[1], Ayumu Saito[1], Ryo Yoshida[2], Tomoyuki Higuchi[2], Takashi Kohno[3], Jun Yokota[3], Satoru Miyano[1], Noriko Gotoh[1]: [1]Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]Institute of Statistical Mathematics, Tokyo, Japan, [3]Biology Division, National Cancer Center Research Institute.

1-1. EGF receptor tyrosine kinase defines critical prognostic genes of stage IA lung adenocarcinoma

Non-small cell lung cancer (NSCLC) is the commonest and the most fatal histological subtype of lung cancer. About 10-30% of stage IA patients die due to recurrence after surgery of curative intent. Therefore, the identification of prognostic biomarkers for stage IA lung adenocarcinoma with poor prognosis is of great importance to select patients who will be benefited by adjuvant therapy. To the best of our knowledge, there is little evidence that gene signatures are useful above the clinical prognostic markers alone, including stage, age and gender. Epidermal growth factor (EGF) signaling pathway is closely related to aggressive phenotypes of lung and other cancers. EGF receptor (EGFR) tyrosine kinase-specific inhibitor, namely gefitinib, is expected to alter the gene expression patterns caused by EGFR tyrosine kinase activity. We performed DNA microarray analysis in order to obtain the comprehensive gene expression profiles in human primary lung epithelial cells that were stimulated with EGF in the presence or absence of gefitinib. The detailed time courses are composed of 19 time points along 48 hours. The data were subjected to a unique mathematical analysis by using the State Space Model (SSM)

in order to select genes of which expression patterns were altered by gefitinib. We indentified such 139 genes as the EGFR tyrosine kinase-influenced key genes. The 139 EGFR tyrosine kinase-influenced key genes were used as expression signatures to train a risk scoring model that classifies patients in high- or low-risk (the risk of death or recurrence in 5 years). This model was trained by using a data set composed of 253 North American patients with lung adenocarcinomas. Then, the predictive ability of the risk scoring model was examined in two independent cohorts composed of North American and Japanese patients. The model enabled the statistically significant identification of high-risk stage IA lung adenocarcinoma in both cohorts, with hazard ratios (HRs) for death of 7.16 ($P=0.029$) for North American and of 10.98 ($P=0.008$) for Japanese.

The set of 139 genes includes many ones that have already been reported in the literature to be involved in biological aspects of cancer phenotypes such as ADAM family genes (ADAM10, ADAM19), matrix metalloprotease (MMP) family genes, NMU and Ube2c, but are yet unknown to be involved in EGF signaling. These results strongly re-emphasizes that EGF signaling status underlies aggressive phenotype of cancer cells, and also suggests the first set of genes that are useful for the identification of high-risk stage IA lung adenocarcinoma patients.

Furthermore, we found that expression levels of a single gene of 72/139 genes are significantly associated with the survival and/or relapse-free survival of the adenocarcinoma patients when stage IA, IB and II patients are combined. Among the 72 genes, expression levels of a single gene of 40 genes are significantly associated with poor prognosis. We are now examining the possibility that there are novel molecular targets for cancer therapy among them.

1-2. Novel molecular mechanisms of acquired resistance to gefitinib in Non-small lung cancer

EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib or erlotinib, were remarkably effective in some patients with NSCLC harboring EGFR kinase domain mutation. Although such NSCLC patients were initially sensitive to EGFR-TKIs, they eventually have acquired resistance to EGFR-TKIs. There are several molecular mechanisms of acquired resistance to EGFR-TKI; a secondary mutation in EGFR(T790M) and the amplification of the MET oncogene are commonly found. However, some NSCLC patients still suffer from gefitinib-resistance caused by unknown mechanisms.

To explore novel molecular mechanisms for gefitinib-resistance, we established the gefitinib-resistant PC9M2 cells that were spontaneously derived from PC9 cells expressing gefitinib-sensitive mutant EGFR, by culturing them in the presence of a low amount of gefitinib for several months. In PC9M2 cells, we found neither the mutation of EGFR T790M nor amplification of MET oncogene and thus the novel mechanisms may underlie the acquired resistance to gefitinib in these cells.

We analyzed gene expression profiles of PC9 and PC9M2 cells in a detailed time course with or without stimulation with EGF in the presence or absence of gefitinib by using DNA microarray. Bioinformatics analysis revealed that expression levels of several genes in Wnt signaling pathways were increased in PC9M2 cells compared with those in PC9 cells. Furthermore, we found the phosphorylation of GSK3 and the nuclear accumulation of β-catenin were increased in PC9M2 cells. Our approach would provide a useful strategy to find appropriate molecular targets for cancer patients who are suffering from acquired resistance to cancer drugs.

1-3. Search for new lung cancer biomarkers and molecular targets

Although patients with lung cancer at the early stages may be curable by surgery, they hardly recognize to have the disease, because there is no symptom. It is thus important to detect lung cancer patients at early stages by examining serum biomarkers. However, useful serum biomarkers that are able to detect lung cancer patients at early stages remain to be identified.

In this study, we searched for new serum biomarkers for the early stage lung cancer by analyzing expression profiles of stage I lung adenocarcinoma tissues derived from ~200 patients who underwent surgery in National Cancer Center Hospital. As a result, 24 candidate molecules were obtained. We collected plasma of all these patients and their plasma samples are subject to enzyme-linked immunosorbent assay (ELISA) to measure expression levels in plasma in order to validate them as candidates.

The protein levels of 23 candidates were examined by ELISA using 10 plasma samples from 2 healthy people and 7 stage I lung adenocarcinoma patients at the first screening. We selected 6 candidate molecules for the second screening. The candidate molecules are CXCL13, ADAM8, COL10A1, FGL1, GPR37 and MUC5B. We used 53 plasma samples from 9 healthy people and

44 stage I lung adenocarcinoma patients. After the second screening, fibrinogen-like 1 (FGL1) turned out to be a strong candidate for the early detection biomarker (AUC＝0.684). According to RefExA database of LSBM, FGL1 mRNA is expressed in normal liver, normal pancreas, fetal liver, a few liver cancer cell lines and some lung cancer cell lines.

2. Breast cancer stem cells

2-1. ErbB/NF-κB signaling regulates breast cancer stem cell-like properties

Kunihiko Hinohara, Seiichiro Kobayashi[4], Arinobu Tojo[4], Jun-ichiro Inoue[5], Noriko Gotoh: [4]Division of Molecular Therapy, [5]Division of Cellular and Molecular Research.

Cancer stem cells, a small proportion of heterogeneous tumor cells, can self-renew and simultaneously produce differentiated tumor cells with strong proliferative activity, and therefore are responsible for tumorigenesis. Although it is important to target cancer stem cells for treatment of cancer patients, it has been difficult to find appropriate target molecules because the mechanisms by which cancer stem cells maintain their ability remain obscure. Here, we identified a molecular mechanism that regulates breast cancer stem cell (BCSC)-like properties. We found that heregulin (HRG), a ligand for ErbB3, induced mammosphere formation in breast cancer stem cells (BCSCs) as well as in breast cancer cell lines. HRG-induced mammosphere formation was reduced by treatment with inhibitors for phosphatidyl inositol 3-kinase (PI3K) or NF-κB and by expression of IκBα-Super Repressor (IκBαSR), a dominant-negative inhibitor for NF-κB. Moreover, the overexpression of IκBαSR in breast cancer cells inhibited tumorigenesis in NOD/SCID mice. Furthermore, we found that the expression of IL 8, a regulator of BCSC self-renewal, was induced by HRG through the activation of the PI3 K/NF-κB pathway. These findings illustrate that HRG/ErbB3 signaling appears to maintain BCSC properties through a PI3K/NF-κB pathway in human breast cancer.

2-2. Identification of ErbB/NF-κB target genes in breast cancer cells

Kunihiko Hinohara, Teppei Shimamura[1], Rui Yamaguchi[1], Masao Nagasaki[1], Seiya Imoto[1], Satoru Miyano[1], Noriko Gotoh

We performed time series microarray analyses to investigate transcriptome dynamics during ErbB/NF-κB activation in breast cancer cells upon stimulation with HRG, and identified 69 genes positively regulated by ErbB/NF-κB signaling. Importantly, treatment of MCF7 cells with several molecules encoded by these genes, including IGF2, SDF1α, SDF1β and IL20, increased mammosphere formation in a dose-dependent manner. These observations suggest that the genes upregulated by ErbB/NF-κB signaling were important for maintaining the mammosphere-forming abilities of breast cancer cells.

3. FRS2 family adaptor protein

A potential role of an adaptor protein FRS2β, a feedback inhibitor for ErbB, for maintenance of dormancy of cancer stem-like cells

Annna Mizutani, Daisuke Iejima, Noriko Gotoh

The adaptor protein FRS2 family comprises two members: FRS2α and FRS2β. Although FRS 2α is a well-known central mediator for FGF signaling, the role of FRS2β for its physiological and pathological significance are still largely unknown. We have previously shown that FRS2β constitutively binds to ErbB family members and inhibits ErbB signaling, resulting in inhibition of cell proliferation. One mechanism is that it serves as a feedback inhibitor by binding to the activated ERK after stimulation with ErbB tyrosine kinase. Another mechanism is that it promotes degradation of ErbB2, resulting in reduced protein amounts of ErbB2. We also showed that expression of FRS2β is restricted to several tissues including epithelial cells in breast and lung tissues.

Immunohistochemical analysis revealed that expression of FRS2β was at low levels in non-pregnant mammary gland tissues, while during pregnancy and lactation, it was increased in restricted areas of luminal epithelial cells in the mammary gland tissues. Expression levels of ErbB2 were also increased during pregnancy and lactation. We found that expression levels of ErbB2 and phospho-histone H3 (pH3), a proliferation marker, were reduced in cells in which expression of FRS2β was up-regulated. This is consistent with the fact that FRS2β downregulates protein levels of ErbB2, leading to inhibition of cell proliferation. These results prompted us to investigate the role of FRS2βduring tumorigenesis in breast tissues.

We generated mutant mice in which *Frs2β* was disrupted by gene targeting. The *Frs2β* knockout mice showed no gross abnormality and healthy and fertile. We crossed the *Frs2β*

knockout mice with mouse mammary tumor virus (MMTV)-ErbB2/Neu transgenic mice in which overexpression of ErbB2 driven by MMTV promoter in mammary tissues induces breast cancer. Consistent with the fact that FRS2β serves as a feedback inhibitor for ErbB signaling, we found earlier onset of appearance of mammary tumors in MMTV-ErbB2(+)/*Frs2β*(−/−) mice than in MMTV-ErbB2(+)/*Frs2β*(+/+) mice. However, tumor growth in MMTV-ErbB2(+)/*Frs2β*(−/−) mice was greatly reduced and eventually, all the MMTV-ErbB2(+)/*Frs2β*(+/+) mice died faster than the MMTV-ErbB2(+)/*Frs2β*(−/−) mice.

Expression levels of ErbB2 were unchanged between cells in which expression levels of FRS2β were up-regulated or not in MMTV-ErbB2(+)/*Frs2β*(−/−) mice, probably due to forced expression of ErbB2. In contrast, expression levels of pH3 were still reduced in cells in which expression of FRS2β was up-regulated, in association with reduced nuclear translocation of ERK, as we previously reported. This strongly suggests that ErbB-ERK axis is still inhibited in cells expressing FRS2β.

Since low levels of cell proliferative activity is a characteristic of tumor-initiating cells or stem/progenitor cells, we examined the possibility that FRS2β plays roles for the functions of maintenance of such cells. We found that the EGF-induced mammosphere forming ability of breast cancer cells and normal breast cells were decreased in MMTV-ErbB2(+)/*Frs2β*(−/−) mice compared with those of MMTV-ErbB2(+)/*Frsβ*(+/+) mice, suggesting that tumor-initiating cells and normal progenitor cells in breast tissues are decreased in MMTV-ErbB2(+)/*Frs2β*(−/−) mice.

It thus appears that inhibition of ErbB-ERK axis is important for the maintenance of both tumor-initiating cells and normal progenitor cells, partly by keeping reduced cell growth activity. Taken together, we propose that ErbB signaling controls tumor-initiating cells and mammosphere formation through multiple ways.

Publications

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