Donation 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)

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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 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 technique and knowledge that have been accumulated through works of haematopoietic systems in our laboratory, we attempt to identify mammalian retinal stem cells and following developmental process 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 has been revealed by microarray analyses. Various signaling molecules and transcriptional factors are under investing for their roles in 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 2010 are as follows.

1. Regulation of red opsin (ROP) expression by COUP-TFII and Thyroid hormone receptor beta2 (TRβ2)

Shinya Satoh, Yo Tanaka, Keiko Akagawa, Sumiko Watanabe

We had previously shown a new molecular cascade involving BMP and COUP-TFs that conveys dorsoventral information to direct the expression of cone opsins during retinal development. Then, we attempted to clarify the molecular mechanism how COUP-TFs regulate the opsin gene. Several lines of evidence indicated that the epigenetical regulation may participate on ROP expression. However, we did not get evidence that modification of COUP-TFII expression levels affect DNA methylation status at *ROP* promoter region. In addition, histone modification status of *ROP* promoter region was also not affected by modulation of COUP-TFII expression.

Importance of thyroid hormone for *ROP* ex-

pression had been known. We cloned various DNA fragments from upstream- and exon/intron-region of human *ROP* gene locus and constructed luciferase reporter plasmids. We found that TRβ2 activates *ROP* proximal promoter and the thyroid hormone responsible element is present within the exon-intron region. Moreover, COUP-TFII suppresses TRβ2- and thyroid hormone-mediated activation of these reporters, suggesting that COUP-TFII suppresses *ROP* expression by suppression of TRβ2 activity. We propose that interaction of TRβ2 and COUP-TFII in *ROP* genome may play critical roles for regulation of *ROP* gene expression and to establish ROP spatial-expression pattern in the retina.

2. Epigenetic regulation of retinal genes during retinal development

Shinya Satoh, Sumiko Watanabe

During retinal development, expression level of many genes dramatically change and play critical roles for cell fate decision of retinal progenitor cells. We examined epigenetic mechanisms, especially histone modifications, for regulation of such temporal change of genes. Using ChIP assay with antibodies against histone H3K 4-me3 and H3 acetylation, that correlate with active gene expression, we found that the level of histone modification status at *Rhodopsin* promoter increases after the neonatal stage. Then, we analyzed repressive histone modification status and found some neurogenesis-inducing factors showed inverse correlation between their level of expression and repressive histone modification status. Taken together, role of active histone modification for *Rhodopsin* gene regulation and negative histone modification of neurogenesis-inducing factors were suggested. Our goal is to reveal a network of epigenetic regulation of gene transcription in retinal development.

3. Role of transcription factor Sox11 in retinal development

Ayumi Usui, Shinya Satoh, Sumiko Watanabe

Sry-related HMG box (Sox) proteins play diverse and critical roles in a variety of morphogenetic processes during embryonic development. We focused on Sox11, which is a member of the SoxC subtype, because of its unique expression pattern revealed by our microarray analysis across different subsets of retinal progenitor cells. Immunohistochemistry and in situ hybridization analyses showed Sox11 is expressed in early progenitor cells and early born retinal neurons. To elucidate the function of Sox 11 in mouse retinal development, we conducted gain- and loss-of-function analyses. Over-expression of Sox11 in retinal progenitor cells resulted in aberrant sub-retinal distribution of cells in explant culture. Moreover, Müller glial cells failed to differentiate. The eyes of Sox11 knockout (KO) mice were far smaller than those of control (WT). Neurogenesis of early arising subtypes was severely suppressed at early stages. However, their differentiation overtakes WT at later developmental stages. We found that the SoxC member, Sox4 is also strongly expressed in developing mouse retina, and its expression level in retina increases as that of Sox 11 subsides. Over-expression of Sox4 in retinal progenitor cells resulted in a phenotype similar to that of Sox11, suggesting redundancy in the activities of Sox4 and Sox11, leading us to propose coordinated regulation of the retina by members of the SoxC gene family.

4. β-Catenin signaling regulates the timing of cell differentiation in mouse retinal progenitor cells

Yasuo Ouchi, Yukihiro Baba, Hideto Koso, Makoto M. Taketo[1], Sumiko Watanabe: [1]Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Wnt signaling is important in development and carcinogenesis. We previously showed that active β-catenin or Lef-1 in the mammalian retinal culture prevents differentiation of retinal cells without modifying cellular proliferation. Then, we investigated the in vivo role of β-catenin in mouse retinal differentiation in transgenic mice, in which retinal-specific activation or inactivation of β-catenin was achieved with Cre recombinase. The gain-of-function mice exhibited small eyes and large cell aggregates consisting of early progenitor cells labeled with SSEA-1 in the peripheral retina. In the loss-of-function mice, we observed a reduced number of SSEA-1-positive progenitor cells and the presence of differentiated cells in the β-catenin ablated retinal region. Interestingly, the number of proliferating cells in the β-catenin gain-of-function mice was highly downregulated, and the proliferation index detected by ki67 expression was slightly lower than that of control mice in the β-catenin loss-of-function mice. The Gsk-3 β inhibitor BIO induced expression of Id3, which was highly expressed in SSEA-positive cells, and transiently maintained SSEA-1 positive retinal progenitor cells (RPCs). Forced expression of Id3 in RPCs mimicked the effects of

BIO. Taken together, β-catenin signaling regulates the timing of differentiation in RPCs by inhibiting premature differentiation of them partly through the regulation of Id3 expression.

5. Dicer is essential for the survival and proliferation of retinal progenitor cells

Atsumi Iida, Toru Shinoe, Yukihiro Baba, Hiroyuki Mano[2], Sumiko Watanabe: [2]Division of Functional Genomics, Jichi Medical University, Tochigi, Japan

Much attention has been paid to the roles of microRNA in developmental and biological processes. A previous study showed that microRNA does not play an important role in retinal morphogenesis, but is essential for the maintenance of retinal structure and function in Chx 10-Cre/Dicer-flox mice. In contrast, Dicer plays essential roles in cell survival and proliferation in other organs, suggesting that retinal development is an exception in terms of the roles of Dicer in organ development. We re-examined the role of Dicer in retinal development using Dkk3-Cre/Dicer-flox (Dicer-CKO) mice. The expected numbers of Dicer-CKO mice based on Mendelian genetics were born, but their eyes never opened. Massive death of retinal progenitor cells occurred during embryogenesis, resulting in microphthalmia, and most retinal cells had disappeared by postnatal day 14. In vitro re-aggregation culture of Dicer-CKO retinal cells showed that cell death and the suppression of proliferation by Dicer inactivation occurred in a cell autonomous manner. Cell differentiation markers were expressed in the Dicer-CKO retina; however, these cells localized abnormally and the inner plexiform layer was absent, suggesting that cell migration and morphological differentiation, especially process extension, are perturbed. Taken together, these results show that Dicer is also essential to early retinal development.

6. Sall3 plays essential roles in horizontal cell maturation through regulation of neurofilament expression levels

Yukihiro Baba, Atsumi Iida, Sumiko Watanabe

The region-specific homeotic gene spalt (sal) gene plays a critical role in *Drosophila* development. The mammalian Sal homologous genes contain four members, and Sall3 is mainly expressed in horizontal cells. In the developing retinas of Sall3 knockout (KO) mice until around birth, horizontal precursor cells developed with comparable numbers and position; the horizontal cell marker NF160 was expressed weakly and neurite-like structure had once formed. Since Sall3 KO mice die at postnatal day 1, subsequent retinal development was examined by *in vitro* retinal explant culture. In the Sall3-KO retina culture, the expression of NF160 was abrogated, and neurite extension was not observed. Furthermore, Sall3-KO horizontal precursors were initially localized at the appropriate horizontal positions, but eventually moved to an abnormal site in the outer nuclear layer. Overexpression of Sall3 in retinal progenitors did not induce differentiation of retinal progenitor cells into the horizontal cell fate, but enhanced NF160 expression and neurite extension. In addition, differentiation into Müller glia was promoted, and rod cells were severely suppressed without perturbing proliferation. In conclusion, Sall3 may not be involved in horizontal cell-fate determination, but rather functions to instruct terminal differentiation of horizontal cells and to maintain NF160 expression.

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Donation Laboratories

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.
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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, H1N1 and HPV vaccines, economic burden of health care on patients, and clinical internship system. In each case, we also study the system of management, information circulation, and network.

【Medical Governance】

Haruka Nakata, Koichiro Yuji[1], Masaharu Tsubokura[2], Mariko Takeuchi[3], Tomoko Matsumura, Yuko Kodama, Masahiro Kami: [1]Department of Medicine (Department of Hematology/Oncology), Institute of Medical Science, The University of Tokyo; [2]Tokyo Metropolitan Cancer and Infectious diseases Center Komagome Hospital; [3]5th grade student, Faculty of Medicine, The University of Tokyo.

We perform individual case studies on H1N1 pandemic, H1N1 and HPV vaccines, 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 (Nakada et al.; Nakada et al.; Tsubokura et al.; Tsubokura et al.; Narimatsu et al.). The results on device, vaccine, and drug lag were particularly successful and are widely published in news papers and popular magazines.

【Simulation of Medical Information】

Haruka Nakada, Hiroki Sato[4], Seiya Imoto[5], Rui Yamaguchi[5], Satoru Miyano[5], Koichiro Yuji, Masahiro Kami: [4]Department of Medical Informatics, National Defense Medical College Hospital [5]Laboratory of DNA Information Analysis

Collaborating with information scientists and public health specialists, we structure the simulation model for a H1N1 virus pandemic and publish the results in scientific journals. (Sato et al.).

【Regulatory Science】

Tetsuya Tanimoto[6], Masaharu Tsubokura[2], Naoko Murashige, Tomoko Matsumura, Yuko Kodama, Masahiro Kami: [6]Pharmaceuticals and Medical Devices Agency, JAPAN

The termination of Bone Marrow Filter supply has adversely affected doctors' ability to provide transplants. We research the effects of regulation

on termination and supply of these filters as well as various anti-cancer medicines. The results are then published in scientific journals (Tanimoto et a l; Tanimoto et al.; Narimatsu et al.).

【Information Circulation on the Advanced Medicine】

Yukiko Sakamoto-Kishi, Masahiro Kami, Tomoko Matsumura,

We investigate the role of the media in the health care (Kishi Y, et al.). We collected the information, which is related to cancer treatment, on major newspapers and weekly magazines to investigate an adequate information provision for the people. In our survey, it was suggested that people accessed a variety of information related to cancer treatment routinely.

We discussed the ideal information circulation through patients, healthcare provider and media.

We continue researches on information provision for the people by patient interview.

【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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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.
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特任准教授 医学博士 後藤典子
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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

Mai Yamauchi, Aska Nakata, Kentaro Ogami, Ikuyo Yoshino, Rui Yamaguchi[1], Masao Nagasaki[1], Teppei Shimamura[1], Seiya Imoto[1], Ayumu Saito[1], Kazuko Ueno[1], Yousuke Hatanaka[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. Receptor tyrosine kinase defines critical prognostic genes of lung adenocarcinoma

There is a great need for diagnostic biomarkers to evaluate the disease aggressiveness of cancer patients, particularly at early stages, to select high-risk patients who will benefit from additional treatments, such as adjuvant chemotherapy, to significantly reduce mortality. Extensive efforts have been made to identify gene signatures (gene sets) as potential diagnostic biomarkers for the prognosis of cancer patients by statistical analysis of gene expression profiles derived from surgically-obtained cancer tissues using DNA microarray. Although several gene signatures for breast cancer prognosis, including a 70-gene signature, are now in clinical trials, there have been still few gene signatures that can be translated into the clinics. As for lung cancer, there is little evidence that gene signatures are useful above the clinical prognostic markers alone, including stage, age and gender. In view of the remarkable progress made in the understanding of the molecular mechanisms of biology and signal transduction over the past few decades, it would be important to find key prognostic genes by a rational approach taking into account the biology and signaling. Along

this line, several gene signatures have been proposed for prognosis prediction of breast cancer, however, none of them have outperformed the 70-gene signature.

We attempted to identify the key genes by a novel method, combining information on intracellular signaling in normal cells *in vivo* to avoid the heterogenous cancer cells, with computational simulation *in silico*. We focused on lung adenocarcinoma (ADC), the most prevalent type of lung cancer. Because epidermal growth factor (EGF) stimulates a variety of cellular responses linked to cancer biology through its receptor tyrosine kinase (RTK), key genes in the EGF signaling seem to represent the cancer aggressiveness. However, there is no appropriate way to identify such genes, since expression levels of enormous amounts of genes show dynamic changes over time in cells stimulated with EGF. Here, we used a State Space Model (SSM), a novel mathematical method, to accurately predict the time-dependent EGF-regulated gene expression patterns *in silico* and to identify 139 key genes under the influence of the EGF RTK. The ability of these genes to predict the prognosis of patients with early stage (stage Ia and Ib) lung adenocarcinoma (ADC) was shown by analyzing publically available data sets and our own profile data set of 162 adjuvant therapy-free cases who received complete resection. We thus showed that individual cancer aggressiveness may be rather simply represented as deregulated patterns of EGF signaling. Therefore, we solved long-standing problems to identify the critical prognostic genes for lung ADC, for clinical use, by using a rational method based on the underlying biology.

1-2. N-cadherin inhibition leads to apoptosis in gefitinib-resistant lung cancer cell line

Non-small cell lung cancers (NSCLCs) with activating epidermal growth factor receptor (EGFR) mutations respond to EGFR-tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib. However, the effect of EGFR-TKI in NSCLCs is often limited by the emergence of drug resistance. Studies over the last few years have identified two different EGFR-TKI resistance mechanisms, a secondary mutation in *EGFR*, *EGFR* T790M, and amplification of the *MET* oncogene. *EGFR* T790M and *MET* amplification account for ~60% to 70% of all known causes of acquired resistance to EGFR-TKIs. In order to overcome the acquired resistance, we analyzed gefitinib-resistant PC9/ZD cells harboring the activating EGFR derived from gefitinib-sensitive parental (PC9) cells by using DNA microarray and confirmed N-cadherin expression was significantly up-regulated in PC9/ZD cells. Interestingly, we identified inhibition of N-cadherin increased the number of cells undergoing apoptosis in PC9/ZD cells. N-cadherin inhibition induced Akt inactivation and the pro-apoptotic factor Bad activation. Furthermore, we confirmed inhibition of Akt or PI3K reduced number of cell viability in PC9/ZD cells. These data suggest that PC9/ZD acquired N-cadherin dependent survival system but not EGFR dependent. Since epithelial mesenchymal transition (EMT) has been reported to be related with reduced sensitivity to EGFR-TKIs, we investigated whether N-cadherin as a one of EMT markers could be a therapeutic target or not in lung cancers. Surprisingly, N-cadherin inhibition resulted in reduction of cell growth in lung cancer cells expressing N-cadherin. In conclusion, our data suggest that N-cadherin inhibition have therapeutic potential for the treatment of EGFR-TKI resistant lung cancers.

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

The main cause of our country's death is cancer, especially lung cancer. 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. There is a big problem to detect lung cancer at the early stages. Moreover, even if they undergo surgery, the significant portion of patients eventually suffer from recurrence. After the surgery, if there is a diagnostic method to select cases with bad prognosis, the adjuvant therapy, such as chemotherapy and radiation, can be added. However, since there is no such diagnostic methods, it is impossible to select such patients accurately. In addition, the therapies with high effect have not existed as an adjuvant therapy yet. Therefore, it is important to discover useful biomarkers that are able to predict relapse of the diseases. It is also important to discover new signaling proteins on which survival of cancer cells are dependent and to develop molecularly targeting drugs for such proteins. We have succeeded in finding 139 key genes in the EGF signaling by using systems biology approach and the accuracy of prognosis prediction was validated using several gene expression profiles of patiens from USA and Japan.

In this study, we searched for new biomarkers and molecular targets of the lung cancer by analyzing expression profiles of lung adenocarcinoma (ADC) tissues derived from ~200 patients who underwent surgery in National Cancer Center. As a result, several candidate molecules were obtained. Because we collected plasma of

all these patients, they are subject to enzyme-linked immunosorbent assay (ELISA) to measure expression levels in plasma in order to validate them as candidates of serum biomarkers to detect lung ADCs. We also validate them as new molecular targets by various methods. Analysis of validation is still ongoing.

2. Molecular mechanisms of breast cancer stem cells

NF-κB and breast cancer stem cells

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 (CSCs), which make up only a small proportion of heterogeneous tumor cells, may possess greater ability to maintain tumorigenesis than do other tumor cell types. It is because cancer stem cells can self-renew and simultaneously produce differentiated daughter cells that strongly proliferate until they reach their final differentiated state. In human breast cancers, the $CD24^{-/low}/CD44^{+}$ cell population was reported to be more highly enriched in breast cancer stem cells than was the $CD24^{+}/CD44^{+}$ cell population. Using gene set enrichment analysis (GSEA), a recently developed analytical method of gene expression profiling data, we have previously demonstrated that several signaling pathways including the NF-κB pathway were enriched in $CD24^{-/low}/CD44^{+}$ cell populations. The NF-κB activity in nuclear extracts was significantly higher in $CD24^{-/low}/CD44^{+}$ cell populations than in $CD24^{+}/CD44^{+}$ cell populations.

To investigate the role of NF-κB in self-renewal of CSCs, we performed mammosphere assay under condition of NF-κB inhibitor DHMEQ treatment. We found that DHMEQ decreased both primary and secondary mammosphere-forming efficiencies. Moreover, mammospheres infected with IκBα-super repressor (IκB-SR), an inhibitor for NF-κB activity, showed a decrease in frequency of sphere formation during serial passages. Moreover, both DHMEQ treatment and IκB-SR infection did not alter proliferation or viability of adherent MCF7 cells. These observations raise a possibility that the self-renewal of breast cancer stem cells is regulated by NF-κB.

Since EGF or bFGF are required for sphere culture of cells, we have tested the hypothesis that EGF receptor tyrosine kinase can regulate self-renewal of breast cancer stem cells through NF-κB activation. Heregulin (HRG) is a ligand for ErbB3, another EGF receptor family members. We found that HRG greatly increased mammosphere-forming efficiency and this mammosphere formation was prevented by DHMEQ and by PI3K inhibitor LY294002 treatment in a dose-dependent manner. These findings suggest that HER/PI3K/Akt/NF-κB signaling pathway plays an important role for the self-renewal of breast cancer stem cells.

3. FRS2 family adaptor protein

3-1. An adaptor protein FRS2beta attenuates ErbB signaling through binding to CIN85/CD2AP-Cbl complex, leading to degradation of the multi-molecular complex including the receptors.

Yuriko Minegishi, Seisuke Hattori[5], Noriko Gotoh: [5]Kitasato University.

The adaptor protein FRS2 family comprises two members: FRS2alpha and FRS2beta. Although FRS2alpha is a well-known central mediator for FGF signaling, the role of FRS2beta in signal transduction and its physiological significance are still largely unknown. We have previously shown that FRS2beta constitutively binds to ErbB family members regardless of receptor activation and acts as a feedback inhibitor without being tyrosine-phosphorylated by ErbB tyrosine kinase. We also showed that expression of FRS2beta is restricted to several tissues including neural tissues and that it colocalizes with lysosomes in neural cells.

To further elucidate the functions of FRS2beta in signal transduction, we examined specific binding partners of FRS2beta by co-immunoprecipitation experiments with FLAG-tagged FRS2beta, followed by LC-MS/MS proteomics analysis. Among the newly identified FRS2beta binding proteins, we focused on Cbl-interaction protein of 85kDa (CIN85) and CD2-associated protein (CD2AP). CIN85 and CD2AP are adaptor proteins, belong to the same family, and are known to be involved in endosomal trafficking. FRS2beta bound to CIN85 or CD2AP via consensus Px(P/A)xxR motif. Overexpression of FRS2beta induced the down-regulation of the protein levels of CIN85, CD2 AP and ErbB2. Co-immunoprecipitation study revealed that E3 ubiquitin-protein ligase Cbl was recruited to FRS2beta through binding to CIN85 or CD2AP. Moreover, knocking down of CIN85 and CD2AP or Cbl, or treatment with lysosomal degradation inhibitors diminished the down-regulation effects of FRS2beta on ErbB2. We thus found one of the molecular mechanisms for attenuation of ErbB2-related signaling

by FRS2beta. It appears that FRS2beta-CIN85/CD2AP-Cbl complex leads to degradation of the multi-molecular complex including the FRS2 beta-binding receptor by negative feedback mechanisms. For further mechanistic insights into the FRS2beta-mediated degradation pathway, we visualized FRS2beta and CIN85 as fluorescent fusion proteins and revealed that these 2 molecules are colocalized and some are also colocalized with lysosome marker LAMP2, while the mutant FRS2beta that lacks the binding ability to CIN85 did not exhibited the colocalization with CIN85 and distributed in diffusely. Knock down of endogenous FRS2beta in primary cultured neural cells, CIN85 and ErbB2 protein levels were increased. In a physiological condition, this knock down of endogenous FRS2 beta increased the colocalization of Tbr2 (progenitor marker) and NeuroD (premature neuron marker). These data suggest that FRS2beta supported the neuronal differentiation by down-regulating ErbB2 expression in neural stem/progenitor cells by inhibiting their proliferative activity. ErbB2 is expressed in embryonic neural tissues for supporting proliferative activity, while some reports gave us some other insights that ErbB2 also related with differentiation and electrophysiology. Whether and how FRS2beta could function in neurophysiology or behavioral research is our current interests for the new phase of FR2beta' in-vivo function analysis.

3-2. A Potential Role of FRS2beta as a Tumor Suppressor for Mammary Tumorigenesis

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We have previously shown that FRS2beta binds to ErbB family members and acts as a feedback inhibitor without being tyrosine-phosphorylated by ErbB tyrosine kinase. We generated mutant mice in which *Frs2beta* was disrupted by gene targeting and analyzed the role of FRS2beta in breast cancer, since it is well-known that overexpression of ErbB2 strongly contributes to malignancy of human breast cancer. We crossed the *FRS2beta* 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. We found earlier onset of appearance of mammary tumors in MMTV(+)/FRS2beta(−/−) mice than in MMTV(+)/FRS2beta(+/+) mice.

Immunohistochemical analysis revealed that expression of FRS2beta was at low levels in non-pregnant mammary gland tissues in wild type mice, while during pregnancy and lactation, it was increased in restricted areas of epithelial cells. Expression levels of ErbB2 were also increased during pregnancy and lactation, however, ErbB2 and Akt expression remained at low levels in cells in which expression of FRS2beta was up-regulated. More, FRS2beta deficiency in MMTV-neu mice further increased cell growth activity and increased nuclear accumulation of ERK. This appears to induce further deregulation of ErbB2 signaling, resulting in the ealier onset of tumorigenesis in mammary tissues than those in MMTV-neu/FRS2beta wild type mice.

These results suggest that FRS2beta negatively regulates ErbB2 signaling by down-regulation of the protein levels in vivo. FRS2beta may contribute to prevention of tumorigenesis induced by overexpressed and hyperactive ErbB2 in the mammary tissues.

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