Donation Laboratories

Division of Cellular Proteomics (BML)
細胞ゲノム動態解析(BML)寄付研究部門

Visiting Professor	Seisuke Hattori, Ph.D.		特任教授(委嘱)	理学博士	服部成介
Visiting Research Associate	Yutaka Harita, MD, Ph.D.		特任助教	医学博士	張田豊

We analyze intracellular signaling pathways using proteomic approaches. Combining enrichment of phosphoproteins with fluorescence difference two-dimensional gel electrophoresis (2-D DIGE), we identified many novel kinase substrates. We also analyze intracellular signaling pathways of kidney podocytes. Nephrin and Neph1 that have been identified as products of nephrosis-causative genes are expressed in podocytes are tyrosine phosphorylated by Fyn and recruits a variety of signaling molecules including Grb2, phospholipase C-γ1, PI3-kinase.

Hidetaka Kosako[1,2], Nozomi Yamaguchi[1,3], Chizuru Aranami[4], Masato Ushiyama[1,5], Shingo Kose[6], Naoko Imamoto[6], Hisaaki Taniguchi[2], Eisuke Nishida[7] & Seisuke Hattori[1,4]: Phosphoproteomic profiling of ERK MAP kinase signaling reveals a role of phosphorylation in the interaction of nucleoporins with transport factors.

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Many ERK MAP kinase substrates have been identified, but the diversity of ERK-mediated processes suggests the existence of additional targets. Here we present a phosphoproteomic approach to identify putative ERK substrates by combining the steroid receptor fusion system, immobilized metal affinity chromatography (IMAC), fluorescent two-dimensional difference gel electrophoresis (2D-DIGE), and phospho-motif-specific antibodies. Purification of phosphoproteins from whole cell lysates by IMAC enabled sensitive detection of minor phosphorylated signaling components that would otherwise be obscured by abundant cellular proteins. Changes in phosphoprotein profiles between selective activation and inhibition of the Raf-MEK-ERK pathway were globally analyzed by 2D-DIGE. Quantitative analysis detected 37 reproducibly changed protein spots, several of which were recognized by the ERK consensus motif-specific antibodies. Mass spectrometric analysis identified 38 proteins as ERK pathway-associated proteins. These included MEK1/2 and ERK1/2 as well as previously known ERK substrates such as RSK2, cPLA2, hnRNP K, caldesmon, cortactin, and vinexin, demonstrating the feasibility of our approach. The remaining 24 proteins were considered candidates for novel ERK targets, which suggest as yet undefined roles for this signaling pathway in cytoskeletal regulation, mRNA processing, vesicle transport, proteolysis, and protein folding. We purified 14 proteins fused to GST, 13 of which were phosphorylated by ERK in vitro. Among them, cytoplasmic dynein intermediate chain 2 and the nucleoporin Nup50/Npap60 were shown to be phosphorylated by ERK in intact cells. ERK phosphorylation of the FG repeat region of Nup50 was found to reduce its affinity

for importin-β. Moreover, the rate of nuclear transport of GFP-fused importin-β and transportin was reduced upon ERK activation. This approach is applicable to other protein kinases and may be useful for large-scale identification of cellular substrates.

Yutaka Harita[1,2], Hidetake Kurihara[3], Hidetaka Kosako[1], Tohru Tezuka[4], Takashi Sekine[2], Takashi Igarashi[2], Ikuroh Ohsawa[5], Shigeo Ohta[5], and Seisuke Hattori1[6,7]: Phosphorylation of Nephrin Triggers Ca^{2+} Signaling by Recruitment and Activation of Phospholipase C-γ1.
[1]Division of Cellular Proteomics (BML), Institute of Medical Science, [2]Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, [3]Department of Anatomy, Juntendo University School of Medicine, [4]Department of Oncology, Institute of Medical Science, [5]Graduate School of Medicine, Nippon Medical School, [6]School of Pharmaceutical Sciences, Kitasato University.

A specialized intercellular junction between podocytes, known as the slit diaphragm (SD), forms the essential structural framework for glomerular filtration in the kidney. In addition, mounting evidence demonstrates that SD also plays a crucial role as a signaling platform in physiological and pathological states. Nephrin, the major component of SD, is tyrosine phosphorylated by a Src-family tyrosine kinase, Fyn, in developing or injured podocytes, recruiting Nck to Nephrin via its SH2 domain to regulate dynamic actin remodeling. Dysregulated Ca^{2+} homeostasis has also been implicated in podocyte damage, but the mechanism of how podocytes respond to injury is largely unknown. Here we have identified phospholipase C-γ1 (PLC-γ1) as a novel phospho-Nephrin binding protein. When HEK293T cells expressing a chimeric protein consisting of CD8 and Nephrin cytoplasmic domain (CD) were treated with anti-CD8 and anti-mouse antibodies, clustering of Nephrin and phosphorylation of Nephrin-CD were induced. Upon this clustering, PLC-γ1 was bound to phosphorylated Nephrin Y1204, which induced translocation of PLC-γ1 from cytoplasm to CD8/Nephrin cluster on the plasma membrane. The recruitement of PLC-γ1 to Nephrin activated PLC-γ1, as detected by phosphorylation of PLC-γ1 Y783 and increase in inositol 1,4,5-trisphosphate (IP3) level. We also found that Nephrin Y1204 phosphorylation triggers the Ca^{2+} response in a PLC-γ1- dependent fashion. Furthermore, PLC-γ1 is significantly activated in injured podocytes *in vivo*. Given the profound effect of PLC-γ1 in diverse cellular functions, regulation of the Ca^{2+} signaling by Nephrin may be important in modulating the glomerular filtration barrier function.

Publications

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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)

Visiting Professor	Sumiko Watanabe, Ph.D.	特任教授　医学博士	渡　辺　すみ子
Project Assistant Professor	Shinya Satoh, Ph.D.	特任助教　工学博士	佐　藤　伸　哉

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. Various signaling molecules and transcriptional factors are under investing 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 2009 are as follows.

1. Role of COUP-TFI and -TFII nuclear receptors in retinal differentiation, and mechanisms regulating retinal specific genes by them

Mariko Inoue, Atsumi Iida, Shinya Satoh, Tatsuhiko Kodama[1], and Sumiko Watanabe: [1]RCAST, Univ. of Tokyo

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are highly conserved members of the steroid/thyroid hormone receptor superfamily throughout evolution. We have shown that two homologous COUP-TF genes, COUP-TFI and COUP-TFII, are expressed in mouse developing retina with unique gradient along with dorsal-ventral axis. We aimed to clarify detailed expression patterns of COUP-TFs in mature retina. Furthermore, their function in retinal progenitor cell differentiation into subtypes of mature retinal cells was examined. COUP-TFI and II were expressed in amacrine cells, especially in a glycinergic subtype in mature mouse retina. Forced expression of COUP-TFI and II promoted amacrine and cone photoreceptor cells differentiation, whereas rod photoreceptors were decreased. Analysis using Y79 retinoblastoma cell line showed that COUP-TFI

and II suppressed transcriptional activation of Nrl gene, which plays essential role for differentiation of rod photoreceptors. Cell proliferation and apoptosis were not affected by perturbation of COUP-TF expression levels. COUP-TFγ expressed mainly in horizontal cells and has weak activity for induction of amacrine cells. Taken together, role of COUP-TF family members in amacrine cell differentiation was revealed.

Mouse cone cells express two types of color photopigment, which are sensitive for short wavelength (S-opsin) and middle wavelength (M-opsin) of lights. Their expression has unique dorsoventral patterning in the retina, and we previously reported that COUP-TFI and COUP-TFII regulate spatial patterning of mouse cone opsin expression; however, its molecular mechanisms have not been clarified. Y79 and WERI-Rb 1 are retinoblastoma cell lines and thought to be derived from immature human cone cells. When endogenous COUP-TFII, but not COUP-TFI, was knocked down in Y79 cells by RNAi, M-opsin expression was up-regulated. It was reported that CpG sites in the M-opsin promoter are hypomethylated in WERI-Rb1 cells, which express high level of M-opsin, and hypermethylated in Y79 cells, which express low level of M-opsin. From these data, we hypothesized that knockdown of COUP-TFII in Y79 cells induced CpG-hypomethylation on the M-opsin promoter, resulting in increase of M-opsin expression. Analysis of effects of knock down of COUP-TFI or COUP-TFII on the CpG methylation pattern of M-opsin promoter in Y79 cells by using bisulfite sequence method showed CpG-hypomethylation on M-opsin promoter when endogenous COUP-TFII was knocked down, suggesting that COUP-TFII regulates M-opsin expression through the regulation of CpG methylation. Furthermore, IP-mass spectrometry analysis showed several interacting molecules with COUP-TFs in mouse retina. These results suggested that COUP-TFs regulate gene expressions through epigenetical mechanisms in addition to direct transcriptional regulation.

2. Identification of CD44 as a cell surface marker for a retinal progenitor cell subset committed to Müller glia fate reveals a signaling mechanism of glial development in the retina

Toru Shinoe, Hiroshi Kuribayashi, Keiko Akagawa, Kyohei Oota[2], Hideyuki Saya[3], Motoharu Seiki[4], Hiroyuki Aburatani[5], and Sumiko Watanabe: [2]Toyo Seikan Kaisha LTD, [3]Graduate School of Medicine, Keio University, [4]Divions of Cancer Cell Research, IMSUT, Univ. of Tokyo, [5]RCAST, Univ. of Tokyo

In the retina, both neurons and glia differentiate from a common progenitor population. CD 44 cell surface antigen is a hyaluronic acid receptor expressed on mature Müller glia in the retina. We found that in the developing mouse retina, expression of CD44 was transiently observed at around birth in a subpopulation of c-kit-positive retinal progenitor cells. Purified CD 44/c-kit-positive retinal progenitor cells exclusively differentiated into Müller glia, not neurons in *in vitro* culture. Although gain-or loss-of-function analyses of CD44 did not alter fate determination in the retinal progenitor cells, overexpression of CD44 inhibited the extension of processes by Müller glia and neurons, and involvement of hyaluronic acid in this process was suggested. Notch signaling is known to be involved in the specification of retinal progenitor into a glial fate. Activation of Notch signaling increased the number of CD44-positive cells in early stage of retina, and treatment with the Notch signal inhibitor, DAPT, at early, but not later, stages of retinal development abolished CD44-positive cells and Müller glia, suggesting that Notch regulates the transition of c-kit single -to CD44/c-kit double-positive cells. BMP receptor is strongly expressed in CD44 positive-cells, and the BMP signal is pivotal for the maturation of Müller glia but not for fate specification of retinal progenitor cells to the glial lineage. Together, these results suggest the identification of CD44 as an early cell surface marker of the Müller glia lineage, plus the sequential requirement of Notch and BMP signals during Müller glia development from retinal progenitor cells.

3. Group E Sox genes, Sox8 and Sox9, are regulated by Notch signaling and required for the Müller glia development in mouse retina

Akihiko Muto, and Sumiko Watanabe

Müller glia plays pivotal roles in vertebrate adult retina, whereas the mechanism regulating the development of Müller glia is poorly understood. Previous studies have revealed that Notch signaling is involved in the multiple steps of retinal development in a stage-specific manner and required for the Müller glia development in postnatal stage. Hes5 was reported to contribute this process, however, presence of Müller glia genesis in Hes5 deficient mice suggested involvement of other molecules in this process. We found that 2 members of group E Sox genes, Sox8 and Sox9, were expressed in proliferating progenitors in embryonic retina and then exclusively in Müller glia at later stages. The expression of shRNA specific to each

significantly decreased the population of Müller glia hence increased rod photoreceptors, but not other retinal neurons, suggesting that these Sox proteins play roles in the specification of Müller glia. We also found that Notch signaling regulated the expression of these Sox genes transcriptionally by using an activated form of Notch and a γ-secretase inhibitor DAPT. This is the first evidence for the role of group E Sox genes in developing vertebrate retina.

4. The forkhead transcription factor foxe1 regulates chondrogenesis in zebrafish

Chisako Nakada, Atsumi Iida, and Sumiko Watanabe

Forkhead transcription factor (Fox) e1 is causative gene for Bamforth-Lazarus syndrome, which is characterized by hypothyroidism and cleft palate. Applying degenerate PCR using primers specific for the conserved forkhead domain, we identified zebrafish *foxe1* (foxe1). Foxe 1 is expressed in the thyroid, pharynx, and pharyngeal skeleton during development; strongly expressed in the gill and weakly expressed in the brain, eye, and heart in adult zebrafish. A loss of the function of foxe1 by morpholino antisense oligo (MO) exhibited abnormal craniofacial development, shortening of Meckel's cartilage and the ceratohyals, and suppressed chondrycytic proliferation. However, at 27 hpf, the *foxe1* MO-injected embryos showed normal *dlx2*, *hoxa2*, and *hoxb2* expression, suggesting that the initial steps of pharyngeal skeletal development, including neural crest migration and specification of the pharyngeal arch occurred normally. In contrast, at 2 dpf, a severe reduction in the expression of *sox9a*, *colIIaI*, and *runx2b*, which play roles in condrocytic proliferation and differentiation, was observed. Interestingly, *fgfr2* was strongly up-regulated in the branchial arches of the *foxe1* MO-injected embryos. Unlike Foxe1-null mice, normal thyroid development in terms of morphology and thyroid-specific marker expression was observed in foxe1 MO-injected zebrafish embryos. Taken together, our results indicate that foxe1 plays an important role in chondrogenesis during development of the pharyngeal skeleton in zebrafish, probably through regulation of *fgfr2* expression. Furthermore, the roles reported for Foxe1 in mammalian thyroid development may have been acquired during evolution.

5. Analysis of syndecan-1 gene promoters during mouse tooth development

Hitomi Ishiguro[6], Yasuo Ouchi, and Sumiko Watanabe: [6]Nippon Dental University School of Life Dentistry

Syndecan-1 is a cell surface heparan sulfate proteoglycan, and plays an important function of cell proliferation in dental papilla during tooth development. We aimed to clarify the transcription mechanisms to regulate the syndecan-1 gene expression in dental papilla and analyzed genomic conservation and putative transcriptional factor binding sites of syndecan-1 gene loci of various animals using the bioinfomatics tool VISTA. To identify the region responsible for syndecan-1 gene expression in mouse dental papilla cells (MDPCs) *in vitro*, the 1.5-kb upstream region of mouse syndecan-1 coding region was inserted upstream of the enhanced green fluorescent protein (EGFP) or luciferase gene, and promoter activity was examined by transient reporter gene expression assay in cultured MDPCs. VISTA analysis showed that the 1.5-kb upstream region was highly conserved between species, and a TATA-box-like- and three GC-rich-motif in this region were identified. Reporter gene assay showed that the 1.5-kb upstream region of mouse syndecan-1 induced reporter gene expression in MDPCs. Deletion of the promoter from 5′ end to 339-bp upstream reduced luciferase activity nearly half of that of 1.5-kb one. Further deletion up to 68-bp resulted in further loss of luciferase activity. The 1.5-kb upstream region of the syndecan-1 gene was sufficient to induce its expression in dental papilla, and multiple putative transcription factor binding sites may co-operate play roles as syndecan-1 enhancer.

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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 Associate Professor	Masahiro Kami, M.D., Ph.D.		特任准教授	医学博士	上　昌広
Project Assistant Professor	Yuji Tanaka, M.D., Ph.D.		特任助教	医学博士	田中祐次
Project Assistant Professor	Tomoko Matsumura, M.D., Ph.D.		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.

• Developing National Consensus on Health Care

Masahiro Kami, Tomoko Matsumura

• National consensus on cancer treatment

Masahiro Kami

We investigated the national consensus on cancer treatment analyzing a concrete case. Information about how the adverse effects of bortezomib were overcome in a short period will prove to be useful in assessing adverse effects of other new drugs. This study showed the important role of the information circulation using email and internet.

• Deficit of the Bone Marrow Filters

Masahiro Kami

We analyzed the impact of the worldwide economic crisis on the medical practice.

Because of the restricting of business, a termination of the supply of the Bone Marrow Collection Kit was identified in Japan. It was predicted that bone marrow transplantation would not be available. We reviewed the information disclosure by relevant parties and media news (Narimatsu et al. 2009, *Biol Blood Marrow Transplant*). This case showed the unfavorable impact on medical practice if the relevant parties did not proactively disclose information to society.

• Information Circulation on the Advanced Medicine

Masahiro Kami, Tomoko Matsumura

We investigate the role of the media in the health care.

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

Masahiro Kami, Yuji Tanaka, Tomoko Matsumura

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 (Yuko Kodama, Project Researcher).

• Patient Recruitment

Masahiro Kami

Since medical practice is rooted in a regional community, investigating patient migration is one of the key factors to promote state-of-the-art practice and clinical researches.

We surveyed hematology practice in Tokushima Prefecture as a model region to investigate optimal coordination among regional medical associations, public administration and regional media, and patient migration (Takita et al. 2009, *Rural Remote Health*). We provided the people with information on regional medical association and its historical background (*Japan Mail Media*).

• Medicolegal Issues

Masahiro Kami, Tomoko Matsumura

• Medical Accident

We verified the impact of the case at Fukushima Prefectural Ohno Hospital on health care. After the case, the number of newspaper articles related to "Health care collapse (*Iryou Houkai* in Japanese)" or "Discontinuation of delivery" dramatically increased. We survey issues on current medical accidents aiming to develop a system for prevention of medical accidents, alternative dispute resolution, and investigation into the cause (Nagamatsu et al. 2009, *Curr Opin Anaesthesiol*).

• Supreme Court Decision about the Join Provision of Medical Treatment (*"Kongou shinryou"* in Japanese)

We investigate the impact of the Decision on health care. A discussion of the advanced medical system and the join provision of medical treatment system is essential to promote clinical researches on cancer peptide vaccine.

• Patient Literacy

Yuji Tanaka

• Patient Associations

We conducted researches on patient feelings with patient associations. We collaborate with the Liaison Council of Inhospital Patient Association Representative which coordinates 20 inhospital patient associations all over Japan and the Ohsawa and Nishihara Laboratory at Faculty of Engineering, the University of Tokyo.

• Pancreatic Islet Transplantation

In our collaborative research with Baylor University in Texas, USA, we studied for improvement in quality of life of patients with type I diabetes before and after pancreatic islet transplantation. We aim to develop an informed consent format for pancreatic islet transplantation which improves patient understanding and better convince them, doing patient surveys to evaluate the current version.

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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.
Project Assistant Professor Mai yamauchi, Ph.D.

特任准教授 医学博士 後藤典子
特任助教 医学博士 山内麻衣

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

Critical prognostic genes for stage I lung adenocarcinoma are identified from normal growth factor-signaling system by overcoming cancer heterogeneity

Mai Yamauchi, Rui Yamaguchi[1], Masao Nagasaki[1], Teppei Shimamura[1], Seiya Imoto[1], Ayumu Saito[1], Kazuko Ueno[1], Yusuke Hatanaka[1], Ryo Yoshida[2], Tomoyuki Higuchi[2], Satoru Miyano[1], and Noriko Gotoh: [1]Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, [2]Institute of Statistical Mathematics, Tokyo, Japan

There is a great need for reliable markers that can assess the aggressiveness of lung cancer, particularly of stage I tumors, to select high-risk patients who will benefit from additional treatments, such as adjuvant chemotherapy. Potential biomarkers have been proposed by analyzing gene-expression profiles of surgically-obtained lung cancer tissues using microarray technologies. However, the performance and general applicability of these signatures for stage I tumors is difficult to establish, because a specific signature might work well in one dataset but not well in another. It appears the controversy has arisen because the reported signatures derived from analysis of cancer tissues have not been properly validated. In fact, there have been no established signatures that accurately predict the survival of stage I lung cancer to date.

Since it has been difficult to identify common key genes reflecting early stage aggressiveness by analyzing cancer tissues because of tremendous heterogeneity, we focused normal lung epithelial cells. Epidermal growth factor (EGF), a major growth factor for lung epithelial cells, stimulates a variety of cellular responses by

regulating expression of many genes known to be involved in cancer aggressiveness. There remains no way to predict gene expression patterns that show dynamic changes over time upon stimulation with growth factors. This hampers the ability to identify key genes regulated by EGF signaling. Here, we applied a State Space Model (SSM), a mathematical model developed for analyzing time-series data, to clarify the EGF-signaling gene regulatory system in normal lung epithelial cells. We accurately simulated the EGF-signaling gene regulatory system *in silico*. Moreover, we identified key genes in the EGF signaling and demonstrated that they accurately predict the survival of patients with stage I lung adenocarcinoma (ADC) with adequate validation using completely independent expression profiling data sets of lung cancer. Most molecules encoded by the key genes are known to play important roles in cancer aggressiveness, providing a theoretical basis for prognosis prediction. We thus solved long-standing problems to identify the good prognostic genes for stage I lung cancer, for clinical use, by sidestepping overwhelming complexities of alterations in cancer tissues *in vivo*.

2. Molecular mechanisms of breast cancer stem cells

A key role of NFκB for self-renewal of breast cancer stem cells

Hinohara Kunihiko, and Noriko Gotoh

Accumulating evidence suggests that Tumor-initiating cells (TICs) or cancer stem cells-which make up only a small proportion of heterogeneous tumor cells-possess a greater ability to maintain tumor formation than other tumor cell types. However, the signaling pathways activated in TICs that contribute to tumorigenesis are not fully understood. In breast cancer cell lines, we found the $CD24^{-/low}/CD44^{+}$ cell populations generated significantly larger tumors than the $CD24^{+}/CD44^{+}$ cell populations in a xenograft model, suggesting that the $CD24^{-/low}/CD44^{+}$ cell populations have the properties of TICs at some extent. To identify expressed genes that were highly enriched in $CD24^{-/low}/CD44^{+}$ cell populations, we next performed DNA microarray analysis using a powerful analytical method called Gene Set Enrichment Analysis (GSEA) for interpreting gene expression data. GSEA revealed that the $CD24^{-/low}/CD44^{+}$ cell populations are enriched for genes involved in a tumor necrosis factor (TNF) or interferon (IFN) response pathway. TNF or IFN response pathway is involved in the expression of many inflammatory cytokines/chemokines, including IL 8 and CCL5. We measured expression levels of IL8 and CCL5 by quantitative RT-PCR (qRT-PCR) and confirmed that they were expressed at significantly higher levels in $CD24^{-/low}/CD44^{+}$ cell populations compared with the $CD24^{+}/CD44^{+}$ cell populations. Moreover, we found the relatively high nuclear factor kappa B (NF-κB) activity in the $CD24^{-/low}/CD44^{+}$ cell populations. In addition, NF-κB was highly activated in mammospheres compared to adherent parental cells. NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) prevented sphere-forming efficiency in vitro and tumorigenesis of $CD24^{-/low}/CD44^{+}$ cell populations in vivo. Our findings suggest that NF-κB play a crucial role for orchestration of transcription factors regulating breast cancer stem cell biology.

3. FRS2 family adaptor protein

3-1. FRS2β

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Fibroblast growth factor receptor substrate 2β (FRS2β) is a member of the FRS2 family of docking/scaffolding adaptor proteins. FRS2β contains N-terminal myristylation sequences for localization to the membrane. FRS2β interacts with a few receptor tyrosine kinases (RTKs) as a docking protein. FRS2β interacts with nonphosphorylated and phosphorylated fibroblast growth factor (FGF) receptors while interaction of FRS2β with neurotrophin receptors (Trks) is tyrosine-phosphorylation dependent. It is also known that anaplastic lymphoma kinase (ALK) in associates with FRS2β both tyrosine-phosphorylated and nonphosphorylated conditions. When the RTKs are activated, they phosphorylate the tyrosine residues of FRS2β, creating binding sites for the adaptor protein Grb2 and Shp2, an SH2 domain-containing tyrosine phosphatase. The FRS2β-Shp2 complex activates the tyrosine phosphorylation of Shp2, resulting in strong activation of ERK in response to FGF. Grb2-SOS complex recruitment via FRS2β is similar to that via FRS2α; FRS2β is believed to relay the signal to the Ras/ERK pathway at moderate levels.

3-2. FRS2β, a potential prognostic gene for non-small cell lung cancer, encodes a feedback inhibitor of EGF receptor family members via ERK binding

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An adaptor protein FRS2β inhibits epidermal growth factor receptor (EGFR) tyrosine kinase without being phosphorylated at tyrosine residues following epidermal growth factor (EGF) stimulation. Although binding to ERK appears to be important for this inhibition, the precise molecular mechanisms and the role of FRS2β in signal transduction mediated by other EGFR family members, as well as its role in human cancer, remain unclear. In the present study, we demonstrate that FRS2β inhibits anchorage-independent cell growth induced by oncogenic ErbB2, another member of EGFR family, and that it inhibits heterodimer formation between EGFR and ErbB2. We mapped the residues important for the FRS2β and ERK interaction to two docking (D) domain-like sequences on FRS2 β and two aspartic acid residues in the common docking (CD) domain of ERK. Moreover, in response to EGF, ERK translocated to the plasma membrane in cells expressing FRS2β. but not an FRS2β mutant in which four arginine residues in the D domains were replaced with alanines, suggesting that FRS2β serves as a plasma membrane anchor for activated ERK. Finally, a low mRNA expression level of FRS2β was significantly correlated with poor prognosis in a cohort of 60 non-small cell lung cancer patients. Therefore, we have identified the molecular mechanisms by which FRS2β acts as a feedback inhibitor of EGFR family members and suggest a role for FRS2β as a tumor suppressor.

4-1. FRS2α

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Fibroblast growth factor receptor substrate 2α (FRS2α) is a member of the FRS2 family of docking/scaffolding adaptor proteins. FRS2α contains N-terminal myristylation sequences and is constitutively anchored to the plasma membrane. It acts as a docking protein downstream of a few receptor tyrosine kinases (RTKs), including fibroblast growth factor (FGF) receptors, neurotrophin receptors, EphA4, RET, and ALK. FRS2α functions as a central mediator for intracellular signalling in response to FGF. When FGFRs are activated, the tyrosine residues of FRS2α are phosphorylated, and this creates binding sites for Grb2 and Shp2. The FRS2α-Shp2 complex induces the tyrosine phosphorylation of Shp2, followed by sustained activation of ERK. Some proteins, namely, SOS, Cbl, and Gab1, are constitutively bound via 2 SH3 domains of Grb2. The ternary complex FRS2α-Grb2-SOS induces modest activation of the Ras/ERK pathway, FRS2α-Grb2-Cbl causes the ubiquitination and degradation of FRS2α and its receptors, and FRS2α-Grb2-Gab1 induces the recruitment of PI-3 kinase and the activation of Akt. Thus, FRS2α is required for various biological activities stimulated by FGF, including the proliferation and migration of cells, outgrowth of neurites, and development of various organs and tissues. FRS2α mutant mice exhibit various developmental defects, including those in anterior-posterior axis formation and the development of the extraembryonic ectoderm, cerebral cortex, eye, carotid body, cardiac outflow tract, prostate, limbs, and skeleton. Many of these defects arise because of defects in FGF signalling during embryogenesis. FRS2α is also important for the maintenance or/and proliferation of several tissue type-specific stem cells, including trophoblast stem cells and neural stem/progenitor cells.

4-2. FRS2α is required for the separation, migration, and survival of pharyngeal-endoderm derived organs including thyroid, ultimobranchial body, parathyroid and thymus.

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In *FRS2α*$^{2F/2F}$ mutant mice at embryonic day (E) 18.5, in which the Shp2-binding sites of FRS2α were disrupted, the thyroid glands were aplastic or hypoplastic. C cells were absent or present in low numbers and rarely formed a compact mass of cells. Parathyroid glands were mostly connected to thymus tissues. At E10.5, the formations of pharyngeal pouches and thyroid primordium were normally initiated in the mutant mice. At E11.5 to E12.5, the thyroid primordium of wild-type embryos was located close to the aortic sac, and the epithelial buds of pharyngeal-derived organs, including the parathyroid gland, thymus and ultimobranchial body, were separated from the epithelium and began to migrate to their final destinations. In the *FRS2α*$^{2F/2F}$ mutants, however, the thyroid primordium became hypoplastic and the pharyngeal-derived organ primordia remained affiliated with the pharyngeal epithelium. At these stages, organ-specific differentiation markers (i.e., NKx2-1/TTF1 for the thyroid lobe and ultimobranchial body; Pax8 for the thyroid lobe; parathormone (PTH), chromogranin A, P75^{NTR}, and S100 protein for the parathyroid gland; and p63 for the thymus) were normally expressed in the mutant tissues. Thus, the separation, migration, and survival of the pharyngeal organs were impaired in the *FRS2α*$^{2F/2F}$ mutants.

Publications

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