Department of Cancer Biology

Division of Cellular and Molecular Biology
分子発癌分野

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Gene expression is largely regulated by signal transduction triggered by various stimulations. Several lines of evidence indicate that genetic defects of molecules involved in the signal transduction or the gene expression lead to abnormal cell differentiation or tumor formation. Our goal is to understand the molecular mechanisms of disease pathogenesis and oncogenesis by elucidating normal regulation of intracellular signal transduction and gene expression involved in cell proliferation and differentiation. We have identified and been interested in Tumor necrosis factor receptor-associated factor 6 (TRAF6), which acts as an E3 ubiquitin ligase to generate Lys63-linked polyubiquitin chains that are crucial for transducing signals emanating from the TNFR superfamily or the TLR/IL-1R family leading to activation of transcription factor NF-κB and AP-1. By generating TRAF6-deficient mice, we found that TRAF6 is essential for osteoclastogenesis, immune self-tolerance, lymph node organogenesis and formation of skin appendices. We are currently focusing on molecular mechanisms underlying TRAF6-mediated activation of signal transduction pathways and how TRAF6 is involved in osteoclastogenesis and self-tolerance. In addition, NF-κB is constitutively activated in various cancer cells and this activation is likely involved in the malignancy of tumors. Thus, we are also investigating the molecular mechanisms of the constitutive activation of NF-κB and how this activation leads to the malignancy of breast cancers and adult T cell leukemia (ATL). In addition, we are investigating novel molecular mechanisms how tumor microenvironments and inflammation are regulated.

1. Molecular mechanism of the regulation of NF-κB transcription factor

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Transcription factor NF-κB binds specifically to a decameric motif of nucleotide, κB site, and activates transcription. The activation of NF-κB has been demonstrated to be carried out post-translationally upon extracellular stimuli through membrane receptors such as members of the TLR/IL-1R family and of TNFR superfamily. In canonical NF-κB pathway, NF-κB forms a complex with regulatory protein, IκB, and is sequestered in the cytoplasm prior to stimulation. Upon stimulation, IκB is rapidly phosphorylated on two specific serine residues by IκB kinase (IKK) complex followed by lysine 48 (K48)-linked ubiquitination and proteasome-dependent degradation of IκB. NF-κB subsequently translocates to the nucleus to activate transcription of target genes. This project is to identify molecules

that regulate signal from membrane receptors to NF-κB/IκB complex. We have previously identified upstream activators of NF-κB, tumor necrosis factor receptor-associated factor (TRAF) 6. TRAF6 contains RING domain in the N-terminus and acts as an E3 ubiquitin-ligase to catalyze the lysine 63 (K63)-linked polyubiquitination of several signaling molecules and TRAF6 itself. To understand the molecular mechanisms of TRAF6-mediated NF-κB activation, we try to identify proteins that are ubiquitinated by TRAF6 upon stimulation. We took advantage of using the peptide that specifically binds K63-linked polyubiquitin chain to purify such proteins. We have confirmed that the peptide-based affinity column is useful for specific concentration of recombinant K63-linked polyubiquitin chain, suggesting that it also works for purification of the proteins of our interest. We are also interested in noncanonical NF-κB pathway, which is crucial for immunity by establishing lymphoid organogenesis and B-cell and dendritic cell (DC) maturation. RelB is a major NF-κB subunit in the pathway. To elucidate the mechanism of the RelB-mediated immune cell maturation, a precise understanding of the relationship between cell maturation and RelB expression and activation at the single-cell level is required. Therefore, we generated knock-in mice expressing a fusion protein between RelB and fluorescent protein (RelB-Venus) from the *Relb* locus. The $Relb^{Venus/Venus}$ mice developed without any abnormalities observed in the $Relb^{-/-}$ mice, allowing us to monitor RelB-Venus expression and nuclear localization as RelB expression and activation. $Relb^{Venus/Venus}$ DC analyses revealed that DCs consist of $RelB^{-}$, $RelB^{low}$ and $RelB^{high}$ populations. The $RelB^{high}$ population, which included mature DCs with projections, displayed RelB nuclear localization, whereas RelB in the $RelB^{low}$ population was in the cytoplasm. Although both the $RelB^{low}$ and $RelB^{-}$ populations barely showed projections, MHC II and co-stimulatory molecule expression were higher in the $RelB^{low}$ than in the $RelB^{-}$ splenic conventional DCs. Taken together, our results identify the $RelB^{low}$ population as a possible novel intermediate maturation stage of cDCs and the $Relb^{Venus/Venus}$ mice as a useful tool to analyze the dynamic regulation of the non-canonical NF-κB pathway.

2. Molecular mechanism of RANK signaling in osteoclastogenesis

Yuu Taguchi, Yui Iwamae, Yuki Nakano, Mikako Suzuki, Saya Bando, Youko Hirayama, Jin Gohda[1], and Jun-ichiro Inoue

Bone is an important organ, which supports body structure and hematopoiesis. Osteoclasts are large multinucleated cells, which have ability to degrade bone matrixes, and play a crucial role in bone homeostasis in concert with osteoblast, which generates bone matrix. As a result of excess formation or activation of osteoclasts, pathological bone resorption is observed in postmenopausal osteoporosis, rheumatoid arthritis and bone metastasis. Therefore, elucidating the molecular mechanism of osteoclastogenesis is important for understanding bone diseases and developing novel strategies to treat such diseases. Osteoclasts are differentiated from hematopoietic stem cells upon stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). It is known that the activation of signal transduction pathway emanating from receptor RANK is essential for osteoclastogenesis. The RANK signal activates transcriptional factors, NF-κB and AP-1, through the E3 ubiquitin ligase TRAF6, and induces activation of PLCγ2-mediated Ca^{2+} signaling pathway. These signals lead to the induction of NFATc1, a master transcriptional factor in osteoclastogenesis. We have previously demonstrated that RANK has a functional amino acid sequences, named Highly Conserved domain in RANK (HCR), which does not have any homology of amino-acid sequence with other proteins. The HCR acts as a platform for formation of signal complex including TRAF6, PLCγ2 and adaptor protein Gab2. This formation of signal complex is involved in sustaining activation of RANK signaling, and is essential for the NFATc1 induction and osteoclastogenesis. To elucidate other functions and the precise molecular mechanism of HCR, we have performed yeast two-hybrid screening and protein-array to identify the interacting protein to receptor RANK including HCR. Some candidate proteins were associated with RANK and HCR, and were involved in the induction of osteoclast-specific gene expression, suggesting that HCR has an additional function other than NFATc1 induction. We are currently investigating the molecular mechanisms of these candidate proteins in osteoclastogenesis. Moreover, to reveal the novel mechanisms involved in osteoclastogenesis, we performed microarray analysis of gene expression levels during osteoclastogenesis. Since some genes were dramatically downregulated in response to RANKL stimulation, we are currently investigating whether these genes are involved in the regulation of osteoclastogenesis *in vitro* and *in vivo*. We also obtained candidate genes involved in osteoclastogenesis by using CRISPR/Cas9-gRNA library screening system. Furthermore, to develop drugs for treatment to osteoporosis, we tried to search candidate compounds which have an ability to suppress osteoclastogenesis. Because some compounds showed inhibitory effect to osteoclastogenesis *in vitro* without arresting cell growth, we are now trying to elucidate mechanisms of inhibition.

3. Intratumoral bidirectional transitions between epithelial and mesenchymal cells in triple-negative breast cancer

Mizuki Yamamoto, Chiho Abe, Aya Watanabe and Jun-ichiro Inoue

Epithelial-mesenchymal transition (EMT) and its reverse process, MET, are crucial in several stages of cancer metastasis. EMT allows cancer cells to move to proximal blood vessels for intravasation. However, because EMT and MET processes are dynamic, mesenchymal cancer cells are likely to undergo MET transiently and subsequently re-undergo EMT to restart the metastatic process. Therefore, spatiotemporally-coordinated mutual regulation between EMT and MET could occur during metastasis.

To elucidate such regulation, we chose HCC38, a human triple-negative breast cancer cell line, because HCC38 is composed of epithelial and mesenchymal populations at a fixed ratio even though mesenchymal cells proliferate significantly more slowly than epithelial cells. We established E-cadherin- and Vimentin-reporter expressing HCC38 cells to analyze EMT status using FACS analysis and live cell imaging. Using this cell, we performed CRISPR/Cas9-mediated screening for intratumoral EMT-regulating genes and found several candidates.

4. Molecular mechanism of the Flavi virus E-protein-mediated membrane fusion.

Mizuki Yamamoto, Yusuke Fujinami, Aya Watanabe and Jun-ichiro Inoue

We have developed a cell-based fusion assay for prME protein of Flavi virus in a low pH-dependent manner, using Aedes albopictus cell line C6/36 cells expressing Renilla luciferase (RL)-based split reporter proteins. Using this assay, we are investigating molecular mechanisms for the E-protein-mediated membrane fusion.

5. Mint3 depletion attenuates proliferation in pancreatic cancer cells

Akane Kanamori, Jun-ichiro Inoue and Takeharu Sakamoto

Pancreatic cancer is one of the deadliest cancers. Although severe hypoxia is characteristic for pancreatic cancer, most cancer cells grow in normoxic and modest hypoxic areas. Hypoxia inducible factor-1 (HIF-1) is a master transcriptional factor for hypoxic response and thought to promote the malignancy of pancreatic cancer not only in severe hypoxic area but also in normoxic and modest hypoxic areas where cancer cells grow. However, the importance of HIF-1 in pancreatic cancer proliferation under the normoxic condition remains unclear. To address this, we focused on Mint3 which activates HIF-1 even in normoxia in cancer cells. Mint3 depletion attenuated proliferation in human pancreatic cancer AsPC1, BxPC3, Panc-1, and MIA-PaCa2 cells. Further analyses revealed that Mint3 depletion caused cell cycle arrest with increased p21 and p27 expression in pancreatic cancer cells in a HIF-1-dependent manner. Mint3 depletion also attenuated orthotopic tumor growth of AsPC1 cells in immunodeficient mice. Thus, Mint3 is a possible target for pancreas cancer treatment.

6. Pathophysiological analyses of the genes related to oxygen-dependent cancer cell proliferation using genetically modified mice

Yuya Fukui, Yoohwa Chung, Jun-ichiro Inoue and Takeharu Sakamoto

Responses to outside stimuli are essential for body homeostasis and dysregulation of these responses can cause pathological conditions such as inflammatory diseases and cancer. We previously surveyed the genes related to oxygen-dependent cancer cell proliferation using genome-wide shRNA libraries and identified 13 genes. Among them, we further focused on two genes, *X* and *Y*, whose pathophysiological roles remain unclear and established conventional and conditional knockout mice of these genes. Gene *X* was expressed ubiquitously in adult mouse tissues and Gene *X* knockout mice showed embryonic lethality. Interestingly, depletion of Gene *X* in myeloid cells promoted acute inflammation in TPA-induced ear inflammation, DSS-induced colitis, and LPS-induced septic shock models without affecting myeloid cell differentiation in the steady state. Meanwhile, Gene *Y* was predominantly expressed in testes among adult mouse tissues. Gene *Y* knockout mice were born less frequently than expected Mendelian ratio. Gene *Y* knockout mice exhibited male infertility. Thus, Gene *Y* contributes to embryogenesis to some extent and male fertility *in vivo*. We further examined the role of Gene *Y* in the tumor microenvironment. Tamoxifen-induced Gene *Y* conditional knockout mice showed attenuated tumor growth of injected murine breast cancer E0771 cells compared with control mice. Further analyses revealed that Gene *Y* was expressed in some of $CD45^+$ cells in E0771 tumor tissues. Thus, Gene *Y* expression in some $CD45^+$ cells might affect tumor growth.

Publications

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