PROGRESS REPORT
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Division of Cancer Cell Biology

Chairman: Daisuke Kitamura, M.D., D.M.S.

Upon encountering antigens through the B-cell receptor (BCR), B cells are activated and take up the antigen, and if the antigen contains a protein ingredient, present the antigen-derived peptide on MHC class II to cognate helper T (Th) cells. In turn, Th cells stimulate B cells through CD40-ligand (CD40L) and cytokines, such as IL-4 and IL-21, to facilitate their proliferation and switching of the BCR isotype from IgM/IgD to IgG, IgA, or IgE (class switching: CS). Some of these B cells then differentiate into short-lived plasma cells (PCs) and move to the extrafollicular region, whereas others further proliferate and form the germinal center (GC) in the B cell follicles. In GC, B cells undergo somatic hypermutation (SHM) of their immunoglobulin (Ig) V region genes to diversify their Ig repertoire. Among the GC B cells, those expressing BCR (typically of IgG classes) that bind to the immunized antigen with high affinity are selected, and they differentiate into memory B cells (MBCs) or long-lived PCs (LLPCs), both contributing to long-lasting humoral immunological memory.

Despite the widely accepted dogma of T-cell-dependent (TD) immune responses, as summarized above, it has been revealed that GC, SHM, and CS are not essential for generating MBCs. One representative finding is that IgG^+ MBCs without SHM could be generated in the absence of GC in mice devoid of Bcl6, a master regulator of GC B cell differentiation (Toyama et al. 2002, *Immunity* 17:329; Kaji et al. 2012, *J. Exp. Med*. 209:2079). In addition, IgM^+ MBCs have been appreciated to exist and to play a role in the humoral immunity (Dogan et al. 2009, *Nat. Immunol*. 10:1292, Pape et al. 2011, *Science* 331:1203). On the other hand, the GC appears necessary for generating LLPC because mice deficient in IL-21/IL-21R signaling show premature contraction of GC and fewer LLPCs (Zotos et al. 2010, *J. Exp. Med*. 207:365; Rasheed et al. 2013, *J. Virol*. 87:7737).

GC is a structure that arises in secondary lymphoid tissues several days after immunization with protein-containing antigens, the so-called TD antigens. GC is mainly composed of intensely proliferating antigen-primed B cells (centroblasts), post-cycled B cells (centrocytes), follicular dendritic cells and follicular helper T (Tfh) cells. Tfh cells produce IL-21, which is critical for the prolonged expansion of GC B cells, as mentioned above, and IL-4, which induces CS to IgG1 (in mice) or IgE. In addition, IL-4 induces epigenomic remodeling of the *Bcl6* locus by reprogramming the TCA cycle and the expression of Bcl6 (Haniuda et al. 2020, *Cell Rep*. 33:108333). It remains unknown which factors are necessary to induce SHM of Ig genes in GC B cells. *In vitro* stimulation of B cells through CD40 or TLR4, together with IL-4 or IL-21, induces massive proliferation and CSR, but not SHM, although it induces the expression of activation-induced cytidine deaminase (AID), which is required for the SHM and CS recombination (CSR) of Ig genes. This suggests that some critical factor(s) required for SHM are missing under this culture condition. It is also unclear how a few high-affinity B cells are selected in GCs among the vast majority of others, including those with considerable affinity to antigens, and how GC B cells differentiate into MBCs or LLPCs. Although the transcription factors Bcl-6 and Blimp1 are known to be necessary for B cells to

differentiate into GC B and PCs, respectively, transcription factors that induce MBC differentiation are unknown. The external stimuli that induce GC-B cell differentiation into MBCs or LLPCs remain elusive.

One reason for the difficulty in elucidating the mechanisms of GC B-cell development could be the lack of appropriate *in vitro* systems that mimic the process of GC B-cell development. To solve this problem, we established a B-cell culture system using an original feeder cell line expressing exogenous CD40L and BAFF (termed 40LB). When naive B cells are cultured sequentially with IL-4 and IL-21 on a 40LB feeder layer, B cells undergo massive expansion, express GC B cell markers, such as GL7 and Fas, and undergo efficient CS either to IgG1 or IgE. We termed these cultured B cells "induced GC B (iGB) cells." This system enabled single B cell culture. Despite extensive proliferation, the SHM of the V region genes could not be detected in iGB cells. After primary culture with IL-4 (iGB-4), the iGB cells can differentiate into memory-like B cells, termed "induced memory B (iMB) cells," after transfer into irradiated mice. When transferred with cognate antigen-primed Th cells to secondary recipient mice, antigen-specific iMB cells quickly respond to soluble cognate antigens to produce IgG1 antibodies, indicating normal memory-recall function of iMB cells. In contrast, iGB cells after secondary culture with IL-21 (iGB-21) failed to develop into iMB cells in the recipients, but instead differentiated into PCs in the bone marrow (Nojima et al. 2011, *Nat. Commun*. 2:465; Haniuda and Kitamura. 2019, *Bio-protocol* 9: e3163).

Thus, iGB cell culture appears to be a promising experimental system for the elucidation of several unsolved questions, including mechanisms for GC formation, generation of SHM and CSR, affinity selection, and B-lymphomagenesis, among others. Indeed, many researchers worldwide have used this system and the results have been reported (Caganova et al. 2013, *J. Clin. Invest*. 123:5009; Wu et al. 2014, *PNAS* 111:e4638; Purwada et al. 2015, *Biomaterials* 63:24; Webb et al. 2016, *J. Immunol*. 196:207; Kuraoka et al. 2016, *Immunity* 44:542; Domeier et al. 2016, *J. Exp. Med.* 213:715; Kuraoka et al. 2017, *Cell Rep*. 18:1627; Lee et al. 2017, *J. Immunol*. 198:1066; Li et al. 2018, *Immunity* 48:530; Le Gallou et al. 2018, *J. Exp. Med*. 215:2035; Litzler et al. 2019, *Nat. Commun*. 10:22; Finney et al. 2019, *J. Immunol*. 203: 3268; Nojima et al. 2020, *J. Immunol*. 205:90; Wigton et al. 2021, *J. Exp. Med*. 218:e20201422; Fukushima et al. *Cell Rep*. 2022; Thomann et al. *PNAS*, 2023; and many more).

Using this system, we have addressed the question of how the generation of SHM is regulated in GC B cells, how the fate of GC B cells toward MBCs is determined (Koike et al. 2019, *eLife* 8:e44245), how the MBC recall response is regulated (Fukao et al. 2014, *J. Immunol*. 193:635; Kodama et al. 2020, *Int. Immunol*. 32:385; Takatsuka et al. 2018, *Nat. Immunol*. 19:1025), and how IgE-producing B cells are restrained (Haniuda et al. 2016, *Nat. Immunol*. 17:1109). Regarding the regulation of MBCs, we found that IL-9 autocrine signaling facilitated proliferation and differentiation toward PCs while suppressing their ICOS-L expression and differentiation toward GC B cells (Takatsuka et al. 2018). We also found that PC development from MBCs was suppressed by the cell-surface inhibitory receptor gp49B (also called Lilrb4), which is selectively expressed on MBCs and marginal zone B cells (Fukao et al. 2014). In addition, our data suggest that MBCs are eliminated when their BCRs bind to specific antigens in the form of T-cell-independent type 2 (TI-2) antigen (Haniuda et al. 2011, *J. Immunol*. 186:5620). Thus, the recall response of MBCs appears to be strictly regulated, probably because MBCs are intrinsically hyper-responsive to antigens, owing to the properties of IgG BCR and the higher expression of MHC class II molecules and co-stimulatory molecules CD80 and CD86 (reviewed in Kitamura 2021, *Int. Immunol*. 33:791).

In addition, we established a system to culture mouse and human B cells for an unlimited length of time using the iGB cell culture system and attempted to establish an in vitro method to generate antibodies against tumor antigens as well as antibody-producing B cells using tumor-infiltrating B cells (Moutai et al. 2014, *PLoS One* 9:e92732; Wang et al. 2021, *PLoS One* 16:e0245608).

Since 2020, an up-and-coming cancer biologist, Dr. Shunsuke Kon, has started his laboratory in this division to study how normal cells, cancer cells, and immune cells interact with each other to maintain a normal cell society and break the norm into malignancy. On this occasion, we have changed the name of this division from Division of Molecular Biology to Division of Cancer Cell Biology.

Commensal bacteria and the lung environment are responsible for natural IgE production in MyD88-deficient mice

IgE antibodies are common mediators of allergic responses and are generally produced in type 2 immune responses to allergens. Allergen stimulation of IgE-bound FcεRI in mast cells or basophils induces the production of chemical mediators and cytokines. In addition, IgE binding to FcεRI without allergens promotes the survival or proliferation of these cells and airway muscle cells. Thus, spontaneously produced natural IgE can increase an individual's susceptibility to allergic diseases. We previously reported that IgE^+ B cells are generated in GCs; however, they immediately differentiate into short-lived PCs and die. This event is determined by the cell surface expression of the IgE-class BCR and subsequent signaling through BLNK and CD19. This is the reason why IgE^+ memory B cells and LLPCs are not generated and blood IgE levels are normally maintained at very low levels (Haniuda et al. 2016, *Nat. Immunol*. 17:1109). However, non-specific or natural IgE levels are often high in individuals who later develop allergic diseases.

Mice deficient in MyD88, a major TLR signaling molecule, have high serum levels of natural IgE, the mechanism of which remains unknown. We found that high serum IgE levels were maintained after weaning by memory B cells but not by LLPCs. IgE from PCs and sera from most *Myd88*$^{-/-}$ mice, but none of the *Myd88*$^{+/-}$ mice, recognized *Streptococcus azizii* (*S. azizii*), a commensal bacterium that is overrepresented in the lungs of *Myd88*$^{-/-}$ mice. $IgG1^+$ MBCs from the spleen also recognize *S. azizii*. Serum IgE levels declined with the administration of antibiotics and were boosted by challenge with *S. azizii* in *Myd88*$^{-/-}$ mice, indicating the contribution of *S. azizii*-specific $IgG1^+$ MBCs to natural IgE production. Th2, but not Th1, Th17, or Treg cells, were increased in the lungs of *Myd88*$^{-/-}$ mice and were activated upon addition of *S. azizii* to lung cells *ex vivo*. Finally, lung non-hematopoietic cells and CSF1 overproduced therefrom were responsible for natural IgE production in *Myd88*$^{-/-}$ mice. Indeed, conventional dendritic cells (cDC2) expressing the CSF1-receptor were increased in the lungs of *Myd88*$^{-/-}$ mice. Thus, CSF1 production in some lung cells is normally restrained by MyD88, which may be stimulated by commensal bacteria interacting with TLRs. When such a signal is depleted, CSF1 is produced and stimulates cDC2 cells that initiate a Th2 response triggered by antigens derived from other commensal bacteria, such as *S. azizii*, which in turn generates memory B cells that continuously produce natural IgE through persistent recall responses (Amano et al. *J. Immunol*. 2023).

Collaborators:

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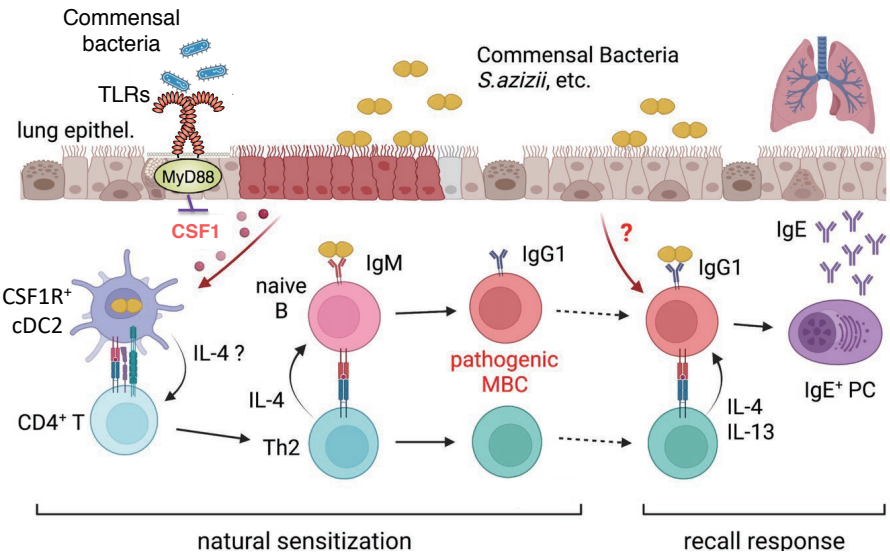

Figure 1. Lung commensal bacteria regulate natural IgE production through MyD88

SpiB regulates the expression of B-cell-related genes and increases the longevity of memory B cells

The generation of memory B cells is one of the key features of adaptive immunity, as they respond rapidly to re-exposure to antigens and generate functional antibodies. Although the functions of memory B cells are becoming clearer, the regulation of memory B cell generation and maintenance are still not well understood. We found that the transcription factor SpiB is expressed in some GC and memory B cells and participates in the maintenance of memory B cells. Overexpression and knockdown analyses revealed that SpiB suppresses PC differentiation by suppressing the expression of Blimp1 while inducing Bach2 in an iGB cell culture system, and that SpiB facilitates the in vivo appearance of iMB cells derived from iGB cells after transfer into mice. Analysis of $IgG1^+$ cell-specific SpiB conditional knockout (cKO) mice showed that SpiB function is critical for the maintenance of late memory B cells, but not for the development of early memory B cells or GC B cells. Gene expression analysis suggested that SpiB-dependent suppression of PC differentiation is independent of the expression of Bach2. We further revealed that SpiB upregulates anti-apoptosis (Bcl_{XL} and Mcl1) and autophagy (Atg5, Atg6, and Atg7) genes to control the survival of memory B cells. These findings indicate that SpiB functions in the generation of long-lasting memory B cells to maintain humoral memory (Horiuchi et al. *Front. Immunol*. 2023).

Collaborators:

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Subcutaneous immunization with zymosan generates immune memory that produces mucosal IgA upon nasal antigen challenge, and protects mice against virus infection

IgA is the most abundant isotype of antibodies and provides the first line of defense at the mucosa against pathogens that invade the host. It is widely accepted that the mucosal IgA response provided by vaccination requires

mucosal inoculation, and intranasal inoculation has been proposed as a vaccine against the influenza virus. However, parenteral vaccination that provides the mucosal IgA response is desirable, considering the difficulty of intranasal vaccination in infants or elderly people. We demonstrated that subcutaneous immunization with zymosan, a yeast cell wall constituent known to be recognized by Dectin-1 and TLR2, potentiates the production of antigen-specific IgA antibodies in the sera and airway mucosa upon intranasal antigen challenge without any adjuvants. We confirmed that antigen-specific IgA-secreting cells accumulated in the lung and nasal-associated lymphoid tissues after the antigen challenge. Such an adjuvant effect of zymosan in the primary immunization for the IgA response depended on Dectin-1 signaling, but not TLR2. The IgA response to antigen challenge required both antigen-specific memory B and T cells, and the generation of memory T cells, but not memory B cells, depended on zymosan as an adjuvant. Finally, we demonstrated that subcutaneous inoculation of inactivated influenza virus with zymosan, but not alum, mostly protected the mice from infection with a lethal dose of a heterologous virus strain. These data suggest that zymosan is a possible adjuvant for parenteral immunization that generates memory IgA responses to respiratory viruses, such as influenza virus (Nihei et al. *Int. Immunol*. 2023).

Collaborators:

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Commensal bacteria-primed production of IgA autoantibodies against glomerular antigens in a mouse model for IgA nephropathy

IgA nephropathy (IgAN) is the most common type of primary glomerulonephritis, with approxymately 40% of patients progressing to renal failure. IgAN is pathologically characterized by the deposition of IgA, IgG, and complement C3 in the glomerular mesangium and proliferative changes in the mesangial cells and matrix. Glomerular IgA deposition has been ascribed to abnormal glycosylation of IgA, namely galactose-deficient (Gd-) IgA1, which tends to form immune complexes (ICs) by self-aggregation or with autoantibodies against Gd-IgA1. However, such ICs would not only be deposited in the mesangial region, but also at various locations in the glomeruli. Additionally, Gd-IgA1 can be found in healthy individuals and in diseases other than IgAN. Therefore, Gd-IgA1 alone cannot explain the mesangium-specific IgA deposition in IgAN. Thus, the mechanisms underlying the selective deposition and generation of pathogenic IgA in IgAN remain unclear.

To address these problems, we used the IgAN mouse model, “gddY” mice. These mice were generated by the selective intercrossing of mice in early onset groups of outbred ddY mice for more than 20 generations. The original ddY mice spontaneously developed IgAN, but with a low incidence and highly variable timing of onset among individuals. In contrast, essentially all gddY mice exhibit proteinuria and IgA deposition in the glomerular mesangium by 8 weeks of age, followed by glomerular injury, resembling that observed in human IgAN (Okazaki et al. 2012, *J. Am. Soc. Nephrol*. 23:1364). Recently, we found anti-mesangium IgA auto-Abs in the sera of gddY mice and human patients with IgAN and identified βII-spectrin as a target antigen in both gddY mice and IgAN patients. IgA^+ plasmablasts (PBs) accumulated in the kidneys of gddY mice and IgA Abs secreted by these PBs also bound to

the mesangium and βII-spectrin. Although βII-spectrin is known as a cytoskeletal protein, it was expressed on the surface of mesangial cells and βII-spectrin-transfected HEK293T cells. A recombinant IgA antibody cloned from the kidney PBs bound to the mesangial regions of the kidney in vivo after i.v. administration. From these data, we propose that the production of IgA autoantibodies against mesangial proteins, such as β2-spectrin, is the first trigger of the pathogenesis of IgAN, and therefore, IgAN is a tissue-specific autoimmune disease (Nihei et al. *Science Adv*. 2023).

Recently, we identified an additional self-antigen (tentatively called TIA66) recognized by recombinant IgA auto-Abs (rAb#66 and others) derived from the kidney PBs of gddY mice. TIA66 is normally an intracellular protein but is detected on the surface of mesangial cells and is recognized by sera from gddY mice and IgAN patients. When BALB/c mice were immunized with TIA66, antigen-specific IgG and IgA antibodies were produced and deposited in the mesangium. Interestingly, the IgA^+ PBs disappeared from the kidneys, glomerular IgA deposition was restrained, proteinuria was reduced, and serum anti-TIA66 IgA disappeared after compound antibiotic treatment of gddY mice, suggesting that commensal bacteria are involved in the production of IgA auto-Abs. In addition, feeding gddY mice an elemental diet caused a similar phenomenon. We found that gddY serum IgA and rAb#66 bound to oral, but not intestinal, commensal bacteria from gddY, but not BALB/c mice, and rAb#66 binding to the oral bacteria was competitively inhibited by the addition of TIA66 protein. We identified a bacterial strain that binds to rAb#66. This was a previously unknown species and tentatively termed "C42." Binding of rAb#66 to C42 was competed by TIA66, suggesting molecular mimicry between C42 and TIA66 epitopes. Immunization of BALB/c mice with C42 induced antibodies that bound to TIA66 and glomerular deposition of IgA in vivo. Finally, C42 was selectively decreased among the oral bacteria in gddY mice fed an elemental diet. Thus, our results indicate that particular strains of oral commensal bacteria can induce an immune response that leads to the production of anti-mesangium IgA auto-Abs in gddY mice, which will facilitate the understanding of IgAN pathogenesis and therapeutic strategies for IgAN (Higashiyama et al. under revision).

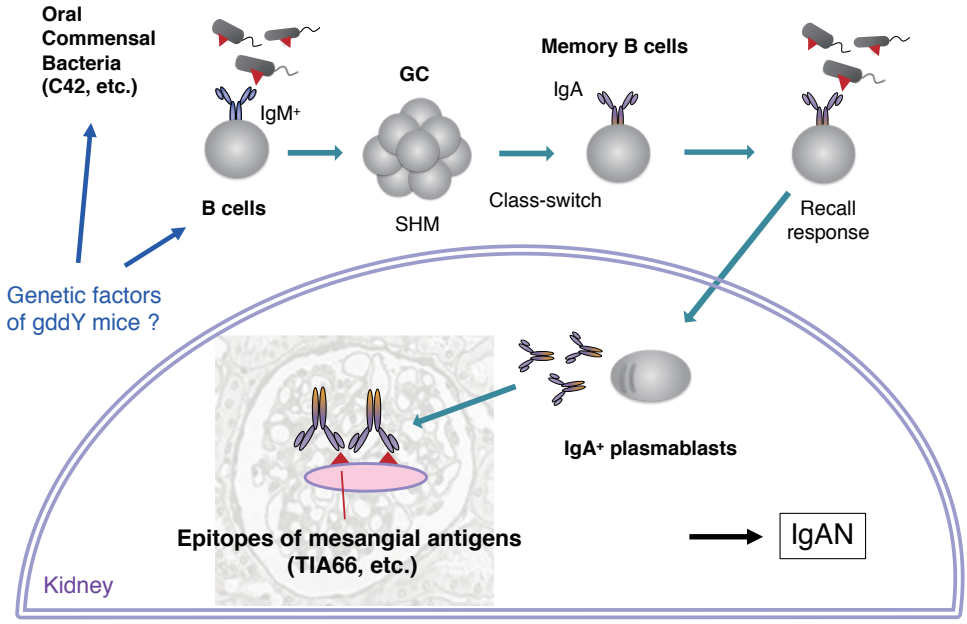

Figure 2. Oral bacteria initiate the response that produces anti-mesangium IgA in mice

Collaborators:

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Daisuke Kitamura, M.D., D.M.S.

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Division of Cancer Cell Biology

Shunsuke Kon, Ph.D.

Cancer is a leading cause of death in Japan, and the establishment of innovative and effective treatments is urgently needed. Upon oncogenesis, a single epithelial cell undergoes an initial transformation due to gene mutations induced by various factors, such as pathogens, sunlight, exposure to carcinogens, and cell division. Subsequently, additional mutational burdens cause these transformed cells to become invasive malignant cells. It is estimated that more than thousands of transformed cells with oncogenic alterations are generated in a body every day. To eliminate these harmful transformed cells that are detrimental to tissues, we employ tumor surveillance systems to fight against emerging transformed cells. Cell competition is an inherent property that living organisms prosses to fulfill this clearance function. When transformed cells incidentally appear within an epithelial tissue, neighboring normal epithelial cells can recognize and eliminate them by pushing them into the apical lumen, effectively squeezing the transformed cells out of the epithelia. This biological phenomenon is referred to as 'apical extrusion' of transformed cells and is one mechanism by which less fit transformed cells are eliminated through cell competition.

Our current primary focus is on understanding how cell competition is mechanically secured and how it is functionally disrupted at the onset of carcinogenesis. We are currently investigating the role of autophagy in cell competition and have discovered that autophagy plays a positive role in the process of apical extrusion of transformed cells. To investigate the functional alterations in cell competition during multi-sequential carcinogenesis, we utilize a cell competition mouse model that we previously established. We examine how the accumulation of genetic mutations affects the fate of transformed cells in a competitive environment. Additionally, we study the molecular mechanisms underlying the deregulation of cell competition caused by the accumulation of genetic insults. Beyond cell competition, we have expanded our research projects to investigate the invasion of cancer cells into lymphatic vessels. This research will contribute a comprehensive understanding of the physiological responses to the production of cancer cells.

Role of autophagy in cell competition

To evaluate autophagic activity in transformed cells surrounded by normal epithelial cells, we established Madin-Darby canine kidney (MDCK) or MDCK-pTRE3G myc-RasV12 cell lines that stably express GFP-LC3. When RasV12 cells are co-cultured with MDCK cells at a ratio of 1:50, the number of GFP-LC3-positive puncta in RasV12 cells is profoundly higher than that in RasV12 cells cultured alone.

Bafilomycin A1 treatment, which does not alter the value, prevents autophagic flux in RasV12 cells non-cell autonomously. This result was supported by experiments using an autophagic flux probe, GFP-LC3-RFP-LC3ΔG, demonstrating that interaction with neighboring normal cells reduces autophagic flux in RasV12 cells. We then explored the fate of undegradable autophagic structures by electron microscopy observations and found that both autophagosomes

and autolysosomes accumulate in RasV12 cells co-cultured with normal cells. To explore the underlying mechanism of hindered autophagic flux, the lysosomal activity of transformed loser cells is carefully evaluated. The incorporation of LysoTracker, which labels acidic, functional lysosomes, is profoundly diminished in RasV12 cells confronted with normal cells. The comparable non-cell autonomous lysosomal dysfunction is also observed when another indicator of lysosomal activity, Magic Red, is exploited. Reduced lysosomal activity is substantially alleviated by the addition of forskolin, which acidifies lysosomes by elevating intracellular cAMP levels, and forskolin treatment inhibits apical extrusion of RasV12 cells in a dose-dependent manner. These results indicate that by perturbing autophagy, lysosomal dysfunction positively regulates cell competition. Previous studies suggest that normal epithelial cells squeeze adjacent transformed cells by locating the cytoskeletal protein filamin at the boundary. This led us to examine the involvement of filamin-mediated mechanical cues in the altered lysosomal-autophagic activity. Filamin-knockdown in normal cells does not induce either decreased lysosomal activity or accumulation of autophagosomes in RasV12 cells, suggesting that mechanical input from surrounding normal cells is required for autophagic perturbation caused by reduced lysosomal activity in transformed cells. We also demonstrated that knockdown of ATG13, a key molecule of the autophagy initiation complex, in RasV12-transformed cells profoundly suppresses apical extrusion of RasV12 cells, signifying that autophagosome formation per se is required for transformed cells to be apically expelled. Furthermore, filamin accumulation in normal cells is substantially abolished when co-cultured with ATG13-knockdown RasV12 cells. Collectively, these findings indicate that there exist mutual regulatory mechanisms between accumulated autophagosomes in RasV12-transformed cells and filamin accumulation in neighboring normal cells, which collaboratively regulate apical elimination of transformed cells.

Finally, we explored autophagic activity in vivo using a cell competition mouse model wherein bicistronic expression of H-RasV12 and eGFP is under the control of a floxed STOP transcriptional cassette. Injection of a low dose of tamoxifen induces the expression of transgenes in a mosaic fashion, and we found that the number of LC3-positive puncta in RasV12 cells confronted with normal cells substantially increases compared to those surrounded by one another. In addition, lysosomal activity is found to be depressed non-cell autonomously, demonstrating that attenuated degradation of autophagosomes caused by lysosomal dysfunction occurs in vivo. Furthermore, we showed that ablation of autophagosome formation by Atg5-knockout in RasV12 cells interferes with apical extrusion in the pancreas. Remaining Atg5-knockout RasV12 cells in pancreatic ducts form an irregular, stratified epithelium with ductal papillary architecture, and eventually cause chronic pancreatitis. Collectively, this study demonstrated that autophagy counters oncogenesis by actively removing transformed cells via cell competition.

Collaborators:

Eilma Akter, Yasuyuki Fujita

Disruption of cell competition during multi-sequential carcinogenesis

We utilized familial adenomatous polyposis (FAP) in humans as a model to study the functional alteration of cell competition in multi-sequential carcinogenesis. During the development of FAP, the initial mutation of the APC gene induces neoplasia, and subsequent activation of the small GTPase Ras leads to carcinoma transition. To mimic the preexisting genetic insults of FAP in mice, we engineered the mice to sustain mosaic, somatic activation of Ras in the background of APC deficiency. As a result,

we found that a significantly higher number of RasV12-transformed cells diffusively invade the basal lamina in APC-deficient mice compared to wild-type mice. In addition, APC/RasV12-transformed cells that have invaded the basal membrane penetrate through the basement membrane and expand in the stroma of mucosal epithelia, without accompanying any papillary adenomatous lesions in surrounding tissues, suggesting that cancer cells are directly generated from the normal mucous membrane through diffuse invasion of transformants (de novo carcinoma). Collectively, we found that Wnt activation induced by APC deficiency causes malfunction of cell competition and potentiates the diffuse invasion of transformed cells, resulting in de novo carcinoma in mice.

Collaborators:

Kazuki Nakai, Hancheng Lin, Kazuki Hachiya, Yasuyuki Fujita

Molecular mechanism underlying diffuse invasion of transformed cells through malfunction of cell competition

This year, we have intensively explored the molecular basis of diffuse invasion of transformed cells caused by the malfunction of cell competition. To phenocopy the diffuse invasion of APC/RasV12 cells observed in mice in vitro, we had previously established Mardin-Darby Canine Kidney (MDCK) cells stably expressing an N-terminally deleted β-catenin mutant (β-catΔN), which is a non-degraded form of β-catenin (β-catΔN cells), and β-catΔN-expressing MDCK cells that express GFP-RasV12 in a tetracycline-dependent manner (β-catΔN/RasV12 cells). We then found that when β-catΔN/RasV12 cells are cultured alone, they remain within the epithelia. In contrast, when β-catΔN/RasV12 cells are co-cultured with β-catΔN cells at a ratio of 1:50, a substantial number of β-catΔN/RasV12 cells diffusively invade into the collagen matrix over time, highlighting the non-cell autonomous diffuse invasion of β-catΔN/RasV12 cells. Thus, we successfully generated an in vitro cell-culture system that recapitulates the salient feature of basal invasion of APC/RasV12 cells. Using this system, we conducted a comprehensive transcriptome analysis to search for molecules whose expression is changed in β-catΔN/RasV12 cells when co-cultured with β-catΔN cells. Consequently, we found that MMP21, a member of the matrix metalloproteinases (MMPs), is profoundly upregulated non-cell autonomously. Furthermore, quantitative PCR analysis revealed that MMP21 is by far the most abundant molecule among MMP isozymes. To characterize the proteolytic capabilities of MMP21, we produced a recombinant protein of the catalytic domain of MMP21 using E. coli and subjected it to incubation with several ECMs, which are principal constituents of connective tissues. Accordingly, the MMP21 catalytic domain extensively degrades collagen type I and collagen type IV, whereas digestion of fibronectin generates both small and large fragments. In contrast, laminin is resistant to hydrolysis, indicating that MMP21 is a competent Zn2+-dependent endoproteinase with unique specificity for collagen type I, collagen type IV, and fibronectin. We then knocked MMP21 down and found that the loss of MMP21 significantly decreases the frequency of non-cell autonomous diffuse invasion of β-catΔN/RasV12 cells, highlighting an active role of MMP21 in the non-cell autonomous basal invasion of transformants. Furthermore, GSEA analysis revealed that the NF-κB signal is activated in β-catΔN/RasV12 cells co-cultured with β-catΔN cells, and inhibition of the NF-κB pathway abrogates both non-cell autonomous MMP21 upregulation and basal invasion of β-catΔN/RasV12 cells. These results indicate that NF-κB signal positively regulates basal invasion of transformed cells through MMP21 upregulation (Figure 1).

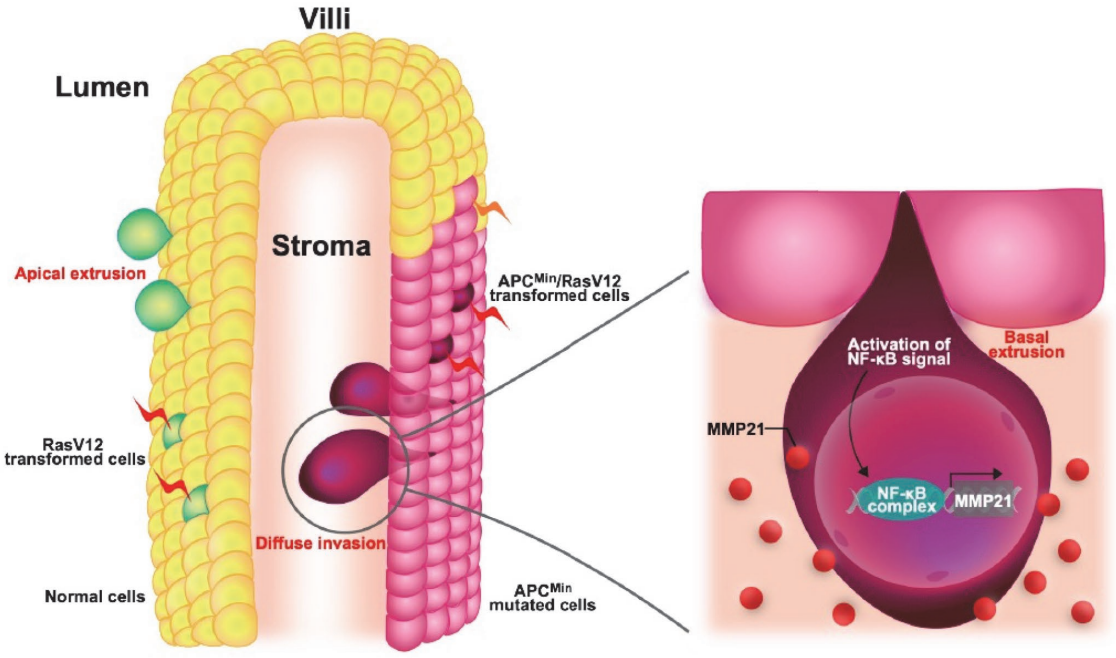

Figure 1. A schematic model for molecular mechanisms of the diffuse invasion of APC^{Min}/RasV12-transformed cells.

Collaborators:

Kazuki Nakai, Hancheng Lin, Junpei Kurauchi, Shinya Tanaka, Yasuyuki Fujita

Dissection of lymphovascular invasion by malignant cells

We have previously observed that APC/RasV12 cells often metastasize and preferentially invade lymphatic vessels but not blood vessels. This prompted us to investigate and uncover the nature of lymphatic invasion by cancer cells, which has been a black box in cancer metastasis. This year, we developed a whole-mount staining method for the small intestine to visualize the 3D structure of vascular networks. Through this method, we discovered that Lyve-1-positive lymphatic vessels gradually disappear after the emergence of APC/RasV12 de novo cancer cells in stromal tissues. This conclusion is based on experiments in which the size, length, and width of lacteals, which make up the lymphovascular system in the intestines, decrease in de novo tumors over time. To assess the function of lymphatic vessels, mice were administered Oil Red O, which is absorbed in intestinal tissues through the lymphatic system. We found that lymphatic function is substantially disturbed in APC/RasV12-induced tumors. Notably, lymphatic endothelial cells close to cancer cells do not show any signs of cell death, suggesting that APC/RasV12 de novo cancer cells induce the regression of lymphatic vessels through a mechanism other than cell death induction. This led us to conduct a Visium analysis, which is a spatio-transcriptional analysis, revealing profound changes in gene expression related to Endothelial-to-Mesenchymal Transition (EndMT) in tumor regions. Immunostaining using EndMT markers such as Transgelin or α-SMA shows that lymphatic endothelial cells surrounded by APC/RasV12 cancer cells are positive for these markers. Furthermore, it was revealed that the expression of Prox1, a master regulator of lymphatic endothelial lineage commitment, diminishes prior to the downregulation of Lyve-1 upon the formation of APC/RasV12-induced tumors. Therefore, these results indicate that APC/RasV12 cancer cells induce the transdifferentiation of lymphatic endothelial cells into a certain type of mesenchymal cells, which leads to the

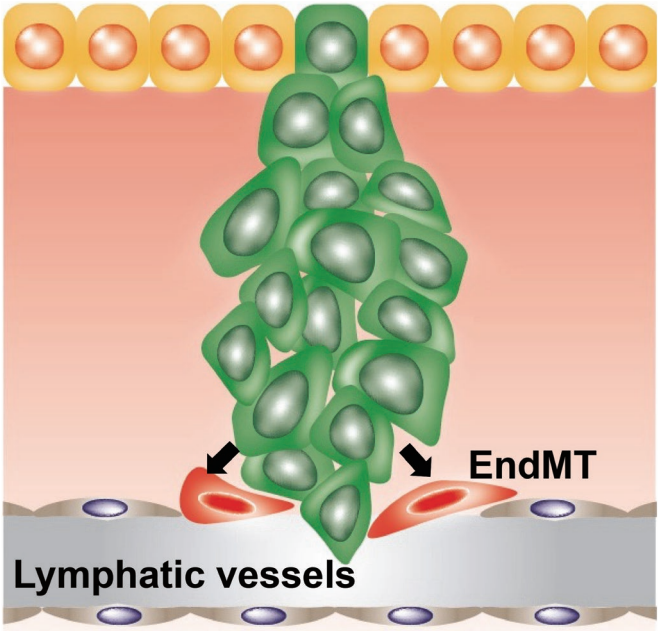

Figure 2. A schematic model for lymphvascular invasion of cancer cells.

disorganization of lymphatic vessels and potentiation of cancer cell invasion (Figure 2). We are now planning to establish an in vitro system to recapitulate EndMT induction induced by malignant cells and identify the key molecules that play a central role in this process in the next year.

Collaborators:

Hancheng Lin, Chenlu Zou, Yuxiao Yang

Identification of a pancreatic cancer-specific 2'O-Methylated microRNA

We have previously reported that RNA modifications play a role in the malignant transformation of gastrointestinal cancers and can serve as biomarkers. We further explored RNA modifications that are specific to pancreatic cancer among gastrointestinal cancers. A database analysis of the expression of RNA modification-related enzymes in pancreatic cancer reveals that the expression of HEN Methyltransferase 1 (HENMT1) is up-regulated in pancreatic cancer compared to normal tissue. HENMT1 has been reported to be an enzyme that 2'O-Methylates the ribose of the 3-terminal base of small RNA. We have also developed a new system for highly sensitive detection of 2'O-methylated microRNAs using a tunnel current sequencer. As a result, we identified several pancreatic cancer-specific 2'O-Methylated miRNAs. We are continuing to conduct functional analysis of these pancreatic cancer-specific 2'O-Methylated miRNAs.

Collaborators:

Masamitsu Konno, Mayuka Ohkawa

Publication

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Toyokuni, S., **Kon, S**., Moriyama, K., Fujita, Y. and Takahashi, A. Hepatocyte growth factor derived from senescent cells attenuates cell competition-induced apical elimination of oncogenic cells. **Nature Communications** 13, s41467. 2022

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4. Mori, Y., Shiratsuchi, N., Sato, N., Chaya, A., Tanimura, N., Ishikawa, S., Kato, M., Kameda, I., **Kon, S**., Haraoka, Y., Ishitani, T. and Fujita, Y. Extracellular ATP facilitates cell extrusion from epithelial layers mediated by cell competition of apoptosis. **Current Biology** 32, 2144-2159. 2022