Extended Abstracts for the 52nd International Symposium of the Princess Takamatsu Cancer Research Fund, 2024

CANCER RESEARCH AND MEDICINE ADVANCED BY EMERGING TECHNOLOGIES AND INNOVATIVE CONCEPTS

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Edited by :
Hideyuki Saya, Charles L. Sawyers, Fuyuhiko Tamanoi, Toru Hirota, and Fumihiko Ishikawa

Princess Takamatsu Cancer Research Fund

CONTENTS

OPENING REMARKS

KEYNOTE LECTURE

MOLECULAR TARGETS

SINGLE CELL APPROACH, LINEAGE TRACING, PROTEOMICS

TUMOR HETEROGENEITY, PATIENT-DERIVED MODELS

DNA BREAKS, RADIATION THERAPY, NUCLEAR MEDICINE

(SPECIAL SESSION) ETHICAL, LEGAL AND SOCIAL ISSUES IN CANCER RESEARCH AND INNOVATION

DATA-DRIVEN CANCER BIOLOGY

MOLECULAR TARGETS : KRAS

GENOME INSTABILITY

IMMUNE RESPONSE

CONCLUDING REMARKS

OPENING REMARKS

Ken Yamaguchi
Chairman, Board of Directors
Princess Takamatsu Cancer Research Fund
(info@ptcrf.or.jp)

The Princess Takamatsu Cancer Research Fund is the oldest and largest cancer research support organization in Japan, and is run as a purely private organization. The founder is Her Late Imperial Highness Princess Kikuko Takamatsu. The Princess Takamatsu hailed from the Tokugawa Shogun's family. The first shogun of the Tokugawa shogunate was Ieyasu Tokugawa, and the shogunate lasted for over 250 years. The last shogun was Yoshinobu Tokugawa, the 15th one, and the Princess Takamatsu was born as his granddaughter.

【Lady Kikuko and the Last Shogun, 1913】

【Prince and Princess Takamatsu, 1930】

At the age of 18, she married His Imperial Highness Prince Nobuhito Takamatsu, the

third son of Emperor Taisho. In 1933, when she was 22, her mother, who was also a member of the Imperial Family, died of cancer at the age of 43. The Princess Takamatsu was keenly aware that there was still so much that was unknown about the disease of cancer, and she decided to devote her life to supporting cancer research. In 1934, she donated therapeutic radioactive radium to the Cancer Institute, Tokyo, and after the World War II, she worked to help the Cancer Institute recover from the damage it had sustained from the wartime bombing.

In 1968, the Nadeshiko-kai (Flower Dianthus Group), a volunteer group made up of the Princess Takamatsu's alumnae from the Gakushuin Girls' School, established the Princess Takamatsu Cancer Research Fund to further strengthen support for cancer research. At the time, then-the Ministry of Education of the Japanese Government also actively supported the establishment of the first private foundation focusing on basic medical research. At the time of the foundation's establishment, the Princess Takamatsu was appointed as Honorary Patroness, and Mrs. Fujiko Iwasaki, a central figure in the activities of the Nadeshiko-kai, was appointed as the first chairperson of the board of directors of the Fund.

The main activities of the foundation include holding international symposia and lectureships, awarding academic prizes, and presenting research grants to Japanese researchers. Of these, the international symposia are the most important activity. At the time of the foundation's establishment, it was not easy for Japanese scientists to attend academic conferences held overseas, but the foundation's international symposia provided young scientists with the opportunity to listen to lectures given by many internationally renowned cancer researchers, and contributed greatly to the development of cancer research in Japan. The late Dr, Takashi Sugimura, the former president of the National Cancer Center, Tokyo, and a major figure in the foundation's management, reminisced about the atmosphere of those days, saying "the foundation opened its doors to international exchange for the first time through the international symposia. Scientists began to trust each other and to advance their researches with hopes for the future. It was a truly wonderful time." Later, the significance of holding international symposia, combined with the progress of cancer research in Japan, came to serve as a forum for exchange between Japanese cancer researchers and those from around the world.

The Princess Takamatsu passed away in 2004, and His Imperial Highness Prince Tomohito of Mikasa, who picked up the torch, made a precious contribution to the Fund's activities as Patron. The Prince Tomohito of Mikasa passed away in 2012, and since then, the foundation has continued to be operated under His Imperial Highness Prince Masahito

Hitachi, who became Patron, and in 2018, the foundation celebrated the 50th anniversary. Looking back over the foundation's half-century history, the activities of this purely private organization have created a new trend in cancer research support, and the Japanese government has followed suit. Furthermore, it has become a model for all volunteer activities in Japan, and its role in pioneering medical research support has been highly evaluated.

The theme of the international symposium is decided by the Scientific Advisory Committee, chaired by Prof. Ryuzo Ueda. It was decided as "Cancer Research and Medicine Advanced by Emerging Technologies and Innovative Concepts" for the 52nd International Symposium. The preparations were carried out by the organizing committee, chaired by Dr. Hideyuki Saya, with Dr. Charles L. Sawyers, Dr. Fuyuhiko Tamanoi, Dr. Toru Hirota and Dr. Fumihiko Ishikawa serving as committee members. A total of 30 prominent researchers were invited to give lectures; they were 13 from the United States, 1 from the United Kingdom, 1 from France, 1 from Germany, 1 from the Netherlands, 1 from Switzerland, and 12 from Japan. In addition, a total of 180 discussants also participated, who all engaged in lively discussions.

Finally, I would like to introduce the words of the Princess Takamatsu, which expressed her thoughts on cancer research. I myself served as the attending physician to the Princess Takamatsu for about 20 years until her passing. During that time, Her Imperial Highness would confide to me on various occasions that "in our fight against cancer, the best results will ultimately be produced through the promotion of scientific research." I sincerely hope that the content presented at this symposium has given the attendees new ideas, that mutual exchange has inspired them for new researches, and that these activities will ultimately lead to the conquest of cancer.

Ken Yamaguchi, MD, PhD

1974	M.D., Keio University School of Medicine, Tokyo
1986-2002	Chief, Endocrinology (Growth Factor) Division, National Cancer Center Research Institute (NCCRI)
1999-2002	Deputy Director, NCCRI
1999-2005	Special Advisor to the Imperial Household
2000-2004	Committee member, Scientific Advisory Committee, International Agency for Research on Cancer, Lyon, France
2002-2023	President, Shizuoka Cancer Center
2017-2023	Council Member, Council for Medical Science and Technology, Ministry of Health, Labour and Welfare (MHLW)
2018-2022	Chairman, Advisory board for Cancer Control in Japan, MHLW
2020-present	Council Member, Council for Genomic Medicine, Cabinet Office, Japanese Government
2023-present	President Emeritus, Shizuoka Cancer Center
2024-present	Chairman, Board of Directors, Princess Takamatsu Cancer Research Fund

LINEAGE PLASTICITY AND CANCER DRUG RESISTANCE

Charles L. Sawyers
Investigator, Howard Hughes Medical Institute
Marie Josee and Henry Kravis Professor
Chair, Human Oncology and Pathogenesis Program
Memorial Sloan Kettering Cancer Center, USA
(sawyersc@mskcc.org)

My laboratory studies prostate cancer and the clinical problem of resistance to therapies targeting the androgen receptor (AR), collectively known as AR signaling inhibitors (ARSI). A major outcome of that work was the discovery that ARSI-resistant prostate cancer (also called castration resistance prostate cancer or CRPC) remains dependent on AR signaling, a finding that we acted on therapeutically by identifying two novel AR inhibitors, enzalutamide and apalutamide, that are now widely used to treat CRPC. The impact is substantial: enzalutamide was just approved for upfront treatment of non-metastatic castration-sensitive disease, based on metastasis free survival of nearly 90% at 5 years.

The underlying rules and patterns of resistance to targeted therapies are now in transition. After two decades of success in targeting oncogenic drivers (numerous kinases, KRAS, etc.) together with improved next generation inhibitors that eliminate target-based resistance mechanisms (e.g., osimertinib for EGFR-mutant lung cancer[8]), we are seeing a shift away from "on target" (mutation-based) resistance to "off target" resistance involving changes in cell state - often called lineage plasticity. Prostate cancer provides a striking example where resistance to ARSIs such as enzalutamide can occur through lineage transition from an epithelial prostate adenocarcinoma (PRAD) to neuroendocrine prostate cancer (NEPC) **(Figure 1)**. There is growing evidence of lineage plasticity in other tumors placed under the selective pressure of targeted therapy (lung, pancreas, melanoma, others).

Our group was among the first to demonstrate that lineage plasticity is an epigenetic, potentially reversible process, and occurs only in tumors with a specific genomic mutational context -- such as loss of the tumor suppressor genes *TP53* and *RB1*. At the time, this phenomenon was a rare consequence of ARSI therapy. But over the ensuing 7 years, the

frequency of this form of escape has increased, coincident with the widespread adoption of next generation ARSI therapy. Whole exome and RNA sequencing of tumors from a cohort of 500 CRPC patients (through a consortium led by Arul Chinnaiyan and me) revealed enrichment for genomic alterations in *TP53*, *PTEN* and *RB1* in tumors with transcriptomic evidence of lineage transition. When we asked which genomic alterations across the entire 500 patient cohort confer an adverse clinical outcome, only one emerged – *RB1* loss – which our studies now show is a major gatekeeper of lineage plasticity. Through analysis of clinical CRPC at a single cell level, we have evidence that lineage plasticity is even more complex, with up to 10 subtypes of PRAD and 3 subtypes of NEPC revealed by gene regulatory network analysis.

In follow-up to our earlier study showing *TP53*/*Rb1* loss can initiate lineage plasticity in human prostate cancer models, we initiated a collaboration with David Goodrich at Roswell Park and Dana Pe'er at MSK to characterize the PRAD to NEPC lineage transition at a single cell level in genetically engineered mouse models (GEMMs) with conditional deletion of *Trp53*/*Rb1*/*Pten* (TKO) or *Rb1*/*Pten* (DKO) in the prostate. This work led to critical new insights that we were unable to appreciate earlier using histology. First, disease initiates with classic appearing PRAD histology but transcriptional profiling revealed a mixed basal-luminal state with elevated inflammatory pathway signaling. Second, this mixed lineage state subsequently gives rise to four distinct tumor-derived lineages defined by master regulator transcription factors (TFs) and their target genes, including NEPC (*Ascl1*), NEPC-like (*Pou2f3*), gastrointestinal (GI)-like (*Tff3*) and mesenchymal (*Twist2*). Among these, the *Ascl1*+ NEPC subpopulation has the highest proliferation rate and eventually dominates the tumor mass. This transition is accelerated by castration, just as in human NEPC. Using a quantitative metric of lineage plasticity developed by Dana Pe'er's lab, we leveraged the single cell data to identify two cell-intrinsic kinases (FGFR and JAK) whose activity is upregulated in the mixed basal luminal state and required for subsequent lineage transition to NEPC. Based on this finding, a clinical trial of a dual FGFR/JAK kinase inhibitor (tinengotinib) was initiated at MSK earlier this year.

The GEMM work was particularly exciting because the mouse phenotypes closely mirror the human disease and provide a model to examine the steps involved in lineage plasticity, as well as opportunities to intervene therapeutically. However, the TKO and DKO models have significant logistical challenges (up to 7 alleles for breeding, 6-9 months readout, etc.) that preclude large scale functional interrogation. Several years ago (initially in collaboration with Hans Clevers), we developed and optimized organoid culture as an *in vitro* tool to study prostate biology. At that time, the prostate field relied heavily on cell lines, which are limited in number and fail to represent the spectrum of cancer phenotypes. We have further optimized the organoid work into a highly efficient *in vivo* platform to study prostate cancer

initiation and progression, while retaining phenotypic similarity to human prostate cancer.

As a first step to explore the feasibility of using organoids to engineer prostate cancer initiation *in vivo*, we reproduced the GEMM phenotype of *Trp53*/*Pten* deletion[22] by co-deletion *Trp53* and *Pten* in freshly derived normal mouse prostate organoids (using CRISPR), followed by orthotopic (OT) transplantation into syngeneic host mice[23]. We have also used this platform to explore cell-of-origin questions using different genetic drivers. For example, luminal cells are the preferred cell of origin over basal cells when *Pten* deletion is paired with *Trp53* loss; however, basal cells are the preferred cell of origin when *Pten* loss is paired with ERG activation, underscoring the importance of cell context for each genomic driver.

After gaining confidence in the fidelity of cancer phenotypes seen in the organoid transplantation model, we invested considerable effort in optimizing the platform to study lineage plasticity at scale. Patient data shows that *TP53, PTEN* and *RB1* loss are all enriched in NEPC, but there has been limited insight into which if any events are required. To address this question, we compared all pairwise (and triple) tumor suppressor combinations (plus a fourth variable of elevated c-MYC expression). The result is definitive: *Rb1* loss is essential for the PRAD to NEPC transition whereas *Pten* loss is not. This likely explains why *RB1* loss is the strongest biomarker of poor clinical outcome, as mentioned earlier. A deeper dive into the kinetics of the RPM ($Rb1^{-/-}$/$Trp53^{-/-}$/Myc^{+}) model identified KRT8+ luminal cells (with stem-like L2 transcriptomic features) as the source of the ASCL1+ cells that subsequently expand and give rise to full blown NEPC. To further investigate the role of the basic helix-loop-helix (bHLH) TF ASCL1 in plasticity, we edited the *Ascl1* locus in RPM organoids prior to transplantation and found, to our amazement, that we completely abrogated all evidence of NEPC transition. In fact, RPM-$Ascl1^{KO}$ organoids give rise to well-differentiated adenocarcinomas (with L1-like luminal features) that are highly castration sensitive. However, when we silence Ascl1 in established NEPC tumors (by building an inducible *Ascl1* rescue allele in *$Ascl1^{KO}$* organoids, then withdrawing doxycycline), tumors regress transiently but then regrow as Ascl1-negative NEPC. Hence, organoid modeling revealed that the timing of intervention in lineage plasticity matters, a study that would have been difficult to perform in GEMMs.

The plasticity work presented so far has focused on the initiation of plasticity. To address the requirements for maintenance of plasticity, we developed a panel of mouse and human tumoroids that stably maintain their parental PRAD or NEPC lineage and can be expanded for large scale chromatin landscape studies, as well as functional screens. We first used a panel of histone marks to examine the chromatin landscape across isogenic pairs of PRAD and NEPC mouse tumoroids and found large differences in enhancer usage. We then used RNA-seq and ATAC-seq to identify 25 NEPC TFs that were more highly expressed in NEPC versus PRAD or displayed selective motif enrichment at NEPC-specific ATAC peaks. To

assess the dependence of NEPC organoids on these 25 TFs, we conducted a mini CRISPR screen and identified several TFs required for proliferation, including *Ascl1*, Foxa1, *Foxa2* as well as others (*Foxa1* is of interest because it displays dependency in PRAD as well as NEPC but regulates a distinct set of target genes in each lineage).

In extending these TF dependency studies to human NEPC tumoroids, we find patient-specific differences that reflect the heterogeneity we described in our recent single cell analysis of human clinical specimens. For example, many of TF dependencies in mouse ASCL1+ tumoroids translate to human ASCL1+ tumoroids, but not to human NEPC tumoroids with mixed PRAD-NEPC phenotypes (e.g. NEUROD1). As I will describe in my talk, it may be possible to address this heterogeneity by targeting chromatin modifying enzymes that these TFs must partner with to drive transcription.

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Charles L. Sawyers, MD

1993-2006	Asst to Full Professor, Dept of Medicine University of California Los Angeles
2002-present	Investigator, Howard Hughes Medical Institute
2006-present	Chair, Human Oncology and Pathogenesis Program Memorial Sloan Kettering Cancer Center

PATIENT-SPECIFIC APOPTOTIC INDUCTION AND IMMUNE CELL-BASED TARGETING OF CELL SURFACE MOLECULES OVERCOME TREATMENT RESISTANCE IN LEUKEMIA

Fumihiko Ishikawa
Department of Comprehensive Pathology, Tokyo Medical and Dental University
1-5-45 Yushima, Bunkyo-ku, Tokyo, JAPAN 113-8510
Laboratory for Human Disease Models, RIKEN
1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, JAPAN 230-0045
(fumihko.ishikawa@riken.jp)

We have aimed to understand how relapse occurs in leukemia and, by understanding mechanisms underlying relapse, our largest goal is to develop new and efficient targeted therapies for clinically-aggressive, treatment-resistant leukemia. To this end, it is crucial to understand biological diversity and genomic complexity of human malignancies then to provide individual patients with most optimal treatment. Among hematologic malignancies, acute myeloid leukemia (AML) has been believed to develop through multiple hits of somatic mutations at immature level of hematopoiesis such as hematopoietic stem/progenitor cells. We have confirmed that transplantation of purified human hematopoietic stem cells (HSCs) and leukemic stem cells into newborn immune-deficient NOD/SCID/Il2rgKO (NSG) mice results in reconstitution of normal human immunity and malignant hematopoiesis, respectively. While we recapitulate patient-specific disease status in mice, we could also functionally define cell populations that are enriched for normal hematopoietic stem cells (HSCs) and leukemia-initiating cells (LICs) present in individual patient samples. Identification of human HSCs and LICs was followed by multi-omics and bioinformatic analysis connecting multiple levels of DNA, RNA and protein. With such integrative omics analysis, we identified molecules and pathways operative in acute myeloid leukemia (AML)-initiating cells as potential therapeutic targets. In particular, we uncovered links between leukemia-initiating genetic events such as mutations and chromosomal aberrations in the nuclei and survival molecules in the mitochondria (BCL-2 and MCL-1) and cytosol (IAPs and AURKB). In vitro killing assay for patient LICs and in vivo treatment studies using NSG xenograft models demonstrated that leukemic cells harboring mutations in NRAS and CBL and chromosomal aberrations such as t(3;3) depend more on IAPs for survival, while those

with IDH1 mutations depend on BCL2. Therefore, inhibition of critical survival molecules such as IAPs and BCL2 enabled us to eradicate patient leukemic cells efficiently in multiple organs such as bone marrow, spleen and peripheral blood of PDX models.

In addition to discovery of association between genetic aberrations and vulnerability molecules, we have sought to take advantage of immune system which can potentially suppress disease activity for longer-term via memory function. Analysis of clinical specimens derived from AML patients who received hematopoietic stem cell transplantation demonstrated a direct correlation between in vivo acquisition of memory T cell phenotype with maintenance of long-term remission, as well as CXCR4 expression with stem cell memory and central memory phenotype in CD4 T cells. In contrast, patients who failed to achieve long-term remission have T cells with exhaustion markers such as cell cycle related molecules, MKI67 and TOP2A, as well as immune-checkpoint molecules, PD1, TIM3 and LAG3. CAR-T cell therapy has emerged as a promising cell therapeutic option especially for patients with B cell leukemia and lymphoma.

We also needed to find cell surface molecules that are expressed by leukemic cells. Among multiple cell surface proteins, we found that expression of CD25 is negatively associated with patient survival indicating that inhibition of the molecules may address clinical unmet needs. Therefore, we have established multiple antibody clones that can firmly bind to CD25, alpha chain of IL2 receptor complex. It is also intriguing because structures of IL2 receptor complex are distinct between leukemic cells and normal cells such as regulatory T cells. Cryo-electron microscopy demonstrated how different antibody clones bind to alpha chain of IL2 receptor. We then designed chimeric antigen receptor (CAR) construct for a lenti-viral vector for specific binding of human T cells to disease hematopoietic c cells. Furthermore, to confer longevity and robustness to engineered T cells, we prepared CXCR4-expressing CAR-T cells targeting CD25/IL2RA. Single intravenous injection of CXCR4-expressing CD25 targeting CAR-T cells into AML PDX models resulted in significantly better leukemia cell elimination than conventional CAR-T cells. Mass cytometry analysis and single cell RNA sequencing analysis demonstrated that CXCR4 expression and signaling helped CAR-T cells achieve memory formation. Moreover, we found that reduction of leukemic cell burden through inhibition of vulnerability molecules followed by CXCR4-expressing CAR-T infusion targeting cell surface molecules led to highly effective leukemia eradication in vivo.

The strategies targeting vulnerability molecules as well as cell surface targets are promising precision therapy options for poor prognosis leukemia with diverse mutational profiles and multiple lineages.

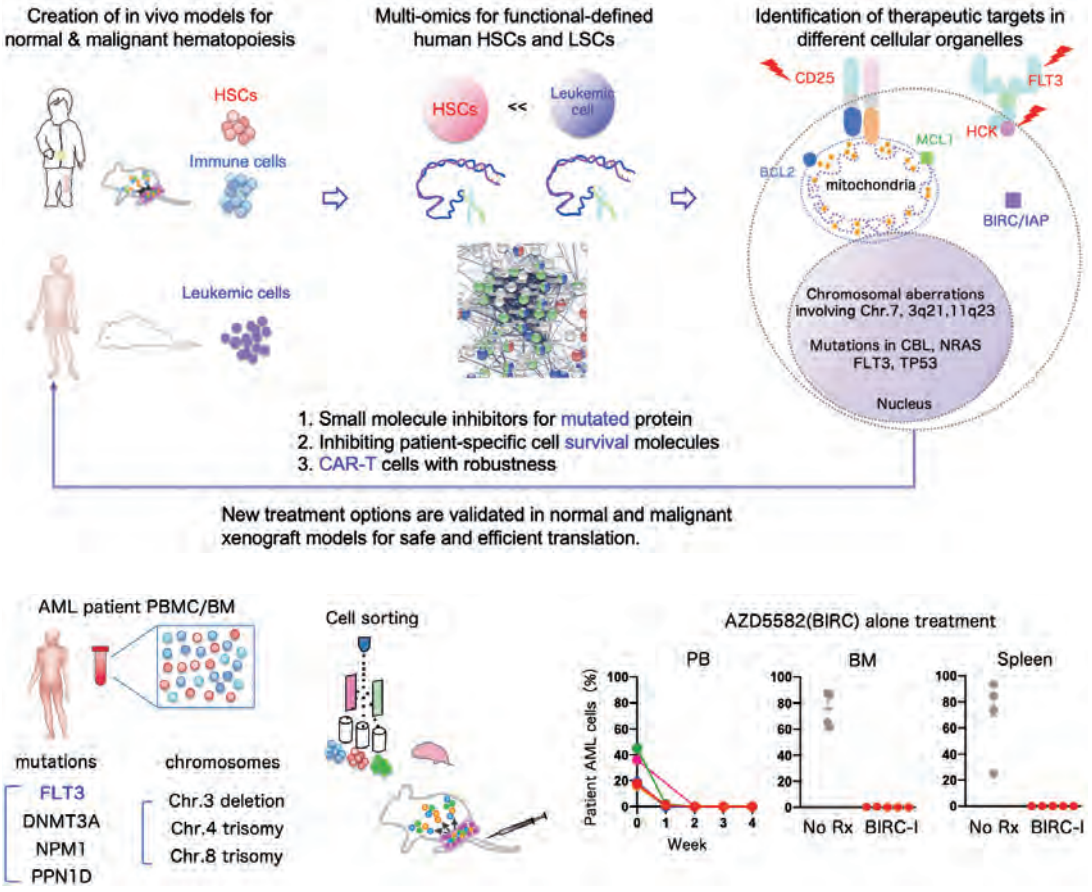

Figure Developing novel precision medicine strategies to improve outcomes in high-risk leukemia through a cross-disciplinary approach

(Upper) Through creation of in vivo models for normal and malignant human hematopoiesis, we identified human stem cells in normal and diseased context. Sequencing of multi-layers and computational analysis led us to find therapeutic targets in cell membrane, mitochondrial inner membrane and cytosol of leukemic cells. We could validate new therapeutic strategies in Humanized Mice and PDX models before translating into clinical medicine.

(Lower) We have recapitulated leukemic status of a patient harboring four mutations and three chromosomal aberrations in NSG PDX model. Inhibition of a critical survival molecule, BIRC gene, resulted in efficient killing of patient-derived leukemic cells in multiple organs of the PDX model.

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Fumihiko Ishikawa, MD, PhD

1998-2002	Postdoctoral Fellow, Makio Ogawa Lab, Medical University of South Carolina
2005-2006	Instructor, First Department of Internal Medicine, Kyushu University
2006-present	PI, Laboratory for Human Disease Models, RIKEN
2023-present	Professor, Department of Comprehensive Pathology, Institute of Science, Tokyo

NOVEL MECHANISMS OF ACUTE MYELOID LEUKEMIA PATHOGENESIS

Margaret A. Goodell
Department of Molecular and Cellular Biology
Baylor College of Medicine
(goodell@bcm.edu)

Acute myeloid leukemia (AML) in adults develops when a series of mutations occur that inhibit cellular differentiation and drive the expansion of progenitor cells. While the common mutations are well known, how they function to drive this malignancy is still poorly understood.

Nucleophosmin 1 (NPM1) is the most frequently mutated gene in adult acute myeloid leukemias (AML), yet the mechanisms through which these mutations drive leukemic transformation remain unresolved. NPM1 is normally found in the nucleolus where it directs active ribosomal biogenesis. Essentially all NPM1 mutations result in a 4-base insertion in one allele of the C-terminal exon, causing a frameshift that generates a novel nuclear export sequence (NES) in mutant NPM1 (termed "NPM1c"). This NES is bound by Exportin 1 (XPO1), the primary exportin, resulting in NPM1c export to the cytoplasm. Despite its eviction from the nucleolus and nucleus, NPM1c paradoxically drives a characteristic HOXA/MEIS1 gene expression program essential for leukemia (Brunetti 2018). Knockout of mutant NPM1 downregulates gene activation, leading to myeloid differentiation and cell growth arrest. Unlike other AML subtypes sharing this gene expression program, such as leukemias driven by nucleoporin (e.g., NUP98) fusions and KMT2A rearrangements, NPM1c does not have a clear association with chromatin regulation or nuclear localization. Notably, these subtypes often share therapeutic sensitivities to MENIN inhibitors (Uckelmann 2023) and XPO1 inhibitors, suggesting a common mechanism underlying leukemogenesis.

Wild-type NPM1 forms biomolecular condensates in the nucleolus. The formation of biomolecular condensates, an area of increasing interest, is thought to be a ubiquitous biophysical phenomenon governing nearly all cellular activities, serving as bioreactors,

sensors, signaling hubs, and more. Examples include nucleoli, PML nuclear bodies, and nuclear pore complexes, which act as organizing centers to enrich the local concentration of specific proteins and facilitate distinct cellular activities. Condensates are liquid-like phases formed from biomolecules, similar to oil phase separating from water but with higher complexity. Much of the field has focused on homotypic interactions involving intrinsically disordered regions (IDRs). However, recent studies have highlighted the importance of heterotypic interactions in driving condensates in cells. Multivalency via oligomerization is a common feature of condensate proteins (Josh paper). For example, NPM1 contains an N-terminal self-pentamerization domain, a large IDR with alternating acidic and basic tracts, and an RNA binding domain (RBD). These motifs are implicated in NPM1 phase separation in vitro, but only the pentamerization domain and RBD are critical for driving heterotypic phase transitions that form the nucleolus in cells. Given that wild-type NPM1 forms biomolecular condensates in the nucleolus, we considered whether NPM1c would also participate in such entities related to AML.

Over the last two decades, several contradictory models have been proposed to connect NPM1c localization and function, including cytoplasmic export of key nuclear proteins, loss of function of wild-type NPM1 (NPM1wt), and direct chromatin binding in the nucleus (Brunetti 2019). Whether NPM1c is meaningfully found in the nucleus and whether it interacts with NPM1wt are ongoing questions requiring rigorous quantitative assessments. Robust data linking any proposed mechanism for NPM1c activity and its influence on cell fitness in models of NPM1-mutant AML is lacking, slowing the development of new targeted therapies.

Furthermore, the connection between NPM1-mutant AML and other subtypes of leukemias driven by nucleoporin and KMT2A oncofusions remains unclear. A major challenge is identifying a common targetable factor underlying leukemogenesis across multiple disease subtypes . In this study, we used high resolution microscopy to study whether NPM1c forms nuclear condensates in models of NPM1-mutant AML. We observed puncta in the nucleus consistent with many properties of phase-separated condensates. The presence of these condensates is linked to some leukemic properties. These condensates have some similarities to other puncta formed by gene fusions involving NUP98 and some other leukemia-associated gene fusions.

Together, our data provide unprecedented clarity into the dynamic role of NPM1c in AML and suggested that biomolecular condensates are a shared feature of additional subtypes of leukemias that may serve as a new therapeutic vulnerability. More broadly, this work highlights important biophysical principles explaining how distinct oncogenic condensates can be driven by relatively small genetic events. Finally, we provide an experimental framework to determine whether multiple reported pathogenic condensates

may coalesce to establish a common targetable mesoscale structure.

How do condensates with NPM1c work? Previous work has shown that NPM1c degradation leads to rapid reduction of nascent transcripts from NPM1c-bound target genes and alters chromatin modifications, yet a specific mechanism of action has not been resolved (brunetti). To that end, we identified several proteins – including XPO1 and others that localize to these puncta and are implicated in its integrity.

Our study reveals new insights into the mechanism of action of targeted therapies with clinical promise in NPM1-mutant AML. XPO1-inhibitors break the NPM1c-XPO1 interaction, leading to the relocalization of NPM1c from the cytoplasm to the nucleolus as previously described (Brunetti 2018). Here we also demonstrate that XPO1-inhibition causes condensate dissolution. Our study suggests that the activity of XPO1-inhibitors in treating disease is a result of condensate disruption, offering new avenues for therapy of this devastating type of leukemia.

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Margaret A. Goodell, Ph.D.

1991-1997	Postdoctoral Fellow, Whitehead Institute for Biomedical Research, Cambridge, MA, and Harvard Medical School, Boston, MA
1997-2002	Assistant Professor, Center for Cell and Gene Therapy, Departments of Pediatrics and Molecular & Human Genetics, Baylor College of Medicine
2002-2006	Associate Professor, Center for Cell and Gene Therapy, Departments of Pediatrics and Molecular & Human Genetics, Baylor College of Medicine
2005-Present	Director, Stem Cells and Regenerative Medicine Center, Baylor College of Medicine
2006-Present	Professor, Departments of Molecular and Cellular Biology, Molecular and Human Genetics, Pediatrics, and Pathology and Immunology; Center for Cell and Gene Therapy, and Dan L. Duncan Cancer Center, Baylor College of Medicine
2019-present	Chair, Department of Molecular and Cellular Biology, Baylor College of Medicine

MOLECULAR MECHANISM GENERATING CANCER-ASSOCIATED FIBROBLASTS AND FIBROTIC DISORDERS

Hideyuki Saya
Oncology Innovation Center, Fujita Health University
1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
(hsaya@fujita-hu.ac.jp)

The tumor microenvironment consists of various cells, among which cancer-associated fibroblasts (CAFs) are known to influence tumor growth and progression by producing inflammatory cytokines, growth factors, and extracellular matrices. Unlike normal fibroblasts, CAFs express α-smooth muscle actin (αSMA) and are thought to originate from resident fibroblasts as well as epithelial cells, mesenchymal stem cells, adipocytes, and even cancer cells. However, the molecular mechanisms underlying this conversion remain unclear. Additionally, many fibrotic disorders that occur in various organs are driven by αSMA-positive activated fibroblasts similar to CAFs, suggesting the involvement of common molecular pathways.

In the microenvironment of a mouse osteosarcoma model [1], we found that numerous adipocytes undergo dedifferentiation and conversion into CAF-like cells. Moreover, we discovered that culture supernatant from highly tumorigenic osteosarcoma cells induces the conversion of adipocytes into CAF-like cells. Through enrichment analysis of gene expression data, we attempted to identify the molecular pathways driving this conversion. We also investigated whether the identified molecules are responsible for inducing activated fibroblasts and promoting fibrosis in adipose and lung tissues.

We previously identified a novel regulatory mechanism of adipocyte differentiation. The regulation of the transcriptional coactivator MKL1 (megakaryoblastic leukemia 1) by actin cytoskeleton dynamics drives adipocyte differentiation mediated by peroxisome proliferator–activated receptor γ (PPARγ), a master transcriptional regulator of adipogenesis [2]. We also found that the disruption of actin stress fibers through the inactivation of RhoA-ROCK signaling induces a rapid increase in monomeric G-actin, leading to the interaction of

G-actin with MKL1. This prevents the nuclear translocation of MKL1, allowing the expression of PPARγ and subsequent adipogenic differentiation. Our findings thus provide new mechanistic insights into the relationship between actin dynamics and transcriptional regulation during cellular differentiation (Figure 1).

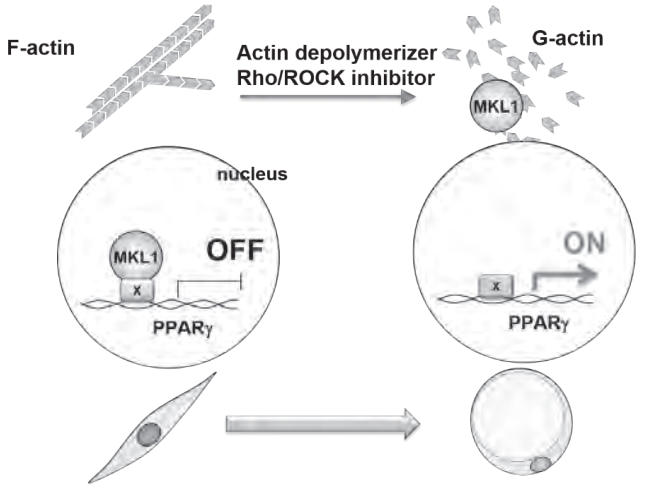

Figure 1 Mechanism of adipocyte differentiation driven by actin cytoskeleton dynamics.

Enrichment analysis of gene expression data revealed that MKL1 plays a major role in converting not only adipocytes but also epithelial cells into CAF-like cells. We created a lineage-tracing mouse model in which MKL1 is activated in adipocytes and observed that these cells undergo lipolysis and rapidly convert into αSMA-positive activated fibroblasts. Furthermore, when fibrosis was induced in lung epithelial cells using bleomycin, we observed an increase in MKL1 activation and the expression of downstream molecules, including αSMA. We also created a mouse model in which MKL1 is activated in lung epithelial cells, successfully inducing a condition similar to human pulmonary fibrosis. These findings demonstrate that the activation of MKL1 through its expression and nuclear translocation is a key driver in the induction of CAFs and fibrotic disorders. In this presentation, I will also discuss the microenvironmental changes that activate MKL1 and identify which downstream molecules play a principal role in the induction of activated fibroblasts.

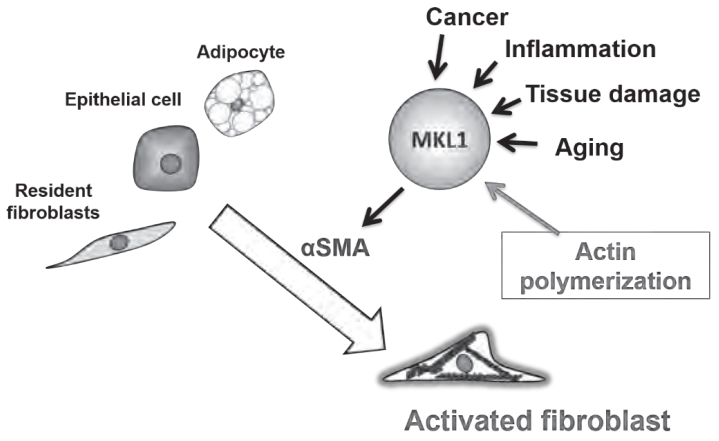

Figure 2 MKL1-mediated induction of activated fibroblasts

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Hideyuki Saya, MD, PhD

1975-1981	Kobe University School of Medicine (MD)
1983-1987	Kobe University Graduate School of Medicine (PhD)
1987-1988	Postdoctoral Fellow, Brain Tumor Research Center, University of California, San Francisco
1988-1994	Assistant Professor, Department of Neuro-Oncology M.D. Anderson Cancer Center
1994-2006	Professor and Chairman, Department of Tumor Genetics and Biology, Kumamoto Univ. School of Medicine
2007-2022	Professor, Institute for Advanced Medical Research Keio Univ. School of Medicine
2021-2023	President, Japanese Cancer Association (JCA)
2022-present	Director, Oncology Innovation Center, Fujita Health University

THE EMERGENCE OF A MALIGNANT NICHE IN CANCER

Dana Pe'er, PhD
Investigator, Howard Hughes Medical Institute
Chair, Program in Computational and Systems Biology Director, Gerry Metastasis and Tumor Ecosystems Center
Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center

Cancer cells face many hurdles during tumor growth and dissemination. They must overcome internal genomic controls on growth and cellular identity, while managing external stresses by adapting to new tissue environments, evading immune surveillance, and resisting therapy. Cellular plasticity is a critical non-genetic driver of disease, providing cancer cells with the ability to develop resistance to therapeutic challenge and to adapt to new environments. Lineage transformation, for example, has been associated with more aggressive metastatic spread in lung cancer, and with resistance to second-line therapies in recurrent prostate cancer [1–3]. It is also becoming clear that cancer cells alter their environment to favor their growth and suppress immunity. Our group has been investigating the origins of plasticity and its consequences on cellular behavior and the emergence of a malignant cellular niche.

We applied single-cell RNA and chromatin accessibility sequencing to study tumor initiation in genetic mouse models of pancreatic ductal adenocarcinoma (PDAC) [4]. This work revealed that oncogenic *Kras* mutation generates a diversity of novel cells states, including a highly plastic neoplastic progenitor population. We quantified epigenetic plasticity as the diversity in transcriptional phenotypes that is enabled or restricted by a given epigenetic accessibility landscape. These plastic cell states are enriched for open chromatin near cell-cell communication genes encoding ligands and cell-surface receptors, suggesting an increased propensity to communicate with the microenvironment. Our algorithm, Calligraphy, identifies modules of covarying receptor and ligand genes, enabling us to find a *Kras*-driven feedback loop between cancer cell and immune subpopulations in the pancreatic epithelium that is initiated by IL33 signaling. Inhibiting IL33 in a mouse

model blocked tumor progression.

To further probe tissue remodeling in tumorigenesis, we are examining the spatial dynamics of *p53* tumor suppressor. We have found that only a small fraction of pancreatic cells activate *p53*. These cells are in a progenitor-like cell state and are associated with a fibrotic, immunosuppressive microenvironment. We developed a mouse model that marks spontaneous *p53* tumor suppressor loss and showed that *p53*-deficient cells co-opt tissue regenerative programs. *p53* inactivation enables these progenitor-like cells to persist, maintain active *Kras* signaling and transform. Using a spatially aware test of communication module enrichment, we find evidence for local spatial feedback between cells. This remodeling can be disrupted by macrophage depletion, and depleting progenitors by inhibiting *Kras* entirely dissipates fibrotic environments, with a significant effect on survival. Thus, plastic progenitor-like cells remodel their microenvironment through robust cell-cell circuits, and removing any component is sufficient to dismantle the entire niche.

Our single-cell genomic studies of metachronously resected primary and metastatic tumors from colorectal cancer patients also provide compelling support for the role of plasticity in metastasis [5]. We observe that primary tumor cells express canonical intestinal fates, but eventually take on unexpected non-intestinal fates associated with diverse differentiated lineages, such as neuroendocrine and squamous identities. Non-canonical fates are greatly enriched in metastases. Their acquisition is associated with poor prognosis and can be accelerated by exposure to chemotherapies in patients and patient-derived organoids. The transition to non-canonical fates occurs via a dedifferentiated fetal-like intermediate state that is itself associated with poor prognosis, and we find that the progression is conserved across our patients as well as an independent cohort. The fetal state displays evidence of peak epigenetic priming for non-canonical states, and a maximal propensity to send and receive signals from the tumor microenvironment, suggesting specific mechanisms for how plasticity is manifested in colorectal cancer. We further identify *PROX1* as a transcription factor that normally functions to repress non-canonical fates, but that allows the expression of non-canonical gene programs when knocked down in patient-derived colorectal cancer organoids.

As a complementary approach, we have assayed primary and multiple metastatic tumors from as individual with PDAC by rapid autopsy. By developing novel computational tools for phylogenetic inference from noisy single-cell gene expression and matched whole-exome sequencing data, we can now compare relationships between cellular populations defined by clonal lineage and those defined by phenotype in this single cancer system. Our work reveals that cancer cells are not constrained by their clonal history; rather, they can take on diverse phenotypes. Using archetypal analysis, we have identified both common and unique gene programs across metastatic organ sites, including shared epithelial-mesenchymal

transition programs and distinct metabolic programs. We find that cancer cells can take on metabolic characteristics of the local neighborhood in the peritoneum and stomach, consistent with a highly plastic ability to activate gene programs not found in the pancreatic epithelium.

Our efforts reveal that cellular plasticity is a key feature of multiple cancer types, which enables cell-fate transitions in tumor progression and metastasis. Plastic progenitor-like cells remodel their local environments through mutually reinforcing feedback from neighboring cells that define a fibrotic and immunosuppressive malignant niche.

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Dana Pe'er, PhD

2022-present	Investigator - Howard Hughes Medical Institute
2019-present	Alan and Sandra Gerry Endowed Chair - Computational and Systems Biology Program, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center
2017-present	Professor - Physiology and Biophysics, Weill-Cornell Graduate School of Medical Sciences
2016-present	Scientific Director - Single-cell Analytics Innovation Lab, Sloan Kettering Institute
2016-presen	Chair and Member - Computational and Systems Biology Program, Sloan Kettering Institute
2016-present	Professor - Gerstner Sloan Kettering Graduate School of Biomedical Sciences
2016-2024	Director - Alan and Sandra Gerry Center for Metastasis and Tumor Ecosystems, Sloan Kettering Institute
2016-2021	Adjunct Professor - Biological Sciences, Columbia University
2016-Professor	Dept of Biological Sciences and Dept of Systems Biology, Columbia University
2012-2016	Associate Professor - Department of Biological Sciences, Columbia University
2006-2012	Assistant Professor - Department of Biological Sciences, Columbia University
2003-2006	Postdoctoral Fellow - Church Lab, Dept of Genetics, Harvard Medical School
1999-2003	PhD Computer Science - Hebrew University of Jerusalem
1996-1999	MSc Computer Science - Hebrew University of Jerusalem
1992-1995	BSc Mathematics - Hebrew University of Jerusalem

MONITORING TUMOR EVOLUTION IN SPACE AND TIME USING A CRISPR-BASED MOLECULAR RECORDER

Jonathan Weissman
Howard Hughes Medical Institute and
Whitehead Institute for Biomedical Research
Suite 661B, 455 Main Street, Cambridge, MA02142, USA
(weissman@wi.mit.edu)

Tumor evolution is driven by progressive acquisition of genetic and epigenetic alterations that enable uncontrolled growth and spread. Accurate reconstruction of cancer phylogenies has long been a goal of cancer research for its promise to reveal the history of these events. Many efforts have provided valuable insights, but have also been technically limited in resolving elaborate subclonal dynamics, deeply rooted phylogenies, and long timescales.

To address these limitations, we have designed, built, and optimized a molecular recorder technology, which makes it possible in principle to capture critical features of a cell's life—environmental insults, developmental decisions, external and internal signals, ancestry and progeny—in a defined and compact region within its genome. These recordings can then be read out in a massively parallel manner using droplet-based single-cell RNA-sequencing technology. Just as the flight recorder of a plane provides critical forensic information about the normal operation of a plane and how these operations failed, our molecular recorder provides an unprecedented view of normal biology and disease. We have built upon our molecular recorder technology in several ways that make it both more easily deployable to study various biological contexts of interest and more information-rich for larger, longer, and deeper recording experiments. We have also further developed a computational pipeline for processing lineage tracing data from large recording experiments, algorithms for phylogenetic tree reconstruction, and a framework for interpreting meaningful biology from lineage tracing data. Our Cas9-based "molecular recorder" technology allows us to reconstruct highly detailed lineages *in vivo* of tens of thousands of cells over months.

We have applied our Cas9-based lineage tracer to study tumor evolution in two setting:

(1) The study of metastatic spread in a lung cancer xenograft mouse model revealing the

underlying rates, routes, and drivers of metastasis (ref. 1). Our studies revealed a stark heterogeneity in metastatic capacity, arising from preexisting and heritable differences in gene expression and identified molecular drivers of metastasis.

(2) The study of tumor evolution in a mouse model of non-small cell lung cancer in which an oncogenic Kras mutation and homozygous loss of the P53 tumor suppressor gene is initiated sporadically in the adult mouse (ref. 2). Our Kras/P53 studies enabled us to track tumor evolution from single transformed cells to metastatic tumors at unprecedented resolution. We explore how tumor plasticity drives intratumoral heterogeneity, the stereotypical trajectories that tumors take during progression, and the origin of metastases. We found that KP tumors are driven by continuous selection and expansion of rare subclones and that loss of the initial, stable alveolar-type2-like state was accompanied by transient increases in plasticity followed by clonal sweep of rare, stable subclones capable of metastasizing to distant sites (Figure). We further showed that tumors develop through stereotypical evolutionary trajectories, and perturbing additional tumor suppressors accelerates tumor progression by creating novel evolutionary paths. Finally, our data indicate that metastases arise from deep within expanded subclones. Overall, our study supports a hierarchical model of tumor evolution, and more broadly enables the in-depth study of tumor progression in vivo.

In the above lineage tracing efforts, we focused on reconstructing lineage from dissociated tumor cells in which all spatial information is lost. Tumor progression, however, is characterized by dynamic interactions between cancer cells and their surrounding tumor microenvironment. Studying the spatiotemporal evolution of tumors can provide insights into how intrinsic changes to cancer cells and extrinsic changes to the microenvironment cooperate to drive different stages of tumor progression. Accordingly, we have more recently coupled high-resolution spatial transcriptomics and evolvable lineage-tracing to elucidate how tumor expansion, plasticity, and metastasis co-evolve with microenvironmental remodeling in a *Kras;p53*-driven model of lung adenocarcinoma. We find that following an initial increase in tumor plasticity, rapid tumor expansion leads to a hypoxic microenvironment that is associated with immunosuppressive and fibrotic cell states. This expansion-associated remodeling of the tumor microenvironment, in turn, is associated with the emergence of a stable, pro-metastatic cancer cell state. Furthermore, spatially-resolved lineage analysis of metastatic progression revealed that metastases arise from a spatially-confined subclone and continue to remodel the distant metastatic niche into a fibrotic, collagen-rich microenvironment. Together, our spatiotemporal tumor analysis describes a rich compendium of data integrating complementary spatial assays and lineage-tracing to profile lung adenocarcinoma and reveals insights into how sequential changes in cancer cell state and microenvironmental structure integrate to promote tumor progression.

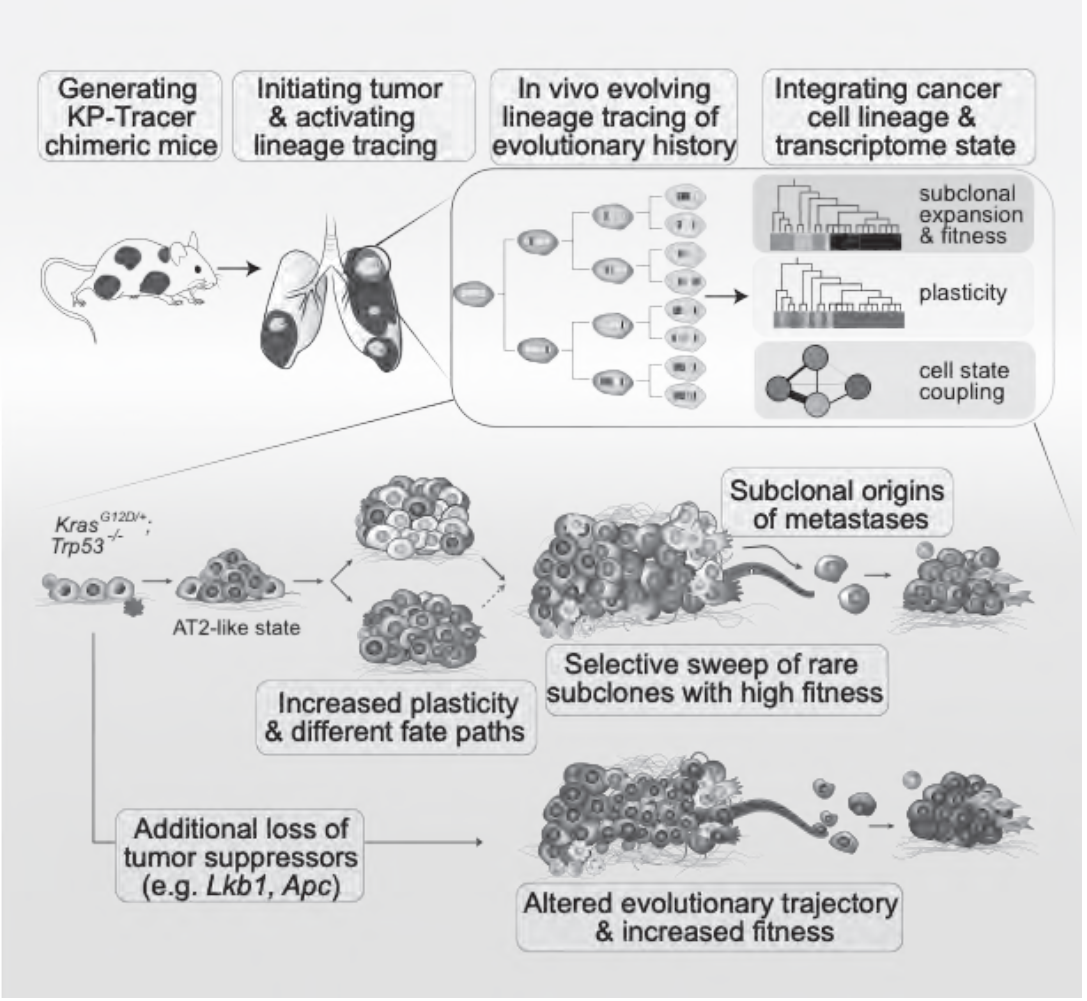

Figure Schematic describing the use of our single cell lineage tracing system to study tumor initiation and progression

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PMID: 35523183; PMCID: PMC9452598.

Jonathan Weissman, PhD

1993-1996	Postdoctoral Fellow, Yale University, New Haven, CT
1996-2000	Assistant Professor, Departments of Cellular & Molecular Pharmacology, and Biochemistry & Biophysics, University of California San Francisco
2000-2003	Associate Professor, Departments of Cellular & Molecular Pharmacology, and Biochemistry & Biophysics, University of California San Francisco
2000-Present	Investigator, Howard Hughes Medical Institute
2000-2020	Professor, Department of Cellular & Molecular Pharmacology, University of California San Francisco
2020-Present	Member and Landon T. Clay Professor of Biology, Whitehead Institute for Biomedical Research

FROM STEM CELLS IN LEUKEMIA TO NEURONS IN PANCREATIC CANCER

Andreas Trumpp[1, 2]

[1]*Division of Stem Cells and Cancer, German Cancer Research Center (DKFZ);*

[2]*Heidelberg Institute for Stem Cell Technology and Experimental Medicine (HI-STEM gGmbH), Heidelberg, Germany.*

(a.trumpp@Dkfz-Heidelberg.de)

In my presentation, I will cover two recent research directions: First, the heterogeneity and role of leukemic stem cells (LSC) in therapy resistance and second a cancer neuroscience study determining the role of individual peripheral neurons in pancreatic cancer (PDAC). **(1) The role of LSC heterogeneity in response and resistance to anti-BCL2 therapy.** The BCL-2 inhibitor Venetoclax (VEN) in combination with 5-AZA is currently transforming Acute Myeloid Leukemia (AML) therapy (1). However, there is a lack of clinically relevant biomarkers that predict response to 5-AZA/VEN. We identified leukemic stem cells (LSC) as primary targets of 5-AZA/VEN whose elimination determined therapy outcome (2). LSCs of 5-AZA/VEN refractory patients displayed perturbed apoptotic dependencies. We developed and validated a flow cytometry-based "MAC-Score" linking the ratio of protein expression of BCL-2, BCL-xL, and MCL-1 in LSCs. MAC-Scoring predicts initial response with a positive predictive-value of >97% associated to increased event-free survival. In summary, combinatorial levels of BCL-2-family members in AML-LSCs are a key denominator of response and MAC-Scoring reliably predicts individual patient response to 5-AZA/VEN. MAC scoring outperforms genetic predictors of poor VEN response such as TP53 mutations or complex karyotype (2). Although mutations do influence the MAC sore of LSCs, additional factors such as differentiation plasticity can mediate secondary VEN resistance and relapse. Along these lines we recently identified four distinct AML-LSC types. First, LSCs mirroring quiescent hematopoietic stem cells (HSC-LSCs) exhibit low apoptotic-priming, contributing to chemotherapy resistance. Conversely, lineage-primed LSCs maintain stemness while exploiting differentiation programs that shift the balance of BCL-2 family expression. For example, during BCL-2 inhibition, LSCs can shift lineage-priming

from BCL-2 dependent Lymphoid-Myeloid-Primed-Progenitor-LSCs (LMPP-LSCs) to Megakaryocytic-Erythrocytic-Progenitor-LSCs (MEP-LSCs). The latter are primed towards MEP-lineage output and show increased BCL-xL expression leading to Venetoclax escape. In rare, aggressive cases, patients possess LSCs outside the classical stem-progenitor state, resembling lineage-committed Monocytic/Dendritic precursors (MoDe-LSCs) expressing high levels of BCL-1. Together, AML encompasses heterogeneous LSC types beyond the canonical HSPC-myeloid state. The lineage priming of these LSCs can adapt plastically under therapeutic pressure, mediating resistance.

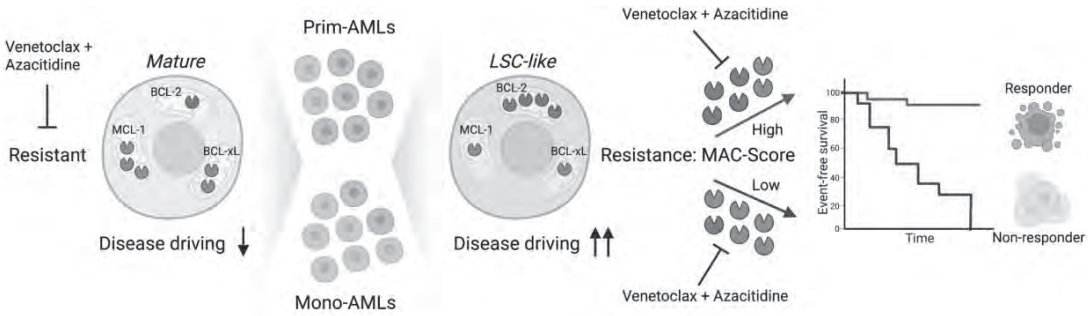

Figure 1 *MAC-scoring in LSCs predicts patient response to Venetoclax/Azacytidine therapy*

(2) Retrograde tracing and molecular analysis of single neurons invading pancreatic cancer reveal actionable dependencies. The peripheral nervous system (PNS) orchestrates organ function in health and disease. Most cancers including pancreatic ductal adenocarcinoma (PDAC) are infiltrated by PNS neurons, contributing to the complex tumor microenvironment (TME). However, neuronal cell bodies reside in various PNS ganglia, far from the tumor mass. Thus, cancer or healthy organ-innervating neurons elude current tissue sequencing datasets. To molecularly characterize PDAC-innervating neurons for the first time at single cell resolution, we developed "Trace-n-seq". This method employs retrograde tracing of axons from tissues to their respective ganglia, followed by single-cell isolation and transcriptomic analysis. By characterizing >5,000 individual sympathetic and sensory neurons with ≈4,000 innervating PDAC or healthy pancreas we reveal novel neuronal cell types and unique molecular networks distinct to pancreatic melanoma metastasis or pancreatitis. We integrate innervating neuron and TME single-cell datasets, delineate cancer mediated reprogramming of neuronal behavior, generate a Pancreatic Cancer-Nerve signature and establish a neuro-cancer-microenvironment interactome. Pharmacological denervation induces a proinflammatory TME and increased immune-checkpoint inhibitor effectiveness. Finally, Nab-paclitaxel caused intra-tumor neuropathy which attenuated PDAC growth and in combination with sympathetic denervation resulted

in synergistic tumor regression (3). Our multi-dimensional data provide new insights into the networks and functions of pancreas- and PDAC-innervating neurons, with direct clinical relevance.

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3. Vera Thiel*, Simon Renders*, Jasper Panten* et al., and Andreas Trumpp (2024). Retrograde tracing and molecular analysis of single neurons invading pancreatic cancer reveal actionable dependencies (in revision).

Andreas Trumpp, PhD

1992	PhD Thesis at EMBL, Heidelberg, Germany
1993-2000	Postdoctoral Research, University of California, San Francisco (with Prof. J. Michael Bishop)
2000-2008	Head of "Genetics and Stem Cell Laboratory, Swiss Institute for Experimental Cancer Research (ISREC), Epalinges/Lausanne, Switzerland
2005-2008	Assistant Professor (PATT), École Polytechnique Fédérale de Lausanne (EPFL), Switzerland
2008-present	Professor and Head of the Division of Stem Cells and Cancer, DKFZ Heidelberg, Germany
2008-present	Managing Director of the Heidelberg Institute for Stem Cell Technology and Experimental Medicine (HI-STEM gGmbH), Heidelberg, Germany

UNDERSTANDING OF GI CANCER INITIATION AND PROGRESSION USING ORGANOIDS

Toshiro Sato
Department of Integrated Medicine and Biochemistry
Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
(t.sato@keio.jp)

In homeostatic adult tissues, niche factors play a crucial role in governing the long-term self-renewal and diverse differentiation potential of tissue stem cells. By recapitulating these niche factors in an in vitro environment, tissue stem cells have demonstrated the capacity to assemble into stereotypic organoid structures, sustaining long-term self-renewal. Notably, a spectrum of tissue-specific niche factors has been identified by our team and others, facilitating the successful propagation of organoids derived from various adult tissues. Human tissue-derived organoids have exhibited the remarkable ability to retain the genetic and epigenetic alterations inherent in the original tissues. Moreover, these organoids have shown disease-relevant biological characteristics both in vitro and in vivo.

Our comprehensive phenotypic analysis of patient-derived organoids has unveiled intricate molecular mechanisms governing genotype-phenotype correlations in human digestive tissue cancers. In parallel, the insights gleaned from genotype-phenotype correlations are harnessed in reverse through genome editing technology. The strategic introduction of genetic mutations into normal organoids has enabled the faithful recapitulation of tumor phenotypes. In this symposium, we will present our recent research showcasing genotype-phenotype associations in patient-derived organoids and engineered counterparts.

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Toshiro Sato, MD, PhD

1991-1997	MD Keio University School of Medicine
1997-1999	Internship, Keio University Hospital
1999-2003	Internal Medicine, Graduate school, School of Medicine, Keio University
2004	PhD
2004-2005	COE Postdoctoral Researcher, Keio University School of Medicine (Supervisor: Hideyuki Okano)
2005-2006	Resident, TEPCO Hospital
2006-2007	Postdoctoral Researcher, Stowers Institute, Kansas City (Supervisor: Linheng Li)
2007-2011	Postdoctoral Researcher, Hubrecht Institute, Utrecht, the Netherlands (Supervisor: Hans Clevers)
2011-2013	Assistant Professor, Department of Gastroenterology, Keio University School of Medicine
2013-2018	Associate Professor, Department of Gastroenterology, Keio University School of Medicine
2018-2023	Professor, Department of Organoid Medicine, Keio University School of Medicine
2023-Present	Professor, Department of Integrated Medicine and Biochemistry, Keio University School of Medicine

ON THE ORIGINS AND CONSEQUENCES OF CHROMOSOMAL INSTABILITY IN CANCER

Geert Kops[1, 2, 3]

[1]**Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW), Utrecht, Netherlands;**

[2]**Oncode Institute, Utrecht, Netherlands,**

[3]**University Medical Center Utrecht; Utrecht, Netherlands**

(g.kops@hubrecht.eu)

Aneuploidy and genomic heterogeneity are hallmarks of human cancer. They are caused by a chromosomal instability (CIN) phenotype: the persistent high frequency of errors in chromosome segregation during cell divisions[1, 2] (*Figure 1*). CIN profoundly impacts tumour evolution and therapy response. We seek to understand the origins of CIN in cancer, and the consequences of CIN on cancer genome evolution, tumor initiation and tumor development. For this we use molecular and genomic approaches in cell lines, human organoid panels, and mice. For example, we showed, using 3D live imaging of patient-derived tumour organoids (tumour PDOs), that CIN is widespread in colorectal carcinomas, regardless of background genetic alterations[3]. Single-cell karyotype sequencing of tumour PDOs revealed heterogeneity in copy number alterations (CNAs) and showed that novel karyotypes evolved over time *in vitro*. The CIN phenotype and the resulting genomic alterations can be profoundly oncogenic, as we showed using our genetically engineered mouse model for CIN[4]. Using single cell karyotype and micronucleus sequencing we furthermore showed that chromosomes have an unequal probability of mis-segregating, based on their location in the interphase nucleus prior to mitosis[5]. This non-random segregation error probability may impact the trajectories of CNA evolution during cancer development. Finally, with super resolution imaging and chromatin configuration capture, we showed that the centromeres of mitotic chromosomes are functionally bipartite structures, which promotes a chromosome-spindle attachment configuration (merotely) that is often seen in cancer cells, suggesting a possible cause for CIN in cancer[6]. In my lecture, I will present our latest efforts to understand the interplay between chromosome segregation mechanisms, CIN, CNA evolution, and cancer.

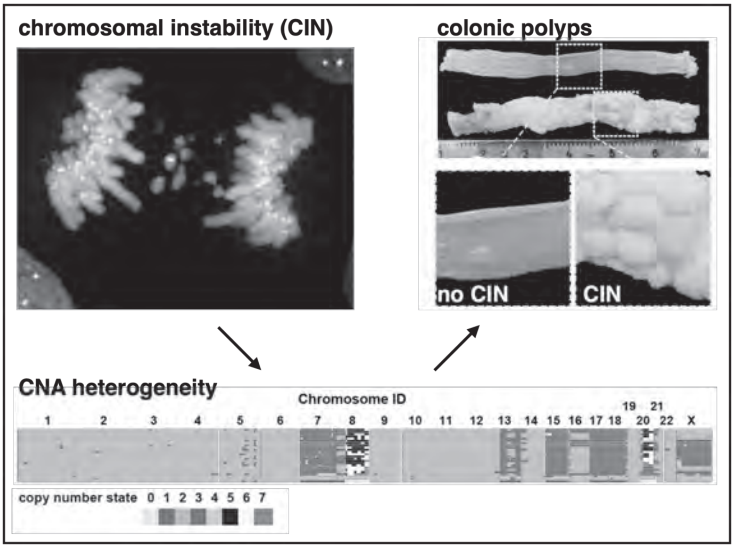

Figure 1 The relation between chromosome segregation errors (CIN), chromosomal copy number alterations (CNAs) and heterogeneity thereof, and cancer.

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Geert JPL Kops, PhD

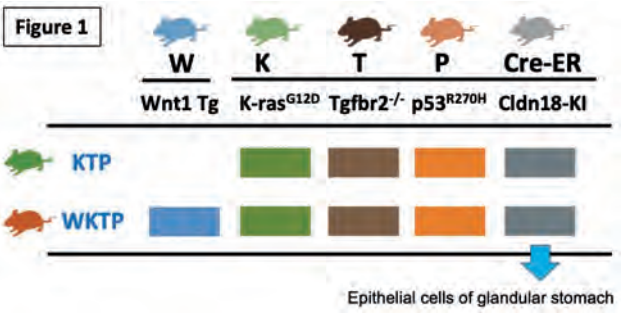
2001-2004	Postdoctoral Fellow, Ludwig Institute for Cancer Research, La Jolla, USA
2004-2011	Assistant/Associate Professor, Department of Molecular Cancer Research, UMC Utrecht, Netherlands
2011-present	Professor of Molecular Tumor Cell Biology, UMC Utrecht, Netherlands
2015-present	Group Leader, Hubrecht Institute, Utrecht, Netherlands
2018-2023	Scientific Director, Oncode Institute, Netherlands
2023-present	Director, Hubrecht Institue, Utrecht, Netherlands

TUMOR AND STROMA SIGNALING INTERACTIONS FOR INVASION AND METASTASIS

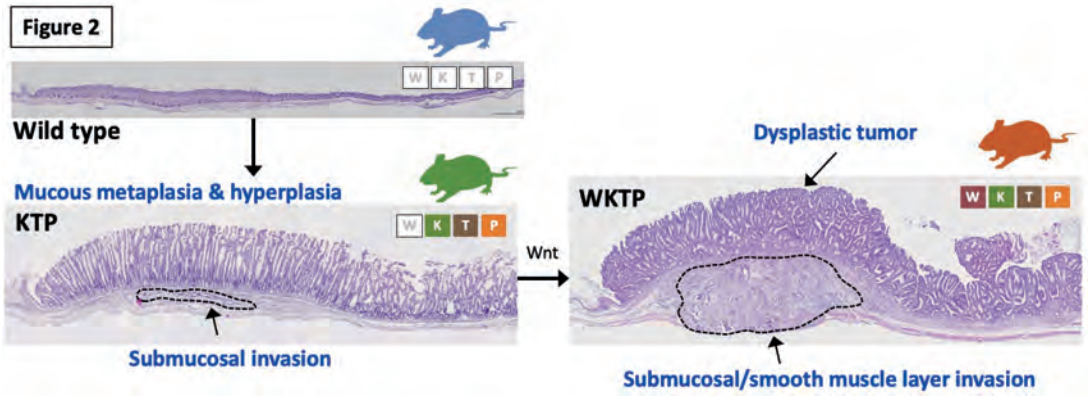
Masanobu Oshima
Division of Genetics, Cancer Research Institute, Kanazawa University
Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan
(oshimam@staff.kanazawa-u.ac.jp)

We previously constructed mouse intestinal tumor-derived organoids and demonstrated that exogenous TGFβ signaling induces cluster migration of tumor cells from the primary site [1, 2]. We also showed that malignant tumor cells activate hepatic stellate cells (HSCs) upon dissemination into the liver sinusoid, creating a fibrotic microenvironment, which is a critical step for metastatic tumor formation [3, 4]. These results indicate that interaction between cancer cells and the microenvironment are essential for malignant progression.

In this study, we examined metastatic process of gastric cancer. Gastric cancer is highly prevalent in East Asia including Japan, and the prognosis for patients with stage IV gastric cancer is alarmingly poor. Therefore, elucidating the molecular mechanisms underlying the malignant progression of gastric cancer is of significant importance. We generated mouse models carrying *Kras*^{G12D}, *Tgfbr2* $^{-/-}$ and *Trp53*^{R270H} mutations in gastric epithelia using Claudin 18-CreER mice, referred to as KTP mice. Subsequently, we crossed KTP mice with *Wnt1* transgenic mice, which express *Wnt1* in gastric mucosa, to create WKTP mice (Fig. 1). Using patient-derived organoids, it has been shown that the majority of gastric cancer cells require ligand-dependent Wnt signaling for survival and proliferation. Thus, we compared the phenotypes of KTP and WKTP mice.



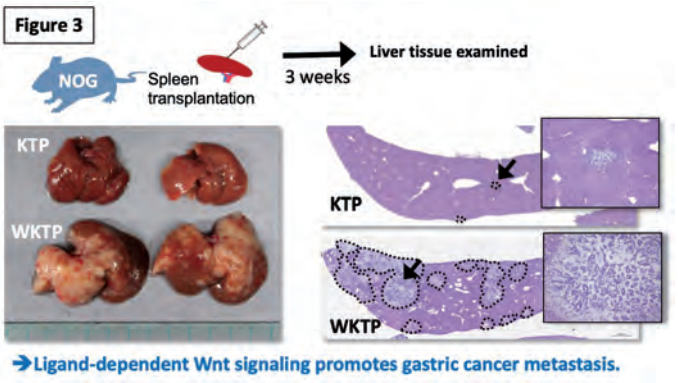
Notably, KTP mice exhibited mucous cell metaplasia and hyperplasia in the glandular



stomach with limited submucosal invasion (Fig. 2). In contrast, WKTP mice developed dysplastic gastric tumors with aggressive submucos al and smooth muscle layer invasion (Fig. 2). These observations suggest that ligand-dependent Wnt signaling plays a critical role in the development of dysplastic primary tumors in the stomach.

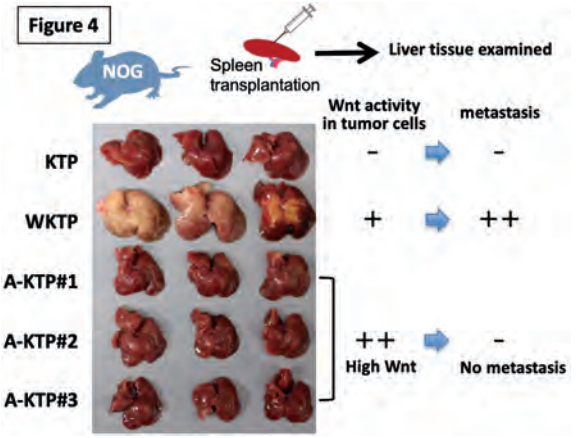
Next, we established organoid lines from gastric epithelia of KTP and WKTP mice. As expected, KTP organoids failed to proliferate in the absence of Wnt ligands in the culture media. Conversely, WKTP organoids continued proliferation without Wnt ligands. However, treatment with a POCRN inhibitor (a Wnt ligand inhibitor) significantly suppressed the proliferation of WKTP organoid cells. These results indicate that while KTP and WKTP organoids depend on Wnt ligand signaling, WKTP cells may utilize self-secreted Wnt ligands through an autocrine or paracrine manner.

To assess metastatic potential, we transplanted KTP and WKTP organoids into the spleen of mice and analyzed liver tissues. Importantly, only WKPT cells formed multiple metastatic tumor foci in the liver, whereas KTP cells did not form liver tumors (Fig. 3). Moreover, treating mice with PORCN inhibitors significa ntly suppressed metastatic tumor formation. Collectively, these results indicate that ligand-dependent Wnt signaling is crucial for gastric cancer metastasis in the liver.

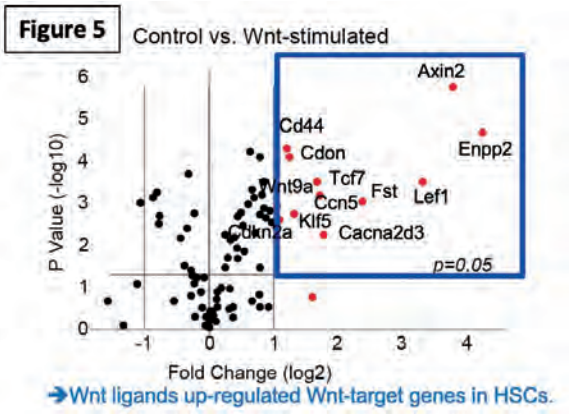


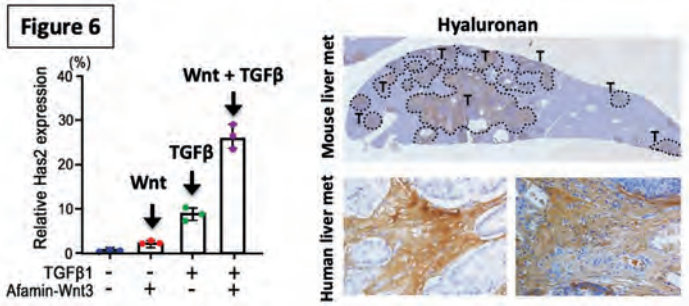
When WKTP cells are disseminated to liver, Wnt signaling may be activated not only in the tumor cells but also in stromal cells through a ligand-dependent mechanism. Therefore,

we hypothesized that Wnt signaling activation in stromal cells is also critical for metastasis. To test this, we thus disrupted *Apc* gene in KTP organoid cells by CRISPR/Cas9 system, generating A-KTP cells. Wnt signaling was constitutively and highly activated in A-KTP cells due to the *Apc* mutation, but not in the surrounding stromal cells. Notably, A-KTP cells failed to form metastatic tumors in the liver after spleen transplantation. These findings suggest that W nt activation in the stroma is essential for metastatic tumor formation, potentially through metastatic niche generation (Fig. 4).



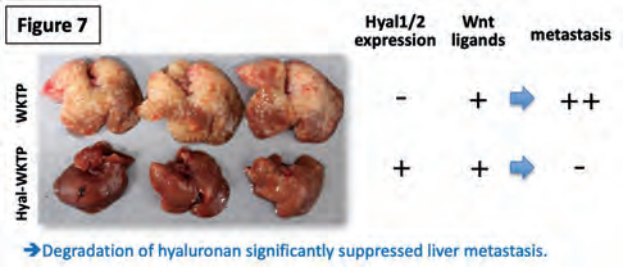
We treated HSCs with Wnt ligands (afamin-Wnt3) and observed that Wnt ligand stimulation upregulated Wnt target genes in HSCs (Fig 5, left). Interestingly, the expression of *Has2*, a hyaluronan synthase, was synergistically upregulated by Wnt ligands in combination with TGFβ. We confirmed the deposition of hyaluronan in the stroma of liver metastatic tumors derived fro m WKTP cells and human gastric cancers (Fig. 6, right). To investigate the role of hyaluronan in metastatic tumor formation, we transfected hyaluronidase, *Hyal1* and *Hyal2*, expression vectors into WKPT cells. We expected that *Hyal1*/2-expressing WKTP cells would degrade hyaluronan in the micr oenvironment. Remarkably, liver metastatic tumor formation of *Hyal1*/2-WKTP cells was significantly suppressed compared to parental WKTP cells (Fig. 7).





In conclusion, Wnt ligands expressed by gastric cancer cells activates HSCs together

with TGFβ upon dissemination to the liver sinusoid. This activation induces *Has2* expression in HSCs, leading to hyaluronan deposition in the tumor stroma, which is a crucial step for metastatic tumor formation in gastric cancer. Therefore, targeting Wnt signaling or *Has2* expression in stromal cells represents a potential preventive or therapeutic strategy against gastric cancer metastasis.



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Masanobu Oshima, DVM, PhD

1988-1992	Research Associate, Chugai Pharmaceutical Co. Ltd., Japan
1992-1997	Research Associate, Banyu Tsukuba Research Institute (Merck), Japan
1997-1999	Postdoctoral Research Fellow, Merck Research Laboratories, PA, USA
2000-2005	Associate Professor, Kyoto University Graduate School of Medicine, Japan
2005-present	Professor, Cancer Research Institute, Kanazawa University, Japan
2017-present	Professor, Nano Life Science Institute (NanoLSI), Kanazawa University, Japan

ROLE OF EXTRACELLULAR VESICLES DURING BYSTANDER CYTOTOXICITY AND BYSTANDER IMMUNITY OF ALPHA-TARGETED RADIONUCLIDE THERAPY

Jean-Pierre Pouget, Timothée David and Julie Constanzo
Institut de Recherche en Cancérologie de Montpellier (IRCM),
Inserm U1194, Institut Régional du Cancer de Montpellier (ICM),
Université de Montpellier, Montpellier, équipe labellisée Ligue contre le cancer, France.
(jean-pierre.pouget@inserm.fr)

The recent approvals by the USA Food and Drug Administration and European Medicines Agency of two new innovative targeted radiotherapies (TRTs) were based on phase 3 clinical trial data demonstrating significant improvements in progression-free survival (PFS) with **[^{177}Lu]Lu-DOTA-TATE (Lutathera™)** for patients with somatostatin receptor (SSTR)-expressing neuroendocrine tumours (NETs; NETTER-1 and NETTER-2 trials) [1, 2], and in overall survival (OS) and PFS with **[^{177}Lu]Lu-PSMA-617 (Pluvicto™)** for patients with prostate-specific membrane antigen (PSMA)-expressing metastatic castration-resistant prostate cancer (mCRPC; VISION trial)[3]. However, half of the patients do not respond to **Pluvicto™**. Furthermore, 80% of patients demonstrate stable disease following Lutathera™, while 20% experience recurrence.

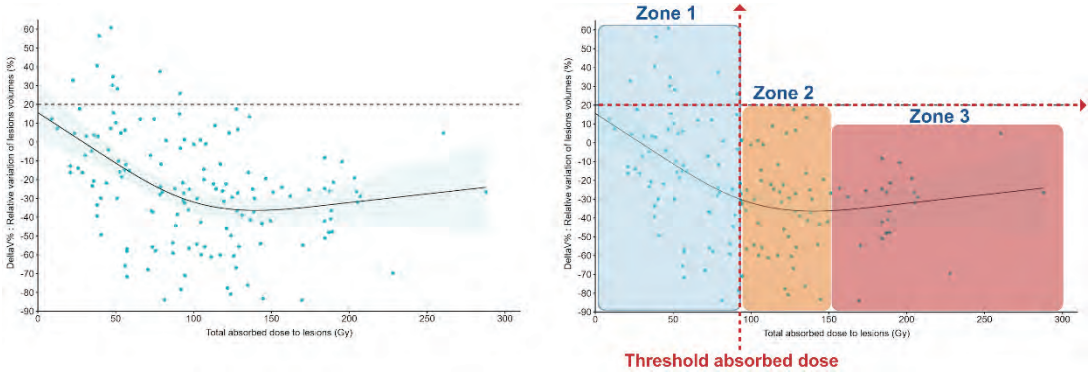

Figure 1 Lesion volume responses based on cumulative absorbed dose, with distinct zones for unpredictable, effective, and plateau zones of response.

To enhance the response to TRT, we first investigated the relationship between the irradiation dose and the response to TRT. In the study run by Hebert et al. 34 patients with neuroendocrine tumors (NETs) were treated with four cycles of **[^{177}Lu]Lu-DOTA-TATE** (7.6 GBq per cycle)[4]. Dosimetry was performed to correlate cumulative absorbed doses with changes in lesion volume measured via CT (before and at the end of treatment). NET control corresponded to volume increase <20%.

We could next categorize Lesions into three zones based on absorbed doses [5]:

1. **Zone 1 – Unpredictable Response Zone:** Absorbed doses below 90–100 Gy, where lesion volume changes do not consistently correlate with dose, and some lesions show >20% progression.
2. **Zone 2 – Effective Response Zone:** Absorbed doses above 90–100 Gy, where all lesion volume changes are <20%, indicating effective treatment.
3. **Zone 3 – Unnecessary Absorbed Dose Zone:** Absorbed doses exceeding 100 Gy show no additional benefit compared to Zone 2, suggesting a response plateau.

Figure 1 shows that TRT faces challenges in treating metastatic disease, as not all lesions reach the therapeutic threshold dose. Increasing treatment cycles (e.g., from 4 to ≥6) can raise absorbed doses for more lesions but also increases toxicity and may still leave some lesions untreated (Figures 1 and 2). Moreover, in higher-dose zones, response plateaus, offering

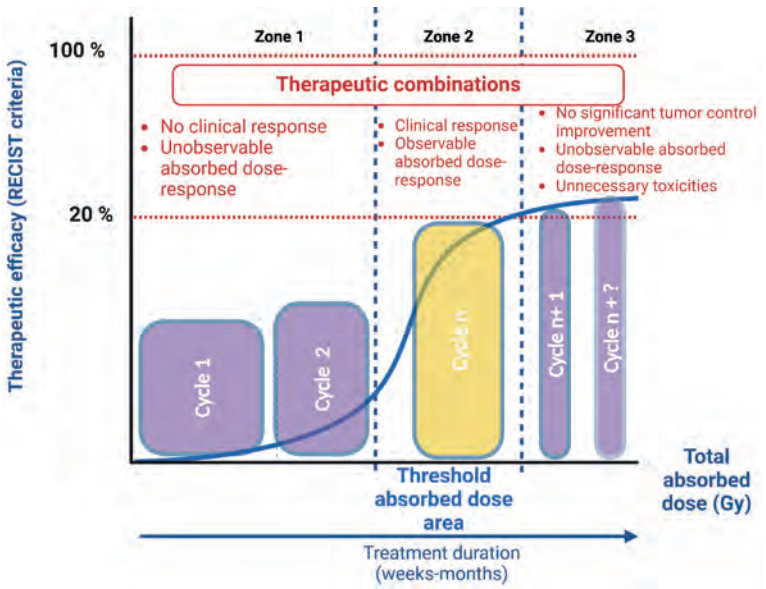

Figure 2 Tumor Control Probability (TCP) during TRT: Influence of the number of cycles to achieve the threshold absorbed dose and the effect of therapeutic combinations.

limited benefit despite higher doses. TRT generally stabilizes rather than cures the disease, with suboptimal tumor control probability (TCP). To improve outcomes, factors like heterogeneity in target expression, disease location, genetic factors, DNA repair capacity, and the tumor microenvironment must be considered to identify better therapeutic strategies (Figure 2).

Immunoactivating effects of TRT

Based on the results of Figures 1 and 2, we investigated the role of the immune system and the use of immune checkpoints as potential candidates for therapeutic combination. We showed that irradiated cells communicate with other neighboring cells, either to destroy them ("bystander cytotoxicity" effects), or to activate them, as in the case of immune cells, in which case we speak of bystander immunity [6, 7]. Bystander cytotoxic effects and bystander immunity are referred to as non-targeted effects because they are not directly observed in irradiated cells but instead occur at a distance from the irradiated cells.

Considering bystander immunity, we showed in a mammary tumour model of mice that beta-TRT using ^{177}Lu-labeled antibody effectively inhibited tutor progression across all activity levels (low, intermediate or high activity). However, even the highest activity, representing the maximal tolerated dose, failed to cure all mice within 74 days post-treatment. In tumour re-challenge experiments, tumour-free mice treated with intermediate or high activities were re-grafted. Intermediate activity provided complete protection in 30% mice, while high activity offered partial protection in 66% mice. These results highlight an inverse relationship between the injected activity level and the immune protection achieved against tumour regrowth (unpublished results). This finding was further supported by tumor microenvironment analysis, performed through immunophenotyping, which provided additional insights into the immune response dynamics. Similarly, we have demonstrated that MHC-I-driven anti-tumor immunity mitigates the effects of low absorbed doses of radiopharmaceutical therapy [8].

Impact of EVs on the immunostimulatory effects of TRT

Finally, our studies highlighted the critical role of extracellular vesicles (EVs) in the immunostimulatory effects of targeted radionuclide therapy (TRT). These small membrane-bound structures, released by irradiated tumor cells, act as carriers of information between tumor and immune cells. EVs contain a variety of biomolecules, including stress proteins, cytokines, and tumor DNA fragments, which function as danger signals to the immune system. They promote the activation of dendritic cells and antigen presentation, thereby enhancing the adaptive immune response. Additionally, EVs modulate the tumor microenvironment by recruiting cytotoxic T lymphocytes and reducing the activity of

suppressive immune cells.

Conclusion: These findings underscore the importance of EVs as key mediators of TRT-induced immunostimulation, paving the way for new therapeutic strategies combining TRT with immunotherapy.

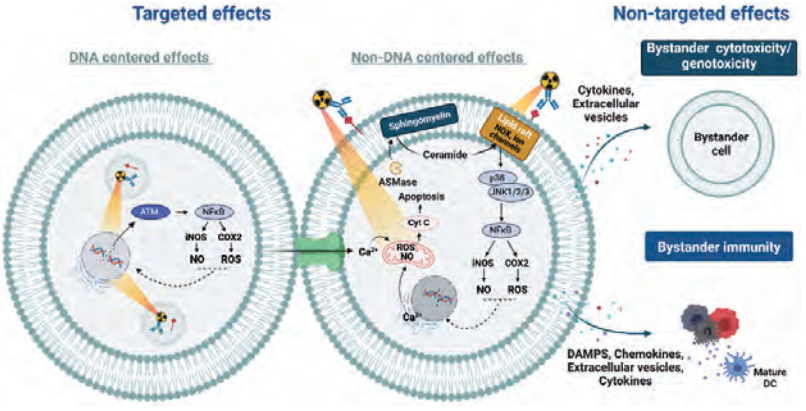

Figure 3 Overview of targeted and non-targeted effects of radiation [7–10]

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Jean-Pierre Pouget, PhD

1997-2000	PhD in Radiobiology (Paris, France)
2000-2001	Post-doctoral fellow at Bart's hospital London, UK
2001-2004	Research scientist at IRSN (Paris, France)
2004-2015	Research Scientist at INSERM (Montpellier, France)
2019	Research Director at INSERM (Montpellier, France)
2015-present	Team leader at INSERM (Montpellier, France)

ULTRA-PRECISE MOLECULARLY TARGETED RADIOPHARMACEUTICAL THERAPY FOR CANCER

Katherine A. Vallis
Department of Oncology, Oxford University
Old Road Campus Research Building, Roosevelt Drive, Oxford OX3 7DQ, UK
(katherine.vallis@oncology.ox.ac.uk)

An essential element of precision medicine is the design of molecularly targeted drugs that provide a clear basis for tumour selective cell cytotoxicity with complete or comparative sparing of normal cells. A particularly attractive strategy to achieve this goal is radiopharmaceutical therapy (RPT) in which a carrier molecule, that seeks functional or molecular targets, is labelled with an isotope that emits charged particles. There are three types of particulate radiation of consequence for RPT; β-particles, α-particles, and Auger electrons (AE), which can irradiate tissue volumes with multicellular, cellular and subcellular dimensions respectively. In some cases, mixed emitters are used to allow both imaging (γ-rays or positrons) and therapy with the same radionuclide i.e. radiotheranostics. In conjunction with SPECT (single-photon emission computed tomography) or PET (positron emission tomography) imaging, radiotheranostics provide assessment of the likelihood of a positive response to therapy before a lengthy course of treatment is undertaken. Recent advances in the field of radionuclide therapy, particularly the success of [^{177}Lu]Lu-DOTATATE for neuroendocrine tumours and [^{177}Lu]Lu-PSMA ligands for metastatic castration-resistant prostate cancer, have underscored the importance of this class of drugs in cancer medicine, and have energized the quest for new and more effective RPTs. Established RPTs and those in development, rely on linkage of the radioisotope to a tumour-seeking moiety such as a small molecule, peptide, antibody or antibody fragment.

β electron-emitters, such as lutetium-177, are widely used clinically due to their moderately high energy (0.1-2.2 MeV) and long range (0.5-10 mm) in tissue. These physical characteristics enable this class of radioisotopes to effectively irradiate tumour masses,

including those where there is heterogeneous expression of the molecular target. The success of radium-223, a calcium mimetic, in the treatment of bone metastases, has stimulated growing interest in the incorporation of other alpha-emitters, such as actinium-225 and lead-212, into RPT design. Alpha particles are emitted with high energy (5-8 MeV) and this, together with their atomic mass of 4 units (about 7300 times greater than a β-electron), accounts for the pattern of dense ionization events along their short track (40-100 μm). Atoms that are unstable due to an excess of protons may decay through nuclear "capture" of an orbital electron. The resulting vacancy is filled by an electron from an outer orbit with release of energy that, when absorbed by another electron, may lead to its ejection. An intense shower of electrons may then occur, in a phenomenon known as the Auger effect.

The advantage of AE-emitting radionuclides is their extremely short range and highly localized energy deposition, with local mean absorbed doses over the range of nm to a few μm in excess of 10 MGy (1). Intranuclear delivery of AE-emitting constructs results in radiobiological effects (RBE) similar to that of α-emitters, but with a reduced crossfire effect compared with α-emitters, making them more suitable for single-cell irradiation (2). The result is that AE-emitting radionuclides are relatively harmless unless they are in close proximity to a radio-sensitive subcellular structure, particularly DNA but also, possibly, the cell membrane or mitochondria (3). This characteristic can be highly advantageous in reducing the side effects of RPT as, unlike α- or β-particles, non-targeted neighbouring cells are unaffected.

The difficulty of delivering sufficient AE-emitting atoms specifically to the interior of cancer cells to cause cytotoxicity following systemic administration is formidable. This challenge accounts for the dearth of clinical studies to have tested this therapeutic modality to date (4). However, we and others have investigated numerous strategies to maximize cancer cell uptake of AE-emitting radioisotopes. One approach is to radiolabel ligands, such as epidermal growth factor (EGF), that naturally internalize on engagement with a cell surface receptor (5). We have explored the integration of EGF into nanoparticles for enhanced payload delivery. In one example, we synthesized poly(lactic-co-glycolic acid) (PLGA) nanoparticles surface conjugated to EGF labelled with an AE-emitter, indium-111 (^{111}In), and encapsulating a ruthenium-based DNA replication inhibitor and radiosensitizer, Ru1 ($Ru(phen)_2(tpphz)^{2+}$; phen = 1,10-phenanthroline, tpphz = tetrapyridophenazine). It was shown that nanoparticle-mediated co-delivery of an AE radionuclide plus the radiosensitizer achieved combinational and targeted therapeutic effects in cancer cells that overexpress EGFR.

Another approach is to radiolabel a DNA-intercalating small molecule for direct insertion of an AE-emitter into the DNA of cancer cells. For this a mismatch DNA binding bis-ruthenium(II) dipyridophenazine (dppz) complex that was radiolabeled with ^{111}In was synthesized. Mismatched DNA base pairs (mismatches) are DNA lesions that are abundant in cells deficient in MMR (mismatch mediated repair) proteins and this form of genetic instability is prevalent in a subset of colorectal cancers. The radioactive construct [^{111}In]In-bisRu(dppz)([^{111}In][In-2]4+) targeted cell nuclei and was radiotoxic towards MMR-deficient human colorectal cancer cells and was substantially less detrimental in a paired cell line with restored MMR function (6).

An alternative strategy to promote AE-emitter uptake into cancer cells is the incorporation of cell-penetrating peptides (CPP) or nuclear localising sequences (NLS) in RPT design. We have achieved cell internalization of radiolabeled antibodies against, for example, Her2-Neu and γH2AX by covalent attachment of the archetypal CPP, Tat (derived from HIV-1 transactivator of transcription protein) (7, 8). A major obstacle to intracellular targeting is, however, the entrapment of macromolecules in endosomes. We have recently shown that branched multimers of arginine rich peptides with cell-penetrating properties, arranged on tetrakis scaffolds, result in efficient delivery and that cyclisation of individual peptides improves uptake and can promote endosomal release (9). We have shown that these compounds, called TriTats, enhance the intracellular delivery of whole IgG molecules as well as smaller antibody or antibody-like formats such as Fab, scFv, iDAbs and DARPins. Once internalized through the action of TriTat these cargo molecules retain the ability to bind to and disrupt the function of their intended intracellular targets. Recently we have applied this technology to the delivery of DARPins and scFv molecules directed against mutant KRas in pancreatic cancer models. Following TriTat enhanced internalization these molecules co-locate with KRas, result in down-regulation of KRas signalling and in radiosensitization. When delivered using TriTat, the AE-emitting radioconjugate, [^{123}I]I-anti-KRas-DARPin-cFarn, resulted in KRas inhibition with simultaneous radiosensitization, was highly cytotoxic and holds promise as a new approach to radionuclide therapy for cancer.

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Katherine A. Vallis, MBBS, PhD, MRCP, FRCR, FRCPC

1995-2006	Assistant/Associate Professor, Radiation Oncology and Medical Biophysics, University of Toronto and Staff Radiation Oncologist, Princess Margaret Comprehensive Cancer Center, Toronto
2006-present	PI, Department of Oncology, Oxford University and Consultant Clinical Oncologist, Oxford University Hospitals NHS Foundation Trust.
2011-present	Professor of Experimental Radiotherapeutics, Oxford University

BORON NEUTRON CAPTURE THERAPY (BNCT): SLC FAMILY MEMBRANE TRANSPORTERS AND UPTAKE OF BORONATED DRUGS

Fuyuhiko Tamanoi
Institute for Integrated Cell-Material Sciences, Institute for Advanced Study, Kyoto University
Yoshida Ushinomiya-cho, Sakyo-ku, Kyoto 606-8501, Japan
(tamanoi.fuyuhiko.2c@kyoto-u.ac.jp)

Boron Neutron Capture Therapy (BNCT) is a cancer therapy that is based on the principle that irradiation of boron-10 with a neutron beam causes boron to split to produce Lithium-7 and Helium-4 [1]. Helium-4 is an alpha-ray that makes DNA double-strand breaks inducing apoptosis of cancer cells (Figure 1). The use of neutron is a key feature of this therapy. Nuclear reactor has been used as a source of neutron beams. More recently, accelerator-based neutron beams have been developed and BNCT clinical therapy has been moving to hospital settings.

The history of BNCT is summarized in Figure 2 which was started in 1932 with the discovery of neutron by Chadwick. The first clinical trials were carried out by MIT and Brookhaven National laboratory in 1951. Since 1970s, Japan has made a number of improvements with neutron beams as well as with boron reagents. Clinical studies with

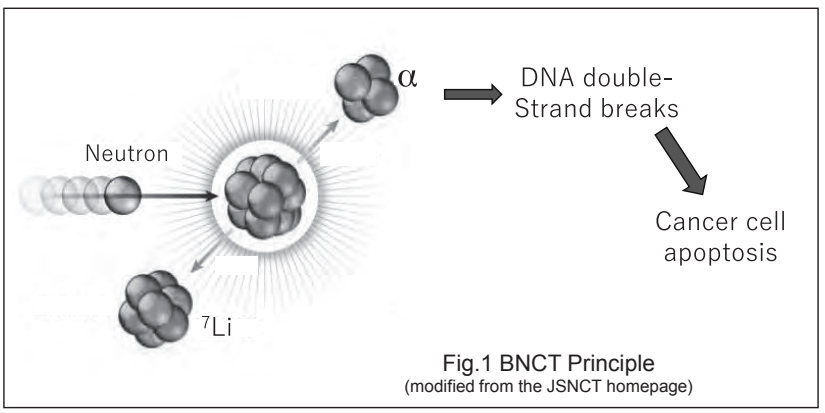


Fig.1 BNCT Principle
(modified from the JSNCT homepage)

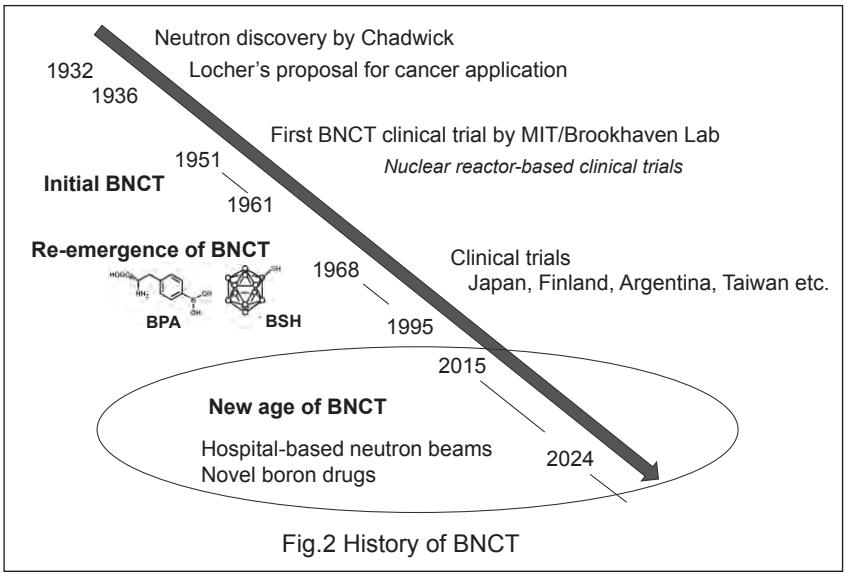
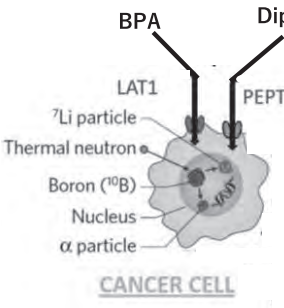


Fig.2 History of BNCT

several hundred cancer patients were carried out in many countries including Japan, Finland, Taiwan and Argentina [2]. The types of cancer include brain tumor and head and neck tumor. Tantalizing results were obtained with more than 60% of patients responding to the treatment. As mentioned above, accelerator-based neutron generators have been developed in Japan, US and Finland. In Japan, BNCT clinical treatment is carried out in several hospitals. Furthermore, insurance coverage for head and neck tumor treatment was approved that opened up the treatment for a large number of cancer patients.

Boronated drugs currently used in BNCT are BPA and BSH. BPA is of particular importance, as selective tumor accumulation of boron is achieved by the use of this compound. BPA is a boronated analog of phenylalanine that is taken up by the Lat1 membrane transporter. Since Lat1 is overexpressed in various cancer cells including head and neck cancer and melanoma, it is possible to achieve preferential tumor accumulation of boron. However, BPA suffers from low solubility and the maximum amount of boron in the tumor is limited.

We have set our effort on finding a drug that exceeds BPA so that higher tumor accumulation of boron can be achieved. In the past several years, we have carried out experiments at the Kyoto University experimental nuclear reactor at Kumatori and identified dipeptides such as BPA-BPA as a different type of boron drug that exhibits activity that exceeds BPA. Dipeptides have higher solubility than BPA and can use a different type of membrane transporters for cellular uptake. While BPA is taken up into cancer cells by the use of Lat1, dipeptides use PepT1 membrane transporter (Figure 3). We have recently found that dipeptides achieve tumor accumulation of boron that exceeds the accumulation



SLC family membrane transporters

Lat1 (SLC7A5)
PepT1 (SLC15A)
PepT2 (SLC15B)

Fig.3 Boron reagents, BPA and dipeptides, are taken up into cancer cells by SLC family membrane transporters

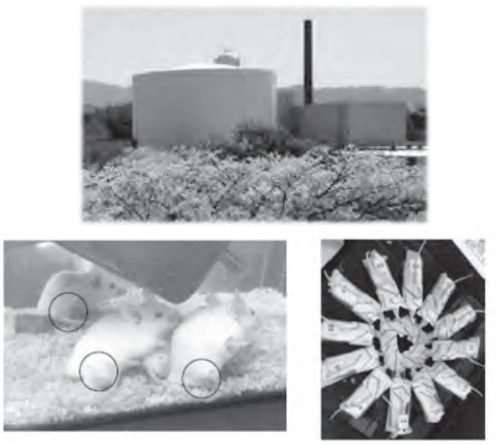


Fig.4 Mouse experiments at Kumatori nuclear reactor

obtained using BPA. Irradiation of xenograft mice after injection of boron-10 containing dipeptides carried out at the Kumatori nuclear reactor (Figure 4) resulted in a dramatic decrease of tumor volume. In fact, we did not see the tumor coming back for 50 days. Analysis of tumor sections showed that cancer cells disappeared. Thus, tumor can be eliminated by the dipeptide-BNCT treatment.

PepT1 and PepT2 transporters belong to a family of SLC (Solute Carrier) family transporters [3]. There are more than 400 members of the SLC family transporters and dipeptide transporters are coupled to proton electrochemical gradient. Structure and mechanism of action of PepT1 and PepT2 have been elucidated. Extensive database analyses have been carried out with tumor tissues, cancer cells. RNA expression analysis using cancer tissue samples revealed that the expression of PepT1 is high in pancreatic cancer and liver

cancer, while PepT2 expression is high in glioma and prostate cancer, respectively. Further studies on the SLC family transporters may significantly broaden the study of BNCT cancer therapy.

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Fuyuhiko Tamanoi, PhD

1980-1985	Senior Staff Investigator, Cold Spring Harbor Laboratory
1985-1993	Assistant Professor, University of Chicago
1993- 1997	Associate Professor, University of California, Los Angeles
1997-2017	Professor, University of California, Los Angeles
2017-present	Professor (Cross-appointment), University of California, Los Angeles
2017-present	Professor, Institute for Integrated Cell-Material Sciences, Institute for Advanced Study, Kyoto University

RESEARCH AND COMMERCIAL USE OF HUMAN SAMPLES AND DATA: ETHICAL AND REGULATORY ISSUES

Megumu Yokono
School of Social Sciences, Waseda University
1-6-1 Nishi-waseda, Shinjuku-ku, Tokyo 169-8050, Japan
(yokono@waseda.jp)

The use of human biological specimens and associated data in research has significantly advanced scientific and medical knowledge, particularly in fields like cancer genomics. However, this practice presents complex ethical, legal, and social challenges as technologies for genetic analysis and data sharing rapidly evolve. This lecture examines key issues surrounding the collection, use, and commercialization of human samples and data for research, focusing on cancer genomics and international sharing practices.

Cancer genomic studies have become crucial in understanding the molecular basis of various cancers and developing targeted therapies. This field raises unique ethical considerations, including privacy concerns, informed consent processes, the return of results, and family implications. The complexity of genomic data raises concerns about re-identification risks and the potential for discrimination or stigma.

The global nature of biomedical research necessitates international collaboration and the sharing of human specimens and data. However, this practice faces challenges due to varying ethical, legal, and practical issues across countries and jurisdictions. Primary concerns include the lack of harmonized international regulations, differing data protection and privacy laws, and ensuring equitable benefit-sharing between developing and developed countries.

This lecture will explore strategies for balancing the potential of cancer genomics research with the need to protect patient rights and interests. It will also discuss efforts toward developing standardized approaches for international specimen and data sharing.

In conclusion, this presentation aims to provide a comprehensive overview of the current ethical, legal, and social landscape in human biological specimen and data research. It

emphasizes the need for continued dialogue among researchers, ethicists, policymakers, patient advocates, and stakeholders to ensure both scientific progress and ethical integrity in this rapidly advancing field.

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Megumu Yokono, LLM

1988-1994	Research Associate, School of Law, Waseda University
2005-2006	JSPS Research Fellow (**PD**)
2006-2011	Assistant Professor, School of Social Sciences, Waseda University
2011-present	Associate Professor, School of Social Sciences, Waseda University

ETHICS OF PATIENT ADVOCACY AND INVOLVEMENT

Kaori Muto
Professor, Department of Public Policy, Human Genome Center,
The Institute of Medical Sciences, The University of Tokyo
4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
(krmt@ims.u-tokyo.ac.jp)

The scope of patient and public involvement (PPI) in research spans all stages of the research process and has broadened to include individuals across the entire healthcare spectrum, from pediatric patients to those receiving palliative care. A recent CIOMS guidebook emphasizes that patient involvement is crucial throughout the entire research ecosystem, from drug development to routine use and into new research and innovation[1].

Cancer research exemplifies the field where patient advocacy in research is most advanced, with efforts to establish partnerships between patients and researchers flourishing. In developed countries, these initiatives include organizing conferences and study groups by patient advocacy groups (PAOs), facilitating dialogue between researchers and PAOs to identify priority research areas and unmet medical needs, research fundraising by PAOs, supporting patient-funded clinical trials, and developing patient experts to take leadership roles.

From an ethical perspective, the promotion of PPI is grounded in two key principles: beneficence, which holds that addressing the needs of the most disadvantaged can lead to significant medical advancements, and justice, which stresses the importance of ensuring equitable access to healthcare and health outcomes for all[2].

However, several concerns persist. One issue is the potential risk that patient-funded research, lacking robust scientific rigor, could ultimately harm research participants and fail to contribute meaningfully to science. Additionally, there is a risk that some patient collaborators may be unduly emphasized. Prioritizing the perspectives of those with a strong interest in participation is critical, but it is equally essential to ensure diversity. Thus, conducting surveys and interviews to understand the views of those less interested is of

paramount importance.

Moreover, there is a need to clearly define the roles and responsibilities that patient collaborators and PAOs should assume. Unlike in Europe and the United States, most PAOs in Japan function as self-help groups and do not rely on government or corporate funding. While an increasing number of PAOs are turning to crowdfunding to conduct small-scale projects, it remains rare for these organizations to have dedicated fundraising teams. In addition, when promoting research that incorporates patient perspectives, it is vital to ensure that patients are not exploited.

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Kaori Muto, PhD

2000-2002	Post-Doctoral Research Fellow, Department of Community Health, Faculty of Medicine, Brown University, RI, USA
2002-2007	Lecturer, Department of Health Sciences, Faculty of Medicine, Shinshu University, Matsumoto, Japan
2007-2013	Associate Professor, Department of Public Policy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
2013-present	Professor, Department of Public Policy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
2023-present	Team Leader, Laboratory of Biomedical Ethics and Co-design (BECoD) , RIKEN Center for Integrative Medical Sciences, Kanagawa, Japan

THE EVOLUTIONARY ORIGINS OF CANCER LETHALITY

Paul C. Boutros
Department of Human Genetics; University of California, Los Angeles
10833 Le Conte Avenue, Los Angeles, California, USA, 90095
(pboutros@mednet.ucla.edu)

At its core, cancer is a disease of evolution. It starts when a single normal cell acquires a competitive advantage over its neighbours. This "fitness advantage" gives the cell the ability to proliferate more rapidly than other cells, or to evade anti-growth signals more effectively, or a combination of the two. As the daughters of this cell become more and more frequent, they acquire further competitive advantages through a combination of adaptation and selection. Eventually, these cells can become cancers, and grow to the size that they are diagnosed and treated.

My research tries to understand the early evolution of cancer. This involves two parts: creating methods and applying them. We create new computational algorithms to quantify different aspects of cancer evolution, ranging from biomarker development to signaling pathway quantitation (Yuan *et al*. Cancer Discovery 2022). Sometimes this also involves benchmarking studies, where we try to create standard approaches for our field (Salcedo *et al*. Nature Biotechnology 2024).

The second part of my research is applying these methods to understand one fundamental question: why some cancers kill and many others do not. Much of our work in this space focuses on cancers of the prostate, where about one third of cancers are non-lethal, another third are localized and curable with surgery or radiation, and the final third are lethal. This variability is determined by genomic instability (Lalonde *et al*. Lancet Oncology 2014), tumour microenvironment (Bhandari *et al*. Nature Genetics 2019) and tumour subclonality (Espiritu *et al*. Cell 2018), amongst others.

In recent work we have made two key advances on this. First, we have demonstrated that exercise influences a wide range of aspects of tumour biology. In a window trial, we show

that pre-surgery exercise is associated with reduced tumour proliferation and burden (Jones *et al*. JAMA Oncology 2024). In molecular correlative studies associated with this trial, we identify exercise-associated changes in tumour signaling, microenvironment and even patient microbiome . With cross-sectional and model system studies we show that long-term exercise is associated with modulations in the fitness landscape experienced by cancer cells, leading to changes in cancer subtype and molecular profile.

The second recent advance has been to understand how cancers can be driven by rare outlier events. We have created a new method to quantify the types of outliers that exist in cancers. We then outline the landscape of these outliers and their origins in genetic and epigenetic events. Finally, we demonstrate that extremely rare outlier events both have clear clinical implications for disease progression and are directly targetable using existing therapies.

Taken together, this work starts to define how cancers evolve to be lethal. They interact with surrounding cells and with the host milieu in a way that shapes the acquisition of both rare and common genetic aberrations. This evolution is powerfully influenced by both genetics and host behaviour, and provides improved opportunities for both interception and treatment.

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Paul C. Boutros, PhD, MBA

2004-2008	PhD Student, University of Toronto
2008-2014	Assistant Professor, Department of Medical Biophysics, University of Toronto
2014-2018	Associate Professor, Department of Medical Biophysics, University of Toronto
2018-present	Professor & Director of Cancer Data Science, University of California, Los Angeles
2022-present	Interim Vice Dean for Research, David Geffen School of Medicine, UCLA

COMPREHENSIVE OMICS LANDSCAPE IN LUNG CANCER ELUCIDATED BY LONG READ SEQUENCING TECHNOLOGIES

Ayako Suzuki
Department of Computational Biology and Medical Sciences,
Graduate School of Frontier Sciences, The University of Tokyo
5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8561, Japan
(asuzuki@edu.k.u-tokyo.ac.jp)

Cancer cells harbor various genomic mutations and other omics aberrations that are deeply associated with disease progression and therapeutic difficulties. Large-scale genomic technologies, such as high-throughput sequencing, enable understanding comprehensive omics features in cancer cells, including genomic and epigenomic alterations. Especially, long read sequencing technologies are useful to elucidate complicated patterns of cancer genome aberrations [1]. Human genomes consists of many repetitive regions, and cancer genomes are highly disrupted with occurrence of structural variants (SVs), both which could be only characterized by long reads precisely [2]. In addition to genomic sequences, nanopore-type long read sequencers provide information on DNA methylation patterns and unveil epigenome aberrations in each molecule. Further, long read information can achieve haplotype phasing of cancer genomes [3, 4]. Backgrounds of omics status are distinctive between two copies of the chromosomes. Characterization of the omics patterns at a haplotype level provides us novel information for understanding mechanisms of aberrant genomic event occurrences in cancer cells.

To identify novel types of cancer omics features only clarified by long read sequencing technologies, we have analyzed long read whole-genome sequencing (WGS) data of lung cancers [4, 5], including non-invasive and invasive adenocarcinoma and other subtypes of lung cancers, such as squamous cell carcinoma (SCC) and large cell neuroendocrine carcinoma (LCNEC). For non-invasive adenocarcinomas including adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA), we have particularly classified the tumor tissues by Noguchi classification. They are correspond to Noguchi type A, B and early C and analyzed for tracing key molecular events associated with stepwise progression of

early lung adenocarcinoma.

From the obtained long read WGS data, we identified various types of SVs from simple large deletions to complicated structures combined with multiple rearrangements. These SVs occasionally occurred in tumor-suppressor genes and would have functional effects for cancer progression. A small number of SVs were detected in early phase of adenocarcinoma while SCC and LCNEC harbored numerous SVs. We also found that genome-wide hypomethylation which was known to associate genome instability proceeded along with adenocarcinoma progression, starting from parts of Noguchi type B and early C cases. In SCC and LCNEC cases, more intense hypomethylation was observed compared with adenocarcinoma.

Moreover, to understand aberrant omics patterns at a haplotype level, mutations and epigenomic aberrations were analyzed in each haplotype on phased blocks that were constructed by haplotype phasing analysis. This analysis would impute order and mechanism of mutation and aberrant DNA methylation occurrences. We also found that lung cancer genomes harbored the regions in which mutations were biasedly enriched in one haplotype, and these regions showed mutation patterns which were related in kataegis and chromothripsis-like events. These distinct regions were observed in *EGFR* mutation-positive adenocarcinoma and detected even in non-invasive cases.

In addition to long read sequencing technologies, we have recently utilized other omics analytical methods, such as single-cell and spatial omics technologies, to identify more comprehensive molecular events occurring in each of the lung cancer cells. Now, we have been integrating omics information which were obtained from diverse advanced omics technologies to identify key molecular events of lung cancer progression at multi-layered levels.

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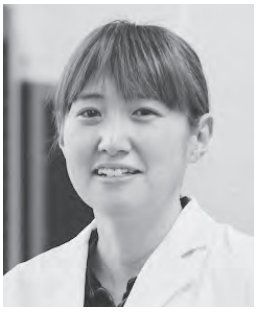

Ayako Suzuki, PhD

2015	Project Assistant Professor, Graduate School of Frontier Sciences, The University of Tokyo
2015-2018	Staff Scientist, Exploratory Oncology Research & Clinical Trial Center (EPOC), National Cancer Center
2018-2021	Project Associate Professor, Graduate School of Frontier Sciences, The University of Tokyo
2021-present	Associate Professor, Graduate School of Frontier Sciences, The University of Tokyo

COMPUTATIONAL AND FUNCTIONAL APPROACES TO UNCOVER SELECTION ADVANTAGES OF CANCER ANEUPLOIDY

Alison M. Taylor
Herbert Irving Comprehensive Cancer Center, Columbia University
1130 St. Nicholas Avenue, Room 309B
New York, NY 10032 USA
(at3488@cumc.columbia.edu)

Aneuploidy, including the gain or loss of whole chromosomes or chromosome arms, is a near-universal feature of cancer. We previously applied methods that define chromosome arm aneuploidy to over 10,000 tumors in the Cancer Genome Atlas (TCGA)[1]. Cancer subtypes are often characterized by tumor specific patterns of chromosome arm copy number alterations and breakpoints; for example, squamous cell carcinomas (SCCs) from different tissues of origin are characterized by chromosome 3p (chr3p) loss and chromosome 3q (chr3q) gain (**Figure 1**). From the TCGA aneuploidy data, we developed an algorithm called BISCUT to distinguish peak regions of aneuploidy breakpoints on each chromosome arm[2]. BISCUT identified loci affected by broad copy number alterations that provide fitness advantages or disadvantages both within individual cancer types and across cancers. Our analyses are consistent with selection being the primary driver of aneuploidy events in cancer.

We next wanted to validate some BISCUT peaks without a known driver, to identify potential gene deletions that are beneficial in cancer cells. We focused on chromosome 8p (chr8p), as this arm is frequently deleted across cancer types, but no strong tumor suppressors have been identified. Recent advances in genome engineering allow generation of large chromosomal alterations and validation of findings from patient genomic data. For this study, we used our CRISPR-Cas9 arm-deletion system to delete chr8p in human immortalized epithelial cells. Cells with chr8p deletion showed lower amounts of cell death in culture. Knockdown of *WRN*, one of the two genes in the smallest chr8p BISCUT peak, was sufficient to reproduce this phenotype, suggesting that *WRN* haploinsufficiency may be beneficial to tumor development.

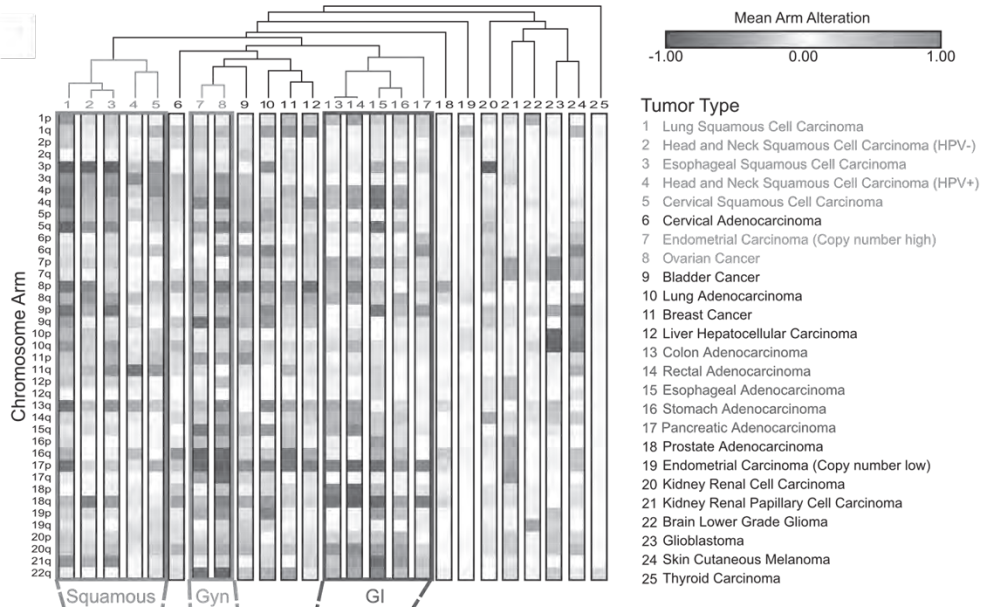

Figure 1 Patterns of aneuploidy occur in tissue-specific patterns[1].

As aneuploidy occurs in tissue-specific patterns, we also wanted to explore the role of chr3 arm aneuploidies in squamous cancers. We again used the CRISPR-Cas9 system to delete one copy of chr3p in a human immortalized lung epithelial cell line similar to the putative cell-of-origin in lung SCC. Consistent with patient data, expression of chr3p genes was decreased upon deletion, as well as increased expression of interferon response genes. Cells with chr3p deletion initially proliferated more slowly than their siblings. Interestingly, after several passages in culture, this proliferation defect was rescued in chr3p deleted cells. Genome sequencing and karyotype analyses suggested that this was partially the result of chr3 duplication, with cells transitioning to a state of chr3q gain.

To test whether chr3p deletion and chr3q gain could promote squamous metaplasia, we grew lung cells in organoid culture. Isolated cells with chr3p deletion and chr3q gain both show deficiencies in proper basal cell differentiation, consistent with their high frequency in squamous cancers (**Figure 2**). Our genome engineering approach to model chromosome arm aneuploidies provides a robust model to validate drivers from aneuploidy events, which will be critical to address the gap in our understanding of aneuploidy in cancer. In addition, our methods have identified consequences of individual aneuploidy events, which will lead to new precision oncology targets for patients.

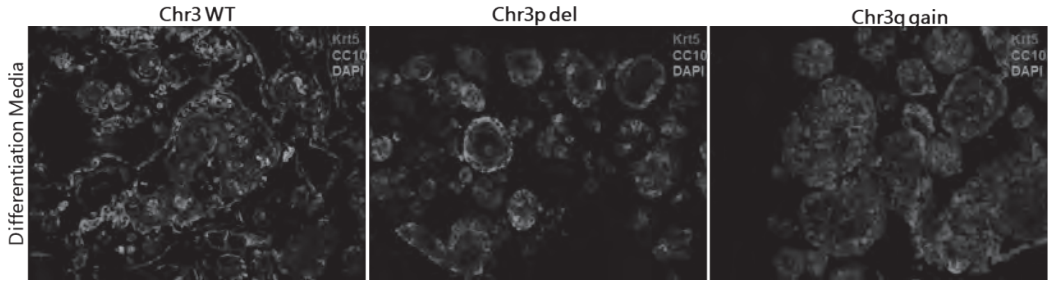

Figure 2 Impact of chr3 arm aneuploidies on differentiation in lung organoid culture.

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Alison M. Taylor, PhD

2013-2019	Postdoctoral Fellow, Dana-Farber Cancer Institute and Broad Institute, Harvard University, Boston
2020-present	Assistant Professor of Pathology and Cell Biology in the Herbert Irving Comprehensive Cancer Center, Columbia University

ENGINEERING AND DEPLOYING T CELLS TO EFFECTIVELY ERADICATE KRAS-EXPRESSING TUMORS

Philip D. Greenberg, Aude Chapuis, E. Gabriela Chiorean, Tijana Martinov, Shannon Oda, Tom Schmitt, Yapeng Su, Ashley Thelen

Program in Immunology, Fred Hutchinson Cancer Center and Departments of Immunology and Medicine, University of Washington

1100 Fairview Ave N., Mail Stop D3-100, Seattle, WA, USA 98109

(pgreenberg@fredhutch.org)

Generating or amplifying T cell responses to tumor-specific neoantigens has proven to be an effective and safe strategy for immunologically targeting tumors. However, as most tumor-specific neoantigens are unique to each patient (private), targeting such antigens requires highly personalized therapy. A smaller subset of neoantigens includes epitopes that span recurrent mutation hotspots, translocations, or gene fusions in oncogenic drivers and tumor suppressors, as well as epitopes that arise from viral oncogenic proteins. These antigens are likely to be shared across patients (public rather than private), required for cancer cell survival and fitness, and uniformly expressed by cells within the tumor. Mutant *KRAS*, the most common human driver oncogene, is one such antigen and our lab has pursued the development of T cell therapies specific for mKRAS epitopes.

Current immunologic and genetic technologies have made it possible to generate T cells reactive to immunogenic neoantigens, isolate high affinity TCRs from the T cells, and then use the TCRs as off-the-shelf reagents to genetically engineer T cells from patients with tumors that express the neoantigen and the HLA-restricting allele recognized by that TCR. We have developed a high-throughput methodology to isolate high affinity TCRs specific for a defined target antigen from CD8 T cells, in which reactive T cells are generated by *in vitro* stimulation, T cells expressing high affinity TCRs selected by stringent cell sorting with limiting amounts of peptide/MHC tetramer, and the TCRs isolated by next-gen sequencing and then validated for expression in patient T cells[1]. Using this approach we have isolated from CD8 T cells and advanced to clinical trials a TCR specific for the $KRAS_{G12V}$ mutation presented by HLA-A11 that efficiently recognizes tumor cells expressing $KRAS_{G12V}$, and have

also isolated candidate TCRs specific for $KRAS_{G12D}$ and $KRAS_{G12C}$ for further development and advancing pending the results of the trial[2].

We have also been performing a clinical trial in which PDA patients who express HLA-A2 are receiving autologous CD8 T cells engineered *ex vivo* to be specific for Mesothelin, a pro-oncogenic protein over-expressed by PDA tumor cells, and this trial is providing insights for the trial targeting mKRAS. Patients are being biopsied pre-infusion of T cells and then at day 21. The trial is based on studies we performed in a genetically engineered murine *KPC* model, in which we demonstrated that such tumor-reactive CD8 T cells specific for Mesothelin efficiently infiltrate into pancreatic tumors and mediate therapeutic benefit. We have completed the first cohort of patients on this trial designed as a cell dose escalation, with patients in this cohort receiving for the initial safety analysis a low dose of 10^9 T cells that is below the expected therapeutic dose, and have been evaluating the results using high-dimensional analyses to compare the findings to what we have observed in the *KPC* model. The infused T cells again infiltrated the tumors, and, despite the low cell dose, transferred T cells were detectable in the blood and remained detectable in the tumor 21 days after administration. The T cells in the tumor were recognizing and being activated by tumor cells. However, several obstacles that will likely impact achieving the desired levels of therapeutic efficacy were revealed. First, many of the infiltrating T cells were likely being deleted at the tumor site, and second the T cells that did persist for 21 days were rendered dysfunctional/exhausted in the tumor microenvironment (TME). The dysfunction appeared to be multi-factorial, which was in part a reflection of chronic TCR signaling at the tumor site resulting in upregulation of the full panel of inhibitory receptors including PD1, Lag3, and Tim3, and expression of genes associated with an exhaustion program such as *TOX*. Additionally, the T cells were being influenced by suppressive factors in the TME, in particular TGFβ. These findings are remarkably similar to what we observed in T cell therapy in the *KPC* model, in which the persisting dysfunctional T cells exhibited not only evidence of exhaustion but also a signature of responding to inhibitory TGFβ signaling, making it feasible for us to evaluate interventions in T cell therapy in the KPC model that will likely be translatable to creation of more effective human therapies.

Our approach to address these obstacles to efficacy has been to pursue further genetic engineering strategies to enhance T cell activity, as this allows creation of cell intrinsic modifications that only impact the tumor-reactive T cells rather than systemically broadly targeting immune pathways or cells, which can have significant toxicities and in particular lead to the development of autoimmune injury. An issue we initially targeted is cell persistence. In the context of persistent/ chronic antigen stimulation in the TME, T cell

survival becomes limited largely due to an event termed activation-induced cell death (AICD), which is mediated via the FasL/Fas death pathway. Therefore, we created a synthetic receptor that retained the Fas receptor ectodomain so that it would still bind and be activated by binding FasL, which is abundant in the TME, but swapped the cytoplasmic death domain signaling tail for the 4-1BB cytoplasmic tail that provides pro-survival and proliferative signals. Both the Fas receptor and 4-1BB naturally form and are activated as homo-trimeric signaling complexes, and we showed that this synthetic module, which we termed an immuno-modulatory fusion protein (IFP), both served as a dominant negative molecule mitigating Fas death signaling mediated by binding FasL, and a costimulatory/survival signal to T cells that are being concurrently activated through the TCR, which enhanced the persistence and therapeutic efficacy of CD8 T cells expressing this IFP in engineered experimental T cell therapy of leukemia, PDA, and ovarian cancer[3, 4].

We next targeted exhaustion and the associated lack of sustained therapeutic activity. The expression of inhibitory receptors on T cells such as PD-1 and CD200R is increased during chronic stimulation and promotes exhaustion. The ligands for these receptors are abundant in the TME, making the complexes active signaling modules. Therefore, we created IFPs that had the ectodomain of these receptors, which signal as homodimers, fused to the cytoplasmic signaling tail of CD28, a costimulatory receptor that also signals as homodimers. These IFPs again not only served as dominant negatives, but also provided costimulatory signals that enhanced T cell function, anti-tumor activity, proliferation, and persistence[5].

Our studies in therapy of PDA in both clinical trials and the mouse *KPC* model, have highlighted the role of TGFβ as a mediator of T cell suppression, and therefore we have pursued strategies to harness TGFβR signaling with an IFP. This is a more complex receptor, as it has two chains, TGβR1 and TGFβR2, that are ligated by TGFβ, which is a homodimeric cytokine that thus creates a 4-chain signaling module resulting from a homodimer of the 2 paired heterodimeric chains. We have demonstrated that fusing the signaling domains of the IL2Rβ and IL2Rγ receptor chains, which are the signaling chains of the IL2R and require formation of oligomeric clusters to signal, to the ectodomains of the TFGβR1 and TGFβR2 chains. This IFP, upon binding TGFβ, induces STAT5 activation and provides authentic IL2 proliferative signals, as well as serves as a dominant negative for natural TGFβR signaling. Thus, T cells expressing this IFP, rather than being suppressed following binding of the abundant TGFβ in the TME, are driven to proliferate and expand.

Modulating the signals that CD8 T cells receive in the TME can enhance T cell function and therapeutic activity, but the requirements for effective immune responses suggest that

there is another fundamental issue that should be addressed. Effective immune responses to pathogens generally require a coordinated CD4 as well as CD8 T cell response, with the CD4 T cells providing helper functions that promote T cell function, proliferation, and survival, effector functions that directly and indirectly via activation of innate effector cells promote tumor cell killing, and supporting functions that prevent or delay T cell exhaustion. Creating engineered cooperative CD4 and CD8 T cell responses has proven feasible with CAR-T cells, as both cells can use the Ab-based recognition module, and administration of both CD4 and CD8 T cells has proven invaluable for achieving therapeutic responses. However, creating such responses with TCRs has been more difficult, due to the fact that CD4 and CD8 T cells recognize distinct antigenic epitopes presented in the context of different Class II or Class I MHC molecules, making matching the responses for each individual patient very complex. Therefore, we have focused on creating a functional CD4 response that can use the same TCR as the one being expressed in CD8 T cells. To accomplish this, we initially isolated TCRs of sufficiently high affinity from CD8 T cells to recognize peptide in Class I MHC molecules in the absence of a contribution by CD8, which normally significantly increases the avidity of the TCR for the peptide/Class I molecule. Expression of such TCRs did induce reactivity by CD4 T cells but was not adequate to sustain CD4 responses. However, expression of the CD8$\alpha\beta$ chains as well as such TCRs in CD4 T cells imparted the CD4 T cells with similar target avidity as TCR-engineered CD8 T cells and enhanced CD4 T cell responses. Moreover, engineered CD4 T cells expressing the TCR and CD8$\alpha\beta$ not only mediated direct anti-tumor activity *in vitro*, but also synergistically enhanced the anti-tumor activity of CD8 T cells, with combined CD4 and CD8 T cells being more effective than either population alone. CD4 T cells are also very dependent upon costimulatory signals for optimal activity, such as provided by our IFPs, and we have shown that also engineering in a costimulatory signal will further augment CD4 function, enhance the ability to promote CD8 T cell responses, and increase the accumulation of CD4 and CD8 T cells in experimental tumors and the resulting anti-tumor activity[1].

There are many other strategies we are currently actively pursuing, including epigenetically and metabolically modifying T cells during the process of cell generation and expansion for therapy to create T cells with enhanced self-renewal qualities and that are better suited for accessing the nutrients available in the TME, as well as utilizing CRISPR technology to identify novel regulatory pathways operative in dysfunctional T cells that can be molecularly targeted to enhance T cell activity. Our plan has been to systematically advance these engineering technologies into T cells that will be used to target *mKRAS*-expressing tumors. We have initiated a trial in which patients with pancreatic, lung, or colon tumors that are $KRAS^+$ $HLA\text{-}11^+$ are being treated by infusion of autologous CD4 and CD8 T

cells that have been engineered to express both a high affinity $KRAS_{G12V}$-specific TCR and CD8αβ and have opened a companion trial in which the engineered CD4 and CD8 T cells are also expressing the Fas/4-1BB IFP. Patients are being actively enrolled, with initial results in treated patients already looking encouraging, affirming the potential benefits of further pursuing these strategies. Treated patients and specimens are now being analyzed and will be discussed.

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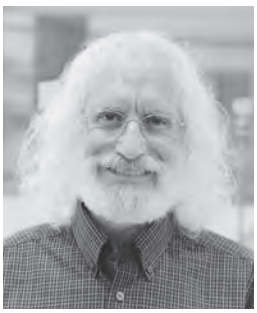

Philip D. Greenberg, MD

1974 to 1976	Postdoctoral Research Fellow, Immunology, UCSD; San Diego, CA
1976 to 1978	Senior Fellow, Oncology, University of Washington (UW) and Fred Hutchinson Cancer Center (FHCC), Seattle, WA
1978 to 1982	Assistant Professor of Medicine, Division of Oncology, UW, and FHCC
1982 to 1988	Associate Professor of Medicine, Division of Oncology, UW, and FHCC
1988 to date	Professor of Medicine, Division of Oncology, UW; and FHCC
1988 to 2003	Director, Immunology Program, UW Center for AIDS Research
1989 to date	Professor of Medicine and Immunology, UW and FHCC
1991 to date	Head, Program in Immunology, FHCC
2016 to date	Investigator, Parker Institute for Cancer Immunotherapy

LOCALIZATION OF RAS AND MECHANISMS OF DRUG RESISTANCE IN RAS/RAF MUTATED CANCERS

Ryohei Katayama
Div of Experimental Chemotherapy, Cancer Chemotherapy Center,
Japanese Foundation for Cancer Research
3-8-31, Ariake, Koto-ku, Tokyo 135-8550, Japan
(ryohei.katayama@jfcr.or.jp)

KRAS mutation is one of the most common driver oncogene across various cancers, and observed in about 15% of non-small cell lung cancer, 35% of colorectal cancer or about 90% of pancreatic cancer. KRAS mutations, which mainly occur in G12, G13 or Q61 mutations, cause aberrant constitutive activation of KRAS-driven growth signaling. To target the mutant KRAS, enormous efforts have been devoted and recently, several KRAS specific inhibitors have been successfully developed. Among them, KRAS G12C mutant specific inhibitor, which directly and covalently bind to mutated cysteine at 12th residue. The development of mutant KRAS specific inhibitors has largely changed the treatment strategies of KRAS G12C positive cancer, especially in non-small cell lung cancer, and will change the treatment strategies of KRAS G12D or other KRAS mutation positive cancer. However, the efficacy of mutant KRAS G12C specific inhibitor such as sotorasib (AMG510) or adagrasib (MRTX1133) have shown very different efficacy between lung, colorectal and pancreatic cancer, and sotorasib is only approved for the treatment of KRAS G12C mutated NSCLC. In particular, KRAS G12C-positive colorectal cancer patients showed very limited efficacy to sotorasib, suggesting intrinsic resistance to KRAS inhibitors in KRAS-positive CRC. In addition, even in KRAS mutated NSCLC patients, most of the patients experience tumor relapse due to acquired resistance mostly within 1 year. We investigated the mechanisms of intrinsic (and acquired) resistance to KRAS inhibitors using commercially available cancer cell lines and our original patient-derived cells (PDCs) established from CRC surgical specimens. To further investigate the mechanisms of intrinsic KRAS inhibitor resistance in CRCs, we evaluated the dependence of KRAS-mediated signaling by treating these KRAS mutant cell lines with KRAS inhibitors or si-KRAS. The growth of most KRAS mutant CRC PDCs was

not significantly suppressed by KRASi monotherapy or KRAS knockdown. To explore the mechanism of intrinsic resistance to KRASi, we then performed inhibitor library screening with or without KRASi and targeted re-sequencing of cancer-associated genes. As a result, KRAS-mutant colorectal PDCs can be classified into at least two groups. One is the KRASi induced EGFR mediated MAPK pathway reactivation group and the other is the KRASi and EGFR inhibitor combination insensitive group. In the latter group, one of the PDC named JC261 was found to show marked sensitivity to PI3K with mTOR inhibitor. In addition, we found that the JC261 revealed that KRAS was mainly localized in the cytoplasm, different from the plasma membrane localization observed in the cells of the former group. Since KRAS was known to be activated at the plasma membrane after appropriate modifications, we evaluated KRAS localization under KRAS or other signaling inhibition using immunofluorescent staining with multiple CRC cells. In JC261 CRC-PDC, KRAS localization was cytoplasmic and not altered to the plasma membrane by KRASi treatment, although KRAS-GTP level was significantly attenuated by KRASi treatment, but knockout of a co-altered oncogene induced plasma membrane localization of KRAS. Furthermore, we found that KRAS localization can be altered by treatment with oncogenic signaling inhibitors in various KRAS mutant cancer cells. Our results suggest that the complex regulation of KRAS localization may influence the dependency of KRAS even in KRAS mutant cancers.

In addition to KRAS mutation, BRAF mutation is second most common in CRC. BRAF mutation, mainly V600E mutation have also been observed in various cancers, melanoma, non-small cell lung cancer or thyroid cancer. Many BRAF V600E mutation specific inhibitors have been developed and currently, BRAF inhibitor with inhibitor against MEK, which is the main downstream molecule of BRAF, have been approved for the treatment of BRAF mutated NSCLC or melanoma. On the other hand, BRAF inhibitor has shown limited effect on BRAF mutant CRC, and to date, many combination therapies have been developed and currently anti-EGFR antibody with BRAF inhibitor co-treatment have been approved for the treatment of BRAF mutant CRC. In this presentation, I'd like to talk about the intrinsic and acquired resistance mechanisms in BRAF-mutant colorectal cancer.

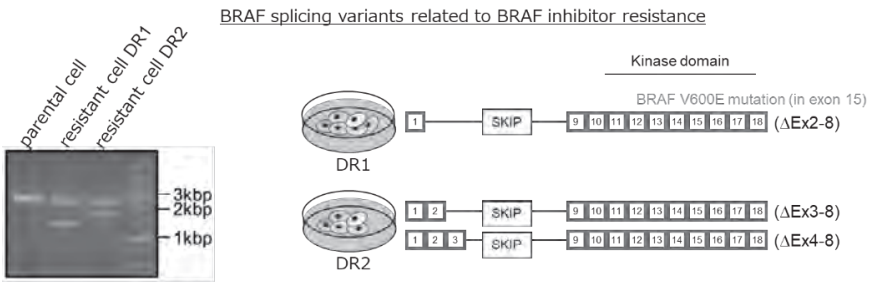

Figure 1 One of the mechanisms of drug resistance in BRAF mutated colorectal cancer.

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Ryohei Katayama, PhD

2006-2010	Staff Scientist, Cancer Chemotherapy Center, JFCR
2010-2012	Postdoc fellow (JSPS research scholar in abroad), Cancer Center, Massachusetts General Hospital, USA
2012-2015	Staff Scientist, Cancer Chemotherapy Center, JFCR
2015-2017	Senior Staff Scientist, Cancer Chemotherapy Center, JFCR
2017-present	Chief, Cancer Chemotherapy Center, JFCR

ENHANCING PDAC IMMUNOGENICITY THROUGH KRAS^{G12D} INHIBITION AND mRNA VACCINE IMMUNIZATION

Caius G. Radu
Professor, Dept. of Molecular & Medical Pharmacology UCLA,
CHS AR-175; Los Angeles, CA90095, USA
(cradu@mednet.ucla.edu)

The emergence of clinical-stage KRAS^{G12D}-selective small molecule inhibitors has provided a means to target constitutively-active KRAS^{G12D}. Preclinical research has shown that inhibiting KRAS^{G12D} renders pancreatic ductal adenocarcinoma (PDAC) more responsive to immunotherapy. However, the impact of KRAS^{G12D} inhibition on antigen presentation by tumor cells remains unclear. To address this, we conducted a multi-omic profiling study to investigate changes in the proteome and peptide MHC ligandome (immunopeptidome) following the treatment of HPAC human cancer cells with RAS(ON) G12D-selective inhibitor RM-044 and/or IFNγ *in vitro*. Our multi-omic profiling revealed significant changes in antigen presentation after KRAS^{G12D} inhibition. Comparison of differentially expressed proteins in treated vs. untreated conditions indicated increased expression of proteins associated with interferon signaling and replication stress, including high expression levels of antigen processing and presentation proteins such as HLA-B and -C alpha chains. The corresponding immunopeptidomic data showed a ~2-fold increase in both immunopeptidome diversity and abundance in treated vs. untreated conditions. Sequence motif enrichment analysis of the identified ligands aligned with the proteomic findings, emphasizing how integrating these two data layers informs the shaping of the immunopeptidomic landscape.

Building on these findings, we hypothesized that KRAS^{G12D} signaling inhibition in preclinical models could enhance immune responses initiated by immunization targeting tumor antigens. We hypothesized that the release of antigens from KRAS^{G12D}-induced cell death, coupled with increased MHC-I expression in the remaining tumor cells, would enhance interactions with T cells activated by immunization. Recognizing the potential of

combining $KRAS^{G12D}$ inhibition with cancer immunization strategies, we turned our focus to the development of nucleoside-modified mRNA vaccines in ionizable lipid-containing nanoparticles (iLNPs), which have opened new avenues for targeting tumor-associated antigens (TAAs). The use of N1-methylpseudouridine (m1ψ) in mRNA vaccines helps avoid innate immune activation, maximize antigen expression, and lead to potent activation of adoptively transferred $CD8^+$ T cells. However, the immunosilent property of m1Ψ-mRNA-iLNP vaccines avoids the release of type I interferon (IFN), impacting T cell functions. We hypothesize that reintroducing type I IFN signaling in m1Ψ-mRNA-iLNP-driven T cell activation enhances the anti-tumor response. Our analysis of T cells expanded by systemic mRNA vaccination supports this, showing the most robust cell expansion and activation in mice vaccinated with m1Ψ-mRNA-iLNP and RNA adjuvants.

To test our hypothesis that targeting mutant KRAS synergizes with our newly developed mRNA vaccines, we orthotopically inoculated C57Bl/6 mice with a murine PDAC-$KRAS^{G12D}$ cell line expressing the model self/tumor-associated antigen gp100. Mice were immunized with empty ionizable lipid nanoparticles (e-iLNPs) or iLNPs packaged with mRNA encoding full-length gp100 (gp100-iLNP) supplemented with novel RNA adjuvants. Following immunization and $KRAS^{G12D}$ inhibitor treatment, mice receiving the mRNA-iLNP immunization followed by $KRAS^{G12D}$ inhibition experienced significant tumor regression compared to those receiving $KRAS^{G12D}$ inhibition alone. These studies provide crucial insights into the signals necessary for anti-tumor $CD8^+$ T cell activation, and they rationalize the combination of allele-specific KRAS inhibition with tumor antigen immunization as a promising strategy for improving outcomes in pancreatic cancer treatment.

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Caius G. Radu, MD

2006-2010	Assistant Professor, University of California, Los Angeles, Department of Molecular and Medical Pharmacology, Los Angeles, CA
2010-2014	Associate Professor, UCLA, Dept. of Molecular and Medical Pharmacology
2014-	Professor, UCLA, Dept. of Molecular and Medical Pharmacology
2018-	Co-Director; Cancer Molecular Imaging, Nanotechnology, and Theranostics Research Program (CMINT) , Jonsson Comprehensive Cancer Center

THERAPEUTIC APPROACHES TO KRAS CANCERS

Frank McCormick
University of California, San Francisco, Helen Diller Family Comprehensive Cancer Center,
1450 Third Street, HD-371, San Francisco, CA 94158
NCI RAS Initiative, Frederick National Laboratory for Cancer Research,
8560 Progress Drive, Frederick, MD 21701 USA
(frank.mccormick@ucsf.edu)

Hyperactive RAS proteins play major roles in human cancer, in Neurofibromatosis Type 1, Noonan syndrome and other germline disorders (1). In cancer, KRAS is the major RAS isoform, and this GTPase has been the subject of intense efforts to develop drugs to treat KRAS-driven cancers. We have focused on developing drugs that target the active, GTP-bound form of KRAS directly. The first of these, BBO-8520, is undergoing clinical trials in patients with KRAS G12C suffering from adenocarcinoma of the lung. The next drug in this series will target multiple forms of cancer, including pancreatic and colorectal cancer, as well as adenocarcinoma of the lung. These compounds inhibit KRAS by trapping the GTP-bound state in a non-productive conformation, referred to as State One, that can no longer interact with downstream effectors (2). Other investigators have developed drugs that trap KRAS in its inactive, GDP-bound state, or block access to effectors through recruitment of cyclophilin A. Progress in these projects will be discussed.

Another approach to targeting RAS proteins involves preventing these proteins activating their downstream effectors, primarily RAS kinases and PI 3′ kinases. Attempts to inhibit Raf kinase activity directly have failed, due to paradoxical activation of kinase activity by these inhibitors. Inhibiting MEK activity is not subject to paradoxical activation, but offers no therapeutic window over normal cells, and so MEK inhibitors are not tolerated at therapeutic doses. However, the process of RAS activation by RAS offers therapeutic opportunities. Activation is a multi-step process, involving recruitment of inactive Raf/14-3-3 complex to the plasma membrane, de-phosphorylation of specific sites on Raf kinases that release 14-3-3, dimerization and re-engagement of 14-3-3 to stabilize the active dimer complex. This process differs in normal cells and cancer cells and suggests ways of preventing Raf

activation in cancer cells selectively.

One critical difference lies in the dependence of this process on a complex consisting of MRAS, SHOC2 and PP1C, which specifically de-phosphorylates residues involved in 14-3-3 binding. We have solved the structure of this complex and proposed mechanisms by which it could be inhibited with selectivity for cancer cells (3). MRAS is the major regulation of the SHOC2.PP1C complex in normal cells, but oncogenic RAS mutants can take over this function in cancer cells, facilitating Raf activation in these abnormal conditions (Figure 1).

The other major effector pathway is the PI3′ kinase pathway. In normal cells, canonical RAS proteins (H-, N- and KRAS) do not engage this pathway. Indeed, Raf activation appears to be the only function of RAS in normal cells. The RRAS family of RAS-related proteins bind and activate PI3′ kinase under normal conditions. However, in cancer cells in which KRAS proteins are hyperactive, and often over-expressed, they appear capable of engaging PI3′ kinase signaling, and this contributes to the malignant phenotype (Figure 1). Furthermore, genetic disruption of the interaction between RAS proteins and PI3′ kinase-a prevents KRAS cancers and causes regressions in mouse models, without causing significant side-effects (4). For these reasons, we have developed a drug, BBO-10203, which binds covalently to PI 3′ kinase at the RAS binding domain and prevents RAS interaction. This compound is in clinical trials for various malignancies, including KRAS mutant cancers. In contrast to drugs that directly inhibit PI3′ kinase, BBO-10203 does not interfere with glucose uptake or homeostasis. This is because these pathways do not depend on RAS proteins and are therefore unaffected by the RAS-PI3′ kinase breaker compound. Effects of this drug on cells and mouse models, as a single agent and in combination therapy, will be presented.

While RRAS proteins (RRAS, RRAS2) activate PI3′ kinase, but not Raf-MAPK in normal cells, mutant versions of these proteins can activate Raf-MAPK (Figure 1). These mutants, such as the Q72L mutant (equivalent to the oncogenic Q61L mutant of canonical RAS proteins) occur in cancer and in Noonan syndrome, a germline condition caused by hyperactivation of Raf-MAPK. These effects appear indirect, however by novel mechanisms that will be discussed, including allosteric activation of SOS1, a protein that stimulates GDP/GTP exchange on canonical RAS proteins.

GDP-bound forms and active GTP-bound forms through the action of GEFs (SOS, for example) and GAPs (NF1, RASA2). MRAS can also be degraded by LZTR1.

In normal cells, HRAS, NRAS and KRAS activate the Raf/MAPK pathway. Oncogenic variants, often over-expressed in cancer cells, can activate effectors of other RAS family members, such as RRAS, RRAS2 and MRAS, leading to inappropriate activation of Pi3′ kinase and SHOC2 in cancer cells (purple arrows).

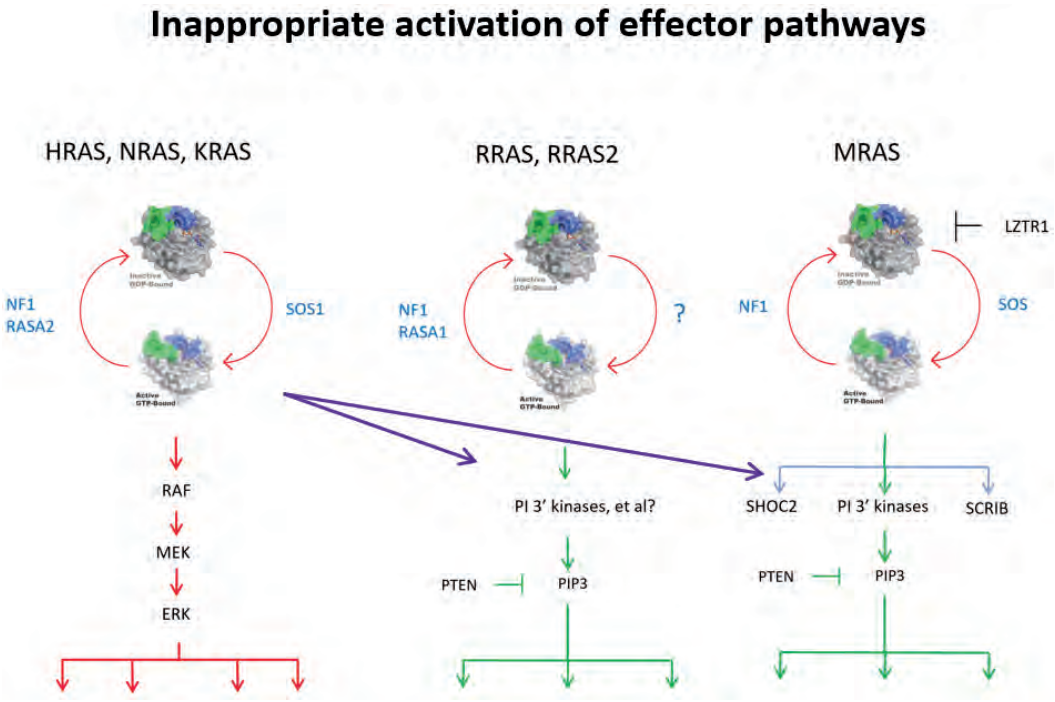

Figure 1 Cross-talk between members of the RAS family. RAS proteins cycle between inactive,

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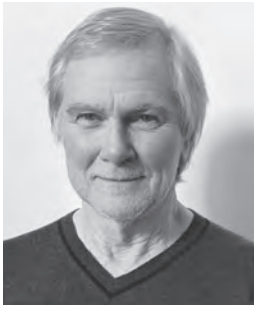

Frank McCormick, PhD, FRS, DSc (Hon)

1975-1978	Postdoctoral Fellow, State University of New York at Stony Brook
1978-1981	Postdoctoral Fellow, Imperial Cancer Research Fund, London
1981-1990	Director of Molecular Biology, Cetus Corporation
1990-1991	Vice President, Research, Cetus Corporation
1991-1992	Vice President, Research, Chiron Corporation
1992-1996	Founder, Chief Scientific Officer, Onyx Pharmaceuticals
1996-present	Fellow of the Royal Society
1997-present	Professor, University of California, San Francisco (UCSF), Helen Diller Family Comprehensive Cancer Center
2013-present	Scientific Director, National Cancer Institute Ras Initiative, Frederick National Laboratory for Cancer Research
2014-present	National Academy of Sciences

CHROMATIN 3D STRUCTURE IN TUMOR EVOLUTION

Elisa Oricchio
Swiss Institute for Experimental Cancer Research (ISREC), EPFL, Lausanne, Switzerland
(elisa.oricchio@epfl.ch)

In the cell nucleus, the genome is organized in tridimensional (3D) functional structures including chromosomes, chromatin compartments, domains, and loops. These structures are important to define and maintain cell identity by regulating gene expression. However, in somatic cells, the accumulation of epigenetic and chromosomal changes alters the 3D organization of the chromatin and can lead to cancer development.

Over the past years, we have been studying the functional interplay between the 3D organization of chromatin and the acquisition of oncogenic genomic lesions. Analyses of the chromatin 3D structure in cancer cells revealed how such structure influences the acquisition of epigenetic modifications and regulates the expression of oncogenes and tumor suppressors. Indeed, we have been able to show that oncogenic mutations of a chromatin remodeling factor like EZH2 in lymphoma cells induce aberrant accumulation of H3K27me3 and conformational changes within chromatin domains, leading to the concurrent silencing of co-regulated tumor suppressor genes (1). Moreover, we reported that the acquisition of chromosomal rearrangements not only results in physical changes in the chromosome structure but promotes the acquisition of allele-specific H3K27ac and 3D loops, which promote monoallelic oncogene expression (2,3). Lastly, we have shown that whole genome duplication induces loss of chromatin segregation between well segregate structures of the chromatin like chromosomes, chromatin compartments, and domains. Increased interactions between these normally segregated structures trigger the acquisition and selection of oncogenic epigenetic and transcriptional changes leading to tumor development (4).

Overall, our studies demonstrate that a functional interplay exists between cancer genomic alterations and the 3D structure of the chromatin: on the one hand, genomic alterations can co-opt the chromatin organization to regulate gene expression and, on the other hand, the 3D structure of the chromatin can favor the selection of specific oncogenic alterations. The analysis of the 3D organization of the chromatin in cancer cells can provide a new lens to understand the effect of genomic alterations on tumor evolution.

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Elisa Oricchio, PhD

9/2008-10/2014	Postdoctoral Fellow, Memorial Sloan Kettering Cancer Center, NY, USA
11/2014-10/2021	Assistant Professor, Swiss Institute of Experimental Cancer Research (ISREC) EPFL
10/2021-present	Associate Professor, Swiss Institute of Experimental Cancer Research (ISREC) EPFL
2022-present	Director Swiss Institute of Experimental Cancer Research (ISREC) EPFL

THE PLASTIC CONTROL OF MITOTIC CHROMOSOME SEGREGATION UNDERLIES CHROMOSOMAL INSTABILITY IN CANCER CELLS

Toru Hirota
Division of Experimental Pathology, Cancer Institute
Japanese Foundation for Cancer Research
3-8-31 Ariake, Koto-ku, Tokyo 135-8550, Japan
(thirota@jfcr.or.jp)

Aneuploidy is a common feature of malignant tumors that arises through persistent chromosome missgregation associated with the pathological condition called chromosomal instability, or CIN. Despite the fact that aneuploidy is a type of genome rearrangement widely found in human cancers, experiments both in vivo and in vitro show that induction of aneuploidy typically leads to a loss of cellular fitness. These inconsistent observations so called aneuploidy paradox have been an outstanding question in the field of cancer biology. To address this issue, we have been studying a cancer stem cell mouse model, which were found to have both high chromosomal instability and high proliferation activity as a population. To investigate the mechanism underlying how these cancer stem cells fluctuate in their level of aneuploidy as they proliferate, we are focusing two possible mechanisms: the internal/external conditions that select cells to proliferate and the level of cellular CIN as they proliferate.

Accurate chromosome segregation depends on proper kinetochore-microtubule (KT-MT) attachments, which is established through the destabilization of incorrect error-prone attachments. The highly conserved chromosomal passenger complex (CPC), containing mitotic kinase Aurora B as a catalytic subunit, ensures faithful chromosome segregation through destabilizing these KT-MT mal-attachments by phosphorylating kinetochore proteins mediating microtubule attachments. We previously found that heterochromatin protein 1 (HP1) is an essential component required for full Aurora B activity. HP1 binding to the CPC becomes particularly important when Aurora B phosphorylates kinetochore targets to eliminate erroneous microtubule attachments. Significantly, a reduced proportion of HP1 bound to CPC is widespread in cancer cells, resulting in insufficient activity of Aurora B. In

light of these findings, we characterized the activity of Aurora B of the cancer stem cell linages, and found that the activity of Aurora B well reflects the level of CIN in cancer stem cells.

Interestingly, We found that the continual Aurora B inhibition paradoxically elevates its activity, altering the degree of aneuploidization. These results led us to find the plastic nature of Aurora B regulation directly impacts the level of CIN. We then attempted to investigate the pathological relevance of CIN through inoculating experiments, and found that cells with different CIN levels lead cells to obtain different malignant phenotypes. We are currently conducting transcriptomic analysis to elucidate the molecular mechanisms in acquiring malignant phenotypes. Our study provides a novel insight into the pathological relevance of CIN, which has long been implicated in advanced cancers.

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Toru Hirota, MD, PhD

1997-2000	Graduate Visiting Student, Kumamoto University, Graduate School of Medical Science, Kumamoto
2001-2003	Postdoctoral Fellow, JSPS Research Fellowship (PD), Kumamoto University School of Medicine
2004-2005	Postdoctoral Fellow, JSPS Research Fellowship (Research Abroad), Research Institute of Molecular Pathology (IMP), Vienna
2005-present	Chief, Division of Experimental Pathology, Cancer Institute, Japanese Foundation for Cancer Research (JFCR)
2024-present	Deputy Director, Cancer Institute, Japanese Foundation for Cancer Research (JFCR)

ONCOHISTONES: EXPOSING THE NUANCES AND VULNERABILITIES OF EPIGENETIC REGULATION

Tom Muir
Princeton University, Department of Chemistry Princeton, NJ 08544 USA
(muir@princeton.edu)

The chromatin landscape governs basic cellular functions that are altered in cancer, including genomic architecture, gene expression patterns, and ultimately developmental pathways. Histone post-translational modifications (PTMs) are central regulators of these processes, and the discovery of dominant-acting mutations in histone proteins (termed 'oncohistones') at or near residues with well-known PTMs, and the aberrant expression of non-histone proteins that resemble mutated histone sequences ('oncohistone mimicry') found in human cancers has reinforced these key regulatory roles. Abnormal development and neoplastic transformation leading to cancer, can be driven by altered epigenetic regulation revealed by these mutations in histone, and non- histone proteins. This makes studying the etiology of oncohistone-associated disease fruitful both from the perspective of providing opportunities for the diagnosis and treatment of cancers, but also for understanding basic chromatin biology [1].

The founding set of oncohistones, centered on the unstructured N-terminal tail region of histone H3, are now known to function as cancer drivers. Remarkably, these mutants act through a gain-of-function mechanism in which they cause reductions in histone methylation marks linked to transcriptional regulation. For example, H3.3K27M, which is found in pediatric gliomas, directly inhibits the methyltransferase, polycomb repressive complex 2 (PRC2), to globally to reduce H3K27me3 levels, while H3K36M directly inhibits SetD2/NSD1 methyltransferases to globally reduce H3K36me3 levels. More specifically, these mutations create pseudo-substrate inhibitors of these methyltransferases, the potency of which is highly sensitive to the local chromatin context [reviewed in 1]. The success of work on the original set of oncohistones has motivated efforts by our group and others to

globally catalogue the histone missense mutational landscape across multiple cancer types [2]. These studies revealed numerous somatic mutations throughout the primary structure of all four core histones as well as the linker histone H1. Studies on these 'novel' oncohistones have revealed multiple modes of action depending on the position and type of mutation (Figure 1). Of particular interest to us, is the impact of these mutations on the activity of ATP-dependent chromatin remodeler enzymes. We have found that mutations can either activate or inhibit the activity of these enzymes, depending on location and remodeler type [3-6]. Moreover, these studies have revealed that a breakdown in the normal two-fold pseudo-symmetry of nucleosomes that results from heterotypic oncohistone incorporation alters the activity of chromatin remodelers. Through this work, we have also come to appreciate that pulling on the 'molecular thread' presented by oncohistones lays bare hitherto unknown aspects of chromatin regulation.

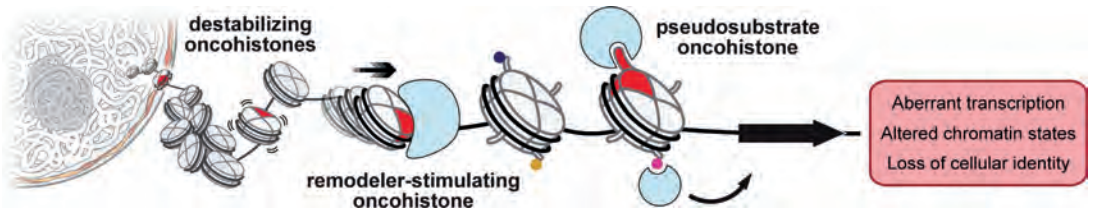

Figure 1 Oncohistones in epigenetic dysregulation. Studies so far have revealed three major modes of action for oncohistones depending on the position and type of mutation; (i) mutations that reduce the intrinsic stability of nucleosomes; (ii) mutations can alter the activity of ATP-dependent chromatin remodelers, and (iii) mutations can create pseudo- substrate inhibitors of histone PTM writer enzymes.

In this presentation, I will describe the development and application of a series of chemical biology approaches that allow the functional effects of oncohistones to be interrogated. In one line of enquiry, I will discuss the use of chemo-proteomics strategies, based on photo-proximity labeling (PPL), to determine how oncohistone incorporation impacts the local constellation of nuclear factors that engage chromatin [4,7]. In one version of this approach, split-intein mediated protein trans-splicing is used to introduce a chemical photocatalyst directly into oncohistones in native chromatin, while in complementary method engineered Light-Oxygen-Voltage (LOV) domains are employed in a fully genetic strategy. These PPL methods have allowed us to show that cancer-associated mutations can dramatically alter chromatin interactomes. For example, oncohistones that map to the so-called 'acidic-patch' of chromatin result in the displacement of various chromatin writer and eraser enzymes that ultimately leads to significant changes in the levels of epigenetic 'marks' such as histone acetylation and DNA methylation. I will also describe an exciting extension of the PPL strategy in which we can impose Boolean logic operations on the process, thereby

allowing interactomes of specific heterotypic complexes to be characterized [8]. In other work, I will introduce a novel protein engineering method that allows internal regions of proteins to be swapped out with a synthetic cassette in a single step [9]. This process is analogous to DNA transposition and employs engineered pairs of split inteins. I will discuss the use of this powerful 'protein transposition' system to study aspects of chromatin (dys) regulation.

In sum, the goal of my presentation is to highlight the emerging field of oncohistones and using advances in chemistry-driven methods, illustrate how these mutants, even at low dose, can disrupt key epigenetic processes leading to cell transformation.

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Tom Muir, PhD

1993-1996	Postdoctoral Associate, The Scripps Research Institute, San Diego, CA
1996-2000	Assistant Professor and Head of Laboratory, Rockefeller University, New York, NY
2000-2002	Associate Professor and Head of Laboratory, Rockefeller University, New York, NY
2002-2005	Professor and Head of the Selma and Lawrence Ruben Laboratory, Rockefeller University, New York, NY
2005-2011	Richard E. Salomon Family Professor /Head of Selma & Lawrence Ruben Laboratory, Rockefeller University, New York, NY
2011-present	Van Zandt Williams Jr. Class of ’65 Professor of Chemistry, Princeton University, Princeton, NJ

Y CHROMOSOME LOSS IN CANCER

Dan Theodorescu, MD, PhD
Cedars-Sinai Cancer Center
8700 Beverly Blvd., Los Angeles, CA 90048 USA
E-mail: dan.theodorescu@cshs.org

The Y chromosome is essential for male sex determination and spermatogenesis[1]. However, it is not widely appreciated that the most common genetic alteration in males is the loss of the Y chromosome (LOY) in peripheral blood mononuclear cells (PBMCs). In fact, it was recently reported that in men over the age of 70 more than 40% of them have detectable LOY, a condition known as mosaic loss of Y chromosome (mLOY). More importantly, mLOY has been associated with increased risk[2, 3] and mortality[4-6] in both solid and hematological malignancies, cardiovascular disease (pulmonary fibrosis, heart failure[7]), and neurological diseases (Alzheimer's) as well as clonal hematopoiesis, a condition associated with specific gene mutations as well as mLOY. PBMC LOY has also been associated with smoking[8].

In parallel studies that found LOY in PBMCs, LOY has also been found in cancer tissues from a variety of tumor types[9]. Interestingly, the risk of development of several tumor types has been associated with LOY in PBMCs, as well as having known LOY in the tumor itself. One such tumor type is bladder cancer (BC), where LOY has been found in 10-40% of tumors regardless of grade and stage[10-18]. This is unsurprising since BC is commonly caused by environmental exposures such as tobacco smoking and industrial chemicals that are known to result in DNA damage in epithelial tissues[19-21] and LOY in PBMCs[8, 22, 23].

Given that BC is the fourth most common cancer in men, we sought to use this cancer type as a model to begin understanding of how LOY in PBMCs contributes to cancer risk and what is the biological implication of this genetic alteration when found in tumor tissues.

The hypothesis guiding our studies was that LOY in both PBMCs and tumors contribute to tumor progression and death in bladder cancer (**Fig 1**).

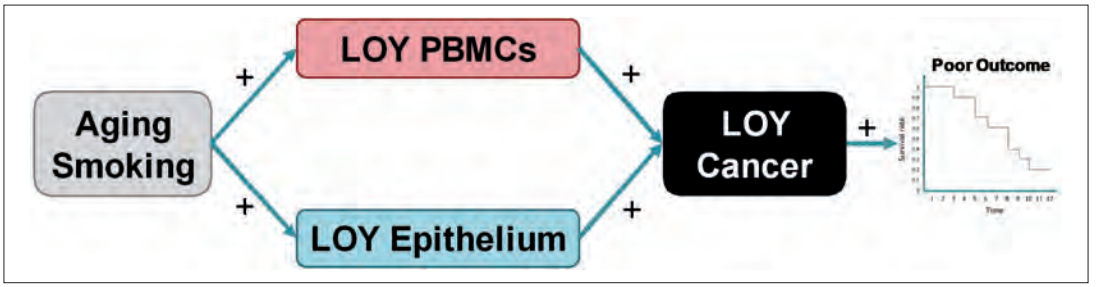

Figure 1 Hypothesis: LOY as a Driver of Bladder Cancer Mortality

To test this hypothesis, we first examined TCGA BC RNAseq data using a Y chromosome gene expression signature and found that patients with LOY had a reduced overall survival following surgery. Given that the MB49 murine BC model is widely used to investigate different aspects of BC biology and has also been shown by others to lose the Y chromosome[24], we used MB49 to isolate two distinct, naturally arising cell populations to represent Y_{low} and Y_{high} tumors. Consistent with human data, MB49 tumors with LOY grew more aggressively. Similar results were seen when knocking out two chromatin modifying genes located on the Y chromosomes, *UTY* and *KDM5D*. Overexpressing these genes in Y- lines regained tumor control in the immune competent host. Loss of *UTY*, the male counterpart of *UTX* (*KDM6A*) located on the X chromosome, has been reported to promote BC development[10, 25]. Unlike UTX, UTY possesses very low demethylase activity for H3K27[26], suggesting that UTY suppresses BC development in a demethylase-independent manner. On the other hand, KDM5D negatively regulates expression of genes involved in tumor cell invasion, such as the matrix metalloproteinase (MMP) family, by demethylation of their H3K4me3 marks. Downregulation of *KDM5D* expression increases H3K4me3 levels at target gene promoter regions, leading to a more aggressive phenotype and the development of metastasis[27].

Through use of mouse models lacking T cells (*Tcrb*/*Tcrd*$^{-/-}$), B cells (*Ighm*$^{-/-}$), or both (*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$, *Rag2*$^{-/-}$), we demonstrated that the differential growth observed between Y- and Y+ MB49 tumors was T cell-dependent. Multispectral flow cytometric analysis of these tumors, as well as in depth analyses of human muscle invasive bladder cancer (MIBC) specimens through snSeq and histological applications, revealed that $CD8^+$ T cells within the Y+ tumors retained their anti-tumor immunity, whereas $CD8^+$ T cells in Y- tumors were phenotypically exhausted. Of significance, TOX, a transcription factor typically known to transcriptionally

and epigenetically program $CD8^+$ T cells towards terminal exhaustion[28, 29], was elevated in both $CD8^+$ and $CD4^+$ T cells at various differentiation states within Y_{low} BC specimens. The molecular mechanism of this process remains unclear. How loss of Y chromosome genes from tumor cells leads to upregulation of TOX in tumor-infiltrating T cells and its role in BC progression warrants further investigation.

Importantly, our findings with naturally arising LOY cell populations were completely recapitulated with CRISPR/Cas9-mediated chromosome depletion of Y+ cells, arguing strongly that the observed phenomenon was driven entirely by loss of Y chromosome, not by any cryptic clonal variations. In essence, we discovered that LOY tumors were able to evade adaptive immunity by promoting $CD8^+$ T cell exhaustion. By doing so, LOY tumors were also more responsive to anti-PD-1 ICB because the primary mode of ICB is driving T cell differentiation from exhaustion to effector function. Taken together, evaluation of Y chromosome gene expression, especially *UTY* and *KDM5D*, could be used to select BC patients for ICB therapy with the expectation of superior response and better survival outcomes. More speculatively, transient pharmacologic inhibition of *UTY* and *KDM5D* may offer enhanced therapeutic benefit for Y_{high} patients undergoing ICB therapy. Future studies are necessary to understand the mechanism of the findings observed here and to specifically define the molecular circuitry that connects the loss of *UTY* and/or *KDM5D* to $CD8^+$ T cell exhaustion in the TME. Since age-related LOY is widespread in lymphocytes[30], it will also be fruitful to investigate the impact of such on immune surveillance. We postulate that early LOY in tumor evolution is an adaptive strategy by tumor cells to evade immunity with deep biological and therapeutical implications.

In conclusion, our study is the first to show that aggressive behavior of LOY BC is a consequence of T cell dysfunction. We report increased tumor-associated macrophages, high levels of immune checkpoint molecules, and $CD8^+$ T cell exhaustion in Y_{low} tumors. Consistent with known mechanisms of response to immunotherapy[31], patients with Y_{low} tumors exhibit a superior response to anti-PD-1/PD-L1 ICB.

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Dan Theodorescu MD PhD

1995-2009	Fellowship, Urologic Oncology, Memorial Sloan Kettering Cancer Center
1995-2009	Assistant Prof to Professor, Dept Urology and Molecular Physiology University of Virginia
1995-2009	Attending Urologist, University of Virginia Hospital
2010-2018	Professor, Departments of Surgery and Pharmacology University of Colorado,
2010-2018	Attending Urologist, University of Colorado Hospital
2010-2018	Director, NCI Comprehensive Cancer Center, University of Colorado
2017-2018	University Professor, University of Colorado
2018-Present	Professor, Departments of Surgery, Urology, and Pathology, Cedars Sinai Health System
2018-Present	Director, Cedars-Sinai Cancer Center

UNDERSTANDING DYNAMICS OF TRANSCRIPTIONAL CONDENSATES AND TARGETING EXTRACHROMOSOMAL DNA (ECDNA) IN CANCER

Hiroshi Suzuki

Division of Molecular Oncology, Center for Neurological Diseases and Cancer,
Nagoya University Graduate School of Medicine
65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan
(hisuzuki@med.nagoya-u.ac.jp)

Abnormalities in the regulation of gene expression are associated with various pathological conditions. Among the distal regulatory elements in the genome, the activation of target genes by enhancers plays a central role in the formation of cell type-specific gene expression patterns. Super-enhancers are a subclass of enhancers that frequently contain multiple enhancer-like elements and are characterized by dense binding of master transcription factors and Mediator complexes and high signals of active histone marks. Super-enhancers have been studied in detail as important regulatory regions that control cell identity and contribute to the pathogenesis of diverse diseases. In cancer, super-enhancers have multifaceted roles by activating various oncogenes and other cancer-related genes and shaping characteristic gene expression patterns in cancer cells. By focusing on quantitative features of microRNA expression and function, we have shown central roles of super-enhancers in cell identity control and cancer pathogenesis [1].

By expanding these studies, we have conducted (1) identification of a novel transcription checkpoint associated with RNA splicing and RNA quality control, which is regulated by Myc oncogene [2], (2) elucidation of functional effects of cohesin dysfunction in myeloid neoplasms [3], (3) discovery of neomorphic mutations of super-enhancer-associated miRNAs in human genetic disease [4], (4) a new direction for shRNA library-based cancer gene screening [5], (5) development of synthetic RNA circuits for cancer immunotherapy [6], and (6) optimization of CRISPR/Cas9-based genome editing [7]. The super-enhancer study has been further expanded to investigate the impacts of biomolecular condensate and liquid-liquid phase separation (LLPS) on gene regulation and extrachromosomal DNA (ecDNA) in cancer biology [8,9] (Figure 1).

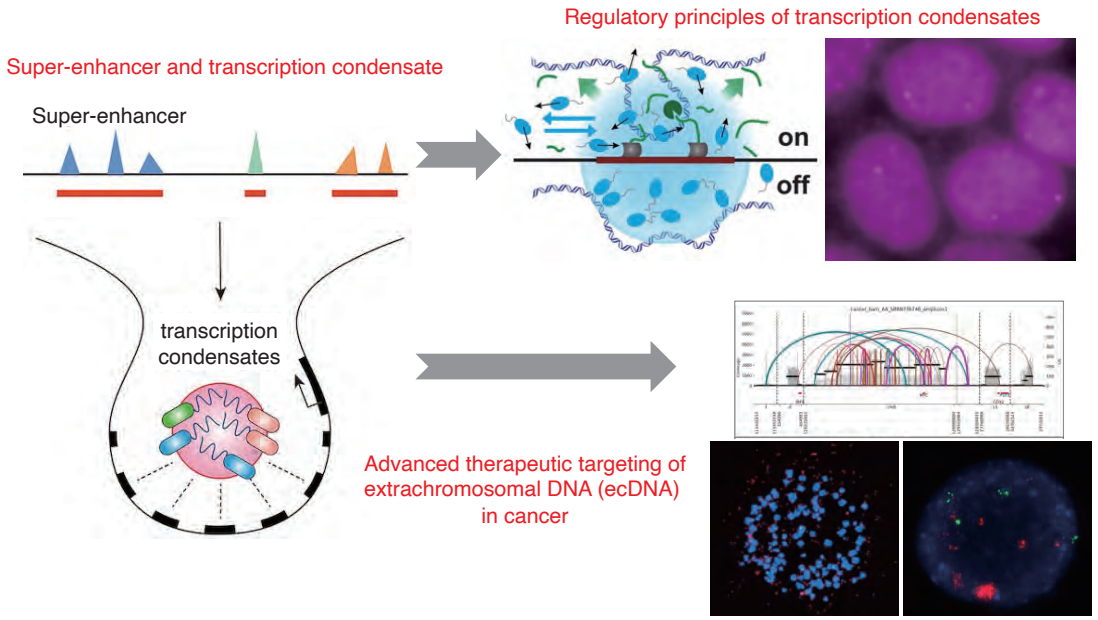

Figure 1 Biomolecular Condensate and Gene Regulation: from Super-enhancer to Extrachromosomal DNA (ecDNA).

Some unique properties of super-enhancers have suggested an intriguing link between gene regulation and membrane-less biomolecular condensates, often induced by liquid-liquid phase separation (LLPS). Recent studies have highlighted alterations in membrane-less biomolecular condensates in cancer cells, which are particularly associated with gene regulation and cell signaling ("transcriptional" and "signaling" condensates) [8,9].

Currently, super-enhancers are considered to promote the formation of transcriptional condensates including transcription factors, cofactors, and RNA polymerase II to activate transcription. By combining multi-omics analysis, cellular imaging, and biophysical simulations, we have recently found a novel regulatory mechanism of super-enhancers and transcriptional condensates. While enhancers typically generate non-coding RNAs (eRNAs) that is rapidly degraded by RNA exosome complex, disturbance of nuclear non-coding RNA homeostasis by conditional depletion of RNA exosome complex resulted in disappearance of large transcriptional condensates formed by Mediator complex and RNA polymerase II. This is further accompanied with alterations in chromatin organization, transcription, transcriptional variability and co-transcription patterns in super-enhancer-associated genes. These findings collectively suggest that dynamic RNA regulation (i.e., non-coding transcription and destabilization) is critical for homeostasis of transcriptional condensates and coordinated transcription of super-enhancer genes. We have also tried to develop new technologies to characterize molecular components of transcriptional condensates.

In addition, altered formation of transcription condensates (transcription hubs) are reportedly associated with extrachromosomal DNA (ecDNA) amplification. As an emerging class of genomic abnormalities, ecDNA is correlated with the evolution, heterogeneity, therapeutic resistance, and poor prognosis in cancer. However, the therapeutic strategies have not been established for ecDNA-positive cancers. To develop ecDNA-targeted cancer therapies, we recently undertook a comprehensive screening approach to combine whole genome sequencing (WGS)-based ecDNA profiling and CRISPR library screening. WGS-based ecDNA profiling in hundreds of cancer cells demonstrated characteristic copy number patterns associated with distinct amplicon classes including linear and circular amplifications. Importantly, we observed that, while about half of cancer cell lines has certain ecDNAs, a fraction of such ecDNA-positive cancer cells exhibits a drastic increase in ecDNA copy numbers, indicative of a fitness advantage of certain ecDNAs. By focusing on such ecDNAs and integrating CRISPR library screening, we explored cell essentiality genes in ecDNA-positive cancers.

In the symposium, we first overview the biology of super-enhancers and biomolecular condensates and will introduce our recent findings on molecular mechanisms underlying RNA-mediated dynamics of transcriptional condensates. We will also discuss our comprehensive approaches of ecDNA-related therapeutic target screening to enable ecDNA-directed next generation cancer therapeutics.

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Hiroshi Suzuki, MD, PhD

2007-2010	Graduate School of Medicine, The University of Tokyo
2010-2014	Project Assistant Professor, Department of Molecular Pathology, Graduate School of Medicine, The University of Tokyo
2014-2020	Visiting Scientist and Research Scientist, Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology (Phillip A. Sharp Laboratory)
2020-present	Professor, Division of Molecular Oncology, Nagoya University Graduate School of Medicine (co-affiliated with iGCORE, One Medicine Translational Research Center (COMIT), and InaRIS)

CAR T-CELL THERAPY FOR CHILDHOOD BRAIN CANCERS

Michelle Monje
Department of Neurology and Neurological Sciences, Stanford University
265 Campus Drive, G3077, Stanford, CA 94305, USA
(mmonje@stanford.edu)

H3K27M-mutant diffuse midline gliomas (DMGs), including diffuse intrinsic pontine glioma (DIPG), are universally lethal pediatric central nervous system tumors. DMGs and other glial malignancies diffusely infiltrate the brain and spinal cord, integrating into neural circuits through neuron-to-glioma synapses[1, 2]. This infiltrative, integrated nature of the disease prevents surgical resection of these lethal brain tumors, and chemotherapies or molecularly-targeted agents have this far proven largely ineffective, highlighting the need for alternative approaches such as immunotherapy. We discovered that DMGs express high levels of the GD2 disialoganglioside, and that chimeric antigen receptor modified T-cells targeting GD2 (GD2-CART) eradicate DMGs in preclinical models[3], providing the rationale for a first-in-human Phase 1 clinical trial (NCT04196413)[4].

Arm A of the trial administered one IV dose of autologous GD2-CART to patients with H3K27M-mutant pontine (DIPG) or spinal (sDMG) diffuse midline glioma at two dose levels (DL1=1e6/kg; DL2=3e6/kg) following lymphodepleting (LD) chemotherapy. Patients with clinical or imaging benefit were eligible for subsequent intracerebroventricular (ICV) GD2-CART infusions (10-30e6 GD2-CART). Because CAR T-cell-induced brainstem inflammation can result in obstructive hydrocephalus, increased intracranial pressure, and dangerous tissue shifts, neurocritical care precautions were incorporated. Primary objectives were manufacturing feasibility, tolerability, and identification of a maximally tolerated dose of IV GD2-CART. Secondary objectives included preliminary assessments of benefit. Thirteen patients enrolled and 11 received IV GD2-CART on study [n=3 DL1(3 DIPG); n=8 DL2(6 DIPG/2 sDMG)]. GD2-CART manufacturing was successful for all patients. No dose-

limiting toxicities (DLTs) occurred on DL1, but three patients experienced DLT on DL2 due to grade 4 cytokine release syndrome (CRS). Nine patients received ICV infusions, which were not associated with DLTs. All patients exhibited tumor inflammation-associated neurotoxicity (TIAN). Four patients demonstrated major volumetric tumor reductions (52%, 54%, 91% and 100%), while an additional four patients exhibited smaller tumor reductions. One patient exhibited a complete response ongoing for >30 months since enrollment. Nine patients demonstrated neurological benefit based upon a protocol-directed Clinical Improvement Score. Sequential IV followed by ICV GD2-CART induced tumor regressions and neurological improvements in patients with DIPG and sDMG. DL1 was established as the maximally tolerated IV GD2-CART dose. Neurotoxicity was safely managed with intensive monitoring and close adherence to a management algorithm[5]. These early results underscore the promise of this approach for H3K27M+ DIPG/DMG therapy and, more broadly, provide lessons for immunotherapy of central nervous system cancers.

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Michelle Monje, MD, PhD

2008-2011	Postdoctoral Fellow, Laboratory of Philip Beachy, PhD, Stanford University
2011-2018	Assistant Professor in the University Tenure Line, Department of Neurology and Neurological Sciences, Stanford University
2018-2021	Associate Professor with tenure, Department of Neurology and Neurological Sciences, Stanford University
2021-present	Professor, Department of Neurology and Neurological Sciences, Stanford University
2021-present	Investigator, Howard Hughes Medical Institute

NEXT-GENERATION CAR-T CELLS TO INDUCE EPITOPE-SPREADING FOR CANCER THERAPY

Koji Tamada
Research Institute of Cell Design Medical Science, Yamaguchi University
1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan
(ktamada@yamaguchi-u.ac.jp)

CAR-T cell therapy against hematological malignancies demonstrated potent therapeutic efficacy. On the other hand, CAR-T cell therapy against solid cancers has yet to be fully developed, as limited cases of early-stage clinical trials have demonstrated the efficacy of CAR-T cells in solid cancers. Potential hurdles for the effects of CAR-T cell therapy in solid cancers include heterogeneity of tumor-associated targets, insufficient migration and infiltration of CAR-T and endogenous immune cells into tumor tissues, and immunosuppressive nature of tumor microenvironment. Novel technologies to overcome these hurdles are highly demanded.

To address these issues, we developed novel CAR technology enabling CAR-T cells to produce both interleukin-7 (IL-7) and CCL19, which induced the efficient accumulation and expansion of the transferred CAR-T cells and host immune cells inside tumor tissues, and demonstrated potent therapeutic efficacy in various mouse solid cancer models [1-7]. In the mice inoculated with OVA-expressing B16F10 melanoma, administration of 7x19 CAR-T cells induced MHC-restricted T cells responses against OVA and intrinsic tumor antigens including TRP-1, TRP-2, and gp100. In these models, the number of CD11b-negative, CD8α-positive dendritic cells (DC), which are known to mediate cross presentation, was increased. In addition, DC harvested from these mice induced the activation of OT-I T cells specific to OVA. The mice inoculated with a mixture of CAR target-positive and negative B16F10, as a model mimicking the heterogeneity of solid cancers, were successfully treated by 7x19 CAR-T cells. Thus, our findings indicated that the epitope spreading is induced in the process of therapeutic responses in solid cancers by 7×19 CAR-T cells, in association with cross presentation by DC (Figure 1).

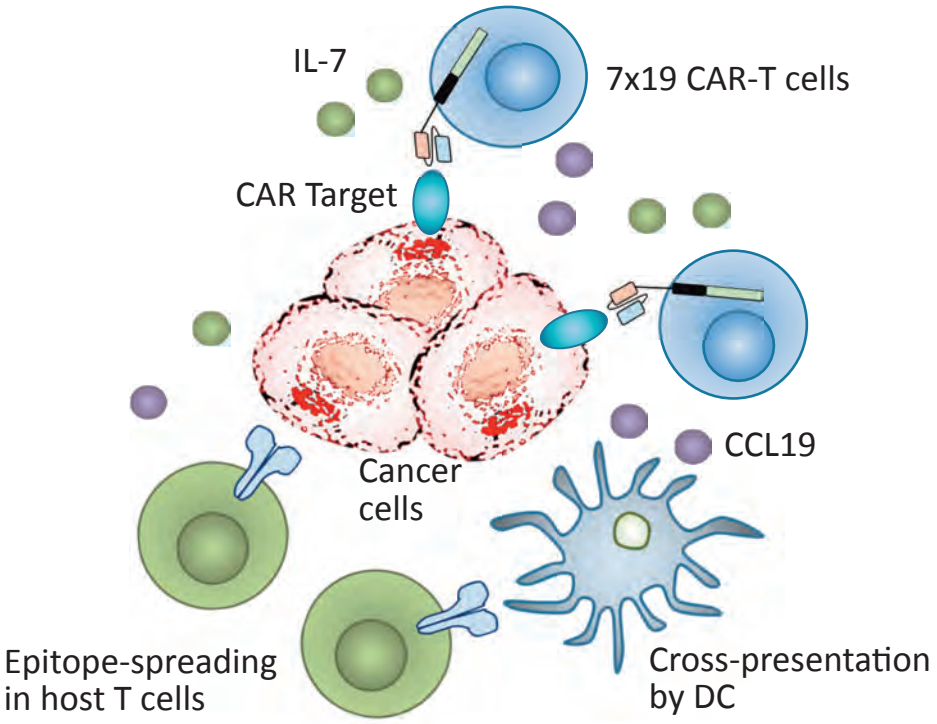

Figure 1 Mechanism of 7x19 CAR-T cells for anti-tumor responses

Technology converting CAR-T cells to express IL-7 and CCL19 simultaneously is a unique and novel approach which efficiently induces the epitope-spreading and to overcome the heterogeneity of solid cancers. Further mechanistic insights and clinical application of 7x19 CAR-T technology are expected.

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Koji Tamada M.D., Ph.D.

1998-2002	Post-doctoral fellow, Mayo Clinic College of Medicine, US
2002-2004	Research Associate, Department of Immunology Mayo Clinic College of Medicine, US
2004-2007	Assistant Professor, Johns Hopkins University School of Medicine, US
2007-2009	Assistant Professor, Marlene and Stewart Greenebaum Cancer Center University of Maryland School of Medicine, US
2009-2011	Associate Professor, Marlene and Stewart Greenebaum Cancer Center University of Maryland School of Medicine, US
2011-present	Professor and Chairman, Department of Immunology Yamaguchi University of Graduate School of Medicine, Japan
2016-present	Adjunct Professor, The Institute of Medical Science, University of Tokyo, Japan
2023-present	Director, Research Institute for Cell Design Medical Science, Yamaguchi University, Japan

RETHINKING CANCER TARGETING IN THE ERA OF SMART CELL THERAPEUTICS

Wendell A. Lim
Cell Design Institute and Dept. of Cellular and Molecular Pharmacology,
University of California San Francisco, 600 16th St., San Francisco, CA94158, USA
(wendell.lim@ucsf.edu)

In the past few decades, cancer therapeutics have increasingly focused on precision targeting of single cancer-associated molecules. Despite great advances, such targeted therapies still show incomplete precision and the eventual development of resistance due to target heterogeneity or mutation. However, the recent development of cell-based therapies such as chimeric antigen receptor (CAR) T cells presents a revolutionary opportunity to reframe strategies for targeting cancers. Immune cells equipped with synthetic circuits are essentially living computers that can be programmed to recognize tumors based on multiple signals, including both tumor cell-intrinsic and microenvironmental features [1-4]. Moreover, cells can be programmed to launch broad but highly localized therapeutic responses that can limit the potential for escape while still maintaining high precision.

For example, we have developed novel multi-step "prime-then-kill" circuits that increase the precision of tumor killing by recognizing two or more antigens [1-2]. Importantly, we can strategically design the circuits such that priming is activated by recognition of a highly specific but less homogeneous antigen (e.g. neoantigen) but killing is triggered by recognition of a more homogeneous but less specific antigen – this partitioning of recognition yields highly specific tumor targeting but more complete and robust killing of even highly heterogeneous tumors [3,4]. We have also developed circuits that allow for targeted remodeling of the tumor microenvironment, via induced delivery of modulating cytokines, chemokines, and other factors that, for example, disrupt an immunosuppressive microenvironment [5].

These types of complex cell sense-and-response circuits can be integrated within a single living cell, resulting in therapeutic agents capable of more sophisticated behaviors that combine nuanced recognition with highly efficacy, in ways that are simply impossible with a molecular agent. In principle, genomic/proteomic tumor data could be used with artificial intelligence to guide how to most effectively design circuits for each disease [6]. We are initiating clinical trials for glioblastoma and pancreatic cancer that harness these types of next generation recognition circuits [5,7].

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Wendell A. Lim, PhD

2020-present	Director, UCSF Cell Design Institute
2020-present	Director, UCSF Center for Synthetic Immunology
2019-present	Byers Distinguished Professorship
2015-2022	Chair, Dept.of Cellular & Molecular Pharm., UCSF
2010-2019	Director, UCSF Center for Sys. & Synthetic Biology
2008-2020	Investigator, Howard Hughes Medical Institute
2006-2016	Director, UCSF/UCB NIH Nanomedicine Center
2006-2015	Deputy Director, NSF Synthetic Biology ERC
2003-present	Professor, Cellular & Molecular Pharm, UCSF
2000-2003	Assoc Professor, Cell. & Molecular Pharm, UCSF
1996-2000	Assist. Professor, Cell. & Molecular Pharm., UCSF

CONCLUDING REMARKS

Hideyuki Saya
Oncology Innovation Center, Fujita Health University
1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
(hsaya@fujita-hu.ac.jp)

The successful completion of the 52nd International Symposium of the Princess Takamatsu Cancer Research Fund (PTCRF) would not have been possible without the invaluable contributions of many individuals and organizations.

First and foremost, we extend our deepest gratitude to all the distinguished speakers and panelists who shared their cutting-edge research and insights. Their expertise and dedication have been instrumental in making this symposium a truly enriching experience. This year's symposium, titled "Cancer Research and Medicine Advanced by Emerging Technologies and Innovative Concepts," was designed to address the increasing fragmentation of cancer research while fostering interdisciplinary exchange. By bringing together leading scientists from diverse fields, we aimed to facilitate discussions on next-generation technologies and concepts that will shape the future of cancer research and treatment.

Throughout the symposium, we explored key topics such as single-cell analysis, tumor heterogeneity, genomic instability, and the integration of molecular, metabolic, and genetic approaches to better understand and combat cancer. Researchers presented pioneering work on data-driven, unbiased methodologies that offer unprecedented insights into tumor biology and its microenvironment. We also examined fundamental cancer properties, including chromosomal instability, DNA damage, and metabolic alterations—factors that play crucial roles in tumor progression and treatment resistance.

We were privileged to host special sessions highlighting the latest developments in KRAS

inhibitors, which have now reached clinical application. The Nakahara Memorial Lecture by Dr. Frank McCormick provided invaluable insights into the journey of KRAS-targeted drug development, while the Keynote Lecture by Dr. Charles Sawyers explored mechanisms of resistance in molecular targeted therapy. Additionally, a dedicated session on research ethics underscored the critical need to address ethical considerations in the rapidly evolving landscape of cancer research, ensuring that scientific advancements are pursued with integrity and responsibility.

We also wish to express our sincere appreciation to all participants, whose engagement and thought-provoking discussions fostered meaningful scientific exchange and collaboration. The dynamic interactions throughout this symposium reflect the strength and diversity of the global cancer research community, and we hope that the ideas shared here will lead to new collaborations and discoveries.

A special acknowledgment goes to the members of the Organizing Committee and all supporting staff who worked tirelessly behind the scenes to ensure the smooth execution of this event. Their commitment and meticulous efforts have been invaluable in creating a platform for productive dialogue and innovation.

Finally, we are profoundly grateful to the Princess Takamatsu Cancer Research Fund for its unwavering support in advancing cancer research through this symposium. Its mission to promote scientific progress and international collaboration remains at the heart of our endeavors.

We hope that the discussions and connections fostered here will lead to new breakthroughs in cancer research and treatment, further strengthening our collective fight against this disease.

Thank you once again to everyone who contributed to this symposium. We look forward to continued collaboration and to welcoming you at future meetings.

Hideyuki Saya, MD, PhD
Chairman, Organizing Committee
52nd International Symposium of the Princess Takamatsu Cancer Research Fund