Extended Abstracts for the 50th Commemorative
International Symposium of
the Princess Takamatsu Cancer Research Fund, 2019

NEW HORIZONS FOR CANCER RESEARCH AND PRECISION MEDICINE

November 12-14, 2019
Tokyo, Japan

Edited by :
Hiroyuki Mano, Elaine R. Mardis, Kohzoh Imai,
Masanobu Oshima and Ryuzo Ueda

Princess Takamatsu Cancer Research Fund

CONTENTS

KEYNOTE LECTURE

PRECISION CANCER MEDICINE

CANCER GENOMICS AND EPIGENOMICS

IMMUNOGENOMICS

CANCER EVOLUTION

NONCODING RNA

METABOLIC TARGETS IN CANCER

CANCER MODELS (ORGANOIDS, PDX, GEM)

TUMOR MICROENVIRONMENT

NAKAHARA MEMORIAL LECTURE

NOVEL IMMUNOTHERAPEUTICS

BASIC MECHANISMS OF CARCINOGENESIS

CONCLUDING REMARKS

PEDIATRIC CANCER PRECISION MEDICINE

Elaine R. Mardis
The Steve and Cindy Rasmussen Institute for Genomic Medicine
Nationwide Children's Hospital
575 Children's Crossroad, Columbus, OH 43215, USA
(elaine.mardis@nationwidechildrens.org)

Pediatric cancer has largely not benefitted from the clinical use of targeted or immunotherapies, due to a lack of clinical trials and other factors including significant differences in the somatic driver alterations when compared to adult cancers. The result of this lack of progress is that cancer remains the leading cause of death by disease in children in first world countries, and those children who do survive their disease face significant adverse health impacts from current therapies for the remainder of their lives, including an elevated risk for secondary cancers.

Our efforts of the past two years have sought to develop and integrate genomic assays of pediatric cancers into the rubric of interdisciplinary molecular tumor boards conducted in collaboration with our oncology providers. These efforts aim to incorporate genomics-based molecular diagnosis, prognosis and therapeutic implications in the context of conventional diagnostics. Of equal importance is the availability of clinical trials that include pediatric cancer patients and provide access to targeted therapies in the setting of recurrent or treatment-refractory cancers. We have developed DNA- and RNA-based assays that provide unbiased evaluation of cancer and germline, and corresponding analytical pipelines that provide a broad range of diagnostically relevant information for each patient studied. To-date, we have studied over 150 patients on a research protocol that identifies DNA level germline and somatic alterations using high depth exome and evaluates all chromosomes for copy number alterations. In addition, we analyze cancer-specific RNAseq data through multiple analytical pipelines to provide information on fusion gene drivers, over-expressed genes, activated and repressed pathways, and the tumor microenvironment. Recently, we have integrated methylation arrays into the

evaluation of these patients, which provide subtyping and copy number alteration data.

The diagnostic yield of this set of assays and analytics is over 75%, which is reasonably high compared to other reports in pediatric cancer applied genomics (Figure 1). We also are beginning to see our diagnoses applied to patient care, which is an important route to ultimately decreasing the impact of treatment-associated sequelae for pediatric cancer survivors. Importantly, the ability to study longitudinal samples from previously banked surgical specimens and our multiplex analyses have provided a virtuous cycle of new research questions that we anticipate will yield new insights into tumor biology. New funding will permit an exploration of our observations of increased immune cell infiltration in recurrent CNS cancers and how this infiltrate might be indicative of an immune-suppressive microenvironment that could be targeted with novel therapeutics. By sharing our data and outcomes, along with our analytical methods, we hope to extend the application of genomics to pediatric precision medicine, thereby introducing assays and information that will guide providers to a more nuanced view of each patient and their possible treatment avenues.

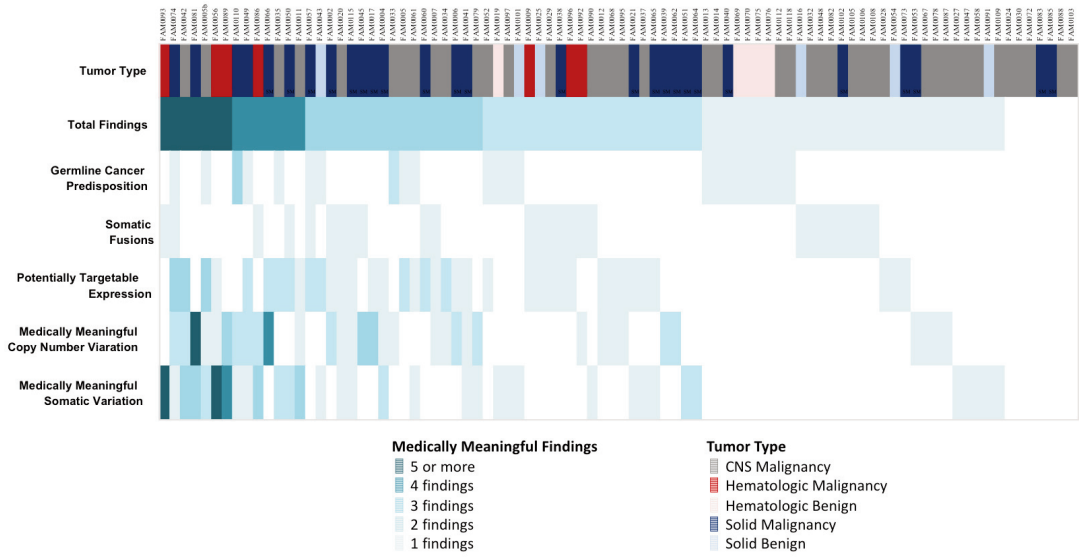


Figure 1 Composite report of medically relevant findings from the Institute for Genomic Medicine Pediatric Cancer Protocol. Medically relevant findings include prognostic, diagnostic and therapeutic indications from genomic testing using DNA and RNA assays of pediatric cancers. The tumor type is broadly indicated by color coding as indicated, with different findings indicated according to the number of findings by different blue shades.

References

1. Schieffer KM, Varga E, Miller KE, Agarwal V, Koboldt DC, Brennan P, Kelly B, Dave-Walla A, Pierson CR, Finlay JL, AbdelBaki MS, White P, Magrini V, Wilson RK, Mardis ER, Cottrell CE. Expanding the clinical history associated with syndromic Klippel-Feil: A unique case of comorbidity with medulloblastoma. *Eur J Med Genet*. 2019 Aug;62(8):103701.
2. Miller KE, Kelly B, Fitch J, Ross N, Avenarius MR, Varga E, Koboldt DC, Boué DR, Magrini V, Coven SL, Finlay JL, Cottrell CE, White P, Gastier-Foster JM, Wilson RK, Leonard J, Mardis ER. Genome sequencing identifies somatic BRAF duplication c1794-1796dupTAC;p.Thr599dup in pediatric patient with low-grade ganglioglioma. Cold Spring Harb Mol Case Stud. 2018 Apr 2;4(2).

Elaine R. Mardis, PhD

1984-1989	Graduated with PhD from University of Oklahoma, Department of Chemistry and Biochemistry
1989-1993	Bio-Rad Laboratories, Senior Scientist, R&D
1993-2016	Faculty member and Co-Director, McDonnell Genome Institute, Washington University School of Medicine
2016-present	Co-Executive Director, The Steve and Cindy Rasmussen Institute for Genomic Medicine at Nationwide Children's Hospital Professor of Pediatrics, The Ohio State University College of Medicine

DEVELOPMENT OF JAPANESE CANCER GENOMIC MEDICINE PLATFORM

Hiroyuki Mano
Center for Cancer Genomics and Advanced Therapeutics/Research Institute
National Cancer Center
5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
(hmano@ncc.go.jp)

Our discovery of *EML4-ALK* oncogene in lung cancer brought highly effective ALK inhibitors to the patients carrying this fusion gene. Identification of other *ALK* fusions in various cancer subtypes further led to realize the importance of beyond-organ, gene-based cancer classification scheme [1], ushering in the "cancer genomic medicine" to the clinics.

To assist clinical sequencing of cancer specimens, we examined a possible caner gene panel test that sensitively and accurately detects gene fusions as well as single nucleotide variations [2]. For detecting gene fusions from formalin–fixed paraffin–embedded (FFPE) specimens, there are principally two approaches; one is to extract genomic DNAs and to capture intronic regions, and the other is to extract RNAs from FFPE and to convert them to cDNA for sequencing. While the latter approach is supposed to be inferior to the former due to RNA degradation in FFPE, we unexpectedly observed that the opposite is the case. Comparison of these two approaches by using fusion–positive FFPE samples revealed that RNA sequencing even from FFPE materials provides much higher sensitivity and specificity for fusion detection.

We thus developed a twin panel system, Todai OncoPanel (TOP), that is comprised of TOP DNA panel interrogating 464 genes and TOP RNA panel examining the fusions of 365 genes and the expression of 109 genes (Figure 1). It can also assess allele–specific chromosome copy number aberrations, microsatellite status and exon skipping. The TOP panel system allows reliable genome profiling even from small lung biopsy specimens [3], and is under Advanced Medical Care as of November 2019 to prove its clinical utility.

Japan planned to adapt gene-panel testing to optimize cancer treatments under the national health insurance system. To discuss a necessary platform to perform such cancer

genomic medicine in a nation–wide manner, The Expert Meeting for Cancer Genomic Medicine Promotion Consortium was held in the Spring of 2017 in The Ministry of Health, Labour and Welfare (MHLW). The Expert Meeting recommends a step-wise adaptation of genomic medicine, *i.e.* such medicine should be first conducted only in designated hospitals, and the number of these hospitals shall be increased gradually. Another important proposal from the Expert Meeting is to set a central datacenter to aggregate genomic as well as clinical information of gene-panel tests. MHLW accordingly designated, in the Spring of 2018, eleven Core Hospitals for Cancer Genomic Medicine and 156 of Cooperative Hospitals for Cancer Genomic Medicine. The Ministry also established, in June 2018, The Center for Cancer Genomics and Advanced Therapeutics (C-CAT) to store and utilize genomic/clinical information (Figure 2).

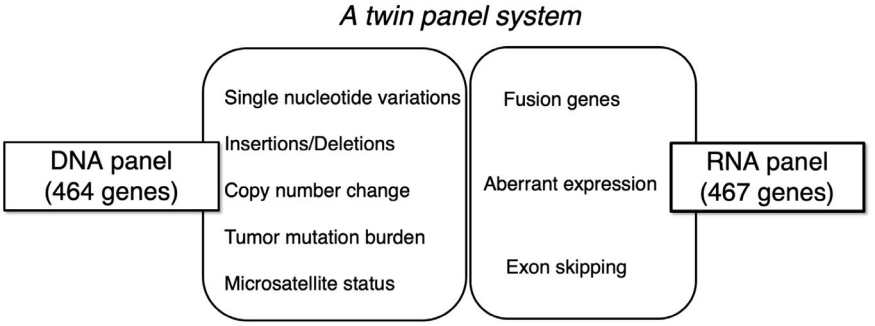


Figure 1 The TOP panel system

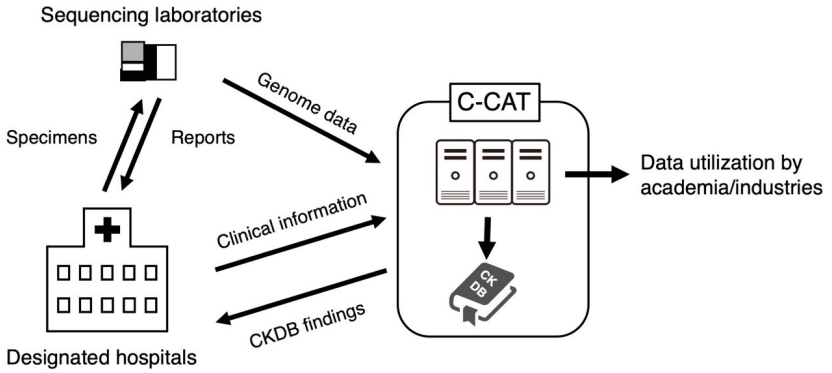


Figure 2 The cancer genomic medicine platform in Japan

The genomic medicine platform in Japan, consisting of these hospitals and C-CAT, officially started on June 1st 2019 with two approved cancer gene panels, "OncoGuide NCC Oncopanel" and "FoundationOne CDx". When patients are admitted to the designated hospitals and give consent to genomic medicine, their specimens will be sent to certified laboratories for sequencing. While the resultant data will be sent back to hospitals, the genomic data will also be sent to C-CAT. Clinical information of patients will be transferred to C-CAT from corresponding hospitals. These clinical and genomic information for every patient will be referred to C-CAT cancer knowledge database (CKDB) to generate "CKDB findings" reports containing the information of matched clinical trials and clinical annotation for each mutation. The patients' data are further utilized, upon their consent, by academia and industries for drug/biomarker development. We are preparing the "C-CAT Cloud" system so that such genomic and clinical information shall become available to academia and third parties.

References

1. Mano H. ALKoma: a cancer subtype with a shared target. Cancer Discov. 2012:2;495-502.
2. Kohsaka S, Tatsuno K, Ueno T, Nagano M, Shinozaki-Ushiku A, Ushiku T, Takai D, Ikegami M, Kobayashi H, Kage H, Ando M, Hata K, Ueda H, Yamamoto S, Kojima S, Oseto K, Akaike K, Suehara Y, Hayashi T, Saito T, Takahashi F, Takahashi K, Takamochi K, Suzuki K, Nagayama S, Oda Y, Mimori K, Ishihara S, Yatomi Y, Nagase T, Nakajima J, Tanaka S, Fukayama M, Oda K, Nangaku M, Miyazono K, Miyagawa K, Aburatani H, Mano H. Comprehensive assay for the molecular profiling of cancer by target enrichment from formalin-fixed paraffin-embedded specimens. Cancer Sci. 2019;110:1464-1479.
3. Kage H, Kohsaka S, Shinozaki-Ushiku A, Hiraishi Y, Sato J, Nagayama K, Ushiku T, Takai D, Nakajima J, Miyagawa K, Aburatani H, Mano H, Nagase T. Small lung tumor biopsy samples are feasible for high quality targeted next generation sequencing. Cancer Sci. 2019;110:2652-2657.

Hiroyuki Mano, MD, PhD

1984	Graduation from School of Medicine, Faculty of Medicine, The University of Tokyo
1986-1989	Clinical Fellow, The Third Department of Internal Medicine, Faculty of Medicine, The University of Tokyo
1989-1991	Postdoctoral Researcher, Department of Biochemistry, St. Jude Children's Research Hospital
1991-1993	Assistant Professor, The Third Department of Internal Medicine, Faculty of Medicine, The University of Tokyo
1993-2001	Associate Professor, Department of Molecular Biology, Jichi Medical University
2001-2013	Professor, Division of Functional Genomics, Jichi Medical University
2013-2018	Professor, Department of Cellular Signaling, Graduate School of Medicine, The University of Tokyo
2016-present	Director, Research Institute, National Cancer Center
2018-present	Director, Center for Cancer Genomics and Advanced Therapeutics, National Cancer Center

INACTIVATION OF DNA REPAIR TO IMPROVE IMMUNE SURVEILLANCE

Alberto Bardelli

Department of Oncology, University of Torino
Laboratory Molecular Oncology, Candiolo Cancer Institute FPO - IRCCS, Candiolo (TO)
EACR (European Association for Cancer Research)
SP 142 km 3,95 - 10060 Candiolo (TO) - Italy
(alberto.bardelli@unito.it)

The mismatch repair (MMR) system, which detects and corrects base mismatches, insertions, and deletions that occur during DNA synthesis, is deregulated in approximately 20% of human cancers. MMR deficient tumors have peculiar properties, including early onset, metastatic potential but generally favorable prognosis, and remarkable response to immune therapy. The functional bases of these atypical clinical features are poorly understood.

We studied how the genomic landscape of MMR deficient tumors affects their biological and clinical behaviors. MutL homolog 1 (MLH1) was genetically inactivated in colorectal, breast and pancreatic mouse cancer cells. The growth of MMR deficient cells was comparable to their proficient counterparts in vitro and upon transplantation in immunocompromised mice. In contrast MMR deficient cancer cells grew poorly when transplanted in syngeneic mice. MMR inactivation increased the mutational burden and led to dynamic mutational profiles, resulting in persistent renewal of neoantigens *in vitro* and *in vivo*, while MMR proficient cells exhibited stable mutational loads and neoantigen pro-files over time. Immune surveillance improved when cancer cells in which MLH1 had been inactivated accumulated neoantigens for several generations.

We next assessed whether the mutational and neoantigen profiles were also modulated in human CRC carrying DNA specific repair defects. To this end Whole Exome Sequencing (WES) and RNA sequencing (RNAseq) were performed in CRC cell lines, *in vitro* and *in vivo*, and in CRC patient-derived xenografts (PDXs) to track longitudinally genomic profiles, clonal evolution, mutational signatures and predicted neoantigens. The majority of CRC models showed remarkably stable mutational and neoantigen profiles, however

those carrying defects in DNA repair genes continuously diversified. Rapidly evolving and evolutionary stable CRCs displayed characteristic genomic signatures, and transcriptional profiles. We further found that molecules implicated in antigen presentation occurred selectively in highly mutated and rapidly evolving CRC.

These results indicate that human CRC cells carrying alterations in DNA repair pathways display dynamic neoantigen patterns that fluctuate over time. Longitudinal monitoring of the neoantigen landscape could be relevant in the context of precision medicine.

Overall these data suggest that targeting DNA repair processes can increase the burden of neoantigens in tumor cells and could be exploited for therapeutic approaches.

References

1. Germano G, Lamba S, Rospo G, Barault L, Magrì A, Maione F, Russo M, Crisafulli G, Bartolini A, Lerda G, Siravegna G, Mussolin B, Frapolli R, Montone M, Morano F, de Braud F, Amirouchene-Angelozzi N, Marsoni S, D'Incalci M, Orlandi A, Giraudo E, Sartore-Bianchi A, Siena S, Pietrantonio F, Di Nicolantonio F, Bardelli A. Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. Nature. 2017 Dec 7;552(7683):116-120. doi: 10.1038/nature24673. Epub 2017 Nov 29. PubMed PMID: 29186113.
2. Rospo G, Lorenzato A, Amirouchene-Angelozzi N, Magrì A, Cancelliere C, Corti G, Negrino C, Amodio V, Montone M, Bartolini A, Barault L, Novara L, Isella C, Medico E, Bertotti A, Trusolino L, Germano G, Di Nicolantonio F, Bardelli A. Evolving neoantigen profiles in colorectal cancers with DNA repair defects. Genome Med. 2019 Jun 28;11(1):42. doi: 10.1186/s13073-019-0654-6. PubMed PMID: 31253177; PubMed Central PMCID: PMC6599263.
3. Truini A, Germano G, Bardelli A. Inactivation of DNA repair-prospects for boosting cancer immune surveillance. Genome Med. 2018 Nov 28;10(1):91. doi: 10.1186/s13073-018-0603-9. PubMed PMID: 30486892; PubMed Central PMCID: PMC6260742.
4. Germano G, Amirouchene-Angelozzi N, Rospo G, Bardelli A. The Clinical Impact of the Genomic Landscape of Mismatch Repair-Deficient Cancers. Cancer Discov. 2018 Dec;8(12):1518-1528. doi: 10.1158/2159-8290.CD-18-0150. Epub 2018 Nov 15. Review. PubMed PMID: 30442708.

Alberto Bardelli, PhD

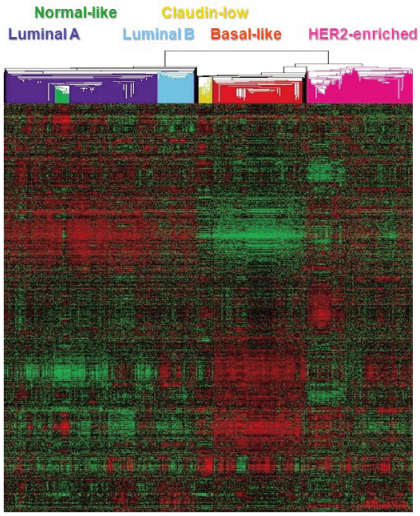
1991-1996	PhD in Biochemistry and Molecular Biology, University College London (UCL). Ludwig Institute for Cancer Research. London (UK). Supervisor: Prof. M. Waterfield.
1996-1999	Postdoctoral Fellow, IRCC and the University of Torino, School of Medicine
1999-2004	Postdoctoral Fellow, The Johns Hopkins University; School of Medicine and the Howard Hughes Medical Institute, Supervisor: Prof. Bert Vogelstein.
2004-present	Director, Laboratory of Molecular Oncology
2016-present	Full Professor, University of Torino, Dept. of Oncology
2018-present	President, EACR - European Association for Cancer Research

QUANTITATIVE MEDICINE FOR BREAST CANCER PATIENTS

Charles M. Perou

Department of Genetics/Computational Medicine Program
Lineberger Comprehensive Cancer Center
Marsico Hall, 5th Floor, CB#7599, 125 Mason Farm Road
The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
(cperou@med.unc.edu)

Gene-expression profiling has had a considerable impact on our understanding of breast cancer biology and clinical care. During the past 20 years, five intrinsic molecular subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like)[1], and a rare subtype with features of stem cells and EMT (Claudin-low)[2], have been identified and intensively studied. Over the years the breast cancer intrinsic subtype designation has been shown to predict multiple therapeutic strategies[3-6], including those outlined in Figure 1; in addition, immune cell types can also be quantitatively identified



Intrinsic Subtype, ROR Score, and/or Immune Signatures provide valuable information for:

1. **The biology of breast cancer**
2. **Baseline prognosis**
3. **Prognosis/Prediction for Endocrine Therapy treated patients**
4. **Prediction of response to Chemotherapy**
5. **Prediction of response to HER2-targeting**
6. **Prediction of response to Immune Checkpoint Inhibitors**

Figure 1

using gene expression profiling[7] and predict prognosis, the benefit of chemotherapy in TNBCs, and may also be linked to an ability to predict response to immune checkpoint blockade[8]. My presentation will briefly touch upon each of these topics with an emphasis on the TNBC/basal-like subtype and it's interactions with the immune system.

The "intrinsic subtype" gene expression patterns were originally identified using DNA microarrays, and have been further developed into a biomarker often referred to as the "PAM50" assay[3], which is currently approved for use in >25 countries around the world. The PAM50 subtyping assay provides important information within Hormone Receptor (HR)-positive breast cancer patients, where the Luminal A and B subtypes represent the majority of cases. Compared to Luminal A, Luminal B tumors are characterized by higher expression of proliferation/cell cycle-related genes and lower expression of several ER-regulated genes such as the progesterone receptor[9]. Clinically, Luminal B tumors show higher pCR rates to neoadjuvant chemotherapy, but worse survival at 5-years and 10-years regardless of adjuvant systemic therapy. The Luminal A vs B distinction, together with tumor size and nodal status, is encompassed within the "PAM50 ROR Score"[3], which quantitatively predicts recurrence and overall survival[10]. In particular, the ROR-PT score is able to identify a low risk group of patients whose 10 year disease free survival is >95% when these patients receive 5 years of endocrine therapy only[11]. Importantly, in the USA the current endocrine therapy recommendation is for an ER/PR+ patient to receive 10 years of therapy, however, the ROR-PT low score patients have an outstanding 10 year survival when receiving only 5 years of endocrine therapy, suggesting that 5 years of

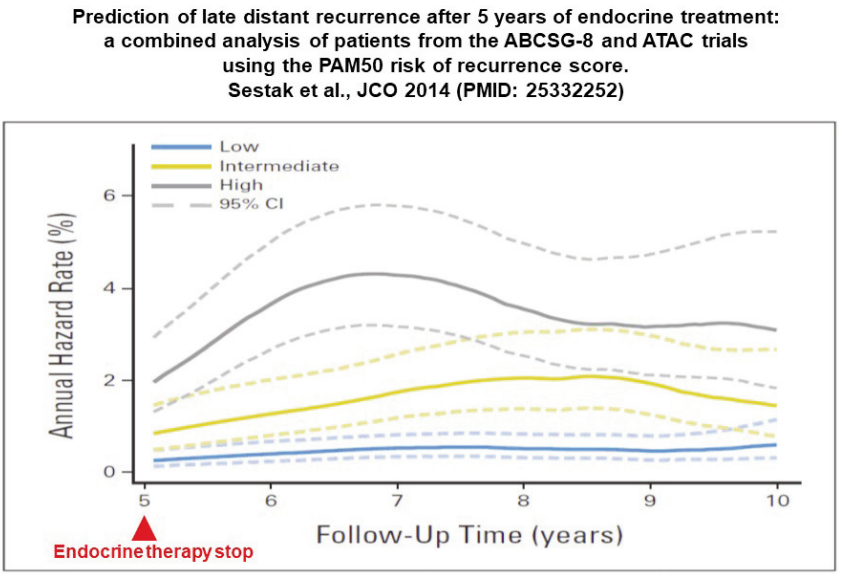


Figure 2 Annual hazard rate curve for all patients according to risk of recurrence groups.

treatment is a good option for these patients. Therefore this gene expression based classification can inform clinical decision making concerning the length of endocrine therapy treatment (i.e. 5 years vs 10 years)[12].

Triple Negative Breast Cancers (TNBC) are amongst the most clinically challenging because of their poor prognosis and paucity of treatment options, which is predominately chemotherapeutics. Through analyses of population-based studies, we and others have determined that TNBC are more common in younger women, and African American women, which contributes to racial disparities in mortality due to breast cancer seen in the USA[13, 14]. Within TNBC, the Basal-like subtype predominates (70-80%)[15], but all the intrinsic subtypes are present. Pan-Cancer analyses of 33 tumor types using 10,000 TCGA tumors revealed that the breast cancer Basal-like subtype is a unique disease entity[16], which is also supported by epidemiological studies[13]. In addition, genomic predictors of chemotherapy benefit for TNBC identify immune cell features as positive predictors of response, thus highlighting the importance of the immune microenvironment in response to chemotherapy (Figure 3 and [17]).

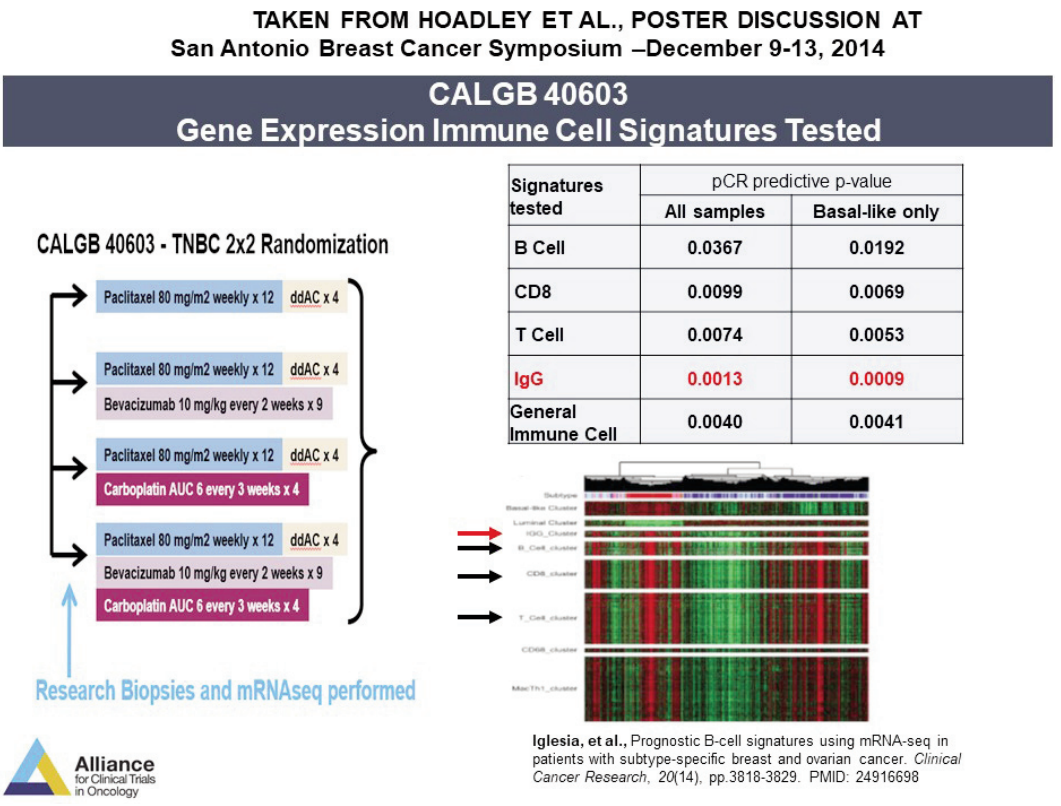


Figure 3

Within clinically HER2+ disease, all the intrinsic subtypes can again be identified, albeit with different proportions when compared to HER2-negative tumors[6]. Among them, the HER2-enriched subtype represents the majority of HER2+ tumors. Recent data suggests that patients with HER2-enriched subtype tumors benefit the most from trastuzumab, and the most from dual HER2 blockade composed of trastuzumab and lapatinib[6]. We again noted that genomic signatures of immune cells predicted a higher pathological complete response rate in the HER2+ neoadjuvant setting, suggesting that tumor subtype plus measures of the immune microenvironment each provide clinically relevant information[18].

Lastly, as was mentioned above, many distinct immune cell types can be identified using gene expression profiling of bulk tumors including B cells, T cells, and Macrophages[7]. In addition, we have shown that these immune cell genomic signatures are prognostic of patient outcomes across most solid epithelial cancers[19]. For TNBC patients, an Immune Checkpoint Inhibitor (ICI) was recently approved using PDL1+ by IHC as the biomarker[20]. To develop improved biomarkers for TNBC patients receiving ICI, we first turned to our murine mammary TNBC models, which showed a low tumor mutation burden (TMB) and low ICI responsiveness. Therefore we developed cell lines from each of two distinct murine TNBC tumors, "mutagenized" these *in vitro* using either ectopic expression of APOBEC3 or UV irradiation, and determined by exome sequencing that we increased the TMB levels by 2-4X; these high TMB mammary tumors were then grown *in vivo* in immune competent mice and we determined that they were highly sensitive to ICIs (Figure 4 and [8]). The results from these "mutagenized" lines have two important implications: first that high TMB/neoantigens is likely a key determinant of ICI responsiveness, and second, that we now have multiple ICI responsive mammary TNBC models for further investigations of the molecular mechanisms of this responsiveness. Building upon this, one of the mechanistic insights coming from these mammary TNBC studies was that genomic signatures of B-cells, in particular B cell signatures containing mature antibody production (so called "IgG signature"), was seen in all responsive models. Interestingly, a human counterpart of this murine "IgG" signature has also been previously identified by ourselves, and many others, and is highly prognostic across multiple human tumor types including breast and lung cancers[19]. Finally, we performed *in vivo* cell depletion studies in these ICI responsive TNBC "mutagenized" models, and noted that depletion of B cells (as well as depletion of CD4+ or CD8+ T Cells) abrogated the response to ICI suggesting that both B cells and T cells are needed for full responsiveness[8].

In closing, much of our current and future research is focused on the development of integrated computational predictors that link together somatic genetics, gene expression features, and pathology features using Machine Learning approaches[18, 21]. Much of our

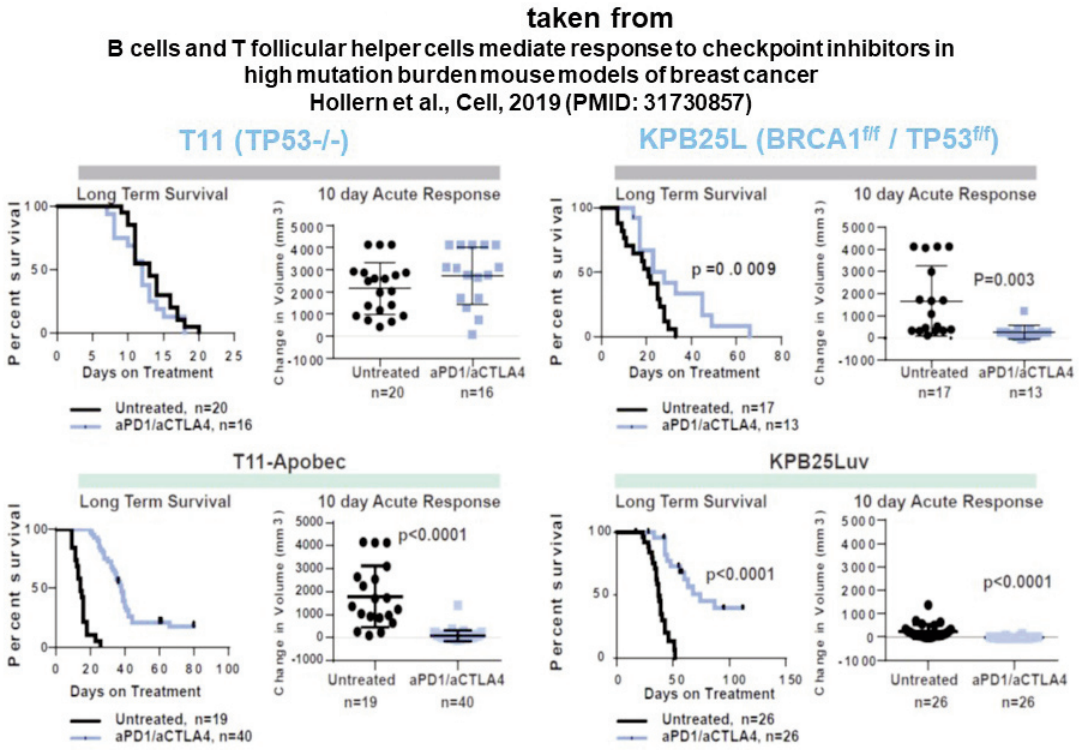


Figure 4 High TMB Mouse Models are Sensitivity to Immune Checkpoint Inhibitors

research has shown that predicting complex phenotypes like drug responsiveness is not simple, and that no single gene/protein can robustly predict response; instead our results suggest that it is genetic features of the tumor (somatic mutations, TMB, Chromosome Copy Number changes), gene expression subtype (cell type of origin, epigenetics), and features of the microenvironment (immune cells, fibroblasts) that each make a predictive contribution. Thus, integrated predictors that can link these features together can provide objective and quantitative assays to help guide treatment escalation, or de-escalation, therefore offering more personalized approaches in the Era of Precision Medicine.

References

1. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. 406(6797): p. 747-52.
2. Prat, A., et al., *Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer.* Breast Cancer Res, 2010. 12(5): p. R68.
3. Parker, J.S., et al., *Supervised Risk Predictor of Breast Cancer Based on Intrinsic Subtypes.* J Clin Oncol, 2009.

4. Nielsen, T.O., et al., *A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer.* Clin Cancer Res, 2010. 16(21): p. 5222-32.
5. Usary, J., et al., *Predicting drug responsiveness in human cancers using genetically engineered mice.* Clin Cancer Res, 2013. 19(17): p. 4889-99.
6. Carey, L.A., et al., *Molecular Heterogeneity and Response to Neoadjuvant Human Epidermal Growth Factor Receptor 2 Targeting in CALGB 40601, a Randomized Phase III Trial of Paclitaxel Plus Trastuzumab With or Without Lapatinib.* J Clin Oncol, 2016. 34(6): p. 542-9.
7. Iglesia, M.D., et al., *Prognostic B-Cell Signatures using mRNA-Seq in Patients with Subtype-Specific Breast and Ovarian Cancer.* Clin Cancer Res, 2014. 20(14): p. 3818-29.
8. Hollern, D.P., et al., *B Cells and T Follicular Helper Cells Mediate Response to Checkpoint Inhibitors in High Mutation Burden Mouse Models of Breast Cancer.* Cell, 2019. 179(5): p. 1191-1206 e21.
9. _CGAN, C.G.A.R. Network, and C.M. Perou, *Comprehensive molecular portraits of human breast tumours.* Nature, 2012. 490(7418): p. 61-70.
10. Wallden, B., et al., *Development and verification of the PAM50-based Prosigna breast cancer gene signature assay.* BMC Med Genomics, 2015. 8: p. 54.
11. Dowsett, M., et al., *Comparison of PAM50 risk of recurrence score with oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy.* J Clin Oncol, 2013. 31(22): p. 2783-90.
12. Sestak, I., et al., *Prediction of Late Distant Recurrence After 5 Years of Endocrine Treatment: A Combined Analysis of Patients From the Austrian Breast and Colorectal Cancer Study Group 8 and Arimidex, Tamoxifen Alone or in Combination Randomized Trials Using the PAM50 Risk of Recurrence Score.* J Clin Oncol, 2014.
13. Millikan, R.C., et al., *Epidemiology of basal-like breast cancer.* Breast Cancer Res Treat, 2008. 109(1): p. 123-39.
14. Carey, L.A., et al., *Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study.* Jama, 2006. 295(21): p. 2492-502.
15. Prat, A., et al., *Molecular characterization of basal-like and non-basal-like triple-negative breast cancer.* Oncologist, 2013. 18(2): p. 123-33.
16. Hoadley, K.A., et al., *Cell-of-Origin Patterns Dominate the Molecular Classification of 10,000 Tumors from 33 Types of Cancer.* Cell, 2018. 173(2): p. 291-304 e6.
17. Hoadley, K., et al. *Multivariate Analysis of Subtype and Gene Expression Signatures Predictive of Pathologic Complete Response (pCR) in Triple-Negative Breast Cancer (TNBC): CALGB 40603 (Alliance).* in *San Antonio Breast Cancer Symposium*. 2016. San Antonio, TX.
18. Tanioka, M., et al., *Integrated Analysis of RNA and DNA from the Phase III Trial CALGB 40601 Identifies Predictors of Response to Trastuzumab-Based Neoadjuvant Chemotherapy in HER2-Positive Breast Cancer.* Clin Cancer Res, 2018.

19. Iglesia, M.D., et al., *Genomic Analysis of Immune Cell Infiltrates Across 11 Tumor Types.* J Natl Cancer Inst, 2016. 108(11).
20. Schmid, P., et al., *Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer.* N Engl J Med, 2018. 379(22): p. 2108-2121.
21. Wong, K.Y., et al., *I-Boost: an integrative boosting approach for predicting survival time with multiple genomics platforms.* Genome Biol, 2019. 20(1): p. 52.

Charles M. Perou, PhD

1984-1987	BS from Bates College
1989-1997	PhD in Experimental Pathology, University of Utah
1997-2000	Postdoc in David Botstein Lab at Stanford University
2000-present	Professor, Department of Genetics, University of North Carolina
2011-present	The May Goldman Shaw Professor of Molecular Oncology
2018-present	Co-Director of the UNC Computational Medicine Program

EPIGENETIC HETEROGENEITY OF HEPATIC LINEAGE TUMORS

Hiroyuki Aburatani
Genome Science Laboratory
Research Center for Advanced Science and Technology
The University of Tokyo
4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan
(haburata-tky@umin.ac.jp)

Genomic intratumoral heterogeneity has been extensively studied in many cancers, but the extent of phenotypic heterogeneity is uncertain and could be caused by epigenetic heterogeneity.

First, we have studied the molecular landscape of liver tumors in adult and children, namely hepatocellular carcinoma and hepatoblastoma (HBL), which is the most common primary liver tumor in children. To uncover HBL, we performed comprehensive analyses of genomic, epigenomic and transcriptomic profiles for the 168 cases in Japan. Consistent with the prior studies, the number of somatic mutational events was very few, while activation of canonical Wnt pathway was observed in >75% cases. DNA methylome profiles focusing on hepatic enhancers identified the distinct subgroups, which is associated with onset age, *TERT* promoter mutations, loss of imprinting of IGF2/H19 and alpha fetoprotein (AFP) production. Motif analysis of subgroup-specific hypomethylated enhancers indicated activation of the transcription factors that may implicate “the cell of origin” of HBLs.

In development, liver arises from foregut, although the precise mechanisms remain to be known. A subgroup of gastric cancer produces AFP and shows aggressive phenotype[1]. Whole exome analysis showed TP53 as the most frequently mutated gene, while DNA methylation profiling demonstrated that AFP-producing gastric tumor has a unique methylation pattern (Figure 1). Single cell transcriptome analysis of AFP-producing tumor organoid cells revealed heterogeneity in cell fate, showing distinct trajectories for AFP-positive and negative cell clusters. RNA velocity (La Manno, Nature 2018) was used to show the branching lineages, hepatic and intestinal, respectively (Figure 2). Integrative

analysis with transcription factor network and chromatin accessibility would elucidate the origin of hepatic lineage cell population from cells of intestinal origin.

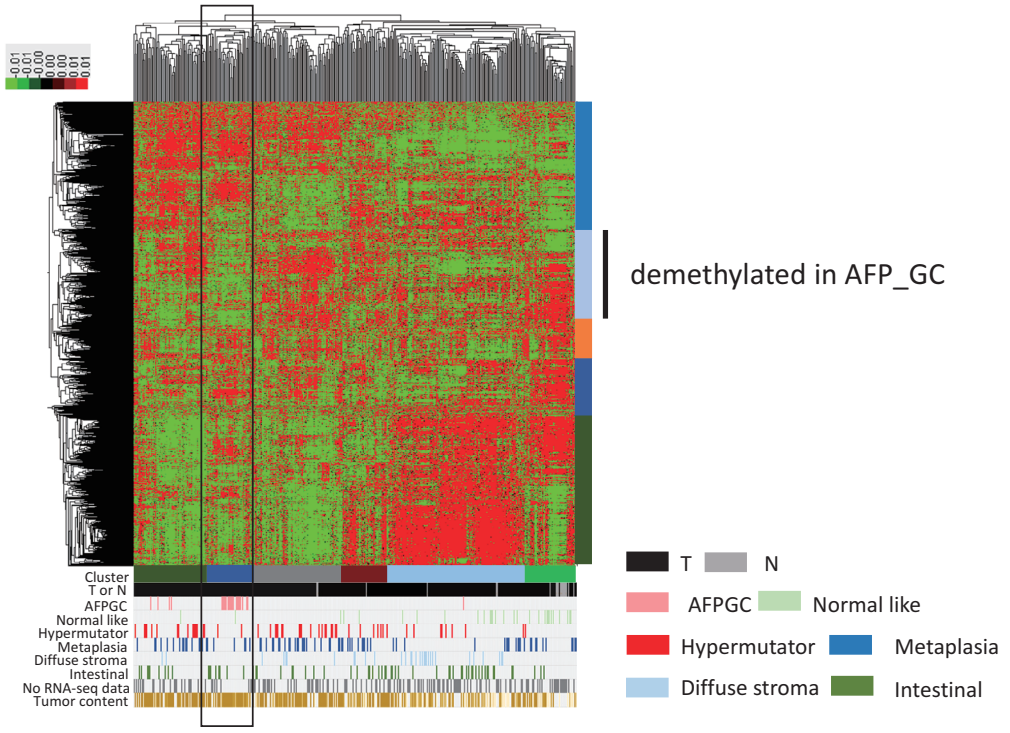


Figure 1 Methylation profiling of gastric cancers
DNA methylation was measured by using Infinium EPIC array (illumina).

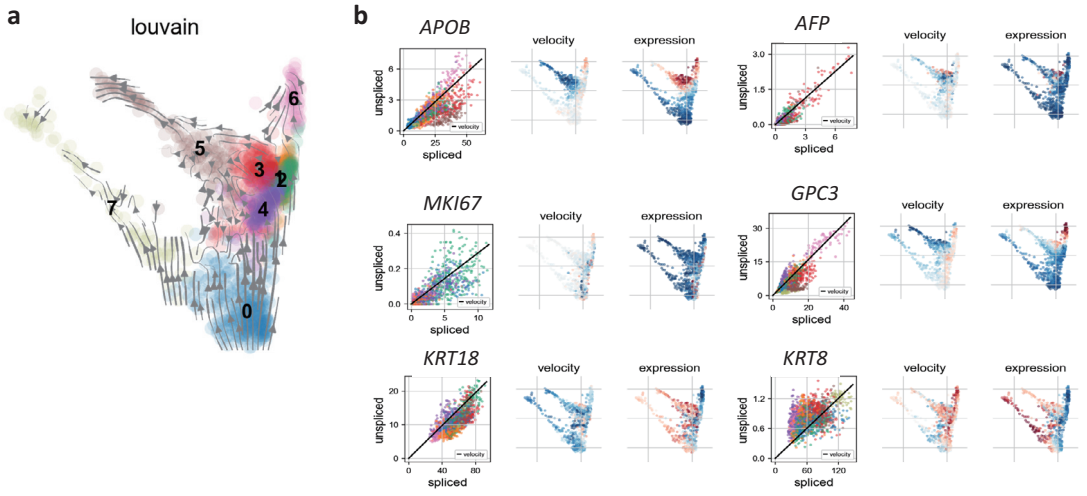


Figure 2 Single cell transcriptome of AFP-producing gastric cancer organoid cells.
a. Diffusion map
b. RNA velocity map of representative genes.

References

1. Yamazawa S, Ushiku T, Shinozaki-Ushiku A, Hayashi A, Iwasaki A, Abe H, Tagashira A, Yamashita H, Seto Y, Aburatani H, Fukayama M. Gastric Cancer With Primitive Enterocyte Phenotype: An Aggressive Subgroup of Intestinal-type Adenocarcinoma. Am J Surg Pathol. 41(7):989-997. 2017

Hiroyuki Aburatani, MD, PhD

1980-1981	Internship in Internal Medicine, University of Tokyo
1983-1988	Clinical Research Fellow, Third Department of Internal Medicine, University of Tokyo
1988-1991	Assistant Professor, Third Department of Internal Medicine, University of Tokyo
1988-1994	Visiting Scientist, Center for Cancer Research, MIT, Cambridge, MA, USA
1995-1999	Assistant Professor, Third Department of Internal Medicine, University of Tokyo
1999-2001	Associate Professor, Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo
2001-present	Professor, Genome Science Division, Research Center for Advanced Science and Technology, University of Tokyo

THE "PETER PAN" SYNDROME: STALLED DEVELOPMENT THROUGH REDISTRIBUTION OF KEY EPIGENETIC MARKS AT THE CORE OF ONCOHISTONE PATHOGENESIS

Nada Jabado
Departments of Pediatrics and Human Genetics, McGill University
Division of Pediatric Hematology-Oncology and CHDD Program
McGill University Health Care Center and MUHC Research Institute
1001 Decarie Boulevard, Montreal, QC H3J1P4 Canada
(Nada.jabado@mcgill.ca)

A growing number of cancers, especially in children and young adults, are due to epigenetic dysfunction, which promotes their genesis, progression and metastatic processes. Our group [1, 2] was one of two[3] to first identify a histone mutation in human disease, irrefutably linking epigenetic deregulation to cancer. High-frequency recurrent somatic mutations at specific residues in histone H3 variants occur in a particularly lethal form of brain tumor, high-grade gliomas (HGG) affecting children and young adults[4-8]. Since this initial discovery, we and others uncovered these "oncohistones" as we label them in bone cancers[9], in posterior-fossa ependymomas group A (PFA-EP) [10], another deadly brain tumor which affects infants and young children, in subsets of head and neck squamous cell carcinomas[11], in undifferentiated sarcomas[12] and in acute myeloid leukemia or myelodysplastic syndromes[13, 14]. Interestingly we helped show how expression of EZHIP (Enhancer of Zeste Homologs Inhibitory Protein, also termed *CXORF67*) facilitates the pathogenesis of PFA-EP[15, 16]. EZHIP has a highly conserved sequence within its C-terminus which acts as an H3K27M oncohistone-mimic and is necessary and sufficient to inhibit the catalytic activity of EZH2/PRC2 *in vitro* and *in vivo[16]*. Thus, the 'K-to-M' paradigm, first established for oncohistones, has now been extended to non-histone substrates (Figure 1). Lastly, recent work from the lab of David Allis uncovered previously unappreciated mutations on core histones in several human cancers[17]. These findings further widen the spectrum of cancer-associated oncohistones and underscore the disease relevance of studying this new mechanism of oncogenesis.

Seminal findings from the Allis lab showed that K-to-M mutations in H3 variants inhibit the methyltransferase activity of the enzyme depositing the respective methyl marks on

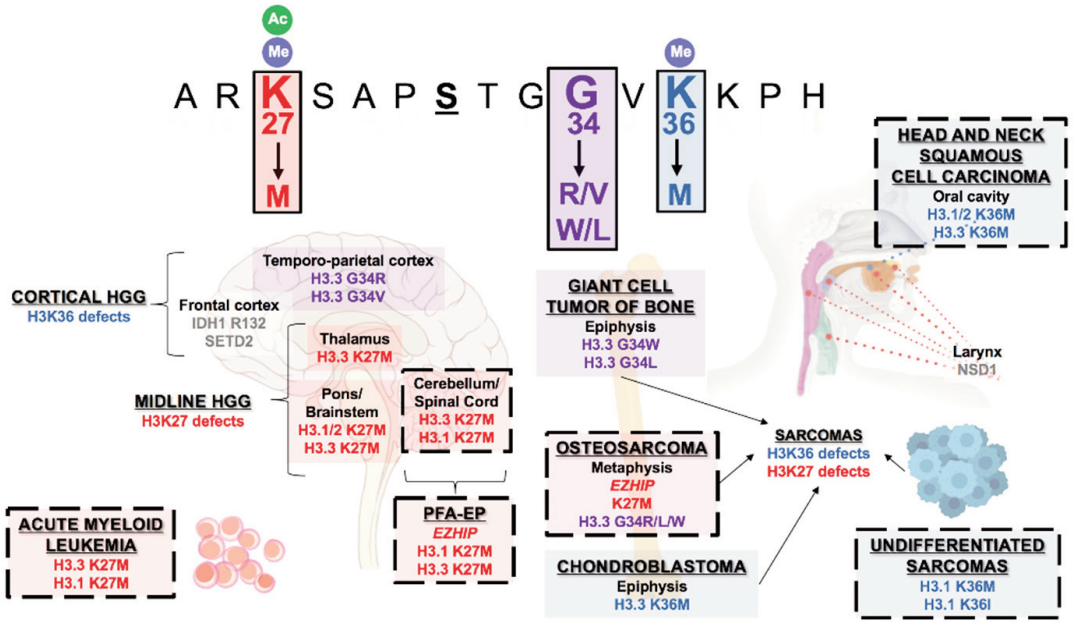


Figure 1 Cancers with Oncohistones and the Oncohistone-mimic EZHIP. Upper panel describes the H3 tail with the three residues where mutations in H3 variants were identified in human disease: H3K27, H3.3G34 and H3K36.

lysine residues in the histone tail[18]. These heterozygote somatic mutations act as dominant negative variants, even if they occur in only one of the 32 genes encoding for an H3. How H3K27M affects tumorigenesis, and its requirement for tumor maintenance, has remained controversial. Mutant H3K27M, which can occur in both the canonical (H3.1 or H3.2) and non-canonical (H3.3) histone variants, contributes to only a fraction of the total H3 pool (3-17%)[18]. However, it has a dominant effect as it drastically reduces overall levels of the repressive H3K27me3 mark in cells[18-20]. *In vitro*, H3K27M has been shown to severely affect the catalytic activity of the Polycomb Repressive Complex (PRC2), which normally catalyzes H3K27 methylation (reviewed in [21]). Crystal structure data suggested that the strong binding of PRC2 to H3K27M-containing nucleosomes potentially sequesters and inactivates PRC2, thereby leading to global reduction of H3K27me3 levels[22, 23]. Beside this model, several others have been proposed to explain how the loss of H3K27me3 results in tumorigenesis. Chan *et al.*[24] compared H3K27M-DIPG lines to neural progenitor cells (NPCs) to argue that H3K27me3 loss in large genomic areas leads to increased gene expression at bivalent promoters (marked, in the normal state, by both H3K27me3 and H3K4me3)[25], while at hundreds of gene loci exhibiting retention of EZH2 (the catalytic subunit of PRC2), H3K27me3 deposition actually increases. Mohammad *et al.*[26] compared joint H3K27M and PDGFB overexpression in mouse NPCs to H3K27M-

DIPG cell lines. They proposed that increased H3K27me3 occurs at several genomic loci that "strongly" bind PRC2, in contrast to "weaker" binding sites that lose the mark. Accordingly, differential effects of H3K27M on distinct genomic loci could be explained by varying levels of PRC2 activity across sites in H3K27M cells [26]. In contrast, Piunti *et al.*[27] suggested specific enrichment of H3.3K27M-carrying nucleosomes in actively transcribed genomic regions where H3.3 is preferentially deposited, which then precludes PRC2 recruitment and leads to heterotypic H3K27ac-K27M nucleosome formation, H3K27ac increase, and bromodomain protein recruitment in tumors. Thus, despite many enticing hypotheses, a unified view on downstream effects of H3K27M was lacking. Since H3K27M is tumorigenic only when introduced in specific neurodevelopmental windows [26-29], while H3K27me3 deposition varies with cellular context and differentiation stage [30], the absence of the appropriate isogenic background likely represents a major confounder. We present herein our findings on K-to-M mutagenesis and show how these mutations act on the chromatin to stall development and differentiation and promote tumor formation. We show that K-to-M mutations (H3K27M and H3K36M) block terminal differentiation of the progenitor cells in which they arise, leading to stalled development - the "Peter Pan Syndrome" - as we name it.

H3K36M mutations are the main driver in chondroblastomas and do not require additional oncogenic partnership to mediate oncogenicity. We thus started with these mutants to investigate how they affect tumor formation. Introducing H3K36M in mouse mesenchymal stem cells led to a block in the differentiation of these progenitors into adipocytes, osteocytes or chondroblast, and induced aggressive sarcomas *in vivo* in NSG mice. We showed that this is achieved through genome-wide rewiring of opposing chromatin marks which leads to increased intergenic H3K27me3 deposition and redistribution and titration of the PRC1 complex. Indeed, H3K36M mutants block the methyltransferase activity of NSD1 and 2, which mediates H3K36 mono and di-methylation, as well as SETD2, which mediates H3K36 trimethylation. We show that the global decrease of the deposition of H3K36me2, a mark of active chromatin which is highly abundant in the intergenome and opposes H3K27me3 deposition is at the core of H3K36M oncogenicity. Indeed, decreased deposition of H3K36me2 leads to increased intergenic deposition of the repressive H3K27me3 mark, which spreads from its boundaries as the PRC2 complex is then unhindered in depositing this repressive mark[12]. This in turn leads to redistribution of PRC1 which is titrated from its normal sites. The net effect is loss of PRC1 suppression as the phenotype we observe in H3K36M is reproduced when we knocked down in mesenchymal stem cells RING1A/B, which are core component of this complex[12]. This led us to investigate other cancers where demethylation of H3K36 may be reduced. We thus identified H3K36M in aggressive undifferentiated sarcomas[12] and in

a subset of squamous cell Head and Neck cancers, where this oncohistone was mutually exclusive with loss-of-function mutations in NSD1[11] (Figure 1). Thus, in H3K36M tumors, defective spread of the abundant H3K36me2 mark leads to pervasive invasion of the repressive H3K27me3 mark, dilution of PRC1 repression and subsequent differentiation blockade[12] (Figure 2).

High-grade gliomas (HGG) defined by histone 3 K27M driver mutations exhibit global loss of H3K27 trimethylation and reciprocal gain of H3K27 acetylation, respectively shaping repressive and active chromatin landscapes. To assess the effects of H3K27M mutagenesis we used an **isogenic tumor model system** where we CRISPR/Cas edited the mutation in primary human glioma cell lines. Building on seminal findings from the Allis lab (*16*), we showed that cells are stuck in a progenitor state of unlimited self-renewal capacity, as they are unable to spread the repressive H3K27me3 and me2 marks to achieve further differentiation (*17*) (Figure 2). Indeed, PRC2 is recruited normally to its nucleation sites at CpG islands (CGI) in the cell/lineage of origin and starts depositing and spreading the H3K27me2 and me3 marks. H3K27me2 can spread outside of the CGI and reach the level of spread of H3K27me3 in isogenic controls, wild type for the K27M mutation, while H3K27me3 deposition is not enough to go beyond these CGIs. This shows that the PRC2

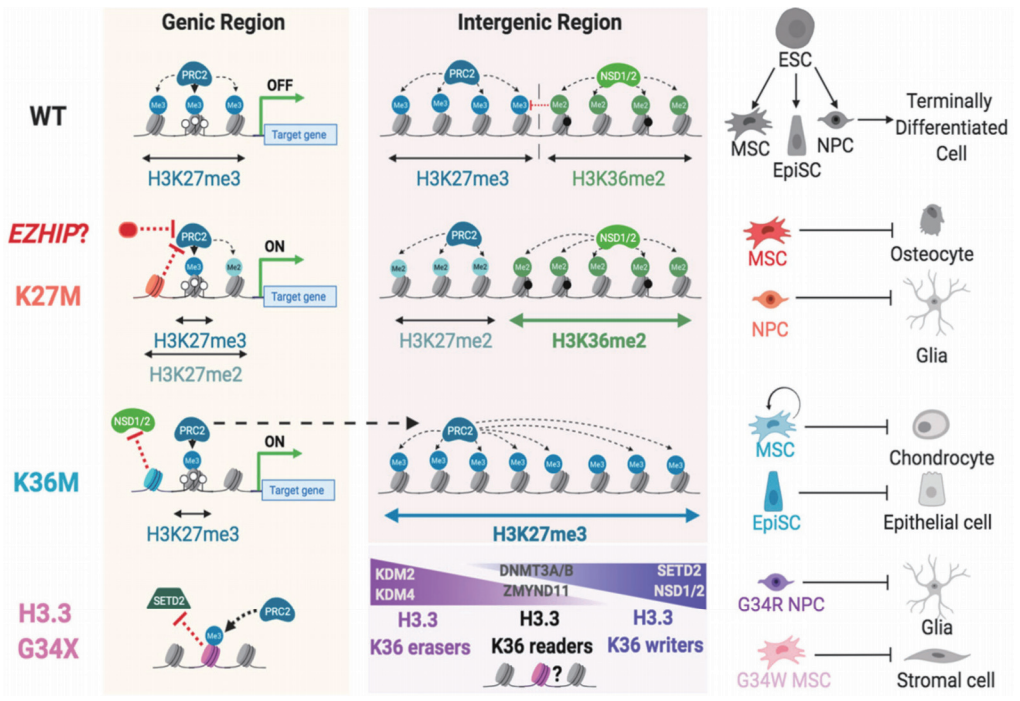


Figure 2 Potential effects of oncohistones and and oncohistone mimics on the chromatin and their role in mediating tumorigenesis.

complex is not blocked on chromatin by mutant nucleosomes as previously suspected, but that the main effect of the mutation is to drastically slow the deposition of the H3K27me3 mark and to a lesser effect that of H3K27me2 in each cell cycle. We presume that this may account why these mutations are mainly found in specific cancers as they can mainly affect progenitors with specific metabolic contingencies, limited H3K27me3 baseline levels and short cell cycles. Possibly, the relative abundance of the H3K327me3 mark with a longer cell cycle in more mature cells can strongly mitigate K27M oncogenicity, making it close to impossible for these mutations to impact more differentiated cells. Furthermore, in keeping with specific progenitors amenable to transformation, we could only generate high-grade gliomas in mice when introducing the H3K27M mutation during a narrow developmental window, between E9.5 and E13.5 of neurogenesis and only when in association with *Tp53* loss[29]. Our work also shows that these mutations are important for initiating and maintaining tumorigenicity, as their removal strongly impairs tumor formation as we and others have shown[31, 32]. Mirroring H3K36M mutagenesis, we show that loss of H3K27me3 leads to redistribution of other epigenetic marks involved in 'crosstalk' with H3K27 and H3K36 methylation, namely H3K36me2 and H3K27ac in H3K27M (*[33]*) and manuscript submitted). Indeed, by isogenic modeling of this mutation in tumor-derived contexts, we show that it leads to pervasive H3K27ac deposition across the genome and its enrichment at repeat elements which normally partly rely on H3K27me3 deposition for their silencing. Conversely, we show that active enhancers and promoters are not created *de novo* and instead reflect genes that define developmental states of origin between H3K27M and H3K27WT HGGs[33] (Figure 2). Deposition of H3K27ac in the intergenome leads to elevated baseline expression of repeat elements in H3K27M cells and a level of viral mimicry, which can be further amplified by DNA demethylation and histone deacetylase inhibitors[9]. These agents may therefore modulate anti-tumor immune responses as a novel therapeutic modality for this untreatable disease. At least two clinical trials by international pediatric brain tumor consortia will open in the next year based on these findings.

The discovery of EZHIP indicates that H3 K-to-M mutants' inhibition of methyltransferases can occur in the absence of nucleosome incorporation. We hypothesize that EZHIP mediates "PRC2 poising" during specific developmental windows and/or progenitor states to prevent untimely repression of developmental transcriptional programs and cell differentiation (Figure 2). This physiological stalling of PRC2 may be co-opted by aberrant/persistent expression of *EZHIP* gene to promote oncogenesis. Whether the extensive chromatin reprogramming induced by K-to-M mutations represents tumor intrinsic and/or immune-related vulnerabilities remains underexplored. Based on our preliminary findings that H3K27M and H3K36M mutations converge on aberrant

activation of repeat elements and activation of innate immune response pathways, we propose new mouse models that allow us to assess the immune infiltration landscape of K-to-M tumors and invent strategies to best therapeutically engage the immune system to treat these cancers.

Last, much less is known on H3.3G34 oncogenesis. We first identified somatic heterozygous mutations in *H3F3A* at H3.3G34 specifically in the parietal and fronto-parietal cortex of 15-35y old patients [3] (Figure 1). 30% of cortical HGGs in these age group harbor G34RH3.3 and less frequently G34VH3.3, and these mutations are always associated with loss-of-function genetic alterations in ATRX and TP53. Another aminoacid substitution occurs in *H3F3A*/*H3F3B* changing glycine to tryptophan (G34W) and rarely to leucine (G34L). This was identified in >90% of GCTBs and is never found in brain tumors [9]. Glycine is the smallest aminoacid and its change to a bulky tryptophan, valine or arginine at position 34 at the base of H3 tail, just 2 residues away from K36, makes it likely that this substitution affects the PTM or the recognition of a post-translationally modified H3.3K36. The fact that this mutation occurs exclusively on H3.3 and accounts for 25% of the total H3.3 pool makes it likely that its effects will be in cis, on the mutated nucleosome incorporated in chromatin, and not genome wide as shown for K-to-M oncohistones. One *in vitro* study described that G34R and V mutants diminish H3K36me2/me3 in cis on the same histone H3 tail but have no dominant effect on blocking K36 methylation on wild type H3 tail [34]. Others found that binding of methyltransferases SETD2 and NSD as well as the K36 demethylase (eraser) KDM4 critically rely on residue G34 for binding [35]. Additionally, a G34R mutant was modeled in fission yeast and found to downregulate both H3K36me3 and H3K36ac levels, while H3K36me2 accumulated on the mutant tail [36]. However, despite these data ongoing efforts are needed to understand how local chromatin changes due to G34 mutants act to promote tumorigenicity and why different mutants are encountered specific cancers (Figures 1 and 2).

In summary, oncohistones are a new model of oncogenesis that shows how the deregulation of the epigenome during specific developmental windows can be co-opted for tumorigenesis. These mutations are unprecedented tools to explore the role of major epigenetic marks during development. Indeed, stalled development is the main recurrent theme that emerges from the studies we and other have performed on these mutations, hence the naming "The Peter Pan Syndrome" as these tumors are stuck in time unable to regress or further "age' and differentiate. We propose that the genome wide rewiring of major opposing marks is at the core of the oncogenic effect of these mutations and creates exquisite vulnerabilities that could be taken advantage of. We suggest that the use of spefic epigenetic drugs targeting these cancers may prove beneficial. Indeed, further inhibition of PRC2 leading to further decrease in H3K27me2/me3 levels will prove mainly toxic for

K27M mutant cells, while the use of DNA demethylating agents can increase ERV expression and further prime these tumors for immune targeting. Investigating the use of combination therapies targeting the epigenome (EZH2 inhibitors, DNA demethylating agents) in combination with agents affecting active pathways in these tumours (PDGFRA, Ras and PI3K signaling, ACVR1 mutations) and/or agents aimed at modulating the immune system (checkpoint inhibitors, vaccines, CART cells) will provide much needed novel therapeutic avenues for patients with these deadly cancers.

References

1. Khuong-Quang, D.A., et al., *K27M mutation in histone H3.3 defines clinically and biologically distinct subgroups of pediatric diffuse intrinsic pontine gliomas.* Acta Neuropathol, 2012. 124(3): p. 439-47.
2. Schwartzentruber, J., et al., *Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma.* Nature, 2012. 482(7384): p. 226-31.
3. Wu, G., et al., *Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas.* Nat Genet, 2012. 44(3): p. 251-3.
4. Fontebasso, A.M., et al., *Epigenetic dysregulation: a novel pathway of oncogenesis in pediatric brain tumors.* Acta Neuropathol, 2014. 128(5): p. 615-27.
5. Fontebasso, A.M., et al., *Mutations in SETD2 and genes affecting histone H3K36 methylation target hemispheric high-grade gliomas.* Acta Neuropathol, 2013. 125(5): p. 659-69.
6. Liu, X.Y., et al., *Frequent ATRX mutations and loss of expression in adult diffuse astrocytic tumors carrying IDH1/IDH2 and TP53 mutations.* Acta Neuropathol, 2012. 124(5): p. 615-25.
7. Fontebasso, A.M., et al., *Recurrent somatic mutations in ACVR1 in pediatric midline high-grade astrocytoma.* Nat Genet, 2014. 46(5): p. 462-6.
8. Sturm, D., et al., *Paediatric and adult glioblastoma: multiform (epi)genomic culprits emerge.* Nat Rev Cancer, 2014. 14(2): p. 92-107.
9. Behjati, S., et al., *Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone.* Nat Genet, 2013. 45(12): p. 1479-82.
10. Gessi, M., et al., *Evidence of H3 K27M mutations in posterior fossa ependymomas.* Acta Neuropathol, 2016. 132(4): p. 635-7.
11. Papillon-Cavanagh, S., et al., *Impaired H3K36 methylation defines a subset of head and neck squamous cell carcinomas.* Nat Genet, 2017. 49(2): p. 180-185.
12. Lu, C., et al., *Histone H3K36 mutations promote sarcomagenesis through altered histone methylation landscape.* Science, 2016. 352(6287): p. 844-9.
13. Boileau, M., et al., *Mutant H3 histones drive human pre-leukemic hematopoietic stem cell expansion and promote leukemic aggressiveness.* Nat Commun, 2019. 10(1): p. 2891.

14. Lehnertz, B., et al., *H3(K27M/I) mutations promote context-dependent transformation in acute myeloid leukemia with RUNX1 alterations.* Blood, 2017. 130(20): p. 2204-2214.
15. Hubner, J.M., et al., *EZHIP / CXorf67 mimics K27M mutated oncohistones and functions as an intrinsic inhibitor of PRC2 function in aggressive posterior fossa ependymoma.* Neuro Oncol, 2019.
16. Jain, S.U., et al., *PFA ependymoma-associated protein EZHIP inhibits PRC2 activity through a H3 K27M-like mechanism.* Nat Commun, 2019. 10(1): p. 2146.
17. Nacev, B.A., et al., *The expanding landscape of 'oncohistone' mutations in human cancers.* Nature, 2019. 567(7749): p. 473-478.
18. Lewis, P.W., et al., *Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma.* Science, 2013. 340(6134): p. 857-61.
19. Bender, S., et al., *Reduced H3K27me3 and DNA hypomethylation are major drivers of gene expression in K27M mutant pediatric high-grade gliomas.* Cancer Cell, 2013. 24(5): p. 660-72.
20. Bechet, D., et al., *Specific detection of methionine 27 mutation in histone 3 variants (H3K27M) in fixed tissue from high-grade astrocytomas.* Acta Neuropathol, 2014. 128(5): p. 733-41.
21. Margueron, R. and D. Reinberg, *The Polycomb complex PRC2 and its mark in life.* Nature, 2011. 469(7330): p. 343-9.
22. Jiao, L. and X. Liu, *Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2.* Science, 2015. 350(6258): p. aac4383.
23. Justin, N., et al., *Structural basis of oncogenic histone H3K27M inhibition of human polycomb repressive complex 2.* Nat Commun, 2016. 7: p. 11316.
24. Chan, K.M., et al., *The histone H3.3K27M mutation in pediatric glioma reprograms H3K27 methylation and gene expression.* Genes Dev, 2013. 27(9): p. 985-90.
25. Bernstein, B.E., et al., *A bivalent chromatin structure marks key developmental genes in embryonic stem cells.* Cell, 2006. 125(2): p. 315-26.
26. Mohammad, F., et al., *EZH2 is a potential therapeutic target for H3K27M-mutant pediatric gliomas.* Nat Med, 2017. 23(4): p. 483-492.
27. Piunti, A., et al., *Therapeutic targeting of polycomb and BET bromodomain proteins in diffuse intrinsic pontine gliomas.* Nat Med, 2017. 23(4): p. 493-500.
28. Funato, K., et al., *Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation.* Science, 2014. 346(6216): p. 1529-33.
29. Pathania, M., et al., *H3.3(K27M) Cooperates with Trp53 Loss and PDGFRA Gain in Mouse Embryonic Neural Progenitor Cells to Induce Invasive High-Grade Gliomas.* Cancer Cell, 2017. 32(5): p. 684-700 e9.
30. Mikkelsen, T.S., et al., *Genome-wide maps of chromatin state in pluripotent and lineage-committed cells.* Nature, 2007. 448(7153): p. 553-60.

31. Harutyunyan, A.S., et al., *H3K27M induces defective chromatin spread of PRC2-mediated repressive H3K27me2/me3 and is essential for glioma tumorigenesis.* Nat Commun, 2019. 10(1): p. 1262.
32. Silveira, A.B., et al., *H3.3 K27M depletion increases differentiation and extends latency of diffuse intrinsic pontine glioma growth in vivo.* Acta Neuropathol, 2019. 137(4): p. 637-655.
33. Krug, B., et al., *Pervasive H3K27 Acetylation Leads to ERV Expression and a Therapeutic Vulnerability in H3K27M Gliomas.* Cancer Cell, 2019. 35(5): p. 782-797 e8.
34. Shi, L., et al., *Histone H3.3 G34 Mutations Alter Histone H3K36 and H3K27 Methylation In Cis.* J Mol Biol, 2018. 430(11): p. 1562-1565.
35. Voon, H.P.J., et al., *Inhibition of a K9/K36 demethylase by an H3.3 point mutation found in paediatric glioblastoma.* Nat Commun, 2018. 9(1): p. 3142.
36. Yadav, R.K., et al., *Histone H3G34R mutation causes replication stress, homologous recombination defects and genomic instability in S. pombe.* Elife, 2017. 6.

Nada Jabado, MD, PhD

1989-1999	Medical Studies and Residency, University of Pierre et Marie Curie (Paris VI), France Graduate School of Medicine University of Paris VI (Pediatrics, Hematology)
1994-1998	Graduate Studies in Immunology, Pierre et Marie Curie (Paris VI), France
1999-2003	Postdoctoral Fellow, Department of Biochemistry, McGill University
2003-present	Professor, Departments of Pediatrics and Human Genetics, McGill University Senior Clinician Scientist, McGill University Health Center

MODELING TUMOR IMMUNITY FROM RNA-SEQ

X. Shirley Liu
Department of Data Sciences
Dana-Farber Cancer Institute, Harvard University
450 Brookline Ave, MC-CLS11007, Boston, MA 02215, USA
(xsliu@ds.dfci.harvard.edu)

I will discuss two algorithms that our laboratory developed to extract useful cancer immunology insights from treatment naïve RNA-seq samples in The Cancer Genome Atlas.

First, we developed a computational method TRUST that can assemble T cell receptor (TCR) and B cell receptor (BCR) complementarity-determining regions (CDR3s) from unselected bulk tumor RNA-seq data (Li et al, Nat Genet 2016; Li et al, Nat Genet 2017). The recent release of the TRUST4 algorithm has improved speed, memory efficiency, sensitivity, and accuracy, as well as the added function to identify full length BCR sequences (https://github.com/liulab-dfci/TRUST4). When applied to over 10K samples in the TCGA, TRUST assembled ~3.3M TCR from TCGA tumor RNA-seq samples and revealed association between tumor infiltrating TCR clonotype diversity and tumor mutational load (Li et al, Nat Genet 2016). TRUST also assembled ~71M BCR CDR3 sequences from TCGA data, and revealed frequent BCR somatic hypermutations and Ig class switch events (Hu et al, Nat Genet 2019). Although there have been inconsistent reports on tumor infiltrating B cells levels in different cancer types, TRUST analyses suggest tumors in general have lower BCR diversity and higher IgG class switch than adjacent normal. Specifically IgG1 and IgG3 B cells are associated with national killer cell activity in the tumors, implicating their important roles in B-cell mediated tumor immunity. Besides analysis of solid tumors, our analysis on acute myeloid leukemia (AML) RNA-seq samples found significantly higher level of B cell activation and more secondary Ig class switch events in adult AML than pediatric AML or non-tumor samples. Furthermore, adult AML with highly expanded IgA2 B cells, which might represent an immunosuppressive microenvironment, are associated with regulatory T cells and worse overall survival.

Second, we derived Tumor Immune Dysfunction and tumor immune Exclusion gene expression signatures (TIDE) from pretreatment tumors to predict patient response to anti-PD1 and anti-CTLA4 treatment (Jiang et al, Nat Med 2018). Recent work has revealed two broad categories of tumor immune evasion with distinct characteristics of the tumor microenvironment (TME). The first subset shows a T cell inflamed phenotype consisting of infiltrating T cells in dysfunctional state. The second subset lacks the T cell infiltration and resists immune attack through T cell exclusion. Based on these two mechanisms, we developed a computational framework, Tumor Immune Dysfunction and Exclusion (TIDE), to identify factors underlying tumor immune escape. To model T-cell dysfunction, we hypothesize that transcriptome profiles of treatment-naïve tumors with patient survival outcome are informative. For example, in the TCGA melanoma tumors, a higher cytotoxic CD8 T lymphocyte (CTL) level, indicates a better patient survival, but only when TGFB1 has a low expression level, as TGFB1 expression indicates T cell dysfunction and tumor immune escape. To systematically evaluate genes with similar behavior as TGFB1, TIDE uses a Cox-PH model to test how the interaction between the expression of a candidate gene P and CTL affects death hazard (estimated from survival) in large-scale tumor profiling cohorts. The TIDE output is a T-cell dysfunction score for every gene in the genome, defined as the interaction coefficient "d" divided by its standard deviation. Ward test in Cox-PH regression is used to identify dysfunction scores with statistically significant p-values. Interestingly, TIDE dysfunctional scores derived from TCGA treatment naive samples show good agreement with signature genes from eight published human or mouse studies on T cell dysfunction, especially fixed state of T cell dysfunction which is resistant to reprogramming by anti-PD1 treatment. To model T-cell exclusion, TIDE considers suppressive factors hypothesized to prevent T cells from infiltrating the tumor, namely tumor-associated macrophage (TAM), myeloid derived suppressor cell (MDSC) and cancer associated fibroblast (CAF). TIDE derives a T-cell exclusion signature by integrating publicly available expression profiles of MDSC, TAM, and CAF, which indeed negatively correlates with CTL in all the TCGA cancer cohorts. When predicting tumor response to ICB from pre-treatment tumor expression profiles, TIDE evaluates whether T cell inflamed (high CTL) tumors have high level of dysfunctional T cells and whether non-inflamed (low CTL) tumors have suppressive cells preventing T cell infiltration, as high correlations with either T cell dysfunction or exclusion signatures predict poor responders. This TIDE framework could predict the outcome of melanoma patients treated with anti-PD1 and anti-CTLA4 with a higher accuracy than other biomarkers such as PD-L1 level and mutation load, as well as recent computational predictions. We also present a data-driven approach integrating large-scale omics data and biomarkers on published immune checkpoint inhibitor trials, non-immunotherapy tumor profiles, and CRISPR screens on a

web platform (http://tide.dfci.harvard.edu). TIDE website has three functional modules to help hypothesis generation, biomarker optimization, and patient stratification respectively, which demonstrates the utility of public data reuse in cancer research.

Together, our work demonstrates that tumor RNA-seq, even on treatment naïve tumors, is cost effective to inform tumor microenvironment and immunity.

References

1. Hu X#, Zhang J#, Wang J, Fu J, Li T, Zheng X, Wang B, Gu S, Jiang P, Fan J, Ying X, Zhang J, Carroll MC, Wucherpfennig KW, Hacohen N, Zhang F, Zhang P, Liu JS*, Li B*, Liu XS*. Landscape of B cell immunity and related immune evasion in human cancers. *Nat Genet*. 2019 Mar;51(3):560-567. 2019.
2. Wang B#, Wang M#, Zhang W#, Xiao T, Chen CH, Wu A, Wu F, Traugh N, Wang X, Li Z, Mei S, Cui Y, Shi S, Lipp JJ, Hinterndorfer M, Zuber J, Brown M, Li W*, Liu XS*. Integrative analysis of pooled CRISPR genetic screens using MAGeCKFlute. Nat Protoc. 2019.
3. Jiang P#, Gu S#, Pan D#, Fu J, Sahu A, Hu X, Li Z, Traugh N, Bu X, Li B, Liu J, Freeman GJ, Brown MA, Wucherpfennig KW*, Liu XS*. Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat Med*. 2018;
4. Hu X, Zhang J, Liu JS, Li B*, Liu XS*. Evaluation of immune repertoire inference methods from RNA-seq data. *Nat Biotechnol*. 2018. 9;36(11):1034.
5. Pan D#, Kobayashi A#, Jiang P#, Ferrari de Andrade L, Tay RE, Luoma A, Tsoucas D, Qiu X, Lim K, Rao P, Long HW, Yuan GC, Doench J, Brown M, Liu XS*, Wucherpfennig KW*. A major chromatin regulator determines resistance of tumor cells to T cell-mediated killing. Science 2018; 359(6377):770-5.
6. Liu XS*, Mardis ER*. Applications of immunogenomics to cancer. *Cell* 2017; 168(4): 600-612
7. Li B*, Li T, Wang B, Dou R, Zhang J, Liu JS, Liu XS*. Ultrasensitive detection of TCR hypervariable-region sequences in solid-tissue RNA-seq data. *Nat Genet* 2017; 49(4):482-3.
8. Li B#, Li T#, Pignon JC, Wang B, Wang J, Shukla SA, Dou R, Chen Q, Hodi FS, Choueiri TK, Wu C, Hacohen N, Signoretti S, Liu JS*, Liu XS*. Landscape of tumor-infiltrating T cell repertoire of human cancers. Nat Genet 2016; 48(7):725-32.

X. Shirley Liu, PhD

1997-2002	PhD in Biomedical Informatics, PhD Minor in Computer Science, Stanford University
2002-2003	Independent Fellow, Department of Biostatistics, Harvard School of Public Health, Dana-Farber Cancer Institute
2003-2012	Assistant Professor and Associate Professor, Department of Biostatistics and Computational Biology, Dana-Farber Cancer Institute, Harvard T.H. Chan School of Public Health
2012-present	Professor, Department of Data Sciences, Dana-Farber Cancer Institute, Harvard University

IMMUNE SUPPRESSIVE MECHANISMS IN THE TUMOR MICROENVIRONMENT

Hiroyoshi Nishikawa
Division of Cancer Immunology, Research Institute/ EPOC, National Cancer Center
5-1-1 Tsukiji, Chuo-Ku, Tokyo 104-0045, Japan
Department of Immunology, Nagoya University Graduate School of Medicine
65 Tsurumai, Showa-Ku, Nagoya 466-8550, Japan
(hnishika@ncc.go.jp / nisihiro@med.nagoya-u.ac.jp)

One of the crucial processes involved in tumor development is a gain of immune escape mechanisms by cancers [1]. Therefore, the induction/recruitment of immunosuppressive cells [e.g., regulatory T (Treg) cells, myeloid-derived suppressor cells and tumor-associated macrophages], and the increased expression of various immunosuppressive molecules [e.g., programmed cell death-1 (PD-1) and PD-ligand 1 (PD-L1)] are hallmarks of cancer development and progression [1], indicating the importance of targeting these immune suppressive machineries in the tumor microenvironment (TME) for successful cancer therapy. The fields of immunology and oncology are now being linked, the clinical application of cancer immunotherapy represented by immune checkpoint blockade (ICB) therapies, such as anti-CTLA-4 monoclonal antibodies (mAbs) and anti-PD-1/PD-L1 mAbs, revealed that the potential antitumor immune responses can be recovered by blocking these immunosuppressive machineries, resulting in tumor regression and leading to a paradigm shift in cancer therapy across multiple cancer types [2]. Yet, more than half of patients treated with ICB therapy fail to respond, even in combination, uncovering a limited window of clinical responses. It is therefore necessary to develop more effective cancer immunotherapeutic strategies and define biomarkers for stratifying responders and non-responders via the detailed analysis of immune responses in patients [2].

There are two types of tumor antigens: tumor-specific antigens (TSAs), which are either oncogenic viral proteins or abnormal proteins stemming from genetic alterations (neoantigens), and tumor-associated antigens (TAAs), which are highly or aberrantly expressed normal proteins. It is not yet determined how the $CD8^+$ T cells that are specific for each type of antigen contribute to tumor regression, and whether activation of these self

vs non-self antigen-specific $CD8^+$ T cells is controlled differently. Treg cells, an immunosuppressive subset of $CD4^+$ T cells characterized by the expression of the master transcription factor forkhead box P3 (FoxP3), are a component of the immune system with essential roles in the maintenance of self-tolerance [3]. Treg cells protect hosts from developing autoimmune diseases and allergies, whereas they promote tumor development and progression by suppressing antitumor immunity. A higher abundance of Treg cells relative to effector T cells in the TME is associated with poor prognosis in patients with various types of cancer [3]. To address the differences in Treg-cell suppression modes, Melan-A (a representative TAA)-, and virus (surrogate TSA)-specific $CD8^+$ T cells were stimulated in the presence of Treg cells. Treg cells rendered Melan-A-specific T cells anergic. In contrast, virus-specific $CD8^+$ T cells proliferated in the presence of Treg cells, yet they highly expressed co-inhibitory molecules such as PD-1 [4], suggesting the potential of PD-blockade for selectively unleashing neoantigen-specific $CD8^+$ T cells.

While PD-1 blockade is a cancer immunotherapy effective in various types of cancer, it sometimes causes rapid cancer progression, called hyper-progressive disease (HPD), in a fraction of treated patients. We explored how PD-1 blockade caused HPD in these patients and how HPD could be treated and prevented. We show that tumor-infiltrating $CD45RA^-FOXP3^{high}CD4^+$ eTreg cells expressed PD-1 at equivalent levels as tumor-infiltrating effector T cells and at much higher levels than eTreg cells in some GC patients. Functionally, $PD\text{-}1^+$ eTreg cells in the TME were highly suppressive and proliferative than $PD\text{-}1^-$ eTreg cells. Furthermore, PD-1 blockade significantly enhanced *in vitro* suppressive activity of eTreg cells. When GC tissue samples were compared between before and after PD-1 blockade, tumor infiltrating $Ki67^+$ eTreg cells were markedly increased by PD-1 blockade in HPD patients, whereas they were reduced in non-HPD patients, indicating that PD-1 blockade may facilitate the proliferation of highly suppressive $PD\text{-}1^+$ eTreg cells in HPDs, resulting in inhibition of antitumor immunity [5] (Figure 1).

Clinical efficacy of ICB against cancers with oncogenic driver gene mutations, while they frequently harbor small numbers of tumor mutation burden (TMB), is variable, suggesting different contributions to immune responses by each driver mutation. We examined immunological phenotypes in the TME of *EGFR*-mutated lung adenocarcinomas (LUADs), to which ICB is largely ineffective [6]. While *EGFR*-mutated LUADs exhibited a non-inflamed TME, $CD4^+$ effector eTreg cells, that are generally detected with effector T cells in the inflamed TME [7], highly infiltrated. EGFR signals activated cJun/JNK and reduced IRF; the former increased CCL22 recruiting eTreg cells and the latter decreased CXCL10 and CCL5 inducing $CD8^+$ T-cell infiltration. EGFR signal inhibitors decreased eTreg cell infiltration in the TME, and combination with ICB provided better antitumor effects compared with either of single treatment. This is a novel mechanism of Treg cell

infiltration into the non-inflamed TME: signaling via driver mutations in EGFR promoted Treg cell infiltration and inhibited effector T-cell infiltration into the TME by directly targeting chemokine expression to establish the immune suppressive TME [8] (Figure 2).

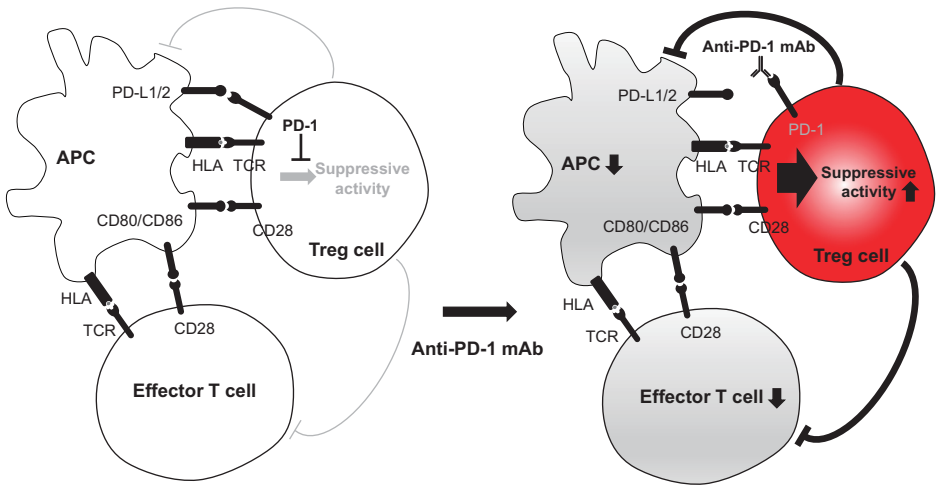


Figure 1 PD-1 expression inhibits TCR and CD28 signals in Treg cells and thereby attenuates Treg-cell-mediated immune suppression (Left). PD-1 blockade by anti-PD-1 antibody increases TCR and CD28 signal in Treg cells, and thereby enhances their proliferation and suppressive activity. Strong immune suppression by such expanded and activated eTreg cells hampers activation of effector T cells such as $CD8^+$ T cells (Right).

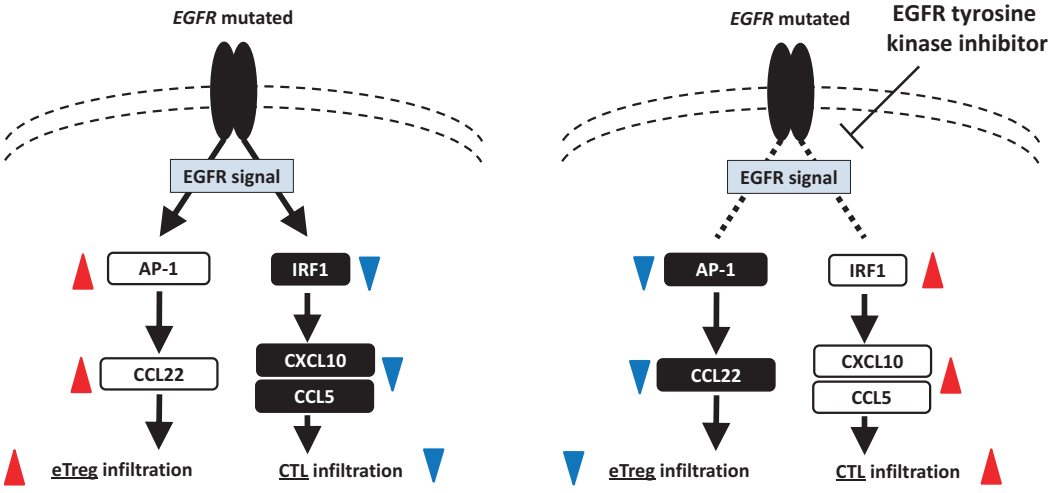


Figure 2 *EGFR*-mutated lung adenocarcinomas cells themselves produce CCL22 to recruit eTreg cells and inhibit to produce CXCL10 and CCL5 to avoid $CD8^+$ T-cell infiltration as a mechanism of immune escape. EGFR-TKI changes the TME more favorable for cancer immunotherapy: high $CD8^+$ T cells and low eTreg cells.

Thus, integrated analyses of immunological and genomic assays are crucial for understanding comprehensive immune suppressive network in the TME. In addition, developing novel strategies to control Treg cells could be important for effective cancer immunotherapies.

References

1. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565-1570.
2. Zou W, Wolchok JD, Chen L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016;8:328rv324.
3. Togashi Y, Shitara K, Nishikawa H. Regulatory T cells in cancer immunosuppression - implications for anticancer therapy. Nat Rev Clin Oncol 2019;16:356-371.
4. Maeda Y, Nishikawa H, Sugiyama D, Ha D, Hamaguchi M, Saito T, Nishioka M, Wing JB, Adeegbe D, Katayama I, Sakaguchi S. Detection of self-reactive $CD8^+$ T cells with an anergic phenotype in healthy individuals. Science 2014;346:1536-1540.
5. Kamada T, Togashi Y, Tay C, Ha D, Sasaki A, Nakamura Y, Sato E, Fukuoka S, Tada Y, Tanaka A, Morikawa H, Kawazoe A, Kinoshita T, Shitara K, Sakaguchi S, Nishikawa H. $PD\text{-}1^+$ regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. Proc Natl Acad Sci USA 2019;116:9999-10008.
6. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhaufl M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crino L, Blumenschein GR, Jr., Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F, Brahmer JR. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. N Engl J Med 2015;373:1627-1639.
7. Spranger S, Spaapen RM, Zha Y, Williams J, Meng Y, Ha TT, Gajewski TF. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by $CD8^+$ T cells. Sci Transl Med 2013;5:200ra116.
8. Sgiyama E, Togashi Y, Takeuchi Y, Shinya S, Tada Y, Kataoka K, Tane K, Sato E, Ishii G, Goto K, Shintani Y, Okumura M, MTsuboi M, Nishikawa H. Blockade of EGFR improves responsiveness to PD1 blockade in EGFR-mutated non-small cell lung cancer. Sci Immunol *In press*.

Hiroyoshi Nishikawa, MD, PhD

2003-2006	Research Fellow, Memorial Sloan Kettering Cancer Center
2006-2010	Assistant Professor, Mie University Graduate School of Medicine
2010-2015	Associate Professor, IFReC, Osaka University
2015-present	Chief, Division of Cancer Immunology, National Cancer Center
2016-present	Professor, Nagoya University Graduate School of Medicine

PROTEOGENOMICS AND IMMUNOPEPTIDOMICS FOR THE DEVELOPMENT OF PERSONALIZED CANCER IMMUNOTHERAPY

Michal Bassani-Sternberg
Laboratory of Immunopeptidomics
Center of Experimental Therapeutics
Department of Oncology UNIL CHUV
Ludwig Institute for Cancer Research Lausanne
Rue du Bugnon 25A, CH-1005 Lausanne, Switzerland
(michal.bassani@chuv.ch)

The remarkable clinical efficacy of the immune checkpoint blockade therapies has motivated researchers to discover immunogenic epitopes and exploit them for personalized vaccines and T cell based therapies. Mutated human leukocyte antigen binding peptides (HLAp) are currently the leading targets. We and others have shown that the direct identification of tissue-derived immunogenic neoantigens by mass spectrometry (MS) is feasible [1]. However, most studies attempt to identify neoantigens based on HLA binding prediction tools. We have compiled a large immunopeptidomics database across dozens of HLA allotypes. By taking advantage of co-occurring HLA-I alleles, we rapidly and accurately identified HLA-I binding motifs. We have shown that training HLA-I ligand predictors on refined motifs significantly improves the identification of neoantigens [2]. Recently, we have acquired the largest reported HLA-II immunopeptidomics dataset. We introduced novel algorithmic tools to analyze this data and developed for the first time HLA-II epitope prediction tool trained on immunopeptidomics data that results in major improvements in prediction accuracy [3].

In contrast to the private neoantigens, tumor-specific antigens that are shared across patients may be more attractive for immunotherapy. Recent studies have focused on the discovery of aberrantly-expressed non-canonical antigens, which expands the repertoire of targetable epitopes through the translation and presentation of presumably non-coding regions. Indeed, translation of 5′UTRs, exon–intron junctions, intronic regions, non-canonical reading frames or antisense transcripts can generate tumor antigens, to elicit specific CD8 T cell responses and to promote tumor regression in humans. However, their identification requires highly sensitive and accurate MS-based proteogenomics approaches.

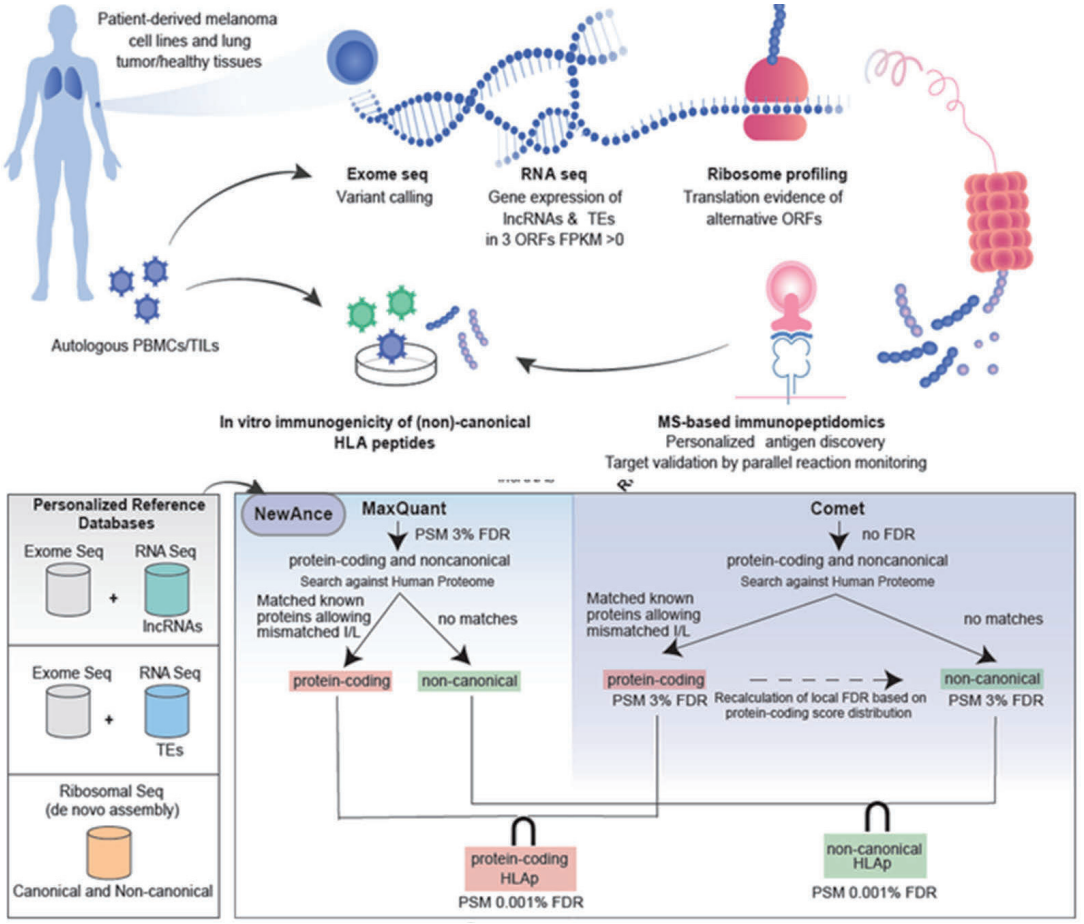


Figure 1 A schematic of the proteo-genomics workflow. Tumor tissue samples or tumor cell lines were obtained and exome, RNA and Ribo-Seq are performed to provide a framework to interrogate the non-canonical antigen repertoire. HLAp are immunoaffinity purified from tumors and analyzed by MS. Immunopeptidomics spectra are searched against RNA and Ribo-Seq based personalized protein sequence databases that contain non-canonical sequences. Identified noncHLAIp are validated by targeted MS and tested for immunogenicity using autologous T cells or PBMCs. Different protein sequence databases combining whole exome sequencing, and inferred from RNA-Seq and Ribo-Seq data are utilized. NewAnce is implemented by retaining the intersection of the two MS search tools MaxQuant and Comet, and applying group-specific FDR calculations for protHLAp and noncHLAp.

We have developed a novel analytical pipeline that can precisely characterize the non-canonical HLAp repertoire [4]. The workflow incorporates whole exome sequencing, both bulk and single cell transcriptomics, ribosome profiling, and a combination of two MS/MS search tools with group-specific false discovery rate calculations for accurate HLAp identification. We identified more than 400 non-canonical HLAp derived from the expressed lncRNAs, transposable elements and alternative open reading frames in nine melanoma and lung cancer samples.

We employed several complementary methods to assess the accuracy of our approach. First, we predicted the binding of peptides to their respective HLA allotypes. Across all 11 investigated samples, 90% of the noncHLAIp and 91% of the protHLAIp we identified, were predicted to bind the HLA allotypes. In addition, we correlated the observed mean retention time (RT) of a given peptide against the predicted hydrophobicity index (HI) and showed that the RT distribution of non-canonical peptides was on the diagonal line, and was not significantly different from the distribution of proteome-derived peptides, supporting their correct identification. We have validated experimentally their common identification across multiple tumors through targeted MS of spiked-in synthetic isotopically heavy labeled peptide counterparts. Furthermore, we validated their translation from the correct open reading frame with Ribo-seq.

We retrieved their RNA expression levels in large databases of healthy tissues, such as GTEx, and observed that about 20% of the non-canonical targets are not expressed in any of the healthy tissues except testis (in GTEx), therefore are likely tumor specific. One non-canonical peptide KYKDRTNILF, derived from a novel downstream ORF (dORF) in the transcript encoding the melanoma stem cell marker ATP-binding cassette sub-family B member 5 (ABCB5) in melanoma primary cell line 0D5P, was found to be immunogenic in both CD8+ TILs and CD8+ T cells from peripheral blood lymphocytes (PBLs). ABCB5 mediates chemotherapeutic drug resistance in stem-like tumor cell subpopulations in human malignant melanoma and is commonly over-expressed on circulating melanoma tumor cells. T cells recognizing the noncHLAIp derived from the dORF of the ABCB5 gene, in both peripheral blood and TILs, suggests no central tolerance, and that this target could allow immune-targeting of melanoma stem cell subpopulation to drastically affect tumor growth. This analytical platform holds great promise for the discovery of novel cancer antigens for cancer immunotherapy.

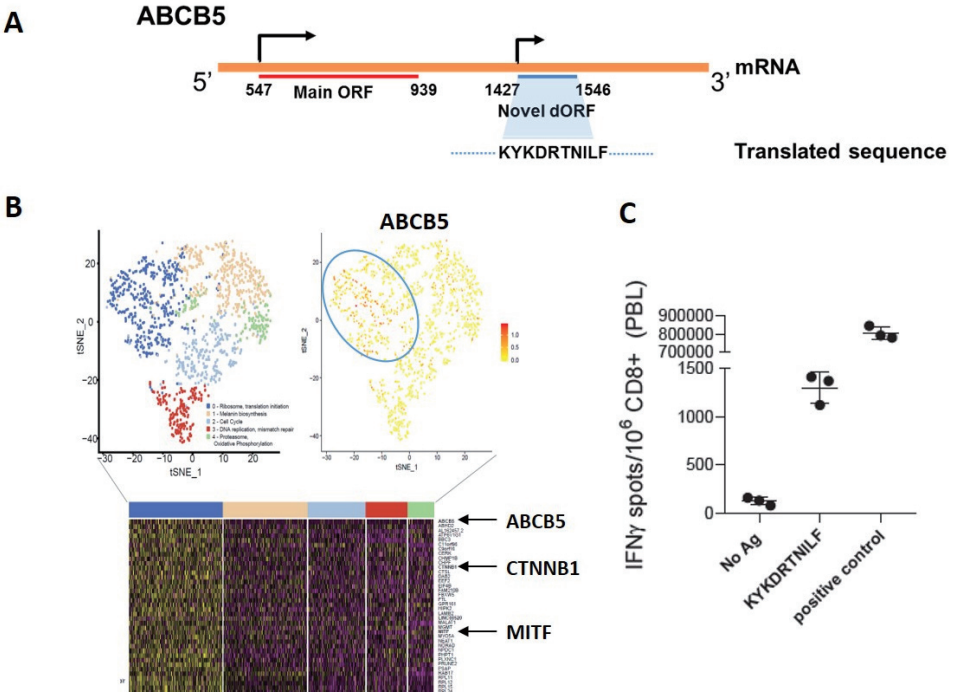


Figure 2 A. Through Ribo-seq analysis, we identified a novel downstream ORF (dORF) in the transcript encoding the melanoma stem cell marker ATP-binding cassette sub-family B member 5 (ABCB5) gene in the melanoma 0D5P sample, which was the source of the peptide KYKDRTNILF. B. ScRNA-Seq revealed non-coding transcriptional heterogeneity in melanoma 0D5P. t-SNE plots of the 1,365 cells colored by the five identified clusters and of the ABCB5 gene expression that is enriched in cluster 0. The Heatmap shows the scaled and centred expressions of marker genes in cluster 0. C. Immune reactivity was measured in melanoma 0D5P by the IFNγ ELISpot assay using CD8+ T cells from PBLs that were re-challenged with autologous CD4+ blasts loaded with 1 μM peptide.

References

1. Bassani-Sternberg, M., Braunlein, E., Klar, R., Engleitner, T., Sinitcyn, P., Audehm, S., Straub, M., Weber, J., Slotta-Huspenina, J., Specht, K., Martignoni, M. E., Werner, A., Hein, R., D, H. Busch, Peschel, C., Rad, R., Cox, J., Mann, M., & Krackhardt, A. M. (2016). Direct identification of clinically relevant neoepitopes presented on native human melanoma tissue by mass spectrometry. *Nat Commun, 7*, 13404. doi:10.1038/ncomms13404
2. Bassani-Sternberg, M., Chong, C., Guillaume, P., Solleder, M., Pak, H., Gannon, P. O., Kandalaft, L. E., Coukos, G., & Gfeller, D. (2017). Deciphering HLA-I motifs across HLA peptidomes improves neo-antigen predictions and identifies allostery regulating HLA specificity. *PLoS Comput Biol, 13*(8), e1005725. doi:10.1371/journal.pcbi.1005725

3. Julien Racle, Justine Michaux, Georg Alexander Rockinger, Marion Arnaud, Philippe Guillaume, Sara Bobisse, George Coukos, Alexandre Harari, Camilla Jandus, Michal Bassani-Sternberg* and David Gfeller* (2019). Deep motif deconvolution of HLA-II peptidomes for robust epitope predictions. *Nature Biotechnology*. doi:10.1038/s41587-019-0289-6
4. Chloe Chong, Markus Müller, HuiSong Pak, Dermot Harnett, Florian Huber, Delphine Grun, Marion Leleu, Aymeric Auger, Brian J. Stevenson, Justine Michaux, Ilija Bilic, Antje Hirsekorn, Lorenzo Calviello, Laia Simó-Riudalbas, Evarist Planet, Jan Lubiński, Marta Bryśkiewicz, Maciej Wiznerowicz, Ioannis Xenarios, Lin Zhang, Didier Trono, Alexandre Harari, Uwe Ohler, George Coukos, Michal Bassani-Sternberg (2019). Integrated Proteogenomic Deep Sequencing and Analytics Accurately Identify the Tumor Non-Canonical Immunopeptidome. BioRxiv.

Michal Bassani-Sternberg, PhD

2004-2009	PhD, Technion, Israel Institute of Technology (Biology)
2010-2011	Postdoctoral researcher, Technion, Israel Institute of Technology (Biology)
2012-2015	Postdoctoral Researcher, The Proteomics and Signal Transduction Department, Max Planck Institute of Biochemistry
2015-present	Head of Immunopeptidomics Unit, the Center of Experimental Therapeutics (CTE) at the University Hospital of Lausanne (CHUV) and The Ludwig Institute for Cancer Research, Lausanne

INTERPRETING CANCER GENOMES THROUGH PHYSICAL AND FUNCTIONAL MODELS OF CANCER CELLS

Trey Ideker
Department of Medicine
University of California at San Diego
9500 Gilman Drive, Mailcode 0688, La Jolla, California 92093, USA
(tideker@ucsd.edu)

Recently we and other laboratories have launched the Cancer Cell Map Initiative [1] (http://ccmi.org/) and have been building momentum. The goal of the CCMI is to produce a complete map of the multi-protein molecular machines that are altered to promote transformation and growth of cancer cells. This map, currently missing, will form a critical component of the next generation of systems to decode a patient's cancer genome in precision medicine applications. CCMI efforts are focused along several lines. The first is administrative, related to coalition building. We have made notable progress in building a dedicated consortium of CCMI investigators and institutions, within as well as external to the University of California (UC) campuses, who provide expertise in data generation and/or development of computational methodology required to build and use cancer cell maps. Currently we have established a major collaborative axis between two UC campuses in particular, UC San Diego and UC San Francisco. These collaborations are also expanding beyond cancer to cover cell mapping for psychiatric disease and other complex disorders [2]. Second, we are establishing platforms for systematic mapping of protein-protein interactions in cancer, based on affinity purification mass spectrometry [3] (AP-MS). Our recent efforts and progress along these lines were the main focus of my presentation, described further below. Third, we are developing technology for mapping gene-gene epistatic interactions rapidly using the CRISPR system. I did not cover this progress in my talk on epistatic interaction mapping, beyond initial pilots which have been published [4, 5] . A fourth focus area is on development of software and database technology to visualize and store cancer cell maps [6, 7]. In terms of algorithm development, we are developing machine learning systems for integrating protein and genetic interaction network data to

create multi-scale models of cancer cells [8] . In a recent paper [9] , we have also shown how a hierarchical map of cell structure can be embedded with a deep neural network, so that the model is able to accurately simulate the effect of mutations in genotype on the cellular phenotype. Our machine learning efforts also include building causal network maps connecting DNA mutations (somatic and germline, coding and noncoding) to the cancer events they induce downstream [10] .

In my talk at the 50th International Symposium of the Princess Takamatsu Cancer Research Fund, I reported recent progress of the CCMI related to the goal of comprehensive protein-protein interaction mapping. Thus far interactions have been mapped for more than 50 cancer proteins, prioritizing those most frequently found to be mutated in breast cancer and head and neck squamous carcinoma [11, 12] . This effort has thus far yielded several thousand new protein interactions purified from a panel of breast and esophageal cell lines representing both tumor and normal cell models. We have combined these data with previously measured protein interactions in the public domain and analyzed this integrated dataset to reveal a hierarchy of protein complexes of different sizes and stringencies of interaction. A significant number of these complexes are somatically mutated in cancer, most in tumor-type specific patterns. The mutated complexes, which number slightly over 300, include many expected based on previous knowledge of cancer pathways and many that represent novel discoveries.

In the resulting discussion period, points were raised about the expression levels of the identified protein complexes and, in particular, whether the complexes found to be mutated in a population of tumors were also found to have high expression levels. In general the answer is yes: the protein complexes mutated in a cancer subtype are also typically expressed at appreciable levels in that subtype. Notable exceptions do emerge, however. In at least one case, the discrepancy could be explained by the fact that a sequenced tumor biopsy typically represents a mixture of cell types and/or tissues, such that the predominant cell type driving the bulk mRNA expression measurement is not the cell type in which the key mutations arise.

Another discussion point related to how we scored somatic mutations: were all single nucleotide changes counted, and how would a more/less conservative policy affect our analysis? Here I replied that we had simply adopted the conventions used in analysis of exomes in The Cancer Genome Atlas (TCGA) projects. These TCGA mutation analyses generally focus on calling non-synonymous mutations in coding regions, which result in a change in the amino-acid sequence, correcting for external factors that have been shown to influence local mutation rates such as gene length, expression level, replication timing, and tumor mutation burden. Thus, the intention of our CCMI analysis was not to recall mutations or change the standard practice in that regard, but to extend the analysis from a

focus on genes and proteins to a focus on the multimeric protein complexes under positive selection pressure for cancer mutations.

It is worth noting the significant synergy between my talk and several others presented in the conference, including that of Dr. Garry Nolan, who spoke in the same session. Nolan covered a series of technological advances for mapping the structure of biological systems, including mapping tissues at single-cell resolution and, remarkably, mapping cells at single-atomic resolution. In both cases, he showed that the data reveal a hierarchy of biological structures and functions and pointed out that this goal of hierarchical mapping was very similar to the analysis of protein interaction networks described in my talk. Particularly if Dr. Nolan is able to develop his new system for mapping the contents of cells at atomic resolution, this technique could be very productively combined with our informatics approaches to model the hierarchy of molecular machines under mutational pressure in cancer, along with their corresponding cell types and locations.

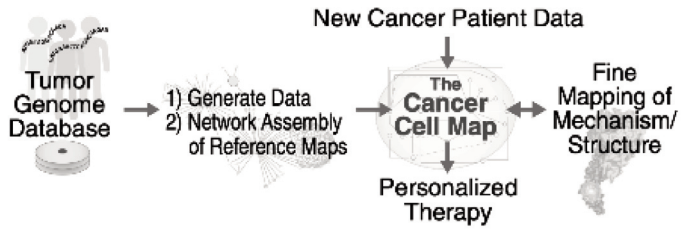


Figure 1 Pipeline for cancer cell network mapping and network interpretation of patient data

Databases of tumor genomes are mined to identify genes and cell types in which alterations drive or predispose to development of cancer. Seeded by this information, systematic network mapping efforts enable assembly of the ''Cancer Cell Map,'' providing a working scaffold of molecular interactions and the cell types and conditions under which they are active. New patient data are assessed by query against this resource, which translates alterations at the genetic and molecular level to reveal the impact these alterations have on the hallmark networks of cancer. Key interactions and network structures are explored for fine mapping of their basis in molecular structure or implications to biomolecular mechanism.

References

1. Krogan, N. J., Lippman, S., Agard, D. A., Ashworth, A. & Ideker, T. The cancer cell mapinitiative: defining the hallmark networks of cancer. *Mol. Cell* 58 , 690-698 (2015).

2. Willsey, A. J. *et al*. The Psychiatric Cell Map Initiative: A Convergent Systems Biological Approach to Illuminating Key Molecular Pathways in Neuropsychiatric Disorders. *Cell* 174 , 505-520 (2018).
3. Eckhardt, M. *et al*. Multiple Routes to Oncogenesis Are Promoted by the Human Papillomavirus?Host Protein Network. *Cancer Discovery* 8 , 1474-1489 (2018).
4. Du, D. *et al*. Genetic interaction mapping in mammalian cells using CRISPR interference. *Nat. Methods* 14 , 577-580 (2017).
5. Shen, J. P. *et al*. Combinatorial CRISPR-Cas9 screens for de novo mapping of genetic interactions. *Nature Methods* 14 , 573-576 (2017).
6. Pratt, D. *et al*. NDEx, the Network Data Exchange. *Cell Syst* 1 , 302-305 (2015).
7. Shannon, P. *et al*. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 13 , 2498-2504 (2003).
8. Yu, M. K. *et al*. Visible Machine Learning for Biomedicine. *Cell* 173 , 1562-1565 (2018).
9. Ma, J. *et al*. Using deep learning to model the hierarchical structure and function of a cell. *Nat. Methods* 15, 290-298 (2018).
10. Zhang, W. *et al*. A global transcriptional network connecting noncoding mutations to changes in tumor gene expression. *Nat. Genet*. 50, 613-620 (2018).
11. Ciriello, G. *et al*. Comprehensive Molecular Portraits of Invasive Lobular Breast Cancer. *Cell* 163 , 506-519 (2015).
12. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* 517, 576-582 (2015).

Trey Ideker, PhD

1994	BS (Electrical Engineering and Computer Science), Massachusetts Institute of Technology
1995	MS (Electrical Engineering and Computer Science), Massachusetts Institute of Technology
2001	PhD (Molecular Biotechnology), University of Washington
2003-present	Fellow, Whitehead Institute for Biomedical Research Pres. Professor, Department of Medicine Adjunct Professor, Departments of Bioengineering & Computer Science, University of California San Diego

ORIGIN OF CANCER IN INFLAMMATORY TISSUES

Seishi Ogawa
Departments of Pathology and Tumor Biology
Institute for the Advanced Study of Human Biology (WPI-ASHBi), Kyoto University
Department of Molecular Hematology, Karolinska Institute
Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
(sogawa-tky@umin.ac.jp)

Chronic inflammation elicits a wide variety of responses in the involved tissues and may cause severe organ damage. However, the tissue response to long-standing inflammation is poorly understood in terms of positive selection in the target tissue. Here we show that in patients with ulcerative colitis (UC), inflamed intestine undergoes widespread remodelling by pervasive clones positively selected by acquiring mutations that down-regulated IL-17 signalling, most frequently affecting NFKBIZ, ZC3H12A (Regnase-1), and PIGR. Substantially different mutation profiles in UC-epithelia and cancer indicate distinct mechanisms of positive selection between both. Particularly, despite highly prevalent in UC, NFKBIZ and ZC3H12A mutations were rarely found in cancer, suggesting a strong negative selection for these mutations during cancer evolution, which was supported by significantly reduced colitis-associated tumour formation in NFKBIZ-deficient mice. Our results provide insight into inflammation-associated tissue remodelling and unmask unique cancer vulnerability, which might be utilized for therapeutics of cancer.

Chronic inflammation is a major cause of morbidity and mortality in the human population and represents a major cancer risk therein. A common feature of chronic inflammation is recurring tissue injury and repair fueled by long-lasting unregulated immunity, terminating in tissue remodeling and debilitating organ dysfunction. Meanwhile, it has recently been demonstrated that extensive tissue remodeling can take place even in physiologically normal tissues in an age-dependent manner, in which clones carrying common cancer-related mutations replace existing normal tissues in the extreme elderly. Substantially promoted by exposure to well-known cancer risks, clonal expansion

of somatically mutated clones may also play a role in inflammation-associated tissue remodeling. However, it is largely unknown whether this actually happens, how frequently and extensively and with what mutations it ever occurs, and how it modifies the inflammatory disease process and cancer development.

Ulcerative colitis (UC) is a common form of inflammatory bowel disease, which is characterized by persistent inflammation involving the large intestine, which causes a severe destruction of intestinal mucosa and intractable ulcer formation. While inflammation-induced mucosal damage is followed by the recruitment of repair mechanisms, the repeated destruction and repair cycles for long years ultimately result in the remodeling of the colorectal epithelium, which is associated with an increased risk (approximately 15-20% depending on disease duration) of developing cancer (colitis-associated colorectal cancer; CAC). In this study, to understand how chronic inflammation shapes tissue remodeling in terms of positive selection and how it correlates with cancer development, we collected multiple specimens of colorectal epithelia from both UC and non-UC individuals, which were analyzed for somatic mutations using unbiased sequencing, followed by functional evaluations of clonal selection.

In the steady state after intestinal development, crypts in adults still divide to balance physiological deficits of crypts; on the basis of sequencing of crypt clusters and assuming an almost constant human colon length (and therefore crypt numbers in the colon), we estimated that annually 100,000 crypts are estimated to be lost and replaced by new crypts generated by fissions of adjacent crypts, which are equivalent to ~4% of the total intestinal crypts per ten years or only ~0.0041 fissions/crypt/year. In UC patients, crypt fission is dramatically accelerated (~0.36 fissions/crypt/year) to amend the large deficits in the injured epithelium, during which strong positive selection operates to allow for the dominance of clones carrying mutations in the IL-17 signalling pathway, although the exact mechanism is still unclear. Importance of the IL-17 signalling in the pathogenesis of UC is also supported by the previous GWAS studies that reported susceptibility associated with the *NFKBIZ* and *TRAF3IP2* loci. These two loci have also been associated with susceptibility to psoriasis, a chronic inflammation in the epidermis, where aberrantly activated IL-17 signalling is implicated on the basis of the critical role of *NFKBIZ* and the efficacy of anti-IL-17 antibody, suggesting a strong link between UC and psoriasis pathophysiology.

As is the case with the remodelling in other tissues, like blood, skin, and oesophageal epithelium, the positive selection also involves mutations commonly affected in CAC, but overall frequencies of their mutations substantially differed between normal and inflammation-associated expansion, and respective cancers. Again, the critical difference in the mechanism between positive selection in cancer and non-cancer tissues is highlighted.

A new finding from the study on UC epithelia would be that the mechanism that allows for positive selection under chronic inflammation is not necessarily operate in neoplastic growth; on the contrary, it imposes strong negative selection and thus, reveals new cancer vulnerability, as evident from extremely rare mutations of *NFKBIZ* and *ZC3H12A* in CAC and sporadic colorectal cancer, despite their pervasive nature in UC non-dysplasia.

Finally, one of the most important implications from our study would be a possibility that chronic inflammation can evoke a tissue response by way of remodelling affected tissues with positively selecting clones that acquired mutations, according to the Darwinian survival-of-the-fittest principle. Chronic inflammation is a highly prevalent disability among the human population, potentially affecting almost every organs and tissues, where a similar mechanism may operate and lead to tissue remodelling depending on the nature of inflammation, which may explain an important part of the disease picture. Understanding of such responses is of potential importance for the better understanding of the pathogenesis of inflammatory diseases and for the development of novel therapeutics.

References

1. Kakiuchi, N., K. Yoshida, M. Uchino, T. Kihara, K. Akaki, Y. Inoue, K. Kawada, S. Nagayama, A. Yokoyama, S. Yamamoto, M. Matsuura, T. Horimatsu, T. Hirano, N. Goto, Y. Takeuchi, Y. Ochi, Y. Shiozawa, Y. Kogure, Y. Watatani, Y. Fujii, S. K. Kim, A. Kon, K. Kataoka, T. Yoshizato, M. M. Nakagawa, A. Yoda, Y. Nanya, H. Makishima, Y. Shiraishi, K. Chiba, H. Tanaka, M. Sanada, E. Sugihara, T. A. Sato, T. Maruyama, H. Miyoshi, M. M. Taketo, J. Oishi, R. Inagaki, Y. Ueda, S. Okamoto, H. Okajima, Y. Sakai, T. Sakurai, H. Haga, S. Hirota, H. Ikeuchi, H. Nakase, H. Marusawa, T. Chiba, O. Takeuchi, S. Miyano, H. Seno, and S. Ogawa. 2020. Frequent mutations that converge on the NFKBIZ pathway in ulcerative colitis, Nature, 577: 260-65.
2. Yokoyama, A., N. Kakiuchi, T. Yoshizato, Y. Nannya, H. Suzuki, Y. Takeuchi, Y. Shiozawa, Y. Sato, K. Aoki, S. K. Kim, Y. Fujii, K. Yoshida, K. Kataoka, M. M. Nakagawa, Y. Inoue, T. Hirano, Y. Shiraishi, K. Chiba, H. Tanaka, M. Sanada, Y. Nishikawa, Y. Amanuma, S. Ohashi, I. Aoyama, T. Horimatsu, S. Miyamoto, S. Tsunoda, Y. Sakai, M. Narahara, J. B. Brown, Y. Sato, G. Sawada, K. Mimori, S. Minamiguchi, H. Haga, H. Seno, S. Miyano, H. Makishima, M. Muto, and S. Ogawa. 2019. Age-related remodelling of oesophageal epithelia by mutated cancer drivers, Nature, 565: 312-17.

Seishi Ogawa, MD, PhD

1989-1993	MD, PhD Program, University of Tokyo
1983-1988	Undergraduate/MD Program, School of Medicine, University of Tokyo
1988-1989	Postgraduate Clinical Training in Internal Medicine
1994-1995	Clinical Associate, University of Tokyo
1995-1996	Research Fellow
1996-2002	Assistant Professor of Medicine, University of Tokyo
2002-2013	Associate Professor, University of Tokyo
2013-present	Professor, Department of Pathology and Tumor Biology, Kyoto University
2017-present	Guest Professor, Department of Molecular Hematology, Karolinska Institute

PATHOLOGY FROM THE MOLECULAR SCALE ON UP

Garry P. Nolan
Rachford and Carlota A Harris Professor
Department of Microbiology & Immunology
Baxter Laboratory for Stem Cell Biology
Stanford University School of Medicine, Stanford, CA 94305, USA
(gnolan@stanford.edu)

High parameter single cell analysis has driven deep understanding of immune processes. Using a next-generation single-cell "mass cytometry" platform we quantify surface and cytokine or drug responsive indices of kinase target with 45 or more parameter analyses (e.g. 45 antibodies, viability, nucleic acid content, and relative cell size). Similarly, we have developed two advanced technologies termed MIBI and CODEX that enable deep phenotyping of solid tissue in both fresh frozen and FFPE formats (50–100 markers). Collectively, the systems allows for subcellular analysis from the 70nm resolution scale to whole tissue in 3D.

I will present evidence of deep internal order in immune functionality demonstrating that differentiation and immune activities have evolved with a definable "shape". Further, specific cellular neighborhoods of immune cells are now definable with unique abilities to affect cellular phenotypes—and these neighborhoods alter in various cancer disease states. In addition to cancer, these shapes and neighborhoods are altered during immune action and "imprinted" during, and after, pathogen attack, traumatic injury, or auto-immune disease. Hierarchies of functionally defined trans-cellular modules are observed that can be used for mechanistic and clinical insights in cancer and immune therapies.

Garry P. Nolan, PhD

1983	BS, Cornell University, Biology/Genetics
1989	PhD, Stanford University, Genetics
1990	Postdoctoral Fellowship, Stanford University, Genetics
1993	Postdoctoral Fellowship, MIT, David Baltimore Laboratory, Biochemistry
1993-1999	Assistant Professor, Department of Molecular Pharmacology, Stanford University School of Medicine
1995-1999	Assistant Professor, Joint Appointment, Department of Microbiology and Immunology, Stanford University School of Medicine
1999-2009	Associate Professor (Tenure), Department of Molecular Pharmacology, Stanford University School of Medicine
2009-present	Professor (Tenure), Department of Microbiology & Immunology, Stanford University School of Medicine
2011-present	Rachford and Carlota A. Harris Professor, Department of Microbiology and Immunology, Stanford University School of Medicine

LONG NON-CODING RNAS AND METASTATIC PROGRESION OF COLORECTAL CANCER

Christopher A. Maher
Departments of Internal Medicine and Biomedical Engineering
McDonnell Genome Institute
Washington University School of Medicine
660 South Euclid Avenue, Campus Box 8069, St. Louis, MO 63110, USA
(christophermaher@wustl.edu)

Colorectal cancer (CRC) is the most common gastrointestinal malignancy in the United States. At the time of initial diagnosis, 20% of patients present with metastasis, and of those patients with primary disease approximately 50% will eventually develop metastatic disease. Although early stage colorectal cancer (CRC) is curable with surgery and adjuvant therapy, metastatic CRC (mCRC) is usually lethal. Further, despite advances in our understanding of primary CRC oncogenesis, the mechanisms of CRC metastasis and subsequent patient death is poorly characterized. To date, CRC research has primarily focused on the deregulation of protein-coding genes to identify oncogenes and tumor suppressors as potential diagnostic and therapeutic targets. Therefore, the characterization of long non-coding RNAs, elucidating their function, and assessing their clinical applicability could significantly impact mCRC diagnosis and treatment. While transcriptome sequencing has provided an unbiased method for discovering lncRNAs, existing large-scale sequencing projects such as The Cancer Genome Atlas Network (TCGA) are comprised of predominantly primary tumors lacking matched metastatic samples. This represents a critical barrier to discovering novel lncRNAs throughout the progression of primary to metastatic disease correlated to treatment response and resistance. To address this, we performed transcriptome sequencing and analysis of normal, primary, and distant metastatic CRC tissues and discovered 148 differentially expressed RNAs Associated with Metastasis (*RAMS*). We prioritized a novel lncRNA, *RAMS11*, due to its association with poor disease-free survival across independent patient cohorts. To understand *RAMS11* functional significance, we created a *RAMS11* knockout model by generating two CRISPR/Cas9 luciferase-tagged cell lines with a genomic deletion

of the last four exons of *RAMS11* in the LoVo metastatic colon cancer cells. We confirmed greater than a 99.9% reduction in our *RAMS11* CRISPR knockout (KO) models relative to wild type cells. We used these genetically engineered cell lines to confirm that the loss of *RAMS11* decreases cellular invasion/migration and anchorage independent growth. Conversely, we overexpressed *RAMS11* in HT29 cell (which lack endogenous *RAMS11* expression) and observed an increase in cellular invasion/migration and anchorage independent growth. Building upon our *in vitro* results, using our genetically engineered cell lines we found that loss of *RAMS11* reduces primary tumor growth and metastasis via three mouse models: (1) a subcutaneous model to study tumor growth and metastases, (2) a tail vein injection model to study the development of lung metastases, and (3) a hemi-splenectomy model to study the development of liver metastases. To understand its role in treatment response, and implicate *RAMS11* with specific signaling pathways, we performed a high-throughput viability assay using FDA-approved drugs to reveal that elevated *RAMS11* expression increased resistance to topoisomerase inhibitors. We subsequently confirmed that manipulating *RAMS11* expression altered *TOP2α* mRNA levels, protein levels, and downstream target gene expression. Due to the nuclear localization of *RAMS11*, we hypothesized that it may interact with epigenetic regulators to transcriptionally activate *TOP2α* expression. We found that *RAMS11* interacts with Chromobox protein 4 (CBX4), which possesses both activation and repressive activities, has been found to interact with lncRNAs, and is known to regulate *TOP2α* expression. Subsequent experiments demonstrated *RAMS11*-dependent recruitment of Chromobox protein 4 (CBX4) to transcriptionally activate Topoisomerase II alpha (TOP2α). A meta-analysis of ~7,000 patients across 22 cancer types revealed *RAMS11* is overexpressed and promotes oncogenic phenotypes in multiple cancer types, thereby broadening the impact of our findings. Overall, our understanding of how lncRNAs promote metastasis in CRC patients may have tremendous biological and clinical significance. To address this, our study used patient samples to characterize the landscape of lncRNA expression throughout the progression of primary to metastatic colorectal cancer. We also show that the novel lncRNA *RAMS11* directly affects mCRC biology, including promoting aggressive phenotypes and correlating with treatment response and resistance.

Christopher A. Maher, PhD

1998-2002	Graduated with BS from Cornell University, Biomedical Engineering
2002-2006	Graduated with PhD from shared program between Stony Brook University and Cold Spring Harbor Laboratory
2007-2009	Postdoctoral researcher, University of Michigan
2009-2011	Research Instructor, University of Michigan
2011-2018	Assistant Professor, Departments of Internal Medicine and Biomedical Engineering, Washington University School of Medicine
2011-present	Assistant Director, McDonnell Genome Institute
2018-present	Associate Professor, Departments of Internal Medicine and Biomedical Engineering, Washington University School of Medicine

ADVANCES IN DIAGNOSTIC AND THERAPEUTIC APPLICATION OF EXTRACELLULAR VESICLES IN CANCER

Takahiro Ochiya
Department of Molecular and Cellular Medicine
Tokyo Medical University
6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
(tochiya@tokyo-med.ac.jp)

Growing evidence indicates that cancer cell-derived extracellular vesicles (EVs) serve as regulatory agents in intercellular communications of the tumor microenvironment. Whether and how this knowledge of EVs, known as small and large exosomes, microvesicles, and oncosomes affect translational research and clinical practices have become pertinent questions.

EVs have been shown to be involved in the development of cancer metastasis [1] via carrying pathogenic components such as proteins, messenger RNAs (mRNAs), microRNAs (miRNAs), DNAs, lipids, and transcriptional factors that can mediate paracrine signaling in the tumor microenvironment [2, 3] (Figure 1). Moreover, these specific encapsulated molecules in EVs derived from high-metastatic cancer cells enable the creation of the appropriate tumor microenvironment for cancer metastasis. Understanding the metastatic

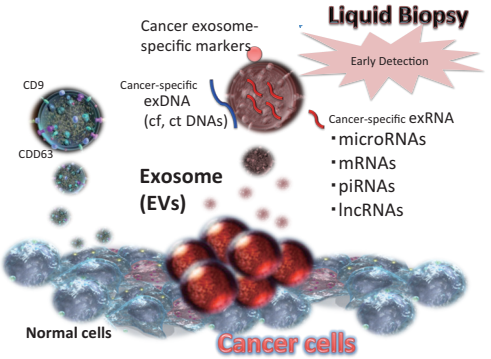


Figure 1 Summary of Extracellular Vesicles for Biomarker Analysis Attached file (PDF)

mechanisms through EV transfer may open up a new avenue for cancer diagnosis and therapeutic strategies [4].

Our current understanding of cancer-EVs functions, cancer-EVs destroy our body's natural barriers and barrier structures. These examples are EVs-mediated brain metastasis of breast cancer cells [5], peritoneal dissemination of highly metastatic ovarian cancer cells [6] and late recurrence of breast cancer [7]. In case of a metastatic model of diffuse-type gastric cancer (DGC), we investigated whether a difference in the fibroblast phenotypes could be observed by comparing between high-metastatic and low-metastatic DGC cells. Our study revealed that cancer cells with high metastatic capacity can generate at least two distinct fibroblast subpopulations: α-SMA-expressing type and chemokine-expressing type [8]. EVs contribute to the formation of a specific subpopulation with chemokine expression for contributing tumor metastasis.

Furthermore, we have demonstrated that RNA transcripts derived from an active human L1 retrotransposon are packaged in EVs and can initiate retrotransposition in recipient cells [9, 10]. Functional RNA intermediates are delivered to recipient cells, which are translated into the encoded proteins, allowing the reverse transcription and genomic integration of EGFP assisted by L1-encoded endonuclease and reverse transcriptase. Characterization of recipient cells by PCR amplification and sequencing analysis confirmed the precise splicing of the intron and the integration of the EGFP gene by retrotransposition. This study demonstrates that an active L1 retrotransposon can be transmitted to neighboring cells without direct cell-to-cell contact mediated by RNA intermediates secreted by the cell. Additionally, we show that RNA transfer may influence recipient transcriptional and post-transcriptional regulation. We demonstrate that L1-derived RNA transcripts and translated proteins are targeted by intrinsic host factors such as APOBEC3 family members, potentially restricting L1 activity to ensure genome stability in the cell. Although further experiments are required to assess the biological consequences of de novo L1 insertions in recipient cells, this study provides evidence for the horizontal transmission of an active L1 retrotransposon mediated by EVs in cultured cells.

The tumor specific methylation of nucleic acids including tissue-specific or circulating DNAs and microRNAs provide various messages on the physiological and pathological status of cancer patients [11]. The "Liquid Methylome" based on our ExoScreen [12] is a method for detecting circulating EVs-based DNA methylation in the patients' body fluids and allows us for developing a novel liquid biopsy for early detection of cancer. We also have several experiences on circulating microRNAs for early detection of cancer [13-16], thus EV is a promising agent for a liquid biopsy.

Currently, histopathological examination by specialists is the only method available for the accurate diagnosis of early detection of tumors, and no biomarkers have been identified

to date. To achieve early and accurate diagnosis of cancers, it will be necessary to develop rapid, noninvasive, and straightforward diagnostic methods. The ideal biomarkers for this purpose should have the capacity to detect cancers regardless of their histological subtype. To this end, it is necessary to develop a biomarker identification strategy based on a novel principle. Currently, circulating non-coding RNAs, including microRNAs and long non-coding RNAs, and the protein components of extracellular vesicles are promising biomarkers for the non-invasive detection of cancer at an early stage. The increasing number of well-designed cancer biomarker-related studies that have been published worldwide. In many of these studies, high diagnostic accuracy, which is represented as the area under the receiver operating characteristic curve being >0.8, could be achieved using combinations of circulating microRNAs. In addition, similar diagnostic accuracies were reported using long non-coding RNAs or proteins present in extracellular vesicles, although these evidences were based on a limited number of studies. In the present study of our japan national project supported by AMED, we examined the expression profiles of serum miRNA that were recently updated in miRBase (release 21) in several very large cohorts that included more than 50,000 cancer patients and 20,000 controls to identify circulating miRNA that can detect 13 species of early stage cancer. Our most current updated result showed the circulating miRNA profiles can be indeed a powerful tool for blood based cancer diagnosis.

Finally, we currently developed a new retrograde virus-vector-based genetic approach to manipulate local autonomic nerves in a tumor-specific and sympathetic or parasympathetic fiber-type-specific manner [17]. A retrospective analysis of breast cancer specimens revealed that increased sympathetic and decreased parasympathetic nerve density in tumors were associated with poor clinical outcomes and correlated with higher expression of immune checkpoint molecules. The EVs-mediated communication of autonomic nervous system and cancer microenvironment is a novel target for controlling cancer metastasis.

References

1. Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., *Ochiya, T.: Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J. Biol. Chem.*, 285: 17442-17452, 2010.
2. Kosaka N, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, *Ochiya T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *J Biol Chem*. 288(15):10849-10859, 2013.

3. Ageta H, Ageta-Ishihara N, Hitachi K, Karayel O, Onouchi T, Yamaguchi H, Kahyo T, Hatanaka K, Ikegami K, Yoshioka Y, Nakamura K, Kosaka N, Nakatani M, Uezumi A, Ide T, Tsutsumi Y, Sugimura H, Kinoshita M, *Ochiya T, Mann M, Setou M, Tsuchida K. UBL3 modification influences protein sorting to small extracellular vesicles. *Nat Commun*. 9(1):3936, 2018
4. Kosaka N, Fujita Y, Yoshioka Y, *Ochiya T. Versatile roles of extracellular vesicles in cancer. *J Clin Invest*, 126(4):1163-1172, 2016
5. Tominaga N, Kosaka N, Ono M, Katsuda T, Yoshioka Y, Tamura K, Lötvall J, Nakagama H, *Ochiya T. Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. *Nat Commun*, 6:6716, 2015
6. Yokoi A, Yoshioka Y, Yamamoto Y, Ishikawa M, Ikeda SI, Kato T, Kiyono T, Takeshita F, Kajiyama H, Kikkawa F, *Ochiya T. Malignant extracellular vesicles carrying MMP1 mRNA facilitate peritoneal dissemination in ovarian cancer. *Nature Commun*, 8, 14470, 2017
7. Ono M, Kosaka N, Tominaga N, Yoshioka Y, Takeshita F, Takahashi RU, Yoshida M, Tsuda H, Tamura K, *Ochiya T. Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci Signal*, 7:ra63, 2014 (2015 Signaling Breakthrough of the Year)
8. Naito Y, Yamamoto Y, Sakamoto N, Shimomura I, Kogure A, Kumazaki M, Yokoi A, Yashiro M, Kiyono T, Yanagihara K, Takahashi RU, Hirakawa K, Yasui W, *Ochiya T. Cancer extracellular vesicles contribute to stromal heterogeneity by inducing chemokines in cancer-associated fibroblasts. *Oncogene*. 38(28): 5566-5579, 2019
9. Kawamura Y, Yamamoto Y, Sato TA, *Ochiya T. Extracellular vesicles as trans-genomic agents: Emerging roles in disease and evolution. *Cancer Sci*. 108: 824-830, 2017
10. Kawamura Y, Sanchez Calle A, Yamamoto Y, Sato TA, *Ochiya T. Extracellular vesicles mediate the horizontal transfer of an active LINE-1 retrotransposon. *J Extracell Vesicles*. 8(1):1643214, 2019
11. Konno M, Koseki J, Asai A, Yamagata A, Shimamura T, Motooka D, Okuzaki D, Kawamoto K, Mizushima T, Eguchi H, Takiguchi S, Satoh T, Mimori K, *Ochiya T, Doki Y, Ofusa K, Mori M, Ishii H. Distinct methylation levels of mature microRNAs in gastrointestinal cancers. *Nat Commun*. 10(1):3888, 2019
12. Yoshioka Y, Kosaka N, Konishi Y, Ohta H, Okamoto H, Sonoda H, Nonaka R, Yamamoto H, Ishii H, Mori M, Furuta K, Nakajima T, Hayashi H, Sugisaki H, Higashimoto H, Kato T, Takeshita F, *Ochiya T. Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. *Nat Commun*, 5:3591, 2014
13. Sudo K, Kato K, Matsuzaki J, Boku N, Abe S, Saito Y, Daiko H, Takizawa S, Aoki Y, Sakamoto H, Niida S, Takeshita F, Fukuda T, *Ochiya T. Development and Validation

of an Esophageal Squamous Cell Carcinoma Detection Model by Large-Scale MicroRNA Profiling. *JAMA Netw Open*. 2(5):e194573, 2019

14. Urabe F, Matsuzaki J, Yamamoto Y, Kimura T, Hara T, Ichikawa M, Takizawa S, Aoki Y, Niida S, Sakamoto H, Kato K, Egawa S, Fujimoto H, *Ochiya T. Large-scale Circulating microRNA Profiling for the Liquid Biopsy of Prostate Cancer. *Clin Cancer Res*. 2019 25(10):3016-3025, 2019
15. Asano N, Matsuzaki J, Ichikawa M, Kawauchi J, Takizawa S, Aoki Y, Sakamoto H, Yoshida A, Kobayashi E, Tanzawa Y, Nakayama R, Morioka H, Matsumoto M, Nakamura M, Kondo T, Kato K, Tsuchiya N, Kawai A, *Ochiya T. A serum microRNA classifier for the diagnosis of sarcomas of various histological subtypes. *Nat Commun*. 10(1):1299, 2019
16. Yokoi A, Matsuzaki J, Yamamoto Y, Yoneoka Y, Takahashi K, Shimizu H, Uehara T, Ishikawa M, Ikeda SI, Sonoda T, Kawauchi J, Takizawa S, Aoki Y, Niida S, Sakamoto H, Kato K, Kato T, *Ochiya T. Integrated extracellular microRNA profiling for ovarian cancer screening. *Nat Commun*. 29(1):4319, 2018
17. Kamiya A, Hayama Y, Kato S, Shimomura A, Shimomura T, Irie K, Kaneko R, Yanagawa Y, Kobayashi K, *Ochiya T. Genetic manipulation of autonomic nerve fiber innervation and activity and its effect on breast cancer progression. *Nat Neurosci*. 22(8):1289-1305, 2019

Takahiro Ochiya, PhD

1988	PhD, Osaka University
1988-1992	Research Assistant, Osaka University
1991-1992	Post-doctoral Fellow, La Jolla Cancer Research Foundation (The SF Burnham Institute)
1992	Chief Researcher, Genetics Div., National Cancer Center Research Institute (NCCRI), Japan
1992-1998	Section Head, Genetics Div., NCCRI
1998-2010	Head, Section for Studies on Metastasis, NCCRI
2010-2018	Chief, Division of Molecular and Cellular Medicine, NCCRI
2018-present	Department of Molecular and Cellular Medicine, Tokyo Medical University

LncRNAs: LOST IN TRANSLATION

Eleonora Leucci
Laboratory for RNA Cancer Biology
Department of Oncology, LKI
KU Leuven, Campus Gasthuisberg
Herestraat 49, O&N1 bus 818, 3000 Leuven, Belgium
(eleonora.leucci@vib-kuleuven.be)

It is now widely accepted that although the eukaryotic genome is pervasively transcribed, less than 2% encodes for proteins [1]. The non-coding genome, hosts the vast majority of recurrent somatic mutations [2], copy number alterations [3] and cancer-related SNPs [4]. The so called "dark matter" of the genome gives rise to a broad spectrum of transcripts including a large class of non-coding RNA longer than 200 nucleotides [5], the lncRNAs. Interestingly, while a minority are broadly expressed and evolutionary conserved [6], the vast majority of them are primate-specific and represent cancer-specific vulnerabilities [5, 7]. The lack of conservation and consequently of appropriate animal models, has hampered the recognition of lncRNAs as key players in cancer. Patient-Derived Xenografts or PDXs, where a human tumour is implanted in an immunocompromised mouse, are increasingly employed as preclinical models [8], because of their ability to recapitulate the therapeutic responses and preserve the morphological and genetic heterogeneity of the original patients. They are therefore emerging as preferential tools to explore the role of primate-specific lncRNAs during cancer progression and therapy responses.

Although recent advances in targeted therapies and immune checkpoint blockade have improve overall survival, cancer remains the second leading cause of death worldwide. Therapy failure is mainly caused by intrinsic or acquired resistance to most anticancer drugs. While most of the work in the field has focused on somatic mutations, emerging evidence indicates that therapy exposure reprograms cancer cells into reversible drug-tolerant states [9, 10]. In fact, as all the evolutionary processes, also cancer progression strongly rely on plasticity to increase heterogeneity and face the environmental insults. Occurring in a contest of global transcriptional repression, reversible reprogramming often

involves the activation of adaptive responses through epigenetic and post-transcriptional events. Although these responses evolved to restore homeostasis following environmental stress, cancer cells hijack them to survive in unfavorable conditions [11]. LncRNA are participating to the regulation of all the epigenetic and post-transcriptional events in the cell and therefore they are key components of the adaptive response pathways [11, 12], as such they are emerging as modulators of therapy responses [13].

Being at the crossroad of several key metabolic processes, mitochondria are essential for the integration of different adaptive responses. Accumulating evidence suggest that they could play a major role in the development of resistance to targeted and immunotherapy [10, 13, 14]. Despite the fact that the metabolic profile of cancer cells varies across patients, tumour types and cells within a tumour, most (if not all) of the cell states within a tumour depend on mitochondria for their plasticity and survival [16]. This is not surprising considering that they are not only the major source of ATP in the cell, but also essential for membrane and nucleotide biosynthesis. Since the mitochondrial genome encodes only 13 subunits of the oxidative phosphorylation (OXPHOS) machinery, a fully functional OXPHOS chain requires, proteins translated both by the mitochondrial and the cellular machinery thus offering a unique opportunity to regulate cellular activities in response to environmental cues [12, 17]. The crosstalk between the cytosol and mitochondria during ribosome biogenesis and translational is essential for sensing and integrating different stress responses [12, 17]. Emerging evidence indicates that several classes of ncRNAs - including lncRNAs- impact indirectly and/or directly on mitochondrial biology [12, 17]. For instance, we have recently demonstrated that the lncRNA SAMMSON is a key guardian of proteostasis in melanoma cells, in which it rewires the compartmentalization of specific RNA binding proteins to coordinately increase translation in the mitochondria and cytosol (Figure 1) [17]. This proof-of-concept study has paved the way to the identification and characterization of other lncRNAs implicated in adaptive stress response and ultimately may lead to the development of novel cancer-specific therapeutic tools that will improve responses and overcome therapy resistance.

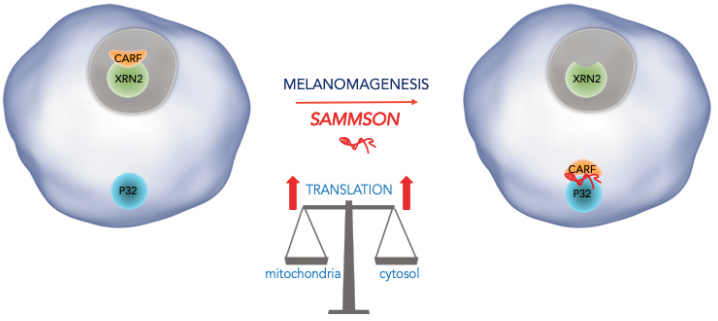


Figure 1 SAMMSON rewire the RNA binding network to promote translation and melanoma growth

References

1. The FANTOM Consortium. The Transcriptional Landscape of the Mammalian Genome. *Science* 309, 1559–1563 (2005).
2. Melton, C. Reuter JA[2], Spacek DV[2], Snyder M[2]. Recurrent somatic mutations in regulatory regions of human cancer genomes. Nat Genet. 2015 Jul;47(7):710-6. doi: 10.1038/ng.3332. Epub 2015 Jun 8.
3. Beroukhim, R. Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, Barretina J, Boehm JS, Dobson J, Urashima M, Mc Henry KT, Pinchback RM, Ligon AH, Cho YJ, Haery L, Greulich H, Reich M, Winckler W, Lawrence MS, Weir BA, Tanaka KE, Chiang DY, Bass AJ, Loo A, Hoffman C, Prensner J, Liefeld T, Gao Q, Yecies D, Signoretti S, Maher E, Kaye FJ, Sasaki H, Tepper JE, Fletcher JA, Tabernero J, Baselga J, Tsao MS, Demichelis F, Rubin MA, Janne PA, Daly MJ, Nucera C, Levine RL, Ebert BL, Gabriel S, Rustgi AK, Antonescu CR, Ladanyi M, Letai A, Garraway LA, Loda M, Beer DG, True LD, Okamoto A, Pomeroy SL, Singer S, Golub TR, Lander ES, Getz G, Sellers WR, Meyerson M. The landscape of somatic copy-number alteration across human cancers. Nature. 2010 Feb 18;463(7283):899-905. doi: 10.1038/nature08822.
4. Cheetham, S.W. Gruhl F, Mattick JS, Dinger ME. Long noncoding RNAs and the genetics of cancer. Br J Cancer. 2013 Jun 25;108(12):2419-25. doi: 10.1038/bjc.2013.233. Epub 2013 May 9.
5. Leucci, E. Cancer development and therapy resistance: spotlights on the dark side of the genome. *Pharmacol. Ther.* 189, 22–30 (2018).
6. Adriaens, C.*, Standaert, L.*, Barra J, Latil M, Verfaillie A, Kalev P, Boeckx B, Wijnhoven PW, Radaelli E, Vermi W, Leucci E, Lapouge G, Beck B, van den Oord J, Nakagawa S, Hirose T, Sablina AA, Lambrechts D, Aerts S, Blanpain C, Marine JC. p53 induces formation of NEAT1 lncRNA-containing paraspeckles that modulate replication stress response and chemosensitivity. Nat Med. 2016 Aug;22(8):861-8. doi: 10.1038/nm.4135. Epub 2016 Jul 4.
7. Leucci, E. Vendramin R, Spinazzi M, Laurette P, Fiers M, Wouters J, Radaelli E, Eyckerman S, Leonelli C, Vanderheyden K, Rogiers A, Hermans E, Baatsen P, Aerts S, Amant F, Van Aelst S, van den Oord J, de Strooper B, Davidson I, Lafontaine DL, Gevaert K, Vandesompele J, Mestdagh P, Marine JC. Melanoma addiction to the long non-coding RNA SAMMSON. *Nature* 531, 518–522 (2016).
8. Byrne A Alférez DG, Amant F, Annibali D, Arribas J, Biankin AV, Bruna A, Budinská E, Caldas C, Chang DK, Clarke RB, Clevers H, Coukos G, Dangles-Marie V, Eckhardt SG, Gonzalez-Suarez E, Hermans E, Hidalgo M, Jarzabek MA, de Jong S, Jonkers J, Kemper K, Lanfrancone L, Mælandsmo GM, Marangoni E, Marine JC, Medico E, Norum JH, Palmer HG, Peeper DS, Pelicci PG, Piris-Gimenez A, Roman-Roman S, Rueda OM,

Seoane J, Serra V, Soucek L, Vanhecke D, Villanueva A, Vinolo E, Bertotti A, Trusolino L.Interrogating open issues in cancer precision medicine with patient-derived xenografts. Nat Rev Cancer. 2017 Apr;17(4):254-268. doi: 10.1038/nrc.2016.140. Epub 2017 Jan 20. Review.

9. Rambow F*, Rogiers A*, Marin-Bejar O, Aibar S, Femel J, Dewaele M, Karras P, Brown D, Chang YH, Debiec-Rychter M, Adriaens C, Radaelli E, Wolter P, Bechter O, Dummer R, Levesque M, Piris A, Frederick DT, Boland G, Flaherty KT, van den Oord J, Voet T, Aerts S, Lund AW, Marine JC. Toward Minimal Residual Disease-Directed Therapy in Melanoma. Cell. 2018 Aug 9;174(4):843-855.e19. doi: 10.1016/j.cell.2018.06.025. Epub 2018 Jul 12.
10. Echeverria GV[1], Ge Z[1,2], Seth S[3,4,5], Zhang X[1], Jeter-Jones S[1], Zhou X[1], Cai S[1], Tu Y[1], McCoy A[1], Peoples M[4,5], Sun Y[4,5], Qiu H[6], Chang Q[4,5], Bristow C[4,5], Carugo A[4,5], Shao J[1], Ma X[4,5], Harris A[4,5], Mundi P[7], Lau R[8], Ramamoorthy V[4,5], Wu Y[8], Alvarez MJ[7,9], Califano A[7], Moulder SL[10], Symmans WF[8], Marszalek JR[4,5], Heffernan TP[4,5], Chang JT[2,6], Piwnica-WormsH[11]. Resistance to neoadjuvant chemotherapy in triple-negative breast cancer mediated by a reversible drug-tolerant state. Sci Transl Med. 2019 Apr 17;11(488). pii: eaav0936. doi: 10.1126/scitranslmed.aav0936.
11. Verheyden Y, Goedert L, Leucci E. Control of nucleolar stress and translational reprogramming by lncRNAs. Cell stress. 2018 Dec 5;3(1):19-26.
12. Vendramin R, Marine JC, Leucci E. Non-coding RNAs: the dark side of nuclear-mitochondrial communication. EMBO J. 2017 May 2;36(9):1123-1133. doi: 10.15252/embj.201695546. Epub 2017 Mar 17. Review.
13. Nath A, Lau EYT, Lee AM, Geeleher P, Cho WCS, Huang RS. Discovering long noncoding RNA predictors of anticancer drug sensitivity beyond protein-coding genes.Proc Natl Acad Sci U S A. 2019 Oct 29;116(44):22020-22029. doi: 10.1073/pnas.1909998116. Epub 2019 Sep 23.
14. Jerby-Arnon L, Shah P, Cuoco MS, Rodman C, Su MJ, Melms JC, Leeson R, Kanodia A, Mei S, Lin JR, Wang S, Rabasha B, Liu D, Zhang G, Margolais C, Ashenberg O, Ott PA, Buchbinder EI, Haq R, Hodi FS, Boland GM, Sullivan RJ, Frederick DT, Miao B, Moll T, Flaherty KT, Herlyn M, Jenkins RW, Thummalapalli R, Kowalczyk MS, Cañadas I, Schilling B, Cartwright ANR, Luoma AM, Malu S, Hwu P, Bernatchez C, Forget MA, Barbie DA, Shalek AK, Tirosh I, Sorger PK, Wucherpfennig K, Van Allen EM, Schadendorf D, Johnson BE, Rotem A, Rozenblatt-Rosen O, Garraway LA, Yoon CH, Izar B, Regev A. A Cancer Cell Program Promotes T Cell Exclusion and Resistance to Checkpoint Blockade. Cell. 2018 Nov 1;175(4):984-997.e24. doi: 10.1016/j.cell.2018.09.006.

15. Poźniak J, Nsengimana J, Laye JP, O'Shea SJ, Diaz JMS, Droop AP, Filia A, Harland M, Davies JR, Mell T, Randerson-Moor JA, Muralidhar S, Hogan SA, Freiberger SN, Levesque MP, Cook GP, Bishop DT, Newton-Bishop J. Genetic and Environmental Determinants of Immune Response to Cutaneous Melanoma. Cancer Res. 2019 May 15;79(10):2684-2696. doi: 10.1158/0008-5472.CAN-18-2864. Epub 2019 Feb 17.
16. Chen H[1], Chan DC[2]. Mitochondrial Dynamics in Regulating the Unique Phenotypes of Cancer and Stem Cells. Cell Metab. 2017 Jul 5;26(1):39-48. doi:10.1016/j.cmet.2017.05.016. Epub 2017 Jun 22.
17. Vendramin R, Verheyden Y, Ishikawa H, Goedert L, Nicolas E, Saraf K, Armaos A, Delli Ponti R, Izumikawa K, Mestdagh P, Lafontaine DLJ, Tartaglia GG, Takahashi N, Marine JC, Leucci E. SAMMSON fosters cancer cell fitness by enhancing concertedly mitochondrial and cytosolic translation. NSMB, 2018 Nov;25(11):1035-1046.

Eleonora Leucci, PhD

2003-2007	PhD in Medical Biotechnology, Dept. of Pathology, University of Siena
2008-2012	Postdoctoral Fellow, BRIC, University of Copenhagen
2012-2016	Postdoctoral Fellow, VIB-KU Leuven
2016-2017	Research Associate, ULB
2017-present	Assistant Professor and Head of Trace, KU Leuven, LKI

PI 3-KINASE AND CANCER METABOLISM

Lewis C. Cantley
Meyer Cancer Center, Weill Cornell Medicine
New York Presbyterian Hospital
Belfer Research Building, 413 East 69th Street, New York, NY 10065, USA
(lcantley@med.cornell.edu)

Phosphoinositide 3-Kinase isoform alpha (PI3Kα, encoded by the PIK3CA gene) is activated by insulin and other growth factors to mediate cell growth during development and regulate whole body glucose homeostasis both during development and in adults. Although multiple PI3K genes exist in humans and other mammals and the enzymes generated from these genes can respond to many types of growth factors and cytokines, the primordial PI3Kα enzymes in lower organisms (flies and worms) are in a single defined genetic pathway downstream of the insulin/IGF1 receptor. Consistent with PI3Kα evolving to mediate insulin/IGF1 signaling, these two growth factors are the most effective way to activate PI3Kα in most tissues and cancer cell lines that have been investigated.

The PIK3CA gene is one of the most frequently mutated oncogenes in human cancer, especially in endometrial, breast, head and neck and bladder cancers. Several of the hot spot mutations have been shown to enhance and/or prolong responses to insulin/IGF1 in cell lines and in mouse tissues. More than 20 PI3K inhibitors have entered clinical trials for treating various cancers and drugs targeting PI3Kδ that drives growth of B cells (encoded by PIK3CD) have been approved for B cell lymphomas. However, with the exception of alpelisib (Piqray), a PI3Kα inhibitor recently approved (in combination with the anti-estrogen receptor drug Fulvestrant) for PIK3CA mutant ER positive, HER2 negative breast cancer, most drugs targeting PI3Kα have failed in clinical trials due to toxicity or insufficient efficacy. The major toxicity observed for drugs targeting PI3Kα is an acute increase in blood glucose levels immediately following drug dosing. This is an on-target effect of turning off wild type PI3Kα in muscle and liver and a consequent release of glucose from these tissues into the blood. This elevation in blood glucose causes a dramatic

elevation in blood insulin levels, that ultimately return blood glucose levels back to normal over the subsequent 3 hours in most patience due to re-activating PI3Kα in liver and muscle, despite the continued presence of the PI3Kα inhibitor.

Many patients with PIK3CA mutations had no benefit in the alpelisib approval trial, and some patients without mutations showed durable responses in phase 2 studies with this drug. In addition, it has been difficult to identify mechanisms of resistance in the various clinical studies that have been published. In contrast to other targeted therapies such as EGFR mutant tumors or BCR-ABL mutant tumors or BRAF mutant tumors, where second mutations in the mutant allele provide resistance to the drugs, resistant mutations in PIK3CA in tumors that acquire resistance to PI3K inhibitors have been difficult to find. These results led us to speculate that variable responses to PI3Kα inhibitors amongst patients with similar mutations and relatively short periods of response might be due to variability in the patients' diets, or due to variability in insulin sensitivity and or variability in therapies used to manage hyperglycemia (several of which act by elevating serum insulin.

Using mouse models of a variety of cancers, we explored the possibility that the elevation in blood insulin following PI3Kα inhibitor therapy might reactivate the mutant PI3Kα in tumors and thereby compromise the effectiveness of these drugs. We found that the concentrations of insulin observed in human blood following PI3Kα inhibitor therapy was high enough to restore PI3K signaling in *ex vivo* studies of human organoids of several cancers, despite the presence of a therapeutic dose of various PI3Kα inhibitors. We explored several approaches to lower blood insulin levels during PI3Kα inhibitor therapy and found that the most effective approach was to place the mice on ketogenic diets (8% carbohydrate, 12% protein and 80% fat) that effectively blunted the elevation of glucose following drug administration. This approach not only prevented reactivation of PI3Kα in the tumors, but also caused dramatic tumor shrinkage in multiple mouse models of cancer, including tumors that lacked PIK3CA mutations.

We also explored the ability of sodium-glucose co-transporter type 2 (SGLT2) inhibitors to lower serum glucose and serum insulin during PI3Kalpha inhibitor therapy. These drugs have been approved for treating insulin resistance and type 2 diabetes and do so by blocking glucose reabsorption into the blood following ultrafiltration in the kidney. We found that these inhibitors were more effective than the anti-diabetes drug metformin in maintaining low glucose and insulin during PI3Kalpha inhibitory therapy and made these drugs more effective in shrinking tumors in mouse models, though they were not as effective as the ketogenic diet.

On the basis of these pre-clinical studies, we are currently collaborating with multiple pharmaceutical companies to execute clinical trials in which PI3Kα inhibitors are combined

with either a ketogenic diet or with an SGLT2 inhibitor to provide more effective and durable responses in patients with PIK3CA mutations. As a minimum we expect that more patients will be able to remain on therapy since hyperglycemia (an on-target toxicity) is the major toxicity observed with PI3Kα inhibitors.

Over the past several decades it has become apparent that increased consumption of sugars, especially in the form of sugary drinks, are driving increases in obesity and consequent increases in insulin resistance and type 2 diabetes. Importantly this increase in sugar consumption also correlates with increases in subsets of cancers, especially endometrial cancer, colorectal cancers and some types of breast cancers. A variety of explanations for this correlation have been proposed, including increased inflammation in the affected tissue, changes in the microbiome and elevations in insulin and IGF1. The studies described above suggest that elevations in insulin and IGF1 in insulin-resistant patients are likely to be causative of some of these cancers due to the ability of these growth factors to stimulate PI3Kα.

We explored the possibility that consumption of sugary drinks could explain the increase in colorectal cancers in young adults that have been observed in the last few decades. We explored this correlation in mice genetically engineered to delete the APC gene in distal small intestine or in the colon. In both models we found that a relatively small bolus of high fructose corn syrup (60/40 fructose/glucose mixture) delivered orally in water (proportionally comparable to a single 12 ounce sugary drink per day in humans) was enough to double the size of polyps and dramatically increase the fraction of invasive polyps in both of the genetic models. This quantity of sugary drink did not cause an increase in weight or induce insulin resistance (though ad lib feeding on high fructose corn syrup in water caused a major increase in obesity and insulin resistance and polyp growth). This result showed that drinking moderate amounts of high fructose corn syrup drinks can cause increased polyp growth independent of obesity or increased serum insulin. Interestingly, the polyps required both sugars simultaneously for accelerated growth. Doubling the quantity of either sugar alone did not accelerate polyp growth compared to a 60/40 mixture of fructose + glucose or a 50/50 mixture of the two monosaccharides.

We utilized isotope tracing with ^{14}C-labeled fructose, ^{13}C-labeled fructose, ^{14}C-labeled glucose, or ^{13}C-labeled glucose to determine how the polyps metabolized these two sugars. Surprisingly, flux of glucose carbons into fatty acid production in the polyps was dramatically accelerated when fructose was present. In contrast, very little fructose carbon was incorporate into fatty acids in the polyps. The fructose was converted to fructose-1-phosphate by ketohexokinase (fructokinase) and this species accumulated to very high levels and correlated with a sharp drop in cellular ATP. The drop in ATP allowed PFK1 (known to be inhibited by cytosolic ATP) to become more active, explaining the

acceleration of glucose flux through glycolysis. Finally, we showed that deleting the ketohexokinase gene in mice eliminated the ability of high fructose corn syrup to drive polyp growth. This result shows that drinking even modest amounts of drinks containing a combination of glucose and fructose (sodas or fruit juice) can drive polyp growth in patients independent of obesity or insulin resistance.

In summary, our studies argue that limiting consumption of sugars is likely to be beneficial in both the prevention and treatment of cancers.

References and Background Reading

1. Phase II trial of AKT inhibitor MK-2206 in patients with advanced breast cancer who have tumors with PIK3CA or AKT mutations, and/or PTEN loss/PTEN mutation. Xing Y, Lin NU, Maurer MA, Chen H, Mahvash A, Sahin A, Akcakanat A, Li Y, Abramson V, Litton J, Chavez-MacGregor M, Valero V, Piha-Paul SA, Hong D, Do KA, Tarco E, Riall D, Eterovic AK, Wulf GM, Cantley LC, Mills GB, Doyle LA, Winer E, Hortobagyi GN, Gonzalez-Angulo AM, Meric-Bernstam F. Breast Cancer Res. 2019 Jul 5;21(1):78. doi: 10.1186/s13058-019-1154-8. PMID: 31277699
2. High-fructose corn syrup enhances intestinal tumor growth in mice. Goncalves MD, Lu C, Tutnauer J, Hartman TE, Hwang SK, Murphy CJ, Pauli C, Morris R, Taylor S, Bosch K, Yang S, Wang Y, Van Riper J, Lekaye HC, Roper J, Kim Y, Chen Q, Gross SS, Rhee KY, Cantley LC, Yun J. Science. 2019 Mar 22;363(6433):1345-1349. doi: 10.1126/science.aat8515. PMID: 30898933
3. Treating cancer with phosphatidylinositol-3-kinase inhibitors: increasing efficacy and overcoming resistance. Paddock MN, Field SJ, Cantley LC. J Lipid Res. 2019 Apr;60(4):747-752. doi: 10.1194/jlr.S092130. Epub 2019 Feb 4. PMID: 30718284
4. Phosphatidylinositol 3-Kinase, Growth Disorders, and Cancer. Goncalves MD, Hopkins BD, Cantley LC. N Engl J Med. 2018 Nov 22;379(21):2052-2062. doi: 10.1056/NEJMra1704560. Review. No abstract available. PMID: 30462943
5. Suppression of insulin feedback enhances the efficacy of PI3K inhibitors. Hopkins BD, Pauli C, Du X, Wang DG, Li X, Wu D, Amadiume SC, Goncalves MD, Hodakoski C, Lundquist MR, Bareja R, Ma Y, Harris EM, Sboner A, Beltran H, Rubin MA, Mukherjee S, Cantley LC. Nature. 2018 Aug;560(7719):499-503. doi: 10.1038/s41586-018-0343-4. Epub 2018 Jul 4. Erratum in: Nature. 2018 Nov;563(7731):E24. doi: 10.1038/s41586-018-0506-3. PMID: 30051890
6. A Phase Ib Study of Alpelisib (BYL719), a PI3Kα-Specific Inhibitor, with Letrozole in ER+/HER2- Metastatic Breast Cancer. Mayer IA, Abramson VG, Formisano L, Balko JM, Estrada MV, Sanders ME, Juric D, Solit D, Berger MF, Won HH, Li Y, Cantley LC,

Winer E, Arteaga CL. Clin Cancer Res. 2017 Jan 1;23(1):26-34. doi: 10.1158/1078-0432.CCR-16-0134. Epub 2016 Apr 28. PMID: 27126994

7. The PI3K Pathway in Human Disease. Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. Cell. 2017 Aug 10;170(4):605-635. doi: 10.1016/j.cell.2017.07.029. Review. PMID: 28802037
8. Phosphorylation of TXNIP by AKT Mediates Acute Influx of Glucose in Response to Insulin. Waldhart AN, Dykstra H, Peck AS, Boguslawski EA, Madaj ZB, Wen J, Veldkamp K, Hollowell M, Zheng B, Cantley LC, McGraw TE, Wu N. Cell Rep. 2017 Jun 6;19(10):2005-2013. doi: 10.1016/j.celrep.2017.05.041. PMID: 28591573

Lewis C. Cantley, PhD

1978-1981	Assistant Professor, Dept. of Biochemistry and Molecular Biology, Harvard Univ.
1981-1985	Associate Professor, Dept. of Biochemistry and Molecular Biology, Harvard Univ.
1985-1992	Professor of Physiology, Tufts University School of Medicine
1992-2003	Professor of Cell Biology, Harvard Medical School
1992-2012	Chief, Division of Signal Transduction, Beth Israel Deaconess Medical Center
2003-2012	Professor of Systems Biology, Harvard Medical School
2007-2012	Director, Beth Israel Deaconess Cancer Center
2012-present	Professor of Medicine, Weill Cornell Medical College
2012-present	Director, Sandra and Edward Meyer Cancer Center at Weill Cornell Medical College/ Ronald P. Stanton Clinical Cancer Program at NewYork-Presbyterian

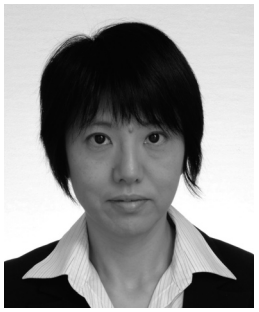
THE MECHANISMS OF OBESITY-ASSOCIATED LIVER CANCER PROGRESSION

Naoko Ohtani
Department of Pathophysiology
Graduate School of Medicine, Osaka City University
1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
(ohtani.naoko@med.osaka-cu.ac.jp)

Recently, emerging evidences revealed that obesity is associated with the development of several types of cancer including hepatocellular carcinoma (HCC). However, the precise mechanisms underlying obesity-associated cancer have remained unclear. We recently reported that the dietary obesity accelerated the development of HCC in mice treated with DMBA, a chemical carcinogen, at neonatal stage, whereas no tumors were developed in mice fed with normal diet. Interestingly, the hepatic stellate cells (HSCs) in tumor microenvironment exhibited signs of cellular senescence and senescence-associated secretory phenotype (SASP), a phenotype that senescent cells secrete a series of proinflammatory cytokines, chemokines or matrix-remodeling factors which could contribute to chronic inflammation and tumorigenesis. We found that mice lacking IL-1β, an upstream regulator of cytokine cascade, showed significantly reduced number of HCC development and the suppression of SASP factors expression in senescent hepatic stellate cells in tumor area, suggesting that IL-1β plays an important role in SASP-associated inflammation and HCC development. Moreover, the enterohepatic circulation of obesity-associated gut microbial metabolite, deoxycholic acid (DCA), provokes DNA damage and cellular senescence and SASP in hepatic stellate cells, thereby creating HCC promoting microenvironment. In this symposium, I have shown that the reciprocal activation of two SASP factors, and a novel network between Treg cells and SASP factor producing senescent hepatic stellate cells in suppressing anti-tumor immunity in liver tumor microenvironment.

References

1. Takahashi A, Loo TM, Okada R, Kamachi F, Watanabe Y, Wakita M, Watanabe S, Kawamoto S, Miyata K, Barber GN, Ohtani N, Hara E. Downregulation of cytoplasmic DNases is implicated in cytoplasmic DNA accumulation and SASP in senescent cells. *Nat Commun*. 2018; 9:1249. doi: 10.1038/s41467-018-03555-8
2. Loo TM, Kamachi F, Watanabe Y, Yoshimoto S, Kanda H, Arai Y, Nakajima-Takagi Y, Iwama A, Koga T, Sugimoto Y, Ozawa T, Nakamura M, Kumagai M, Watashi K, Taketo MM , Aoki T, Narumiya S, Oshima M, Arita M, Hara E and Ohtani N. Gut microbiota promotes obesity-associated liver cancer through PGE2-mediated suppression of anti-tumor immunity. *Cancer Discovery* 7, 522-538, 2017 doi: 10.1158/2159-8290.CD-16-0932
3. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, Iwakura Y, Oshima K, Morita H, Hattori M, Honda K, Ishikawa Y, Hara E, Ohtani N. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499, 97-101 (2013)



Naoko Ohtani, MD, PhD

1995-1998	Assistant Professor, Kyoto Prefectural University of Medicine
1998-2003	Postdoctoral Fellow, University of Manchester, Paterson Institute for Cancer Research
2003-2007	Lecturer and Associate Professor, Institute for Genome Research, University of Tokushima
2008-2014	Senior Staff Scientist, Cancer Institute, Japanese Foundation for Cancer Research
2014-2017	Professor, Dept. Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science
2017-present	Professor, Department of Pathophysiology, Graduate School of Medicine, Osaka City University

METABOLIC PHENOTYPES AND CANCER PROGRESSION

Ralph J. DeBerardinis
Children's Medical Center Research Institute (CRI)
University of Texas Southwestern Medical Center
5323 Harry Hines Boulevard, Dallas, TX 75390-8502, USA
(ralph.deberardinis@utsouthwestern.edu)

Metabolic reprogramming – the regulated alteration of metabolic activities in cancer cells – is viewed as an essential component of malignancy and a potential source of therapeutic targets. Classical cancer metabolism studies have focused for the most part on cell-autonomous aspects of reprogramming, often as a consequence of oncogenic mutations, that confer enhanced cell survival and cell growth in the treatment-naïve state. However, tumor metabolism responds to a combination of factors either intrinsic (e.g. the oncogenotype) or extrinsic (e.g. the tumor microenvironment) to malignant cells, and these factors culminate in marked metabolic heterogeneity of primary tumors within the tissue of origin (1). Emerging evidence also suggests that metabolic preferences and liabilities evolve throughout the course of cancer progression. A broad understanding of cancer metabolism will therefore require approaches to understand how metabolic phenotypes are established in disease-relevant environments, and how these phenotypes relate to therapeutic opportunities.

To develop a clinically-relevant understanding of tumor metabolism, we developed methods to assess metabolic phenotypes in primary human cancers using intra-operative infusions with stable isotope-labeled nutrients during surgical resection of solid tumors (Figure 1). During the infusion, the labeled nutrient is taken up by the tumor and adjacent tissue, and the isotope label is distributed to numerous intracellular metabolites as a consequence of metabolic activity within the tissue. This allows pathway utilization to be compared between tumors and adjacent, nonmalignant tissue; between fragments acquired from different tumors; and among regions with different biological properties within the same tumor. The method is well-tolerated and sufficiently versatile to apply to many different types of human cancer and several different metabolic tracers.

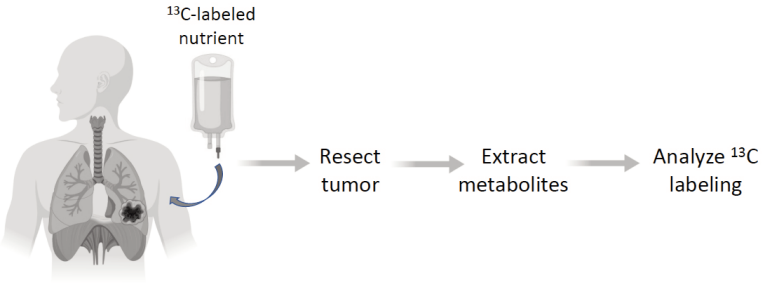


Figure 1 Clinical cancer metabolism studies with intra-operative ^{13}C infusions

Our work to this point demonstrates extensive metabolic heterogeneity among tumors arising at different anatomic sites, and suggests that heterogeneity arises from a combination of factors extrinsic and intrinsic to malignant cells. For example, tumors in the brain and lung avidly oxidize fuels like glucose imported from the circulation (2-4). In contrast, clear cell renal cell carcinomas (ccRCCs) exhibit glycolytic metabolism with reduced glucose oxidation, similar to the classical description of aerobic glycolysis first postulated by Warburg in the 1920s (Figure 2). Glycolytic metabolism in ccRCCs is likely related to the loss of the *Von Hippel Lindau* (*VHL*) tumor suppressor in these tumors, imposing a chronic pseudohypoxic state associated with elevated glycolysis and suppressed oxidative metabolism in preclinical models (5). Thus, this isotope infusion approach is sensitive enough to detect heterogeneous metabolic preferences among different human tumors in vivo. Data from the cancers analyzed so far suggest that a systematic, in vivo assessment of metabolic phenotypes may help predict which tumors are most likely to respond to therapies aimed against particular pathways.

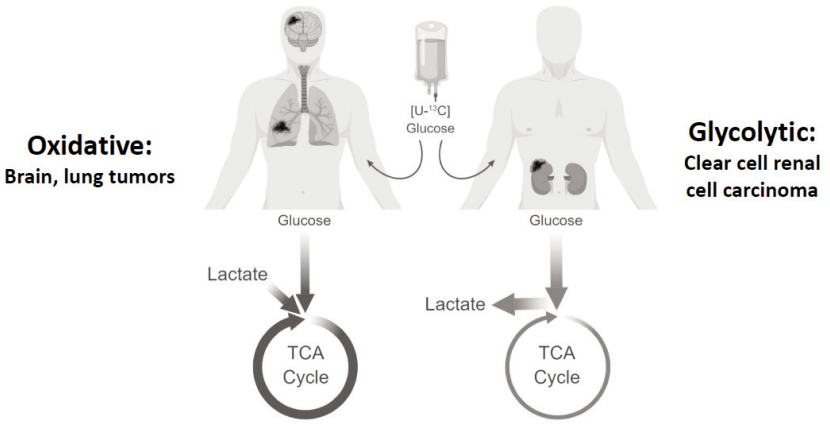


Figure 2 Metabolic heterogeneity of human tumors growing at different sites

One surprising finding arising from analysis of human non-small cell lung cancer is that a subset of these tumors take up lactate from the circulation and use it as a carbon source for oxidative metabolism (Figure 3) (Faubert et al., 2017). Patients whose tumors had evidence of lactate import tended to undergo clinical progression (generally to metastatic cancer) more rapidly than patients whose tumors lacked this metabolic property. This was striking because the isotope infusions were performed months-to-years earlier than the clinical appearance of metastatic disease. I will discuss efforts to reverse translate this finding into mouse models to test whether the ability to import lactate supports metastatic efficiency.

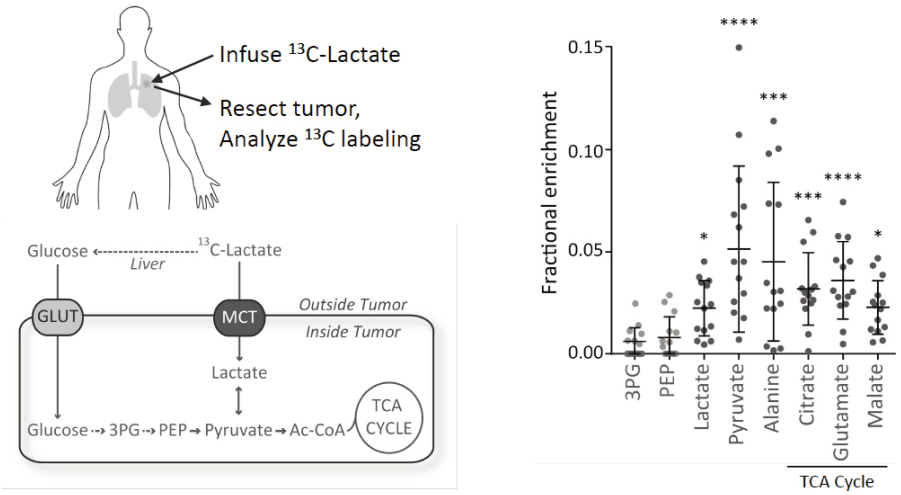


Figure 3 Evidence for lactate oxidation by human non-small cell lung cancers

References

1. Kim, J., and DeBerardinis, R.J. Mechanisms and Implications of Metabolic Heterogeneity in Cancer. Cell Metabolism 2019; 30:434-446.
2. Hensley, C.T., Faubert, B., Yuan, Q., Lev-Cohain, N., Jin, E., Kim, J., Jiang, L., Ko, B., Skelton, R., Loudat, L., Wodzak, M., Klimko, C., McMillan, E., Butt, Y., Ni, M., Oliver, D., Torrealba, J., Malloy, C.R., Kernstine, K., Lenkinski, R.E. and DeBerardinis, R.J. Metabolic Heterogeneity in Human Lung Tumors. Cell 2016; 164:681-694.
3. Maher, E.A., Marin-Valencia, I., Bachoo, R.M., Mashimo, T., Raisanen, J., Hatanpaa, K.J., Jindal, A., Jeffrey, F.M., Choi, C., Madden, C., Mathews, D., Pascual, J.M., Mickey, B.E., Malloy, C.R. and DeBerardinis, R.J. Metabolism of [U-13 C]glucose in human brain tumors in vivo. NMR Biomed 2012; 25:1234-1244.

4. Mashimo, T., Pichumani, K., Vemireddy, V., Hatanpaa, K.J., Singh, D.K., Sirasanagandla, S., Nannepaga, S., Piccirillo, S.G., Kovacs, Z., Foong, C., Huang, Z., Barnett, S., Mickey, B.E., DeBerardinis, R.J., Tu, B.P., Maher, E.A. and Bachoo, R.M.. Acetate is a bioenergetic substrate for human glioblastoma and brain metastases. Cell 2014:159;1603-1614.
5. Courtney, K.D., Bezwada, D., Mashimo, T., Pichumani, K., Vemireddy, V., Funk, A.M., Wimberly, J., McNeil, S.S., Kapur, P., Lotan, Y., Margulis, V., Caddedu, J.A., Pedrosa, I., DeBerardinis, R.J., Malloy, C.R., Bachoo, R.M. and Maher, E.A. Isotope Tracing of Human Clear Cell Renal Cell Carcinomas Demonstrates Suppressed Glucose Oxidation In Vivo. Cell Metabolism 2018; 28:793-800 e792.
6. Faubert, B., Li, K.Y., Cai, L., Hensley, C.T., Kim, J., Zacharias, L.G., Yang, C., Do, Q.N., Doucette, S., Burguete, D., Li, H., Huet, G., Yuan, Q., Wigal, T., Butt, Y., Ni, M., Torrealba, J., Oliver, D., Lenkinski, R.E., Malloy, C.R., Wachsmann, J.W., Young, J.D., Kernstine, K. and DeBerardinis, R.J. Lactate Metabolism in Human Lung Tumors. Cell 2017; 171:358-371 e359.

Ralph J. DeBerardinis, MD, PhD

2008-2013	Assistant Professor, Department of Pediatrics, UT Southwestern
2013-2017	Associate Professor, CRI, UT Southwestern
2013-present	Director, Genetic and Metabolic Disease Program and Chief, Pediatric Genetics & Metabolism, UT Southwestern
2017-present	Robert L. Moody, Sr. Faculty Scholar
2017-present	Professor, CRI, UT Southwestern
2018-present	Investigator, Howard Hughes Medical Institute

MECHANISMS OF DRUG RESISTANCE IN LUNG CANCER

Katerina Politi
Departments of Pathology and Medicine (Section of Medical Oncology)
Yale Cancer Center
Yale University School of Medicine
333 Cedar Street, SHM-I 234D, New Haven, CT 06510, USA
(katerina.politi@yale.edu)

Targeted therapies have transformed the landscape for the diagnosis and treatment of metastatic lung cancer. These tumors are now routinely tested for the presence of mutations or rearrangements in specific oncogenic drivers that, if present, predict sensitivity to targeted therapies directed to the genomic alterations present. Genotype-directed therapies have improved outcomes in specific subsets of patients with metastatic lung cancer. Despite this success, targeted therapies are not curative and acquired resistance is a major impediment to cures for patients treated with these therapies. Moreover, there is heterogeneity in the durability and depth of responses between patients and at different metastatic sites within individual patients.

A paradigm for the success of targeted therapies in lung cancer, comes from *E*pidermal *G*rowth *F*actor *R*eceptor (*EGFR)* mutant lung cancer. Mutations in exons encoding the tyrosine kinase domain of EGFR confer sensitivity to tyrosine kinase inhibitors (TKIs) [1-3] and several TKIs (e.g. erlotinib, gefitinib, afatinib and, most recently, osimertinib) are currently approved for the first-line treatment of *EGFR* mutant lung cancer. Acquired drug resistance, however, is a major challenge with all of these TKIs and especially for osimertinib we have very limited knowledge of the mechanisms of resistance given its recent adoption in the clinic (Figure 1). Without knowledge about resistance mechanisms, optimal post-osimertinib treatment strategies remain to be defined.

Several years ago, we developed genetically engineered mouse models of EGFR mutant lung cancer (Figure 2) [4]. In these models, we showed that tumors are dependent on the EGFR oncogene for their survival and that they regress upon TKI treatment. Moreover, long-term treatment of mice with EGFR-directed therapies led to the development of drug

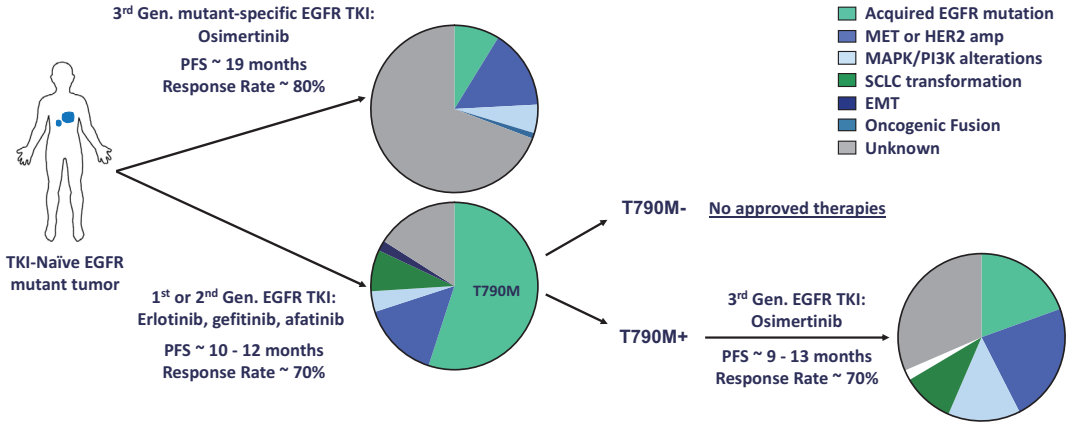


Figure 1 Treatment Paradigm for *EGFR* Mutant Lung Cancer

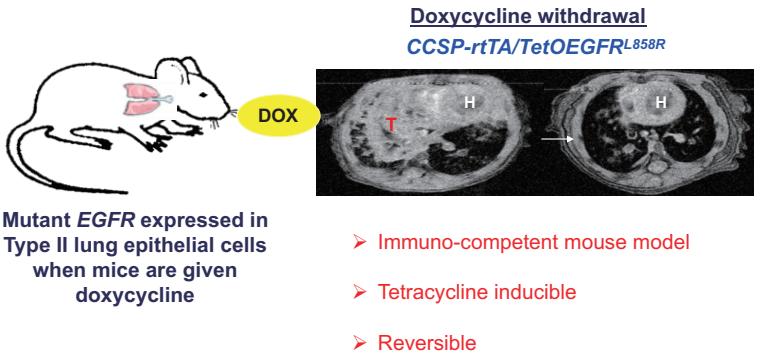


Figure 2 EGFR Mutant Tumors Depend on the Driver Oncogene

resistance mostly due to the emergence of the $EGFR^{T790M}$ mutation [5]. Since then we have used these models to evaluate therapeutic strategies to overcome TKI resistance and identify mechanisms of resistance to these new therapies [6-9]. Given that osimertinib is now emerging as the preferred option for the first-line treatment of the disease, we used these mice to model acquired resistance to first-line osimertinib treatment in transgenic mouse models of $EGFR^{L858R}$-induced lung adenocarcinoma. In these studies we found that it is mediated largely through secondary mutations in *EGFR*-either C797S or L718V/Q or *Kras* mutations. Therapeutic testing in mice revealed that both erlotinib and afatinib caused regression of osimertinib-resistant C797S-containing tumors, whereas only afatinib was effective in L718Q mutant tumors. Further, combination first-line osimertinib plus erlotinib treatment prevented the emergence of secondary mutations in *EGFR*.

These results highlight how these models can be valuable to identify mutational on-target mechanisms of resistance to TKIs *in vivo* that recapitulate the human disease. Moreover, they can be used to test therapeutic strategies to target these *EGFR* resistance mutations. However, in these models, the spectrum of resistance mechanisms observed, is limited whilst the spectrum of resistance mechanisms in patients is broader and the molecular properties of these resistance mechanisms are poorly understood. In patients, the depth and duration of response is also more variable than in the transgenic mouse models. It is increasingly becoming apparent that tumors without on-target EGFR resistance mutations have worst outcomes [10] therefore understanding the biology that underlies this variability is critically important. We hypothesize that the presence of co-occurring genetic alterations contributes to a more complex tumor phenotype and spectrum of resistance mechanisms *in vivo*. To test this, we have developed new mouse models to specifically evaluate the consequences of co-occurring tumor suppressor gene alterations on the progression and TKI sensitivity of *EGFR* mutant tumors. In the new models, viral delivery of *cre* recombinase leads to the expression of mutant EGFR in the lung epithelium and concomitant deletion of the *Trp53* tumor suppressor gene. *TP53* is mutated or lost in over 50% of advanced EGFR mutant lung adenocarcinomas. As predicted, in the *EGFR; p53* model, tumors develop that are more aggressive than in the p53 WT model. To further include layer on additional alterations, we generated mice that carry a conditional *Cas9* allele. This system allows us to deliver virus carrying both *cre* recombinase and an sgRNA targeting a gene of interest. Using this new model, we tested a pool of lentiviruses targeting 10 commonly mutated tumor suppressor genes in lung adenocarcinoma. We found that the effects of tumor suppressor gene inactivation fall into three different categories: 1) promoting tumor growth, 2) no effect on tumor growth or 3) a detrimental effect on tumor growth.

Collectively, our findings highlight how genetically engineered mouse models of lung cancer, including those with complex genotypes, can be leveraged to study tumor progression and drug resistance *in vivo*.

References

1. Lynch, T.J., et al., *Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib.* N Engl J Med, 2004. 350(21): p. 2129-39.
2. Paez, J.G., et al., *EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy.* Science, 2004. 304(5676): p. 1497-500.

3. Pao, W., et al., *EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib.* Proc Natl Acad Sci U S A, 2004. 101(36): p. 13306-11.
4. Politi, K., et al., *Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors.* Genes Dev, 2006. 20(11): p. 1496-510.
5. Politi, K., et al., *Erlotinib resistance in mouse models of epidermal growth factor receptor-induced lung adenocarcinoma.* Dis Model Mech, 2010. 3(1-2): p. 111-9.
6. Pirazzoli, V., et al., *Afatinib plus Cetuximab Delays Resistance Compared to Single-Agent Erlotinib or Afatinib in Mouse Models of TKI-Naive EGFR L858R-Induced Lung Adenocarcinoma.* Clin Cancer Res, 2016. 22(2): p. 426-35.
7. Pirazzoli, V., et al., *Acquired Resistance of EGFR-Mutant Lung Adenocarcinomas to Afatinib plus Cetuximab Is Associated with Activation of mTORC1.* Cell Rep, 2014. 7(4): p. 999-1008.
8. Pirazzoli, V. and K. Politi, *Generation of drug-resistant tumors using intermittent dosing of tyrosine kinase inhibitors in mouse.* Cold Spring Harb Protoc, 2014. 2014(2): p. 178-81.
9. Takezawa, K., et al., *HER2 amplification: a potential mechanism of acquired resistance to EGFR inhibition in EGFR-mutant lung cancers that lack the second-site EGFRT790M mutation.* Cancer Discov, 2012. 2(10): p. 922-33.
10. Oxnard, G.R., et al., *Assessment of Resistance Mechanisms and Clinical Implications in Patients With EGFR T790M-Positive Lung Cancer and Acquired Resistance to Osimertinib.* JAMA Oncol, 2018. 4(11): p. 1527-1534.

Katerina Politi, PhD

2003	PhD in Genetics and Development, Columbia University
2003-2010	Postdoctoral Researcher, Memorial Sloan Kettering Cancer Center
2010-present	Assistant /Associate (2016-) Professor, Department of Pathology, Yale School of Medicine
2010-present	Member, Research Program Leader (2019-), Yale Cancer Center

BIOLOGICAL MECHANISM OF POLYCLONAL METASTASIS OF COLORECTAL CANCER

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

Wnt signaling, TGF-β pathway, KRAS and p53 functions are frequently dysregulated in human colorectal cancer (CRCs) caused by the driver gene mutations, which induces development and malignant progression of CRCs. However, mutation patterns vary in individual patients, and the combinations of mutations that are responsible for the promotion of each step of malignant progression, including invasion, intravasation and metastasis, have not been fully understood. To understand biological mechanisms of CRC development *in vivo*, we constructed mouse models that carried multiple driver mutations in various combination by crossing GEM models of $Apc^{\Delta 716}$ (A), $Kras^{G12D}$ (K), $Tgfbr2^{-/-}$ (T), $Trp53^{R270H}$ (P) or $Fbxw7^{-/-}$ (F) mutant mice. All mutant mice developed intestinal tumors because of germline mutation of *Apc* gene (Figure 1A).

The initial histologically identified malignant phenotype is submucosal invasion. We found that mice that carried AT or AP mutations in combination in their genotypes showed submucosal invasion of intestinal tumors, indicating that either suppression of the TGF-β pathway or expression of gain-of-function (GOF) mutant p53 plays a role in submucosal invasion (Figure 1B) (1, 2). Moreover, additional $Kras^{G12D}$ activation mutation (*i.e.*, AKT or AKP combination) accelerates the EMT-like morphology and intravasation into microcapillary vessels, although AK mutation only causes mucosal benign adenoma development (Figure 1C) (3). These results indicate that Kras activation is responsible for the important malignant phenotypes when combined with mutations in the TGF-β pathway or p53.

We next established tumor-derived organoids from mice with all possible mutant combinations and transplanted them to the mouse spleen to examine liver metastasis.

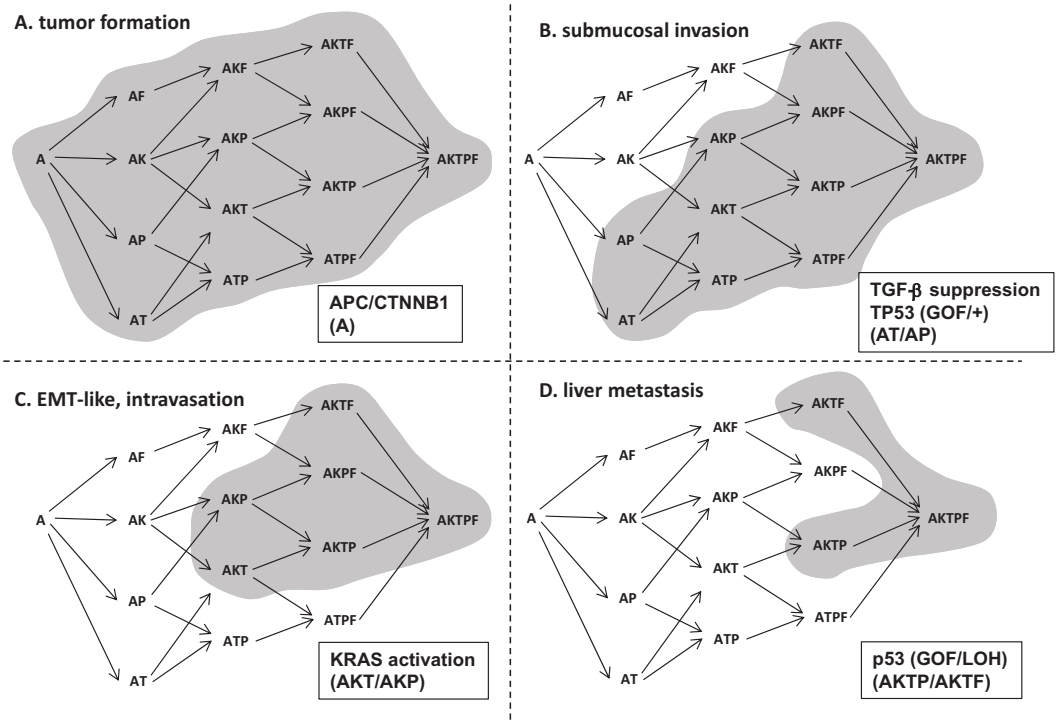


Figure 1 Genotype-phenotype relations of intestinal tumors developed in mouse models. A, K, T, P, and F indicate genetic mutations for *Apc*, *Kras*, *Tgfbr2*, *Trp53* and *Fbxw7* in mouse models. Genotypes with gray color indicate mice that showed tumor/malignant phenotypes. A. All genotype mice carrying *Apc* mutation (A) developed intestinal tumors. B. TGF-β suppression or p53 GOF mutation (AT or AP) induced submucosal invasion. C. Additional Kras mutation (AKT or AKP) caused EMT-like morphology and intravasation. D. Combination of AKTP with loss of wild-type p53 (LOH) or AKTF mutation induced liver metastasis with high multiplicity.

Similar to the primary tumors, tumor organoids with the combination of AKT or AKP in their genotypes formed liver metastasis (3). Moreover, organoids with additional mutations of AKTP, AKTF and AKTPF showed increased multiplicity of liver metastasis with advanced malignant histology (Figure 1D). Using these organoid cells, we have recently developed CRISPR/Cas9 screening system to identify novel driver genes (4). We further identified that AKTP cells with loss of wild-type p53 gene by LOH were enriched in liver metastatic lesions (Nakayama et al., in revision). These results indicate a genotype-phenotype relationship in the primary intestinal tumors (Figure 1A-D).

We next examined metastasis mechanisms using established organoid systems. Recently, it has been shown that tumor cell clusters detached from the primary sites can metastasize to the distant organ more efficiently when compared with single cells. To

examine a polyclonal metastasis mechanism *in vivo*, we differentially labeled AKTP organoids and non-metastatic tumor-derived organoids with GFP and TdTomato, respectively. When adenoma-derived organoid cells were injected to the spleen together with metastatic AKTP cells, only AKTP cells formed metastatic foci in the liver. However, to our surprise, adenocarcinoma-derived non-metastatic cells formed chimeric metastatic foci when co-injected with AKTP cells (Figure 2). These results indicate that non-metastatic cells can metastasize by polyclonal mechanism if they form polyclonal cell clusters with metastatic cells by cooperative invasion.

We finally examined whether AKTP cells are continuously required for survival and proliferation of non-metastatic cells in the liver lesions by depletion of AKTP cells from the metastatic lesions by the DT-DTR system. Importantly, non-metastatic cells continued proliferation and formed large tumors even after depletion of metastatic cells (Figure 2). These results indicate that metastasis process cannot be explained not only by a concept of genetic alterations and clonal selections. Further study to understand a mechanistic insight of polyclonal metastasis is required for establishment of novel clinical strategy against metastasis.

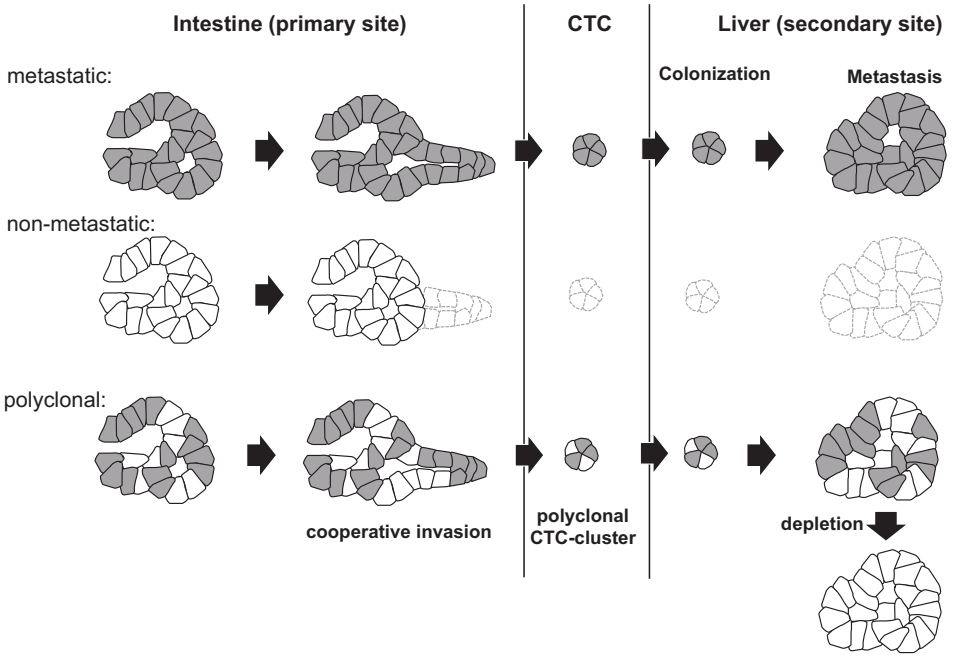


Figure 2 Schematic drawing of metastasis of intestinal tumors from the primary site (left) to the liver (right) via circulating tumor cells (CTC). When metastatic cells (gray, top) and non-metastatic cells (white, middle) are co-localized in the primary tumors (mixed, bottom), cell clusters containing both cell types can circulate as CTC-cluster, disseminate to the liver and form polyclonal metastatic foci. Once polyclonal metastasis lesions are developed, non-metastatic cells no longer require support of metastatic cells.

References

1. Oshima H, Nakayama M, Han TS, Naoi K, Ju X, Maeda Y, Robine S, Tsuchiya K, Sato T, Taketo MM, Oshima M. Suppressing TGF-β signaling in regenerating epithelia in an inflammatory microenvironment is sufficient to cause invasive intestinal cancer. Cancer Res. 2015;75: 754-765.
2. Nakayama M, Sakai E, Echizen K, Yamada Y, Oshima H, Han TS, Ohki R, Fujii F, Ochiai A, Robine S, Voon DC, Tanaka T, Taketo MM, Oshima M. Intestinal cancer progression by mutant p53 through the acquisition of invasiveness associated with complex glandular formation. Oncogene. 2017;36:5885-5896.
3. Sakai E, Nakayama M, Oshima H, Kouyama Y, Niida A, Fujii S, Ochiai A, Nakayama KI, Mimori K, Suzuki Y, Hong CP, Ock CY, Kim SJ, Oshima M. Combined mutation of *Apc*, *Kras* and *Tgfbr2* effectively drives metastasis of intestinal cancer. Cancer Res. 2018;78:1334-1346.
4. Takeda H, Kataoka S, Nakayama M, Ali MAE, Oshima H, Yamamoto D, Park JW, Takegami Y, An T, Jenkins NA, Copeland NG, Oshima M. CRISPR-Cas9 mediated gene knockout in intestinal tumor organoids provides functional validation for colorectal cancer driver genes. Proc Natl Acad Sci USA. 2019;116:15635-15644.

Masanobu Oshima, DVM, PhD

1986-1988	Graduate School of Veterinary Medicine (MS), Hokkaido Univ.
1988-1991	Chugai Pharmaceutical Co., Ltd.
1992-1997	Banyu Pharmaceutical, Co., Ltd.
1997-1999	Research Associate, Merck Research Laboratories (Merck)
2000-2005	Associate Professor, Kyoto University Graduate School of Medicine
2005-present	Professor, Cancer Research Institute, Kanazawa University
2013-2017	Director, Cancer Research Institute, Kanazawa University

SINGLE CELL ANALYSIS OF DIFFERENTIATION AND DRUG RESPONSE IN GLIOBLASTOMA

Peter A. Sims
Department of Systems Biology
Columbia University Irving Medical Center
3960 Broadway, Lasker 203AC, New York, NY 10032, USA
(pas2182@cumc.columbia.edu)

Glioblastoma (GBM) is a deadly form of malignant brain tumor against which conventional targeted therapies and immunotherapies have had limited success. Both intratumoral heterogeneity and the complexities of drug delivery in the brain are formidable challenges in neuro-oncology. Furthermore, patient-derived models of GBM often bear little resemblance to the corresponding primary tumor tissue and do not recapitulate the microenvironment. Recent technological advances in microfluidic implementations of single-cell RNA-seq (scRNA-seq) have enabled detailed analysis of gene expression across thousands of individual cells. While scRNA-seq is revolutionizing phenotypic characterization of complex tumors, it also opens new avenues for evaluating patient-derived models. We recently reported the broad extent of neural lineage identity and differentiation associated with gliomas based on scRNA-seq [1, 2]. In a parallel effort, we have analyzed matched, patient-derived glioma neurospheres from these tumors. Glioma neurospheres represent the state-of-the-art patient-derived model of gliomas and the basis of most drug screening and xenografts. While they are adept at preserving the genotypic diversity of gliomas, we found that they fail to recapitulate the phenotypic diversity of transformed cells in the tissue from which they originate.

To address these issues, we have developed a new approach to analyzing drug response in primary GBM tissue with a readout that tracks the response of different cellular subpopulations within the tumor. We are conducting small-scale drug screens in acute slice cultures of GBM tissue and using scRNA-seq to deconvolve the cell type-specific responses to drugs. We find that acute slice cultures recapitulate the phenotypic diversity of GBM tissue, harbor an intact immune microenvironment, and allow us to inexpensively deliver

single-cell resolved drug response information on up to 10 drugs in parallel within 72 hours.

Most recently, we have developed two experimental designs to facilitate analysis of cell type-specific drug response in patient-derived acute slice cultures. To identify conserved effects, we screen several drugs per resection along with controls where biological replicates are distributed across patients. For analysis of cell type-specific responses within a patient, which could enable precision medicine, we screen a smaller number of drugs with multiple biological replicates per patient resection.

Our most comprehensive analysis in acute slice culture to date has focused on the conserved effects of etoposide and panobinostat across four patients. Etoposide is a chemotherapy and topoisomerase inhibitor that is thought to primarily target cycling cells. Indeed, in acute slice cultures of both murine and human gliomas, we find robust loss of proliferating tumor cells in slice cultures treated with etoposide and limited effects on non-neoplastic cells in the microenvironment relative to control slices. Panobinostat is a histone deacetylase inhibitor, and in stark contrast to etoposide, has significant effects on gene expression in all detectable cell types in acute slice cultures. Among the malignantly transformed glioma cells, panobinostat appears to act as a differentiation therapy, and we observe widespread induction of mature neuronal gene expression. More remarkably, panobinostat significantly alters the composition of the immune microenvironment of treated slice cultures. The two major immune populations in GBM are microglia, which are commonly found in a pro-inflammatory state, and macrophages, which are thought to be immunosuppressive and associated with poor survival. While both of these myeloid populations are detected in slice cultures from all four patients, macrophages are selectively depleted from the panobinostat-treated slices, raising the possibility that a single agent could drive differentiation of the transformed cells while ablating immunosuppressive myeloid cells.

Overall, these initial studies show that scRNA-seq is a powerful tool not only for characterizing complex solid tumors, but also for assessing patient-derived models. We find that acute slice cultures of human glioma surgical specimens effectively recapitulate the cellular composition of the tumor tissue from which they are derived. In combination with small-scale drug screening in acute slice cultures, scRNA-seq can be used to assess the cell type-specific response of tumor tissue to drugs, including both malignantly transformed cells and non-neoplastic cells in the microenvironment. While promising, these studies also highlight a number of important analytical challenges with the acute slice culture approach. For some drugs, rapid alteration of gene expression obscures the original cellular identity of the tumor cells, complicating the determination of which cell types in the untreated tumor tissue are perturbed by the drug. To address this issue, we are

developing computational methods for projecting scRNA-seq profiles of perturbed cells onto untreated control profiles. As we extend this platform to more patients and test its predictive power, we envision potential utility for informing clinical trial enrollment and novel drug combinations.

References

1 Yuan, J., Levitin, H. M., Frattini, V., Bush, E. C., Boyett, D. M., Samanamud, J., Ceccarelli, M., Dovas, A., Zanazzi, G., Canoll, P., Bruce, J. N., Lasorella, A., Iavarone, A. & Sims, P. A. Single-cell transcriptome analysis of lineage diversity in high-grade glioma. *Genome Med* 10, 57, (2018).

2 Levitin, H. M., Yuan, J., Cheng, Y. L., Ruiz, F. J. R., Bush, E. C., Bruce, J. N., Canoll, P., Iavarone, A., Lasorella, A., Blei, D. M. & Sims, P. A. De novo Gene Signature Identification from Single-Cell RNA-seq with Hierarchical Poisson Factorization. *Molecular Systems Biology* 15 (2019).

Peter A. Sims, PhD

2004-2009	PhD, Chemistry, Harvard University
2009-2011	Postdoctoral Fellow, Dept. of Chemistry & Chemical Biology, Harvard University
2012-present	Assistant Professor, Department of Systems Biology, Columbia University Irving Medical Center

NEUTRALIZING PROTUMOR INFLAMMATION: LESSONS LEARNED FORM PRECLINICAL MOUSE MODELS

Lisa M. Coussens
Department of Cell, Developmental & Cancer Biology
Basic Research, Knight Cancer Institute
Oregon Health & Science University
Knight Cancer Research Building, Room 3030 | Mail Code KR-CDCB
2720 SW Moody Avenue, Portland, OR 97201, USA
(coussenl@ohsu.edu)

The concept that leukocytes are critical components of solid tumors is now generally accepted, however, their role(s) in regulating aspects of neoplastic progression, and/or response to cytotoxic, targeted and/or immune therapy is only beginning to be understood. Utilizing de novo mouse models of organ-specific solid tumor development (mammary, cutaneous, and pancreas carcinomas and mesothelioma), we now appreciate that adaptive leukocytes differentially regulate myeloid cell recruitment, activation and effector function, and in turn, activated tumor-infiltrating myeloid cells engage tissue-based programs to foster malignancy, and repress anti-tumor immunity by a diversity of mechanisms. Treatment of tumor-bearing mice with therapeutic agents that disrupt lymphocyte-myeloid cell interaction, myeloid cell activation, or myeloid cell functionality generally slow primary tumor growth kinetics when combined with cytotoxic therapy; however, their impact on metastases is variable. Similar to organ-specific regulatory programs co-opted to foster primary tumor growth, regulation of metastatic seeding and outgrowth is also regulated by tissue- and organ-specific mechanisms. Based on this, it stands to reason that therapeutic strategies may not be efficacious in both primary and metastatic locations. To be presented will be our recent insights into organ and tissue-specific regulation of primary and metastatic cancer development by adaptive and innate immune cells, how systemic regulation of humoral immunity and complement-mediated pathways regulate pro- versus anti-tumor immune responses, and new studies evaluating how attenuating protumor properties of select lymphoid and myeloid cells can be exploited to enhance therapeutic responses to cytotoxic and immune-based therapy.

LMC acknowledges generous support from the NIH / NCI, the Department of Defense Breast Cancer Research Program, Susan G. Komen Foundation, the Breast Cancer Research Foundation, the Brenden-Colson Center for Pancreatic Health.

Lisa M. Coussens, PhD

1980	BA, San Francisco State University, San Francisco (Biology)
1993	PhD, University of California, Los Angeles (Biological Chemistry)
1997	Postdoctoral Scholar, University of California, San Francisco (Tumor Biology)
2011-present	Professor and Chairwoman, Cell, Developmental and Cancer Biology, Oregon Health and Science University (OHSU), Associate Director for Basic Research, Knight Cancer Institute, OHSU

ROLE OF INTRAEPITHELIAL LYMPHOCYTES IN COLON CANCER

Bernardo Reis and Daniel Mucida
Laboratory of Mucosal Immunology
The Rockefeller University
1230 York Avenue, New York, NY 10065, USA
(mucida@mail.rockefeller.edu)

Colorectal cancer (CRC) is the 4th most common cancer in the US. Although this cancer arises at a key interface between the host and the environment, the role of the tumor microenvironment and microbiome in CRC remains largely unexplored (Lawler et al., 2018). Up to 20% of IBD patients develop cancer within 30 years of disease onset and up to 50% of these patients die of CRC. Nevertheless, the majority of CRCs develop in patients without underlying inflammation, primarily due to loss of tumor suppressor genes. In both cases of CRC, tumor-elicited inflammation triggers epithelial cell damage resulting in adenoma invasion by microbes, in turn driving tumor growth by sustaining inflammation (Grivennikov et al., 2012). Therefore, immune surveillance of the epithelial barrier may help prevent damaged epithelial cells from progressing into cancer.

Intestinal intraepithelial lymphocytes (IELs) comprise a large T cell population that occupies the intestinal epithelium. Dysregulation of IELs correlates with loss of mucosal barrier and inflammatory bowel diseases (IBD)(Jabri and Sollid, 2009). IELs are a heterogeneous group of T lymphocytes characterized by high expression levels of activation markers, gut-homing molecules, NK-like receptors and cytotoxic T lymphocyte (CTL)-molecules such as granzyme B and perforin (Denning et al., 2007). Another common characteristic of IELs is the surface expression of CD8αα homodimers, which can bind both to classical MHC-I and to epithelial cell-associated non-classical MHC-I molecules, presumably working as TCR "co-repressors" (Cheroutre and Lambolez, 2008; Cheroutre et al., 2011; Guy-Grand et al., 1991; Guy-Grand et al., 2013). Dissecting the role played by these cells under homeostatic and pathophysiological conditions, including cancer, has been hampered by a lack of genetic models for specific control of IEL development and

function. Additionally, poor survival of IELs in culture and failure to simulate their location within the epithelial compartment has further added to the difficulty in assessing IEL function.

TCRγδ^{+} cells form a major subpopulation of IELs, but their function is incompletely understood [5, 8]. Nonetheless, studies using TCRγδ$^{-/-}$ mice have reported increased susceptibility to very distinct pathogens [5, 8], while studies investigating the use of TCRγδ^{+} cells for cellular immunotherapy have confirmed their potent tumor reactivity in several different malignancies, including CRC (Scheper et al., 2014). The use of multi-photon intra-vital microscopy (IVM) has provided insights into cell-cell dynamics and behavior at the intestinal epithelium that could not be achieved with previous imaging techniques. Although the use of multi-photon microscopy in the gut is complicated by peristalsis and difficult accessibility, we are able to track a large number of IELs in multiple villi simultaneously. Using this method, we have observed that under homeostatic conditions TCRγδ^{+} IELs migrate rapidly within the intraepithelial (IE) compartment and survey the entire villous epithelium. This motility pattern is accompanied by continuous IEL movement into the lateral intercellular spaces (we denominated this pattern "flossing"). Additionally, we found that different enteric pathogens, including *Salmonella* and *Toxoplasma*, trigger increased IEL flossing rates (Hoytema van Konijnenburg et al., 2017).

Importantly, a γδ T cell gene signature was the most favorable prognostic factor among tumor-infiltrating leukocytes across cancer types (Gentles et al., 2015). In addition, recent studies in human CRC showed a favorable prognosis for tumors enriched in T cells expressing cytotoxic genes normally associated with IEL function (Cheroutre et al., 2011; Galon et al., 2006; Muller et al., 2000; Pages et al., 2010). We postulated that IELs play an important role in surveillance of intestinal epithelium against initiation, or progression of CRC. IELs provide a first line of immune defense against pathogens due to their location at the critical interface between the intestinal lumen and the core of the body. Hence, IEL immune surveillance of the epithelial layer may also help preventing damaged epithelial cells (ECs) from progressing into cancer. We addressed whether a coordinated EC-IEL response to environmental perturbation results in activation of IELs leading to removal (killing) of damaged or transformed epithelial cells.

To address EC-IEL dynamics *in vivo* during CRC, we used multi-photon intra-vital microscopy as well as tissue clearing associated with light sheet microscopy. Using these techniques, we tracked IEL dynamics simultaneously in multiple villi during cancer or in the steady state, and observed drastic changes in IEL behavior in tumor-bearing areas. We observed significant changes in γδ IEL movement patterns in tumor areas (disorganized, heterogenous) when compared to healthy areas (highly organized, homogenous). Of note, we also observed an accumulation of some γδ IELs moving with very reduced speed within

tumor areas. Additionally, we observed a sharp increase in IEL inter-epithelial movements (flossing), especially around tumor areas. The observation of segregated behavior of γδ IELs in CRC indicated the existence of potentially responsive subsets. Flow cytometry analysis indicated that, in naïve animals, there is a gradual increase in the frequency of CD8αα$^-$ TCRγδ cells towards the distal small intestine and colon. While the small intestine epithelium is primarily occupied by Vγ7$^+$ IELs, we found that colon Vγ usage is more diverse, including populations expressing Vγ1, Vγ4 and additional Vγs; CD8αα$^-$ γδ IELs rarely express the Vγ7 chain. Additionally, by utilizing a cleared-tissue based imaging analysis, we found that CD8αα$^+$ γδ IELs predominantly populate the tip of the villi, in close proximity to the intestinal lumen, while CD8αα$^-$ γδ IELs accumulate around the intestinal crypts. Together, these data suggest regional specialization in IEL subset function(Di Marco Barros et al., 2016), which could also be of relevance during cancer surveillance.

Further supporting this possibility, γδ IELs infiltrating tumor regions significantly expressed the immunoregulatory checkpoint molecule PD-1, while IELs in the neighboring healthy tissue showed very low levels of PD-1 expression. Additionally, heightened PD-1 expression was mostly restricted to the CD8αα (and Vγ7$^-$) population, pointing to a possible distinct role for these cells in anti-tumor immunity, and also suggesting possible new targets for immunotherapy intervention. These preliminary data also highlight the merit of multi-photon microscopy to gain insights into IEL dynamics and function *in vivo* during epithelial transformation. Coupled with gene expression analysis, metabolic profiling and novel genetic manipulation of IELs, these approaches will allow the characterization of novel mechanisms of epithelial surveillance. In preliminary observations, we found that mice lacking TCRγδ IELs display enhanced inflammation (reduced colon length, enhanced lipocalin-2) and develop tumors in a low-dose DSS (AOM) model, while wild-type mice do not (at low 1% DSS dose), suggesting a crucial role for TCRγδ IELs in controlling tumor development and/or progression.

Overall, our data suggests a "division of labor" among IEL subsets in the regulation of CRC development or progression; particular IEL subsets may be involved in the prevention of tumor initiation, while migrating IEL subsets that infiltrate the tumor may boos tumor progression. Our data expands our understanding of adaptive immunity at the gut epithelial barrier, contributing valuable information regarding the role of epithelial immunity in cancer.

References

1. Cheroutre, H., and Lambolez, F. (2008). Doubting the TCR coreceptor function of CD8alphaalpha. Immunity *28*, 149-159.

2. Cheroutre, H., Lambolez, F., and Mucida, D. (2011). The light and dark sides of intestinal intraepithelial lymphocytes. Nat. Rev. Immunol. *11*, 445-456.
3. Denning, T.L., Granger, S.W., Mucida, D., Graddy, R., Leclercq, G., Zhang, W., Honey, K., Rasmussen, J.P., Cheroutre, H., Rudensky, A.Y., *et al.* (2007). Mouse TCRalphabeta+CD8alphaalpha intraepithelial lymphocytes express genes that down-regulate their antigen reactivity and suppress immune responses. J. Immunol. *178*, 4230-4239.
4. Di Marco Barros, R., Roberts, N.A., Dart, R.J., Vantourout, P., Jandke, A., Nussbaumer, O., Deban, L., Cipolat, S., Hart, R., Iannitto, M.L., *et al.* (2016). Epithelia Use Butyrophilin-like Molecules to Shape Organ-Specific gammadelta T Cell Compartments. Cell *167*, 203-218 e217.
5. Galon, J., Costes, A., Sanchez-Cabo, F., Kirilovsky, A., Mlecnik, B., Lagorce-Pages, C., Tosolini, M., Camus, M., Berger, A., Wind, P., *et al.* (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science *313*, 1960-1964.
6. Gentles, A.J., Newman, A.M., Liu, C.L., Bratman, S.V., Feng, W., Kim, D., Nair, V.S., Xu, Y., Khuong, A., Hoang, C.D., *et al.* (2015). The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat. Med. *21*, 938-945.
7. Grivennikov, S.I., Wang, K., Mucida, D., Stewart, C.A., Schnabl, B., Jauch, D., Taniguchi, K., Yu, G.Y., Osterreicher, C.H., Hung, K.E., *et al.* (2012). Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature *491*, 254-258.
8. Guy-Grand, D., Malassis-Seris, M., Briottet, C., and Vassalli, P. (1991). Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocytes is induced locally. Correlation between functional assays, presence of perforin and granzyme transcripts, and cytoplasmic granules. J. Exp. Med. *173*, 1549-1552.
9. Guy-Grand, D., Vassalli, P., Eberl, G., Pereira, P., Burlen-Defranoux, O., Lemaitre, F., Di Santo, J.P., Freitas, A.A., Cumano, A., and Bandeira, A. (2013). Origin, trafficking, and intraepithelial fate of gut-tropic T cells. J. Exp. Med. *210*, 1839-1854.
10. Hoytema van Konijnenburg, D.P., Reis, B.S., Pedicord, V.A., Farache, J., Victora, G.D., and Mucida, D. (2017). Intestinal Epithelial and Intraepithelial T Cell Crosstalk Mediates a Dynamic Response to Infection. Cell.
11. Jabri, B., and Sollid, L.M. (2009). Tissue-mediated control of immunopathology in coeliac disease. Nat. Rev. Immunol. *9*, 858-870.
12. Lawler, M., Alsina, D., Adams, R.A., Anderson, A.S., Brown, G., Fearnhead, N.S., Fenwick, S.W., Halloran, S.P., Hochhauser, D., Hull, M.A., *et al.* (2018). Critical research gaps and recommendations to inform research prioritisation for more effective prevention and improved outcomes in colorectal cancer. Gut *67*, 179-193.

13. Muller, S., Buhler-Jungo, M., and Mueller, C. (2000). Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. J. Immunol. *164*, 1986-1994.
14. Pages, F., Galon, J., Dieu-Nosjean, M.C., Tartour, E., Sautes-Fridman, C., and Fridman, W.H. (2010). Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene *29*, 1093-1102.
15. Scheper, W., Sebestyen, Z., and Kuball, J. (2014). Cancer Immunotherapy Using gammadeltaT Cells: Dealing with Diversity. Front. Immunol. *5*, 601.

Daniel Mucida, PhD

2000	BS in biochemistry and immunology, Federal University of Minas Gerais
2005	PhD, University of São Paulo and New York University
2006-2010	La Jolla Institute for Allergy and Immunology
2010-2016	Assistant Professor, The Rockefeller University
2016-Present	Associate Professor, The Rockefeller University

TGF-β FAMILY SIGNALING IN PROGRESSION OF CANCER

Kohei Miyazono
Department of Molecular Pathology, Graduate School of Medicine
The University of Tokyo
Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan
(miyazono@m.u-tokyo.ac.jp)

Transforming growth factor-βs (TGF-βs) are multifunctional proteins that elicit both anti-tumorigenic and pro-tumorigenic functions during progression of cancer. TGF-β serves as an anti-oncogenic factor through growth inhibition and apoptosis induction of various types of cells, including epithelial cells and endothelial cells. However, TGF-β exhibits pro-tumorigenic functions by inducing epithelial-to-mesenchymal transition (EMT), as well as immune suppression and angiogenesis. Here, we present our findings on TGF-β signaling in progression of cancer, focusing on induction of EMT by TGF-β. We also show our findings on the role of bone morphogenetic proteins (BMPs) in the most malignant type of brain tumors, glioblastoma multiforme.

TGF-β-induced EMT in progression of cancer: TGF-β induces EMT through activation of Smad and non-Smad signaling pathways [1]. Through inhibition of TGF-β signaling pathways by an inhibitory Smad, Smad7, cancer metastasis can be prevented in a mouse model using mouse breast cancer JygMC(A) cells. TGF-β induces the expression of EMT-transcription factors (EMT-TFs), such as Snail, Slug, ZEB1, and ZEB2; these EMT-TFs play crucial roles in induction of EMT. Importantly, Smad signaling cross-talks with K-Ras signaling activated in some cancer cells, and induces certain crucial genes involved in EMT. For example, in the presence of active K-Ras, TGF-β upregulates the expression of Snail, and thus induces an EMT phenotype in pancreatic cancer PANC1 cells. Although mutations of the *SMAD4*/*DPC4* gene can be found in ~50% of advanced stages of pancreatic cancer patients, mutations of *KRAS* occur in earlier phase of ~90% of pancreatic cancer patients; therefore, TGF-β may mainly play pro-tumorigenic functions during progression of pancreatic cancer through cooperation with K-Ras signaling [1].

We have developed a tissue-clearing technology, termed CUBIC-based cancer (CUBIC-Cancer) analysis. CUBIC-Cancer analysis allows whole-body/organ imaging of macroscopic to microscopic analyses of cancer with single-cell resolution. By CUBIC-Cancer analysis using human lung adenocarcinoma A549 cells, we revealed that EMT induced by TGF-β accelerates not only the process of extravasation, but also cell survival and extravasation of cancer cells at metastatic sites [2]. Moreover, TGF-β-treated A549 cells with mesenchymal phenotype appeared to express E-cadherin at metastatic foci, suggesting that these cells had undergone mesenchymal-to-epithelial transition (MET) when they formed metastatic foci [2].

EMT is known to be a reversible biological process. We found that chronic TGF-β treatment for more than 12 days induced stabilized EMT in mammary epithelial cells transfected with H-Ras [3]. In the stabilized EMT, mammary carcinoma cells acquired stem cell-like properties and chemoresistance. Chronic TGF-β treatment showed enhanced mTOR signaling, and inhibition of mTOR signaling resulted in suppression of stem cell-like properties and chemoresistance. Moreover, inhibition of mTOR signaling reduced tumorigenicity of breast carcinoma cells in vivo, suggesting that mTOR signaling pathway is a potentially interesting target for treatment of cancer metastasis [3].

Roles of BMPs in glioblastoma: BMPs, members of the TGF-β family, are also multifunctional proteins; in addition to induce bone and cartilage formation in vivo, BMPs are involved in morphogenesis of various organs and tissues, including neuronal tissues, blood and lymph vessels, and hair follicles. Recent findings revealed that BMPs play critical roles in regulation of the progression of various cancers [4]. BMPs induce growth arrest, apoptosis, and differentiation of glioma-initiating cells (GICs). Similar to TGF-βs, BMPs transduce signals through type II and type I receptors and downstream Smad and non-Smad signaling pathways. Among four different type I receptors for BMPs, we found that one of the BMP type I receptor known as ALK-2 (activin receptor-like kinase 2) plays a critical role in induction of apoptosis and differentiation of GICs [5].

Although BMPs exhibit anti-tumorigenic functions in GICs, BMPs do not always prevent progression of glioblastoma in vivo, possibly because glioblastoma tissues are composed of heterogenous cell populations, and some glioblastoma cells do not respond to BMPs. To further investigate the function of BMPs in GICs, we searched for genes and proteins regulated by BMPs using RNA-seq analysis and some other methods. We found that several nuclear proteins, such as Distal-less homeobox 2 (DLX2) and paired related homeobox 1 (PRRX1), were induced by BMPs. DLX2 induced differentiation and apoptosis of GICs [5]. Inoculation of GICs with ectopic expression of ALK-2 or DLX2 resulted in prolonged survival of nude mice.

Using a phospho-receptor tyrosine kinase array, we also found that phosphorylation of erythropoietin-producing hepatocellular carcinoma receptor A6 (EphA6) is induced by

BMP signaling [6]. Through a complex formation with ALK-2, EphA6 enhanced the signaling activity of ALK-2. Although ALK-2 kinase activity was essential for induction of GIC apoptosis, kinase activity of EphA6 was not required. Injection of GICs with ectopic EphA6 resulted in prolonged survival of mice compared to that with control GICs, suggesting that EphA6 is a putative tumor suppressor of glioblastoma. We have also identified some genes regulated by BMP signaling in GICs. Further analyses may allow us to identify potential target(s) for treatment of glioblastoma.

References

1. Miyazono K, Katsuno Y, Koinuma D, Ehata S, Morikawa M. Intracellular and extracellular TGF-β signaling in cancer: some recent topics. Front Med. 2018;12:387-411.
2. Kubota SI, Takahashi K, Nishida J, Morishita Y, Ehata S, Tainaka K, Miyazono K, Ueda HR. Whole-body profiling of cancer metastasis with single-cell resolution. Cell Rep. 2017;20:236-250.
3. Katsuno Y, Meyer DS, Zhang Z, Shokat KM, Akhurst RJ, Miyazono K, Derynck R. Chronic TGF-β exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. Sci Signal. 2019;12:pii: eaau8544.
4. Davis H, Raja E, Miyazono K, Tsubakihara Y, Moustakas A. Mechanisms of action of bone morphogenetic proteins in cancer. Cytokine Growth Factor Rev. 2016;27:81-92.
5. Raja E, Komuro A, Tanabe R, Sakai S, Ino Y, Saito N, Todo T, Morikawa M, Aburatani H, Koinuma D, Iwata C, Miyazono K. Bone morphogenetic protein signaling mediated by ALK-2 and DLX2 regulates apoptosis in glioma-initiating cells. Oncogene, 2017;36:4963-4974.
6. Raja E, Morikawa M, Nishida J, Tanabe R, Takahashi K, Seeherman HJ, Saito N, Todo T, Miyazono K. Tyrosine kinase Eph receptor A6 sensitizes glioma-initiating cells towards bone morphogenetic protein-induced apoptosis. Cancer Sci. 2019;110:3486-3496.

Kohei Miyazono, MD, PhD

1988-1990	Assistant of Professor, Third Dept. Internal Medicine, The Univ. of Tokyo
1990-1995	Assistant Member, Ludwig Institute for Cancer Research, Uppsala, Sweden
1995-2000	Chief, Department of Biochemistry, The Cancer Institute, Japanese Foundation for Cancer Research
2000-present	Professor, Department of Molecular Pathology, Graduate School of Medicine, The Univ. of Tokyo

CAR T CELLS: THE EMERGENCE OF SYNTHETIC IMMUNITY

Michel Sadelain
Center for Cell Engineering
Memorial Sloan-Kettering Cancer Center
1275 York Avenue, New York, NY 10065, USA
(m-sadelain@ski.mskcc.org)

Natural immune responses fall short of eradicating tumors in most cancer patients. T cell engineering offers a means to repurpose immune cells to perform enhanced therapeutic functions. Chimeric antigen receptors (CARs) are synthetic receptors that redirect and reprogram T cells to mediate tumor rejection. CARs that target CD19 offer the prospect of complete remissions in patients with chemorefractory, relapsed B cell malignancies, especially acute lymphoblastic leukemia (ALL). In the first 53 adult patients with refractory and relapsed ALL we treated with autologous peripheral blood T cells expressing the 19-28z CAR, a complete remission (CR) was obtained in 83%. The US Food and Drug Administration approved the first CD19 CAR therapies in 2017. Despite high CR rates, a number of patients will eventually relapse, pointing to the need to further improve CAR design and T cell engineering strategies to increase the functional persistence of CAR T cells and reduce their toxicities. Using CRISPR/Cas9, we found that directing a CAR to the T cell receptor alpha chain (*TRAC*) locus not only results in uniform CAR expression in human peripheral blood T cells, but enhances T cell potency by attenuating CAR tonic signaling and T cell exhaustion, enabling *TRAC*-CAR T cells to outperform conventionally engineered CAR T cells. Further analyses of the signaling properties of the 19-28z CAR have identified signaling components that more effectively control T cell persistence and acquisition of effector functions, yielding new CAR designs that are better suited to balance T cell memory and anti-tumor activity. Recent studies reveal that CAR T cells acquire the CAR target and many other molecules from the tumor cell via trogocytosis, providing new insights into the biology of CAR T cells and evolving strategies to further perfect the design of engineered T cell immunity to tackle solid tumors.

Michel Sadelain, MD, PhD

1984	MD, University of Paris
1989	PhD, University of Alberta
1989	Postdoctoral Fellow, Whitehead Institute, MIT
1994	Assistant Professor, Memorial Sloan Kettering Cancer Center
2007-present	Director, Center for Cell Engineering; Stephen and Barbara Friedman Chair, Memorial Sloan Kettering Cancer Center

ADVANCES OF CAR-T CELL THERAPY FOR SOLID TUMORS

Koji Tamada
Department of Immunology
Yamaguchi University Graduate School of Medicine
1-1-1 Minami Kogushi, Ube, Yamaguchi 755-8505, Japan
(ktamada@yamaguchi-u.ac.jp)

Gene-modified T cells utilizing tumor-specific T cell receptor (TCR) or chimeric antigen receptor (CAR) have been rapidly developed and translated into clinic in recent years. In particular, cancer immunotherapy using CAR-T cells has demonstrated the outstanding clinical effects in B cell hematological malignancies, resulting in FDA and PMDA approval of CD19 CAR-T cell therapy for children and young adult, refractory or relapsing B cell acute lymphoblastic leukemia (ALL) and adult patients with relapsed or refractory B cell lymphoma in 2017 and 2019, respectively. On the other hand, CAR-T cell therapy against solid tumors has yet to be fully developed, as only a few exceptional cases have been reported to achieve clinical efficacy. Potential hurdles for CAR-T cell therapy in solid tumors 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. Various modifications and improvement of CAR-T cells to overcome these hurdles have been actively investigated by worldwide researchers.

To this end, our group has developed novel CAR technology which enables CAR-T cells to simultaneously produce interleukin-7 (IL-7) and CCL19, aiming at efficient accumulation, expansion, and survival of immune cells inside solid tumor tissues (Figure 1). Treatment of pre-established solid tumors with IL-7/CCL19-producing CAR-T cells achieved complete regression followed by prolonged mouse survival and long-term memory response specific to tumor (Figure 2). Tumor tissues from the mice treated with IL-7/CCL19-producing CAR-T cells demonstrated massive accumulation of immune cells including both the transferred CAR-T cells and endogenous T cells and DC (Figure 3). An

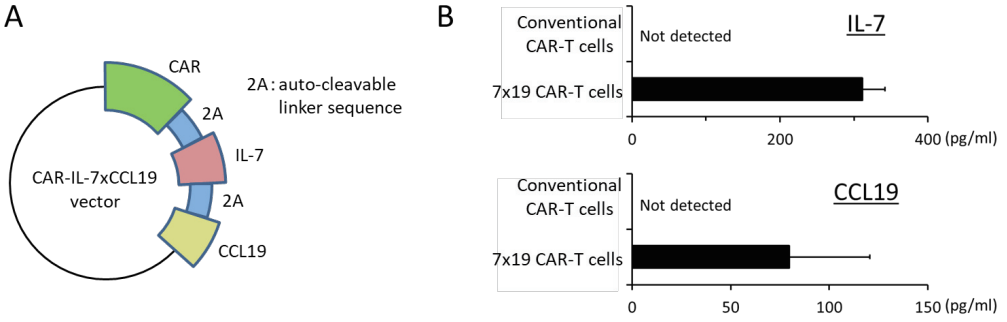


Figure 1 Generation of IL-7/CCL19-producing CAR-T cells.
(A) Schema of vector encoding IL-7/CCL19-producing CAR. (B) Production levels of IL-7 and CCL19 in the culture supernatants of IL-7/CCL19-producing CAR-T cells (hereafter referred to as 7x19 CAR-T cells)

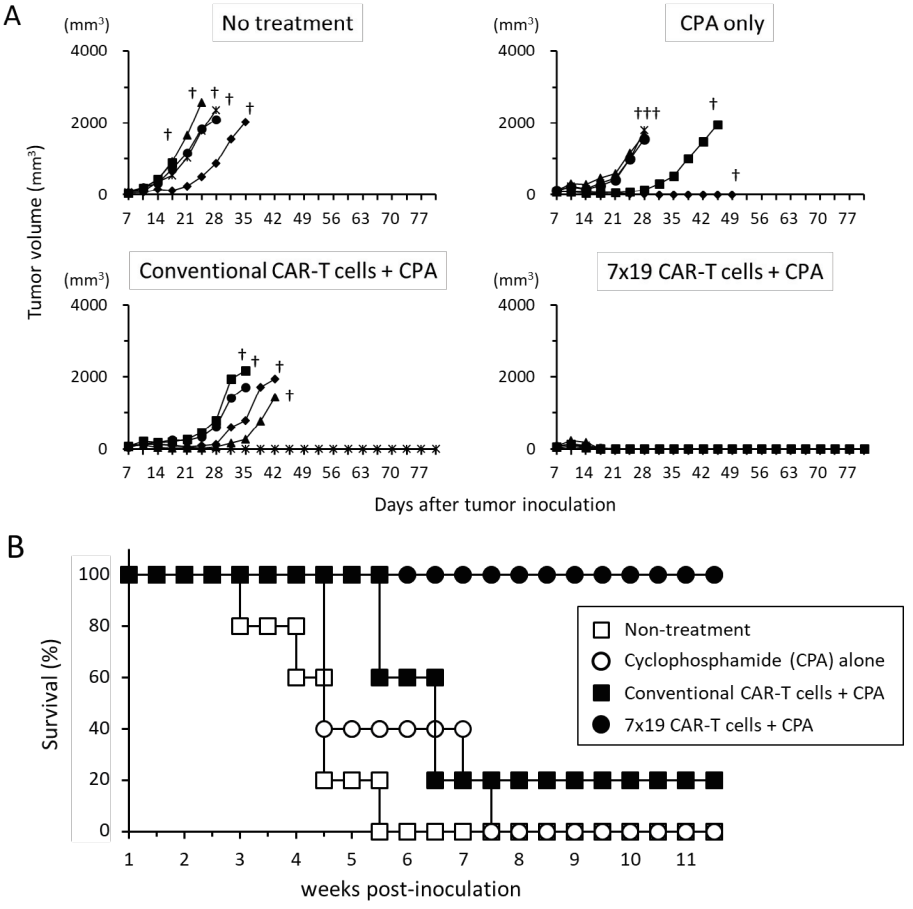


Figure 2 Anti-tumor effects of IL-7/CCL19-producing CAR-T cells.
(A) Tumor growth in the mice treated with conventional CAR-T cells or IL-7/CCL19-producing CAR-T cells is shown. As control, tumor growth in the mice without treatment or those treated with cyclophosphamide alone is shown. (B) Survival of the mice is shown.

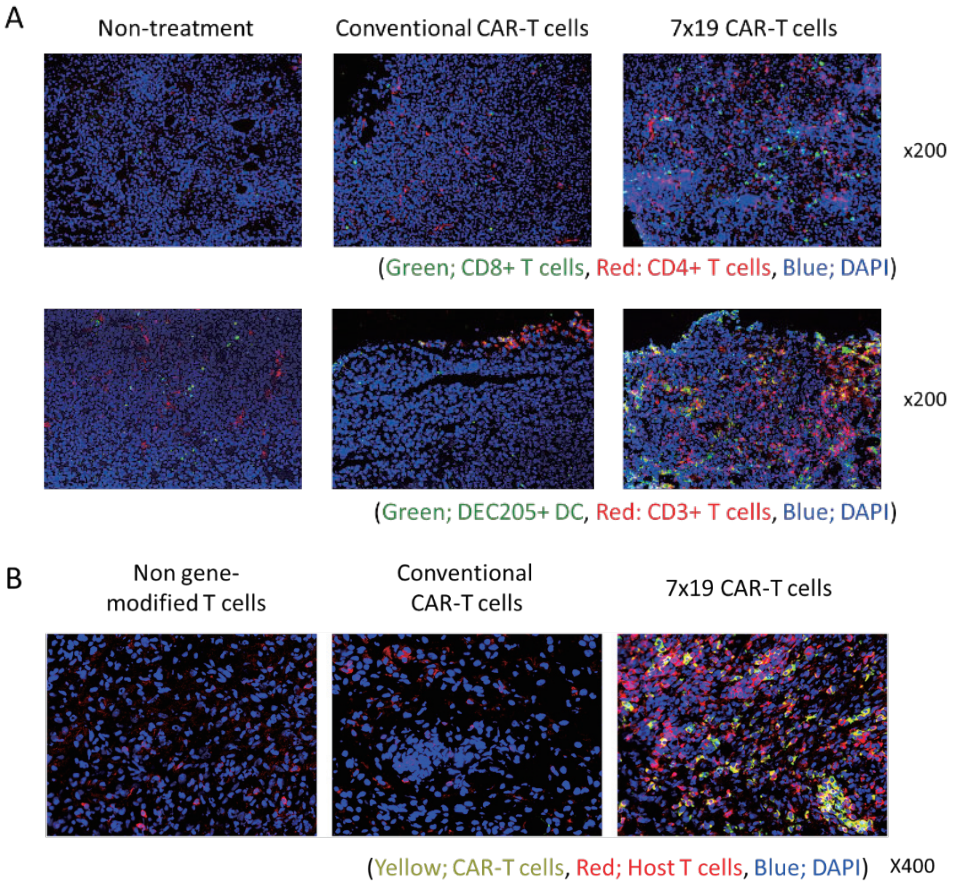


Figure 3 Immune cell infiltration in tumor tissues of the mice treated with IL-7/CCL19-producing CAR-T cells.
(A) Infiltration of CD4+ and CD8+ T cells (upper panels) and DEC205+ DC and CD3+ T cells (lower panels) in the tumor tissues analyzed by IHC staining is shown. (B) Infiltration of transferred CAR-T cells and host T cells in the tumor tissues analyzed by IHC staining is shown.

important role of endogenous T cells in the anti-tumor effects of IL-7/CCL19-producing CAR-T cells was confirmed (Figure 4).

As another approach, we also developed CAR-T cells which produce anti-PD-1 scFv to attenuate PD-1 signal (Figure 5). Anti-PD-1 scFv-producing CAR-T cells demonstrated improved therapeutic efficacy against solid tumors, along with enhanced responses of endogenous tumor-specific T cells (Figure 6).

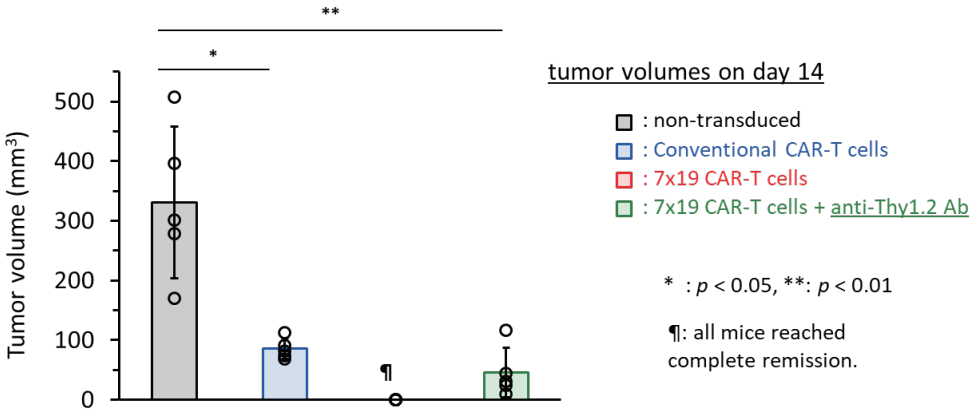


Figure 4 An important role of host T cells in the treatment efficacy of IL-7/CCL19-producing CAR-T cells.
Tumor growth in the mice treated with 7x19 CAR-T cells with or without host T cell depletion is shown. As control, tumor growth in the mice without treatment or treated with conventional CAR-T cells is shown.

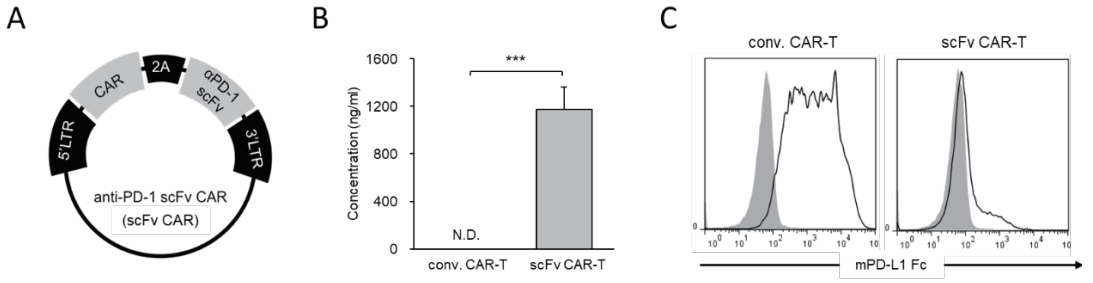


Figure 5 Generation of anti-PD-1 scFv-producing CAR-T cells.
(A) Schema of vector encoding anti-PD-1 scFv-producing CAR (hereafter referred to as scFv CAR). (B) Concentration of anti-PD-1 scFv in culture supernatant conventional or scFv CAR-T cells is shown. ***; P<0.001. (C) Blockade of PD-L1/PD-1 interaction by an addition of the culture supernatant from conventional or scFv CAR-T cells is shown.

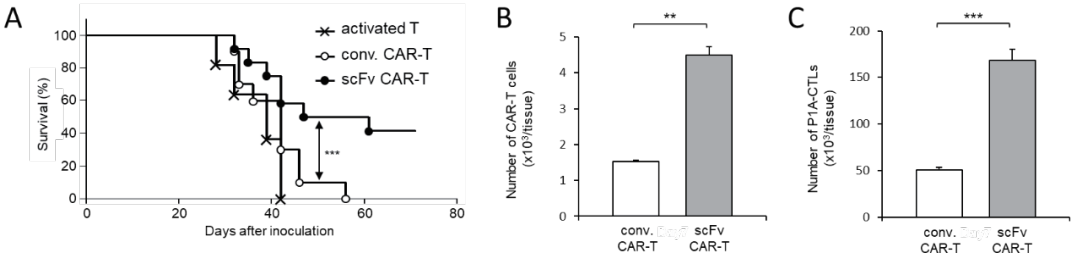


Figure 6 Anti-tumor effect of anti-PD-1 scFv-producing CAR-T cells.
(A) Survival of mice treated with scFv CAR-T cells is shown. As control, survival of mice treated with activated T cells (non-CAR-T cells) or conventional CAR-T cells is shown. (B) Number of CAR-T cells in the tumor tissues of the mice treated with conventional or scFv CAR-T cells is shown. (C) P1A-specific TCR-transgenic T cells were injected into the mice and considered as host-derived tumor-specific T cells. After the treatment with conventional or scFv CAR-T cells, the number of P1A-specific TCR-transgenic T cells in the tumor tissues is shown. ***; P<0.001. **; P<0.005.

Thus, we developed next-generation CAR-T cell technologies effective for solid tumors. Out studies suggest the importance of accumulation of anti-tumor immune cells in tumor tissues, induction of endogenous polyclonal T cell responses against tumor likely due to epitope spreading, and attenuation of immune-inhibitory tumor microenvironment in the treatment of solid tumors.

Koji Tamada, MD, PhD

1986-1992	MD, Kyushu University
1994-1998	PhD, Kyushu University, Graduate School of Medicine
1998-2005	Postdoctoral researcher, Mayo Clinic
2005-2008	Assistant Professor, Johns Hopkins University
2008-2011	Associate Professor, University of Maryland
2011-present	Professor, Department of Immunology, Yamaguchi University Graduate School of Medicine

THE SYNTHETIC FUTURE OF CAR T CELL CANCER IMMUNOTHERAPY

Michael C. Jensen
University of Washington School of Medicine
Department of Pediatrics, Hematology-Oncology
Ben Towne Center for Childhood Cancer Research/Seattle Children's Research Institute
1100 Olive Way, Suite 100, Seattle, WA 98101, USA
(michael.jensen@seattlechildrens.org)

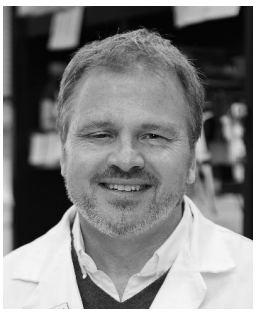
CD19-specific chimeric antigen receptor (CAR)-expressing autologous T cells administered following lymphodepleting chemotherapy can induce clinical remissions in B-lineage malignancies including refractory pediatric acute lymphoblastic leukemia (B-ALL), irrespective of disease burden and anatomic dissemination. Obstacles to broad clinical deployment of this treatment are (1) a high failure rate in CAR-T cell manufacturing, (2) heterogeneity of anti-tumor responses, and (3) severe modality-associated toxicities in some patients. While structure-function attributes of the various CD19-specific CARs have been delineated in preclinical models, variations among the polyclonal transduced CAR-T cell products tested in clinical trials have limited opportunities for the systematic study of product attributes related to cellular composition, differentiation, and transgene expression that impact therapeutic potency and safety.

We engineered a CAR-T cell product that comprises a defined 1:1 ratio of $CD4^+/CD8^+$ CAR-T cells, selects for uniform high-level CAR expression, and limits activation-induced differentiation of $CD4^+/CD8^+$ T cells by using homeostatic cytokines for CAR-T cell expansion. Herein we report a robust intent-to-treat (ITT) product manufacturing success rate in minimally selected heavily pre-treated patients, a 93% MRD-negative (MRD-neg) complete remission (CR) rate resulting in 89% overall efficacy based on the ITT population, with a tolerable side effect profile. We report on duration of leukemic remissions, and the impact of lymphodepleting regimen and CD19 antigen burden on sustained CAR engraftment and durable remissions. These data establish the feasibility of this advanced manufacturing platform and support further study of this highly defined CD19 CAR-T cell product. This study also has revealed shortcomings of the technology in its current iteration. Short engraftment as measured by duration of B cell aplasia less than 63 days is a

risk factor for relapse (RR>33) and low CD19 antigen burden at the time of T cell transfer is a risk for short BCA. Patients with longer engraftment are still at risk for CD19 antigen escape relapse. I will describe new technologies such as T cell antigen presenting cells that express CD19 as a booster product to reproliferate CD19CAR T cells in patients and our strategy to manufacture CD19xCD22 dual specific CAR T products that are anticipated to reduce antigen loss escape. These next generation trials have commenced at Seattle Children's and their current status will be described.

References

1. Finney, O.C. et al., 2019. CD19 CAR T cell product and disease attributes predict leukemia remission durability. The Journal of Clinical Investigation, 130.
2. Gardner, R. et al., 2016. Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy. Blood, 127(20), pp.2406-2410.
3. Gardner, R.A. et al., 2017. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. Blood, 129(25), pp.3322-3331.
4. Gust, J. et al., 2019. Glial injury in neurotoxicity after pediatric CD19-directed CAR-T cell therapy. Annals of Neurology.
5. Lu, Y.J. et al., 2019. Preclinical Evaluation of Bispecific Adaptor Molecule Controlled Folate Receptor CAR-T Cell Therapy With Special Focus on Pediatric Malignancies. Frontiers in oncology, 9, p.151.
6. Taraseviciute, A. et al., 2018. Chimeric Antigen Receptor T Cell-Mediated Neurotoxicity in Nonhuman Primates. Cancer discovery, 8(6), pp.750-763.
7. Turtle, C.J. et al., 2016. CD19 CAR-T cells of defined CD4+:CD8+ composition in adult B cell ALL patients. The Journal of Clinical Investigation, 126(6), pp.2123-2138.



Michael C. Jensen, MD

1986-1990	MD; University of Pennsylvania School of Medicine
1990-1993	Intern and Resident in Pediatrics, Children's Hospital of Northern California
1993-1997	Fellow in Pediatric Hematology-Oncology, University of Washington and the Fred Hutchinson Cancer Research Center
2010-present	Jim & Jan Sinegal Endowed Professor, Pediatrics, University of Washington School of Medicine
2011-present	Director, Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute

DECIPHERING AND TARGETING CLONAL EVOLUTION IN MYELOID MALIGNANCIES

Linde A. Miles, Robert L. Bowman, Aaron Viny, and Ross L. Levine
Human Oncology & Pathogenesis Program
Department of Medicine
Center for Hematologic Malignancies
Memorial Sloan Kettering Cancer Center
1275 York Avenue, New York, NY 10065, USA
(leviner@mskcc.org)

Genomic studies of myeloid malignancies (MM), including acute myeloid leukemia (AML), myeloproliferative neoplasms (MPN) and myelodysplasia (MDS), identified mutations with different allele frequencies. Recent studies of clonal hematopoiesis (CH) discovered a subset of MM disease alleles, while other alleles are only observed in overt MM. These observations suggest an important pathogenetic role for the chronology of mutational acquisition. The most common CH mutations are in DNMT3A, TET2, and ASXL1. Subjects with CH have reduced overall survival and carry an increased risk of subsequent hematologic malignancies and increased cardiovascular risk21,22. CHIP is observed in 25% of advanced solid tumor patients and is associated with an increased risk of progression of their primary malignancy and with increased risk for subsequent myeloid malignancies. These data resonate with in vivo studies showing that alterations in Tet2, Dnmt3A, and Asxl1 in murine models lead to stem cell expansion.

Green and colleagues have used genomic studies of clonal colonies in MPN patients with concurrent JAK2 and TET2 mutations to show that the order of mutational acquisition can differ, such that either JAK2 or TET2 mutations could be the initiating or subsequent event29. They also showed that the order of mutations (e.g. JAK2 first vs. TET2 first) impacted clinical parameters, sensitivity to ruxolitinib in vitro, and transcriptional programs. This seminal study demonstrates that the order of genetic events is a critical feature governing MPN pathogenesis and progression. Our studies aim to use similar approaches to map evolution and assess the functional and therapeutic relevance of evolution from CH to MPN and to AML. Although bulk sequencing informs prognostication, it cannot distinguish which mutations occur in the same clone and cannot

offer definitive evidence of mutational order. Delineation of clonal architecture at the single cell level is key to understanding how the sequential/parallel acquisition of somatic mutations contributes to myeloid transformation.

In order to elucidate the clonal structure of MM, we designed a custom single cell 109 amplicon panel of the most frequently mutated amplicons in 50 MM genes using the Mission Bio Tapestri v2 platform. Viable cells were sorted from 90 samples from 78 patients with CH, AML, and MPN/post-MPN AML followed by single cell amplification/sequencing. Mutation calls were filtered based on read depth, quality, and alleles genotyped per cell. We reconstructed a random distribution of clones by permuting genotype calls across cells and generated empirical p values for each clone. To identify dominant clones, we used a Poisson test to determine clones were significantly enriched compared to the mean clone size. Clones with significant p-values ($p < 0.05$) were used to generate plots of clonal architecture of each sample. Despite significant clonal complexity, the majority of MM patients (80%;72/90) present with one (51/90; 56.7%) or two (21/90; 23.3%) dominant clones. These data show there are specific genotypic combinations which lead to clonal dominance with increased fitness relative to other clones and/or suppression of minor clones by dominant clone(s). We next investigated whether specific molecularly defined AML subtypes had increased clonal complexity. *FLT3-ITD* mutant AML samples had a significantly greater number of clones ($p < 0.002$) compared to AML samples with multiple epigenetic modifier mutations. Similar findings were not observed when comparing AML samples with epigenetic mutations to *RAS* pathway mutant samples.

We next investigated whether specific mutations were likely to co-occur/be mutually exclusive at a single cell level. We observed evidence of oligoclonality in CH, including parallel acquisition of *DNMT3A* mutations and clones with multiple mutations in the absence of progression to MM. By contrast, in MM the dominant clone(s) almost always harbored multiple epigenetic modifier mutations, suggesting cooperative epigenetic remodeling in myeloid transformation. Mutations in signaling effectors (*FLT3-ITD/TKD; RAS/RAS*) were mutually exclusive. We observed distinct *FLT3*-mutant clones in *FLT3*-mutant AML patients and parallel acquisition of different *RAS* pathway mutations. We used this data to develop clonal architecture trees in each patient, giving us a definitive picture of mutational acquisition and transformation at a single cell level. We calculated a Shannon diversity score and observed an increase in clonal complexity with disease evolution; CH samples had the lowest clonal diversity and *FLT3-ITD* AML patients the highest clonal diversity.We extended our findings by combining cell surface marker assessment and single cell mutational analysis. Patient samples were stained with an antibody cocktail of 6 oligo-conjugated antibodies with barcode tags prior to single cell sequencing, which allowed simultaneous acquisition of single cell immunophenotypic and

genotypic data. This allows us to identify distinct populations of stem/progenitor cells with distinct clonal/mutational repertoires. This includes studies of CD34+ and CD34- AML, which show striking differences in mutational representation in different stem/progenitor compartments. In summary, these studies of clonal architecture at a single cell level provide us novel insights into the pathogenesis of myeloid transformation and give us new insights into how clonal complexity contributes to disease progression.

We have also sought to identify novel targets in CH and in overt myeloid malignancies. We have begun to investigate whether CHIP disease alleles render cells sensitive to specific therapeutic targets. We recently described a conditional *Jak1* allele and showed that Jak1 is critical for stress hematopoiesis and for inflammatory signaling in hematopoietic stem/progenitor cells[34]. We therefore investigated whether *Jak1* was a liability in *Jak2V617F* and *Tet2* mutant stem cells by crossing *Jak1* knockout mice to both disease alleles and assessing stem cell function in competitive transplant assays. *Jak1* loss lead to a reversal of the fitness advantage of *Jak2V617F* and *Tet2* mutant stem cells, consistent with Jak1 dependency in both models of CHIP. We have now initiated preclinical therapeutic studies in collaboration with Incyte (MTA) to test a clinical Jak1inhibitor (INCB39110) in order to build towards the first therapeutic trial in CHIP.

We have also adopted a stromal co-culture system developed by Shahih Rafii and Jason Butler, which allows us to culture wild-type and CH/MPN/AML cells with defined genotypes in culture for 2-3 weeks, with 10-100 fold expansion and retained ability for long-term engraftment and disease propagation *in vivo*. This system can be used to test candidate therapeutic agents and genetic targets (CRISPR screens) for their ability to abrogate the fitness advantage of CH/MPN/AML cells relative to wild-type cells *in vitro* and *in vivo*. We can do this in CD45.2/1 co-culture systems where CH/MPN/AML are studied in the same *in vitro*/*in vivo* context as wild-type cells, or in systems with >1 CH/MPN/AML clone in order to delineate therapeutic dependencies in a complex clonal milieu.

In summary, our studies delineate the trajectories which drive hematopoietic stem/progenitor cells towards CH and overt myeloid malignancies, and we are using this insight to identify novel therapeutic targets aimed to intercept clonal evolution for therapeutic impact.

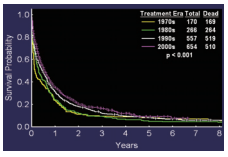
Genetics and Therapy of Clonal Hematopoiesis and Myeloid Malignancies

Ross L. Levine M.D.
Molecular Cancer Medicine Service
Human Oncology and Pathogenesis Program
Leukemia Service, Department of Medicine
Center for Hematologic Malignancies
Center for Epigenetics Research
Memorial Sloan Kettering Cancer Center

Disclosures

- Supervisory Board/BoD (equity): Qiagen
- Scientific Advisory Board (equity): Loxo (Lilly), C4 Therapeutics, Imago, Isoplexis, Auron
- Consultant/Honoraria: Roche, Celgene, Novartis, Gilead, Incyte, Janssen, Morphosys, Astellas
- Grant Support: Roche, Celgene, Prelude

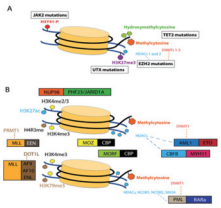
Acute Myeloid Leukemia: Poor Overall Survival



- Even with intensive induction chemotherapy/transplantation most patients die of their disease →new insights are needed
- Only 2 classes of agents approved for AML 1980-2017 (hypomethylating agents, gemtuzumab)
- AML is a disease of the aging population: median age 68 years, worsening prognosis with increasing age

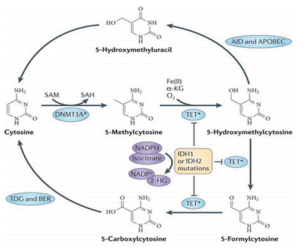
Issa, Kantarjian et al, Cancer 2008

AML Mutations and Their Effect on the Epigenetic state



- Mutations can indirectly or directly alter the epigenetic state of cancer cells: leukemia and human malignancies in general
- Many are associated with adverse outcome- need for novel biologic and therapeutic insights
- Mutations in epigenetic modifiers and gene regulatory factors increase in incidence with age as prognosis worsens
  - DNA Modifications: TET2, IDH1/2, DNMT3A
  - Chromatin Modulators: EZH2, MLL-PTD
  - DNA topology: cohesin mutations
  - RNA splicing: SRSF1, SF3B1…

Mutations in Genes Which Regulate DNA Modifications in AML



- Mutations in TET2, DNMT3A are common in AML (MDS, MPN…)
- Age-dependent incidence (<5% pediatric AML, 40-50% of AML in older adults)
- How do these alleles contribute to transformation?
- Are there therapeutic targets specific to AML with mutations in epigenetic regulators?

Clonal Hematopoiesis

- Nonrandom X-inactivation rations in blood cells of female elderly subjects consistent with clonal outgrowth (Busque, Gilliland)
- Increased myeloid bias, risk of myeloid leukemias with increasing age
- Multiple hypotheses for mechanism:
  - Caused by mutations conferring selective growth advantage in stem cells.
  - Stochastic clonal dominance secondary to stem cell depletion
  - Genetic trait
- Hypothesized clonal hematopoiesis due to somatic mutations-> exome sequencing of granulocyte/normal DNA on elderly subjects with clonal hematopoiesis

*Busque, Patel, *et al. Nature Genetics* 2012

Somatic TET2 Mutations in Clonal Hematopoiesis

Nucleotide substitution*	Amino acid substitution	Chromosome	Position
c.286_298delCCCACAGTTAGTG	p.Arg96Asnfs*12	4	106155385
c.1330delA	p.Thr444Hisfs*6	4	106156429
c.1348delA	p.Lys450Lysfs*2	4	106156447
c.1547delC	p.Pro516Hisfs*16	4	106156646
c.1630C>T	p.Arg544*	4	106156729
c.3311_3312insAT	p.Phe1104Leufs*3	4	106158411
c.3991A>C	p.Thr1331Pro	4	106182952
c.5200delA	p.Met1734Leufs*11	4	106196867
c.5575insT	p.Ile1859tyrfs*16	4	106197239
c.5725G>T	p.Glu1909*	4	106197392

TET2 is a commonly mutated leukemia gene (8-10% of AML) with age-dependent incidence

3-5% of healthy subjects>60 have TET2 mutations

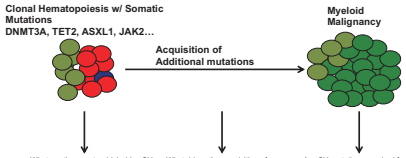
Identical loss-of-function mutations as those seen in AML, MDS, MPN

*Busque, Patel, *et al. Nature Genetics* 2012

Clonal Hematopoiesis and Somatic Mutations

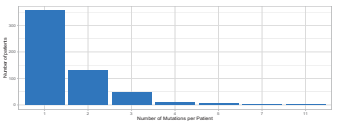
- Premalignant clonal state induced by somatic mutations in hematopoietic stem cells
  - Mutations in TET2 in CH (Busque et al. Nature Genetics 2012)
  - DNMT3A, TET2, IDH1/2 mutations in preleukemic stem cells (Jan *et al.* STM 2012, Shlush *et al.* Nature 2014)
  - Somatic mutations in blood cells in population based cohorts (Jaiswal/Ebert *et al.*, McCarroll *et al.* NEJM)
- Implications of this on genomic profiling in the clinical setting are expanding
  - Identification of somatic mutations in known leukemia disease alleles in patients with solid tumors
  - Following MRD: cannot follow a single mutation as residual CHmay not always drive relapse
  - Increased risk of cardiovascular disease (Ebert et al, Fuster/Walsh et al.)
- May represent the first frontier for effective malignancy prevention studies
  - Identify and intervene on patients with somatic mutations to prevent hematopoietic malignancies and other sequelae

Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies



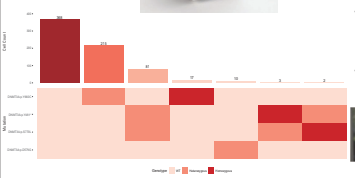
- What are the events which drive CH
- What is the implication of CH on clinical outcome, including cardiovascular disease and epithelial tumors
- How do we manage/follow patients who are found with CH in the clinical context
- What drives the acquisition of additional mutations (mutagenesis, selection)
- Does the stem/progenitor compartment which acquires additional mutations dictate risk of transformation
- What microenvironmental factors promote expansion of specific clones (Meisel et al. Nature 2018)
- Are CH mutations required for maintenance of the leukemic clone
- How do subsequent mutations cooperate with CH alleles to drive disease phenotype
- Are there therapeutic implications of targeting different mutant alleles including CH mutations

Delineating Clonal Heterogeneity



- See CH/leukemia patients with 2, 3, 4+ mutations including co-occurring mutations in known, validated leukemia alleles
- Can we delineate if in same/different clone->does this have biologic or clinical relevance
- Can we use this to better model/understand pathogenesis of myeloid malignancies

Mission Bio Tapestri-Based DNA-sequencing To Delineate Mutations in Single Cells

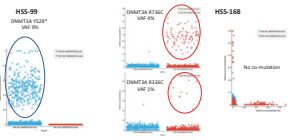


- Barcode based sequencing of single cells
- Designed a custom panel of 100+ amplicons for top CH/myeloid genes
- Can be used for informative samples to provide single cell evidence of clonal architecture and mutant order
- Delineate clonal trajectories and architecture from HSC->CH->myeloid malignancies


Linde Miles


Bobby Bowman

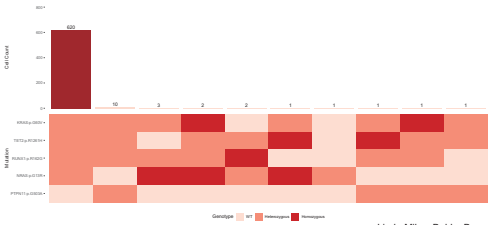
Single Cell Cell DNA Sequencing in Clonal Hematopoiesis



- Analysis of CH patients with multiple mutations to provide definitive evidence of clonal architecture and mutant order
  - mutational evolution in a single clone vs. expansion of >1 clone->incidence, clinical relevance

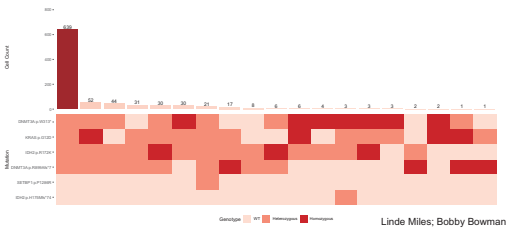
What We've Learned So Far With Single Cell DNA Sequencing

1. The majority of AML patients have 1-2 dominant clone(s)



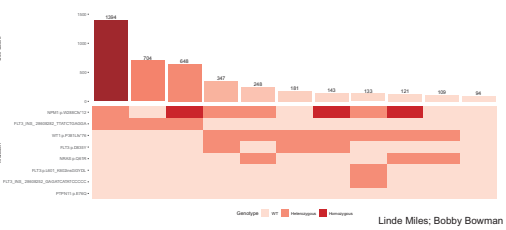
Linde Miles; Bobby Bowman

2. Patients with ≥ 2 mutations in epigenetic modifiers – mutations are most commonly in the same cell and same dominant clone->can track order of mutational acquisition



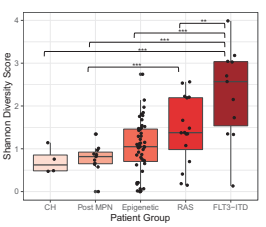
Linde Miles; Bobby Bowman

3. Patterns assumed from biological studies hold true – FLT3-ITD, FLT3-TKD, RAS pathway mutations mutually exclusive



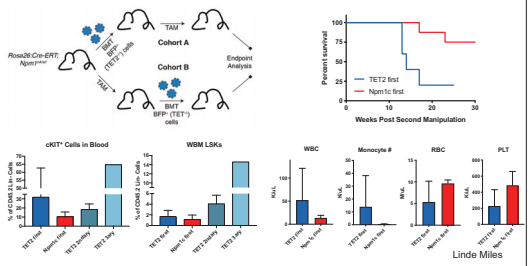
Linde Miles; Bobby Bowman

4. Clonal Diversity Increases with Disease Progression/Evolution



Linde Miles; Bobby Bowman

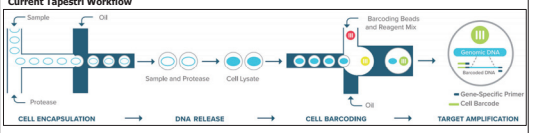
Does Mutational Order Matter: Mutational Order Modulates Transformation by Concurrent TET2/NPM1c Mutations



Linde Miles

Where We're Going->Single Cell Mutational/Cell Surface Marker Expression

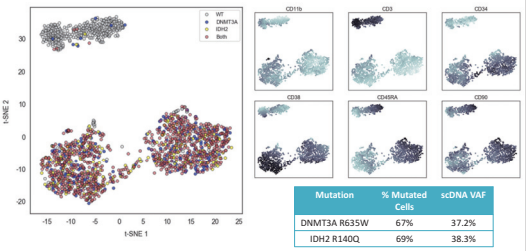
Current Tapestri Workflow



- Merge Ab-Seq technology with Tapestri workflow
- Stain cells with oligo-conjugated antibodies
- Perform library prep on both protein-derived library and DNA-derived library

Mission Bio

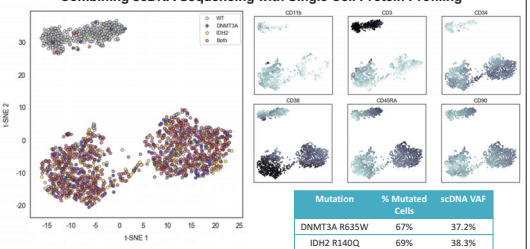
Combining scDNA Sequencing with Single Cell Protein Profiling



Mutation	% Mutated Cells	scDNA VAF
DNMT3A R635W	67%	37.2%
IDH2 R140Q	69%	38.3%

Linde Miles; Aik Ooi (MB); Pedro Mendez (MB)

Combining scDNA Sequencing with Single Cell Protein Profiling



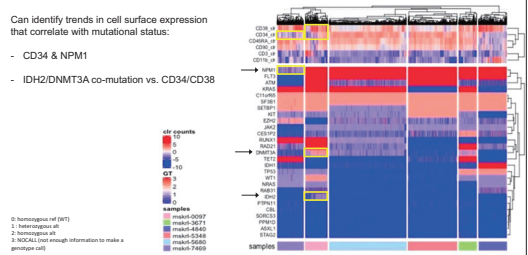
Mutation	% Mutated Cells	scDNA VAF
DNMT3A R635W	67%	37.2%
IDH2 R140Q	69%	38.3%

Linde Miles; Aik Ooi (MB); Pedro Mendez (MB)

Integrated Analysis of Surface Marker/Mutational Data

Can identify trends in cell surface expression that correlate with mutational status:

- CD34 & NPM1
- IDH2/DNMT3A co-mutation vs. CD34/CD38



Lessons from Functional Studies of CH Alleles to Date

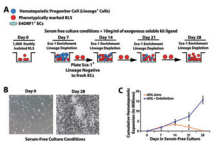
- DNMT3A, TET2, ASXL1 loss leads to enhanced stem/progenitor numbers in vivo and to myeloid bias->critical features of CH
  - Variable phenotype in classical self-renewal assays
  - Not enough work done on specific mutations seen in CH/myeloid malignancies
- Murine studies have been the backbone of mechanistic work to date->need similar work in human systems (though murine phenotypes are consistent!)
- Little is known about what are the actual downstream targets which mediate enhanced fitness/self-renewal by these specific mutations->bulk RNA has not been highly instructive to date

Need for Better In Vitro Systems for CH/Leukemia Biology

- Murine/Human hematopoietic cells historically do not culture well
- Other than MLL-derived models, leukemia cells do not transplant back into recipients after extended culture/transduction
- Many biologic/therapeutic perturbations have longer-term readouts than current culture systems except methocellulose which has limited throughput/scale

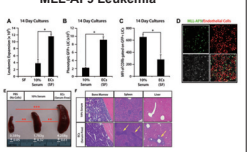
Extended Culture over Bone Marrow Endothelial Cells Maintains Stem Cell Self-Renewal

Normal HSPC



Butler et al Rafii, Cell Stem Cell 2010

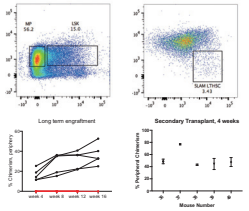
MLL-AF9 Leukemia



Poulos et al, Exp Hema 2014

Jason Butler, Shahin Rafii

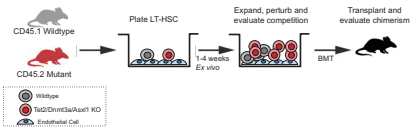
BMEC Cultures Preserve/Expand LT-HSCs With Preserved Self-Renewal



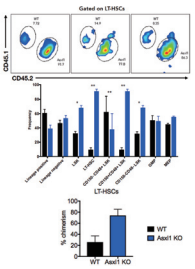
- Can optimize expansion/transplantability by altering cytokines in culture
- Robust engraftment in secondary/tertiary transplants

Bobby Bowman

Assessing Relative Impact of CH Alleles on Fitness in Competitive BMEC Assays

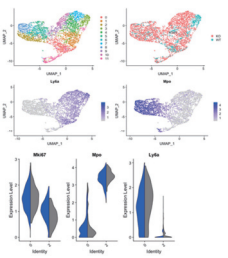


ASXL Loss Promotes Expansion of LT-HSC>Progenitors



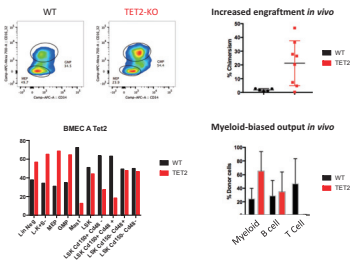
- Can read out relative fitness of mutant/WT cells in vitro->in vivo
- Can be done in competitive setting with full assessment of hematopoietic ontogeny

scRNA-seq of ASXL1 KO/WT BMEC Cultures

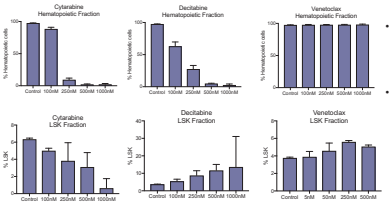


- Can see differences in specific cell populations in BMEC cultures
- Higher Ki67, lower MPO in ASXL1 KO myeloid progenitors

TET2 KO Cells Have Distinct Phenotype In BMEC-> In Vivo



Therapeutic Studies in BMEC Co-Cultures



- Assess efficacy of a panel of anti-leukemic therapies in defined CH/AML genotypes
- Read out mutant vs. wild-type impact on viability, differentiation including in defined stem/progenitor states

All experiments can proceed from ex vivo->in vivo readouts given transplantability is maintained

Lessons from BMEC Studies to Date

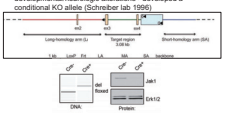
- Can investigate the biological function of specific CH/myeloid malignancy alleles in vitro on an extended time-frame, allowing studies of differentiation, self-renewal
- Can maintain in vivo propagation after extended culture->allows for in vitro perturbations followed by longer term in vivo readouts
- Can co-culture mutant/wild-type cells and look at relative effect of specific perturbations (therapeutics, CRISPR) on specific genotypes in vitro->in vivo
- Can use this as a system to efficiently delete/activate knock-in/knock-out alleles

How Can We Therapeutically Target Clonal Hematopoiesis and Prevent Leukemia?

- Therapies which restore wild-type CH TSG function (vitamin C in Tet2-mutant patients) or target mutant specific dependencies
- Therapies which target mutant oncogenes which drive CH (JAK2V617F, IDHm)
- Reduce use of chemoradiotherapy in solid tumor patients at highest risk of leukemia->need prospective studies
- Alter the fitness landscape and reduce the clonal advantage of mutant CH clones
  - Microbial induced inflammation increases fitness advantage of TET2-mutant CH (Jabri)
  - Can we specifically target inflammation and abrogate the fitness advantage of CH clones?
    - Small molecules which attenuate inflammation
    - Life-style/exercise interventions (Lee Jones)

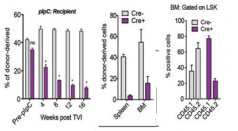
Studies of JAK1 loss in the Hematopoietic Compartment->Potential New Therapeutic Opportunity in CH?

- Germline *Jak1* knock-out mice die shortly after death due to developmental/neurologic alterations->developed a conditional KO allele (Schreiber lab 1996)



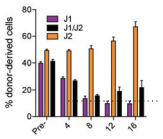
Kleppe et al. Cell Stem Cell 2017

JAK1 Loss Results in Reduced Competitive Transplantation Capacity



- Similar results with RosaCreRT – not a pIpC effect
- Complete absence of JAK1-deficient stem cells in secondary/serial transplant assays and marked reduction in colony output in vitro
- Reduced survival/protection in setting of 5FU-induced myelosuppression

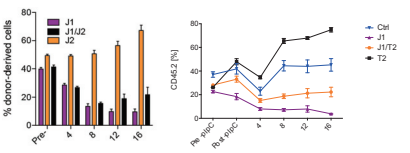
Constitutive Jak2 activation Cannot Fully Rescue Jak1-mediated Stem Cell Defect



- Polycythemic disease in primary mice is minimally affected by JAK1 loss
- Transplantablity of disease is markedly attenuated
- Complete loss of MPN cells in secondary->tertiary recipients

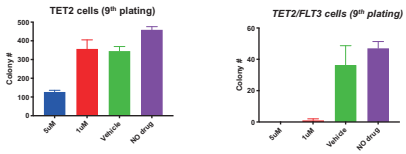
Conclusion: JAK1 is required for survival of JAK2-mutant MPN stem cells

How do we Target TET2 Mutant Pre-Leukemic/Leukemic cells: JAK1/cytokine Signaling



- JAK1 loss abrogates competitive advantage of TET2-mutant stem cells
- Clinical JAK1 (JAK2 sparing) inhibitors are in clinical development->can see if genetic requirement translates to pharmacologic inhibitors

JAK1 Inhibition Reduces Viability of TET2 and TET2/FLT3 Mutant Cells in Vitro



- In vivo studies currently ongoing in TET2-mutant stem cells and TET2-mutant leuekemia cells with aim to move to clinical trials in CH setting
- Testing in myeloid malignancies->including in combination with chemotherapy to see if cytokine/JAK1 signaling is used to allow AML cells to survive in setting of therapeutic stressors

Conclusions

- Clonal hematopoiesis is common in aging healthy subjects and in patients with concurrent solid tumors
  - Likely contribute to pathogenesis of many aging associated diseases
  - Common pre-leukemic state
- The mechanisms by which CH mutations promote clonal expansion and leukemic transformation in an aging hematopoietic environment remain to be delineated
- There may be specific therapeutic dependences in CH which can be used to abrogate this interaction and to reduce the risk of secondary sequelae including secondary malignancies, cardiovascular events, and other aging-related diseases

Acknowledgements

Levine Lab
- **Bobby Bowman**
- **Linde Miles**
- **Michael Waarts**
- **Wenbin Xiao**
- **Pablo Sanchez Vella**
- **Aaron Viny**
- Alan Shih
- Andrew Dunbar
- Fredericke Pastore
- Sheng Cai
- Tiffany Merlinsky
- Nicole Delgaudio
- Shira Redlich
- Isabelle Csete

MSKCC
- **Mike Berger**
- **Ahmet Zemir**
- **Kelly Bolton**
- **Larry Norton**
- **Elizabeth Comen**
- **Elli Papaemanuil**
- **Luis Diaz**

- Jason Butler (Hackensack)
- Sara Meyer (Jefferson)
- Martin Carroll (Penn)
- Iannis Aifantis (NYU)

Mission Bio
- Robert Durruthy-Durruthy
- Aik Ooi
- Pedro Mendez

NCI (R35, RO1), NIH (Mouse Models U54)
MSK Precision Interception Program
Waxman Foundation

References

1. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat Genet. 2012;44(11):1179-1181.
2. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med. 2014;20(12):1472-1478.
3. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;371(26):2488-2498.
4. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;371(26):2477-2487.
5. McKerrell T, Park N, Moreno T, et al. Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis. Cell Rep. 2015;10(8):1239-1245.
6. Jaiswal S, Natarajan P, Silver AJ, et al. Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. N Engl J Med. 2017.
7. Coombs CC, Zehir A, Devlin SM, et al. Therapy-Related Clonal Hematopoiesis in Patients with Non-hematologic Cancers Is Common and Associated with Adverse Clinical Outcomes. Cell Stem Cell. 2017;21(3):374-382 e374.
8. Abdel-Wahab O, Gao J, Adli M, et al. Deletion of Asxl1 results in myelodysplasia and severe developmental defects in vivo. J Exp Med. 2013;210(12):2641-2659.
9. Ortmann CA, Kent DG, Nangalia J, et al. Effect of mutation order on myeloproliferative neoplasms. N Engl J Med. 2015;372(7):601-612.
10. Kleppe M, Spitzer MH, Li S, et al. Jak1 Integrates Cytokine Sensing to Regulate Hematopoietic Stem Cell Function and Stress Hematopoiesis. Cell Stem Cell. 2017;21(4):489-501 e487.
11. Butler JM, Rafii S. Generation of a vascular niche for studying stem cell homeostasis. Methods Mol Biol. 2012;904:221-233.

Ross L. Levine, MD

2007-2011	Assistant Member, Memorial Sloan Kettering Cancer Center, Assistant Professor of Medicine, Weill Cornell Medical College
2011-2014	Associate Member, Memorial Sloan Kettering Cancer Center
2011-2015	Associate Professor of Medicine, Weill Cornell Medical College
2014-present	Member, Memorial Sloan Kettering Cancer Center, Tenure, Full Time
2015-present	Professor of Medicine, Weill Cornell Medical College
2015-present	Center for Epigenetics Research, MSKCC
2016-present	Director, MSK Center for Hematologic Malignancies

CELL COMPETITION DURING CANCER DEVELOPMENT

Yasuyuki Fujita
Division of Molecular Oncology
Institute for Genetic Medicine, Hokkaido University
Kita-15, Nishi-8, Kita-ku, Sapporo 060-0815, Japan
(yasu@igm.hokudai.ac.jp)

At the initial step of carcinogenesis, transformation occurs in a single cell within an epithelial sheet, and the emerging transformed cells grow while being surrounded by normal epithelial cells. However, it was not clear what happens at the boundary between normal and transformed cells. Using newly established cell culture and mouse model systems, we have shown that various phenomena can occur at the interface between normal and transformed epithelial cells [1-3]. For example, when Ras- or Src-transformed cells are surrounded by normal epithelial cells, various signaling pathways are activated in the transformed cells and they are often eliminated from the apical surface of the epithelial monolayer (Figure 1) [4,5]. These phenomena are not observed when transformed cells alone are present, suggesting that the presence of surrounding normal cells affects the signaling pathways and fate of transformed cells. Furthermore, we have demonstrated that normal epithelial cells can recognize and actively eliminate neighboring transformed cells and named this process EDAC (Epithelial Defense Against Cancer) (Figure 2) [6].

For cancer progression, an additional oncogenic mutation should occur within transformed clones, acquiring more malignant phenotypes. During cancer development, double-transformed cells would expand their territory while being surrounded by single-transformed cells; however, it remains elusive what occurs at the boundary between them. Recently, we have found that when RasV12 expression is induced within a monolayer of Scribble-knockdown epithelial cells, the substantial number of Scribble-knockdown cells surrounding the RasV12/Scribble-knockdown cells undergo apoptosis with fragmented and/or condensed nuclei. Moreover, the dead Scribble-knockdown cells are often engulfed by the neighboring RasV12/Scribble-knockdown. These results suggest that the sequential accumulation of oncogenic mutations can affect the consequence of cell competition.

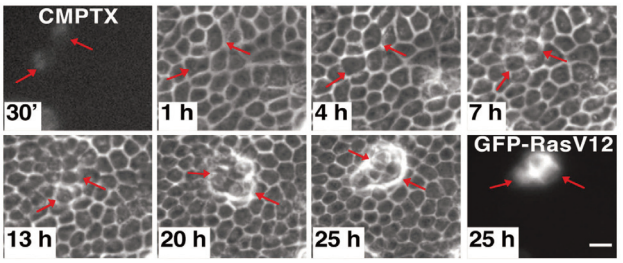


Figure 1 Apical extrusion of RasV12-transformed cells from a monolayer of normal epithelial cells. Arrows indicate RasV12-transformed cells that are apically extruded.

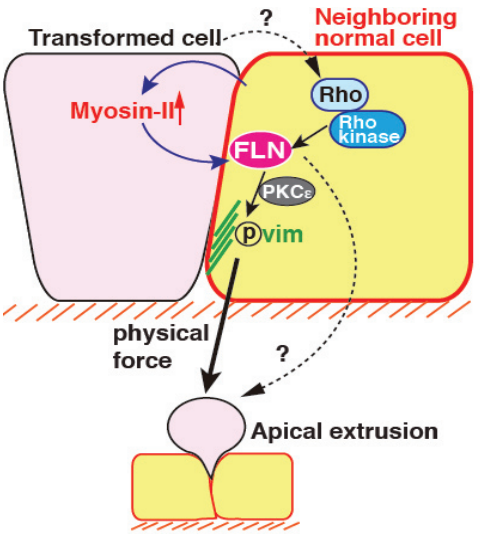


Figure 2 Schematics for EDAC (Epithelial Defense Against Cancer)

A series of recent studies using next-generation sequencing have revealed that in various epithelial tissues of the adult human there are a number of precancerous lesions with a single or double oncogenic mutation(s) that apparently look normal. Indeed, this issue was intensively discussed between the speakers and audience during the symposium. The notion that epithelia possess the anti-tumor activity EDAC implies that cell competition study can be potentially applied to cancer prevention: early diagnosis and prophylactic eradication of these precancerous lesions. Several cytosolic proteins such as filamin and vimentin accumulate at the interface between normal and transformed cells [6], suggesting the presence of plasma membrane proteins that recruit them to the intercellular adhesion sites. If such membrane proteins are identified, they can be used as a boundary marker to detect the outer margin of pre-malignant cell groups. The establishment of

diagnostic scheme for precancerous lesions would profoundly influence pathological and clinical science. It will enable us not only to detect precancerous lesions in our bodies, but also to monitor their fate upon prophylactic treatment. That would boost the search for chemical drugs against the early stage of tumors that has just recently begun. For this end, EDAC can be a potent candidate for drug target. Indeed, a cell competition-based high-throughput screening platform has been established for chemical compounds that enhance elimination of transformed cells from the monolayer of normal epithelial cells [7]. Further advance of cell competition studies would create a brand-new dimension in cancer biology and clinical science.

References

1. Maruyama, T. and Fujita, Y. Cell competition in mammals – novel homeostatic machinery for embryonic development and cancer prevention. *Current Opinion in Cell Biology* 2017, 15;48:106-112.
2. Sasaki, A., Nagatake,T., Egami, R., Gu, G., Takigawa, I., Ikeda, W., Nakatani, T., Kunisawa, J. and Fujita, Y. Obesity suppresses cell competition-mediated apical elimination of RasV12-transformed cells from epithelial tissues. *Cell Reports* 2018, 23(4):974-982.
3. Kon, S., Ishibashi, K., Katoh, H., Kitamoto, S., Shirai, T., Tanaka, S., Kajita, M., Ishikawa, S., Yamauchi, H., Yako, Y., Kamasaki, T., Matsumoto, T., Watanabe, H., Egami, R., Sasaki, A., Nishikawa, A., Kameda, I., Maruyama, T., Narumi, R., Morita, T., Sasaki, Y., Enoki, R., Honma, S., Imamura, H., Oshima, M., Soga, T., Miyazaki, J., Duchen, M. R., Nam, J.-M., Onodera, Y., Yoshioka, S., Kikuta, J., Ishii, M., Imajo, M., Nishida, E., Fujioka, Y., Ohba, Y., Sato, T., and Fujita, Y. Cell competition with normal epithelial cells promotes apical extrusion of transformed cells through metabolic changes. *Nature Cell Biology* 2017, 19(5):530-541.
4. Hogan, C., Dupré-Crochet, S., Norman, M., Kajita, M., Zimmermann, C., Pelling, A.E., Piddini, E., Baena-López, L.A., Vincent, J. P., Hosoya, H., Itoh, Y., Pichaud, F. and Fujita, Y. Characterization of the interface between normal and transformed epithelial cells. *Nature Cell Biology* 2009; 11 (4), 460-467.
5. Kajita, M., Hogan, C., Harris, A. R., Dupre-Crochet, S., Itasaki, N., Kawakami, K., Charras, G., Tada, M., and Fujita, Y. Interaction with surrounding normal cells influences signaling pathways and behavior of Src-transformed cells. *Journal of Cell Science* 2010; 123 (Pt2): 171-80.

6. Kajita M., Sugimura, K., Ohoka, A., Burden, J., Suganuma, H., Ikegawa, M., Shimada, T., Kitamura, T., Shindoh, M., Ishikawa, S., Yamamoto, S., Saitoh, S., Yako, Y., Takahashi, R., Okajima, T., Kikuta, J., Maijima, Y., Ishii, M., Tada, M., and Fujita, Y. Filamin acts as a key regulator in epithelial defence against transformed cells. *Nature Communications* 2015; 5:4428
7. Yamauchi, H., Matsumaru, T., Morita, T., Ishikawa, S., Maenaka, K., Takigawa, I., Semba, K., Kon, S. and Fujita, Y. (2015) The cell competition-based high-throughput screening identifies small compounds that promote the elimination of RasV12-transformed cells from epithelia. *Scientific Reports*, 5:15336. doi: 10.1038/srep15336.

Yasuyuki Fujita, MD, PhD

2002-2011	Group Leader, MRC, LMCB, University College London
2010-present	Professor, Hokkaido University, Institute for Genetic Medicine, Division of Molecular Oncology

NEW CANCER VULNERABILITIES AND THERAPEUTICS IN THE RNA WORLD

Trey Westbrook
Therapeutic Innovation Center
Department of Molecular & Human Genetics
Baylor College of Medicine
Houston, TX 77030, USA
(thomasw@bcm.edu)

The Westbrook team leverages functional genomic and chemical biology approaches to discover the gene networks driving breast cancer pathogenesis as well as tumor-selective vulnerabilities. During tumorigenesis, breast cancers acquire genetic alterations that confer widespread changes in transcriptional, metabolic, and biosynthetic processes. Such processes fuel uncontrolled proliferation and other hallmarks of cancer. However, these large-scale changes in the synthesis of macromolecules like RNA and protein also create new dependencies in breast cancer and other malignancies. Recently, the Westbrook lab and others discovered a new cellular stress in cancer that we term RNA splicing stress, or RSS (see Kessler et al, Science; Hsu et al, Nature). Many oncogenes and tumor suppressors drive collateral RSS, and genetic or pharmacologic inhibition of RNA processing enzymes can enhance this stress, thus tipping the balance toward selective cancer cell death and inhibiting progression of primary and metastatic breast cancer. Importantly, new therapeutics that prime RSS have been developed and are advancing in the clinic. In the current study, we have identified a surprising mechanism by which spliceosome-targeted therapies engage the immune system and selectively kill breast cancers (Figure 1). Specifically, we have discovered that spliceosome-targeted therapies induce a tumor-selective activation of antiviral signaling and downstream recruitment of adaptive immune compartments including CD8+ CTLs. This presentation will elaborate on the mechanisms of RSS, the cancer genomic indications that drive RSS, and how this tumor-intrinsic stress can be leveraged therapeutically for breast cancer patients.

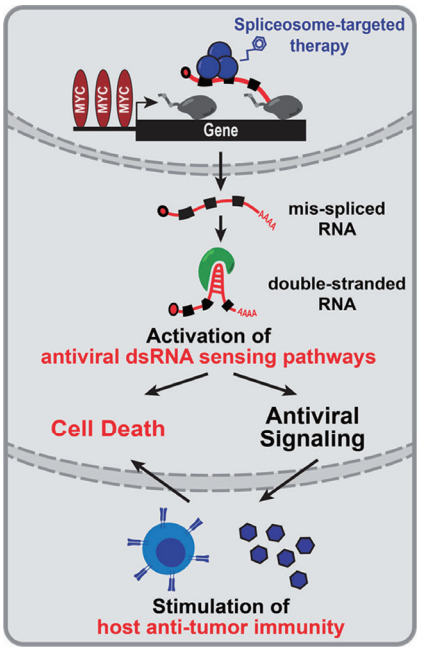


Figure 1 Spliceosome-targeted therapies (STTs) kill triple-negative breast cancer (TNBC) through antiviral (tumor-cell autonomous) signaling and downstream recruitment of adaptive anti-tumor immune cells.

Trey Westbrook, PhD

1997-2002	University of Rochester, School of Medicine, Program: Molecular Virology & Immunology
2003-2007	Postdoctoral Fellow (Mentor: S. Elledge) Dept. of Genetics, Harvard Medical School
2008-present	Professor, Dept. of Molecular & Human Genetics, Baylor College of Medicine
2017-present	Executive Director, Therapeutic Innovation Center (THINC), Baylor College of Medicine

CONCLUDING REMARKS

Hiroyuki Mano
Center for Cancer Genomics and Advanced Therapeutics/Research Institute
National Cancer Center
5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
(hmano@ncc.go.jp)

On 12th–14th November 2019, we had a pleasure to hold the 50th Commemorative International Symposium of The Princess Takamatsu Cancer Research Fund with 30 distinguished speakers and about 200 of discussants. We cancer researchers deeply appreciate the long-standing support from The Princess Takamatsu Cancer Research Fund in many activities. To celebrate this 50th Symposium, the organizers decided to make this event special, not dealing with a specific topic, but including a wide range of areas from ground-breaking basic research to cutting-edge precision medicine techniques. The main theme of the 50th Symposium was thus determined as "*New Horizons for Cancer Research and Precision Medicine*". It was also our privilege to welcome Dr. Margaret Foti, the CEO of American Association for Cancer Research (AACR). It was also our joy to award The Nakahara Memorial Lecture Prize to Dr. Kohei Miyazono for his unique and world-leading research of the TGF-β world.

The distance from basic research to patients has never been closer than today. The way to diagnose and treat cancer patients is incessantly revolutionized, and such accomplishments are swiftly translated to the clinics. In our Symposium, a novel and comprehensive approach of precision medicine for pediatric cancer in USA was demonstrated, and a nation-wide precision medicine initiative under the Japanese health insurance system was discussed. Also, the results of large-scale clinical studies to stratify breast cancer based on gene expression profiles were demonstrated, proving the utility of the outcome prediction with expression patterns.

Many facets of cancer genome/epigenome were discussed. The mismatch repair system was shown to play a critical role in producing neoantigens and to be potentially regulated

by therapeutic compounds. Chronic inflammation was proved to be a driver in producing somatic mutations in colorectal carcinoma. Further, genomic/epigenomic status enabled the classification of the tumors of hepatic lineage. Variants of histone H3 possess recurrent somatic mutations in some brain tumors, and the role of such genetic anomalies was hypothesized to shift global gene expression profiles. Resistance to targeted drugs for cancer is almost inevitable. We discussed how secondary mutations responsible for drug resistance can be monitored and, potentially, be overcome.

Although cancer immunology is probably the most transformed field in the last decade both in research and clinics, lack of reliable biomarkers and presence of untargetable tumors is still the major hurdle in this field. So, many innovative research activities were presented in our Symposium, from biomarker analyses in tumor microenvironment to novel CAR-T therapies. Further novel approaches for single cell bioimaging were proposed.

Noncoding RNAs have been shown to play a plethora of function in onset and progression of cancer. MicroRNAs can, for instance, affect microenvironment through extracellular vesicles, and some long noncoding RNAs may regulate cancer metabolism and metastases. *PIK3CA* is one of the most frequently mutated genes in cancer, and many inhibitors have been tried to suppress the growth of tumors with activating mutations within *PIK3CA*. Recent studies indicate that treatments with such inhibitors result in an increase in blood glucose levels, thereby stimulating insulin receptors and inducing resistance to the inhibitors. Other metabolites such as deoxycholic acid were shown to confer carcinogenesis. Establishing cancer model systems is critical to simulate multistep carcinogenesis and to screen effective drugs. In addition to cell lines, many other systems have been developed to recapitulate cancer environment in non-human conditions. In our Symposium, insights obtained from such model systems were presented.

Lastly, but never the least, as the chairman of the organizing committee, I deeply thank our co-organizer, Dr. Elaine R. Mardis, for her dedication to make this Symposium so special. I also thank the other members of the committee to support this Symposium; Drs. Kohzo Imai, Masanobu Oshima and Ryuzo Ueda. We owe entirely our Secretary General, Dr. Toshikazu Ushijima, for running the Symposium flawlessly. We are deeply thankful to the office of The Princess Takamatsu Cancer Research Fund and its chairman Dr. Takao Sekiya to support this wonderful Symposium. We also appreciate young researchers in National Cancer Center and Kanazawa University for helping the logistics of this Symposium. We believe this meeting was priceless for such young scientists as well.

Finally, we deeply appreciate all speakers and discussants who made our Symposium so memorable. We hope this Symposium has evoked further discussion and collaboration.