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ONCO-METABOLOMICS; A NEW CLUE TO UNDERSTAND CARCINOGENESIS, CANCER BIOLOGY AND TO DEVELOP NOVEL DIAGNOSTICS AND THERAPEUTICS

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CANCER METABOLISM: BACK TO THE FUTURE

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1. Introduction

Why has it been so difficult to cure cancer? The answer lies in our inability to define all the elements that contribute to the transformation and survival of tumor cells, and challenges in dissecting the body's responses to these malignant growths. As far back as 1924, Otto Warburg proposed that "The cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar", a concept now widely known as the "Warburg effect"[1,2]. Because this change would not alter the "antigenic face" of a cancer cell, the immune system would not be able to recognize these aberrant cells and remove them, necessitating externally imposed therapies. Oncologists of the day therefore focused on eliminating all fast-replicating cells, normal or cancerous, by radiation or chemotherapy, a trend that remained firmly in place into the 1990s (Table 1). In the 1970s, Warburg's hypothesis was sidelined as scientists became convinced that

Table 1 Chemotherapeutic agents approved.

Year Approved	Agent
1942	Nitrogen mustard
1948	6-Mercaptopurine
1958	Methotrexate
1959	Cyclophosphamide
1975	5-Fluorouracil
1978	Cisplatin
1992	Pacitaxel
1996	Gemcitabine
1996	Topotecan
2004	Pemetrexed

the underlying cause of cancer was aberrant function of either oncogenes or tumor suppressor genes. This mindset led to the development of numerous therapeutic agents targeting specific oncogenes or other relevant molecules or structures (Table 2). Although anti-oncogene agents are undeniably helpful, it has become clear that the cancer cell genome is too varied and oncogenes too numerous for these strategies to be able to eradicate all tumors. Researchers have instead returned to Warburg's hypothesis in their search for ways to disrupt tumor cell metabolism and mitosis.

Table 2 Targeted agents approved.

Year of approval	Target	Drug name
1998	Her-2	Herceptin
2001	Bcr-abl	Imatinib
2003	EGFR	Gefitinib
2003	Proteasome	Bortezomib
2004	VEGF	Bevacizumab
2006	HDAC	Vorinostat
2007	mTOR	Temsirolimus
2011	CTLA-4	Ipilimumab
2011	ALK	Crizotinib
2011	B-Raf	Vemurafenib
2012	Hedgehog	Vismodegib
2013	Btk	Ibrutinib
2014	PI3K-δ	Idelalisib
2014	anti-PD1	Nivolumab
2014	PARPi	Olaparib
2015	CDK4/6	Abemaciclib

2. Targeting Cancer Cell Metabolism

In addition to their genetic and epigenetic alterations, cells undergoing transformation implement specific metabolic adaptations that are induced by their altered microenvironment. These adaptations lead to upregulation of signaling pathways that are not inherently tumorigenic but allow developing tumor cells to survive under conditions that would kill normal cells[3]. This "metabolic addiction" of precancerous and ultimately cancerous cells provides new opportunities for therapeutic intervention, since normal cells, which have not had to endure the same stresses, should be unaffected by agents targeting cancer cell metabolic adaptations.

2.1 Targeting Carnitine Palmitoyltransferase-1C (CPT1C)

CPT1C, a brain-specific metabolic enzyme, may be involved in tumor cell metabolic adaptation to heightened stress[4]. CPT1C expression correlates inversely with mTOR pathway activation in tumor cells[4,5] and contributes to rapamycin resistance in murine primary tumors. CPT1C is overexpressed in human non-small-cell lung carcinomas[4], and increases fatty acid oxidation, ATP production, and resistance to glucose deprivation or hypoxia in cultured human cancer cells[4]. CPT1C depletion reduces tumor growth in mouse xenograft models[4] and increases mouse survival in a neurofibromatosis type I tumor model[6-8]. CPT1C is a p53 target gene, and CPT1C expression is induced by hypoxia or glucose deprivation in a p53- and AMPK-dependent manner[8]. Thus, p53 may initially protect cells from metabolic stress via CPT1C induction, but excessive CPT1C expression then promotes carcinogenesis (Figure 1)[8]. Because CPT1C expression is normally restricted to the brain, and most drugs cannot penetrate the blood–brain barrier, a small-molecule CPT1C inhibitor may be ideal for treatment of hypoxic and otherwise treatment-resistant cancers[5,8].

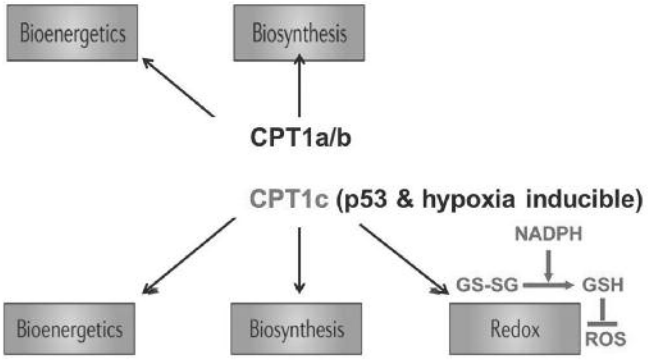

Figure 1 The role of CPT1c in tumor cell metabolic adaptation.

2.2 Targeting Mutated Isocitrate Dehydrogenases (IDH)

IDH1 and IDH2 govern the NADP/NADPH ratio in a cell's cytoplasm and mitochondria, respectively. IDH1/2 normally convert isocitrate to α-ketoglutarate (αKG) while reducing NADP to NADPH and liberating CO_2[9]. Oncogenic IDH mutations were first discovered during cancer genome sequencing projects[10-12]. IDH1 mutations occur at high frequency in glioblastoma multiforme (GBM)[11], acute myeloid leukemia (AML)[10], cholangiocarcinoma[13,14], and chondrosarcoma[15], and less frequently in melanoma, NSLLC, and prostate and colon cancers[16]. IDH2 mutations[9,17] occur in cholangiocarcinoma[13,14], myelodysplastic syndrome (MDS) and myeloproliferative disorder (MPD)[18-20], AML[10],

chondrosarcoma[15], angioimmunoblastic T-cell lymphoma (AITL)[21], and D-2HG aciduria[22] (Figure 2). In 2009, scientists at Agios Pharmaceuticals made the breakthrough discovery that the tumorigenic effect of IDH1/2 mutations is not due to a loss-of-function of these proteins. Instead, the mutant IDH enzymes acquire a neomorphic activity in which the normal product αKG is converted to 2-hydroxyglutarate (D2HG) in a reaction that consumes, rather than produces, NADPH[9,23]. D2HG competitively inhibits 2-OG-dependent dioxygenases (2OGD)[9], more than 60 of which are involved in collagen biosynthesis, fatty acid metabolism, DNA repair, RNA and chromatin modifications, and hypoxia detection[24]. Additional potential targets of D2HG inhibition include TET proteins involved in DNA methylation, JumonjiC domain-containing histone demethylases, prolyl hydroxylases (PHD) and lysyl hydroxylases (LHD) required for collagen folding and maturation, and PHDs that regulate hypoxia-inducible factor (HIF) signaling[9].

Our group generated conditional knock-in mouse strains allowing expression of the mutant IDH1 protein from the endogenous locus[25]. Systemic expression of mutant IDH1 leads to D2HG production that is embryonic lethal[9]. When expressed solely in the myeloid compartment, mutant IDH1 causes splenomegaly, decreased bone marrow cellularity, and extramedullary hematopoiesis[25]. Mutant hematopoietic progenitors show increased hypermethylation of CpG sites and histones[25], consistent with the DNA methylation changes observed in human IDH1/2 gliomas[26] and AML[27]. In early 2014, an oral inhibitor (AG-221) of mutant IDH2 underwent clinical evaluation for patients with advanced IDH2-mutant hematologic malignancies. AG-221 treatment reduced D2HG levels and demonstrated a dose-dependent survival benefit[28]. Pursuit of a similar compound to combat IDH1-mutant cancers is ongoing.

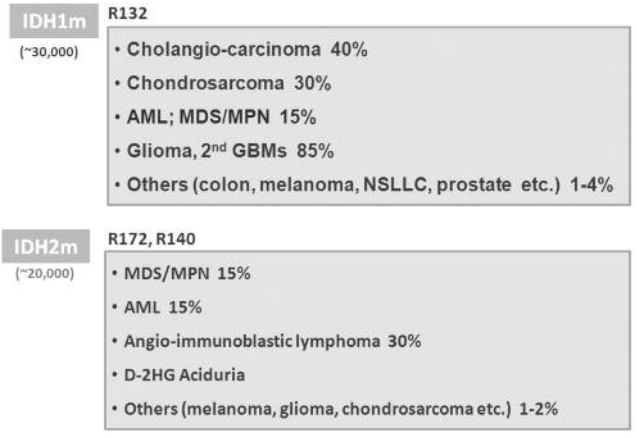

Figure 2 Percentages of patients with the indicated disorders that have the indicated IDH1 or IDH2 mutations.

3. Targeting Reactive Oxygen Species (ROS)

An important stressor in cancer cells is the level of reactive oxygen species (ROS). ROS regulation is critical for normal cellular functions and survival, and the accelerated growth of tumor cells generates increased ROS. Cancers therefore need to adjust signaling pathways linked to ROS regulation to cope with their enhanced ROS. Elevated ROS are generated by hypoxia, defective metabolism, endoplasmic reticulum (ER) stress, and oncogene activity[29]. Conversely, ROS are eliminated routinely via NADPH, glutathione and dietary antioxidants, and under stress conditions through the activation of transcription factors such as NRF2 and the activity of tumor suppressors such as BRCA1, p53, PTEN and ATM[29]. During transformation, cellular ROS levels progressively increase, triggering the cell to upregulate antioxidant pathways. Targeted therapeutics that interfere with this upregulation may thus induce apoptotic death. Alternatively, agents that increase ROS production beyond the capacity of the upregulated antioxidant mechanisms to cope may kill the tumor cell while sparing normal cells in which these pathways are not activated.

Our group has shown that ROS regulation may explain the tissue specificity of BRCA1-related cancers, which occur almost exclusively in breast and ovary. BRCA1 deficiency enhances ROS levels in breast cancer cells and impairs Nrf2-driven antioxidant pathways[30]. BRCA1 directly interacts with Nrf2 in a manner that affects Keap1-mediated Nrf2 ubiquitination, stability and activation[30]. Estrogen treatment partially restores Nrf2 levels and enhances tumor growth in the absence of BRCA1[30,31]. We hypothesize that, in tissues lacking estrogen, BRCA1 deficiency impairs Nrf2 antioxidant signaling, leading to an accumulation of ROS in BRCA1-deficient cells that kills them. However, in breast and ovary, estrogen activates Nrf2 via a mechanism that depends on PI3K–AKT and protects BRCA1-deficient cells from ROS-induced death. If such a BRCA1-deficient cell also loses PTEN, the PI3K–AKT pathway may reinforce estrogen-mediated Nrf2 signaling. Mitogenic and antioxidant pathways acting downstream of AKT, coupled with the genomic instability caused by a lack of BRCA1-mediated DNA repair, might then drive complete transformation of BRCA1-deficient cells[31] (Figure 3). Exploitation of the altered ROS regulation in these cells may point to an effective future therapy.

Other antioxidant pathways appear to play the opposite role. We have demonstrated that GCLM-driven synthesis of the antioxidant glutathione (GSH) is required for cancer initiation. Genetic loss of Gclm in mice prevents precancerous cells from undergoing transformation. Buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, has the same anticancer effect but only if delivered prior to tumor onset. This result suggests that GSH becomes dispensable during tumor progression, perhaps due to compensation by alternative antioxidant pathways. Combined inhibition of GSH and thioredoxin (TXN)

antioxidant pathways leads to synergistic cancer cell death *in vitro* and *in vivo*, pinpointing these two antioxidants as potential therapeutic targets[32]. In the clinic, use of BSO alone to inhibit GSH synthesis and treat malignancies has failed. However, we have demonstrated in mice that use of sulfasalazine (SSA) and auranofin (AUR), which are TXN inhibitors currently used to treat inflammatory diseases, can be combined with BSO to reduce tumorigenesis[32].

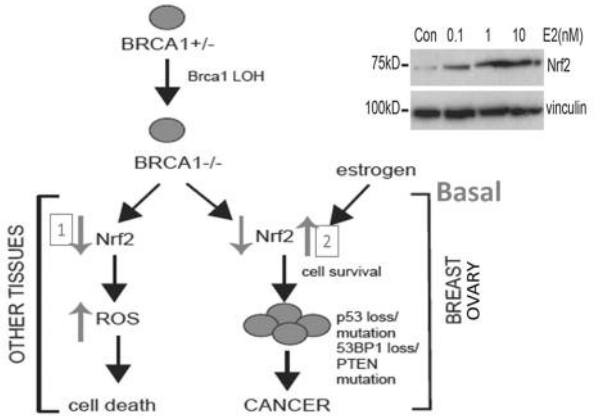

Figure 3 Model of the roles of Nrf2, estrogen, ROS and p53 in the survival of BRCA1-deficient cells in breast/ovary vs other tissues. Estrogen-mediated support of antioxidation allows precancerous BRCA1-deficient cells to survive high ROS levels and progress to breast or ovarian cancer.

4. Exploiting Cancer Cell Aneuploidy

An alteration shared by many advanced cancer cells but not found in normal cells is aneuploidy. By combining RNAi screening with gene expression analysis in human breast cancers and cell lines while focusing on cancer cell aneuploidy, we identified polo-like kinase-4 (PLK4), an enzyme critical for aneuploidy maintenance, as a promising therapeutic target[33]. Our drug discovery program culminated in the isolation of CFI-400945, a potent and selective small-molecule PLK4 inhibitor[33]. *In vitro* treatment of human cancer cells with CFI-400945 results in mitotic defects, centriole duplication and cell death[33]. In *in vivo* mouse models based on human ovarian or breast cancer xenografts, tumor growth is significantly inhibited by CFI-400945 in a manner influenced by the PTEN status of the tumor[33]. PTEN-deficient xenografts show a greater response to CFI-400945 than xenografts expressing wild type PTEN, making PTEN status a potential predictive biomarker for therapy with this first-in-class agent[33].

A second target emerging from our screen was TTK (MPS1), which is a conserved dual-specificity kinase essential for spindle assembly checkpoint (SAC) maintenance during cell

division[34]. TTK is overexpressed in many solid tumors exhibiting genomic instability or aneuploidy[35-41]. Elevated TTK levels correlate with aggressive subtypes and high histological grade[36], and are associated with adverse outcome in ER+ breast cancer[42]. Overexpression of TTK may promote both tumor initiation and survival of genomically unstable and aneuploid breast cancer cells[43,44]. TTK also has several non-mitotic roles, including participation in DNA damage signaling pathways[45-48]. We have generated a series of small-molecule TTK inhibitors[49,50] that not only have potential utility as single anticancer agents but also demonstrate synergy with taxanes *in vitro*. Taxanes trigger SAC activation but this signal is overridden by TTK inhibition, resulting in chromosome missegregation and mitotic catastrophe. This chain of events has been independently identified using a chemically distant TTK inhibitor[51] and is under investigation in a Phase I clinical trial[52]. Preclinical toxicology studies of our TTK inhibitor CFI-402257 are now complete and FDA/Health Canada IND/CTA submissions are in preparation. Phase I trials are slated for 2016 (Figure 4).

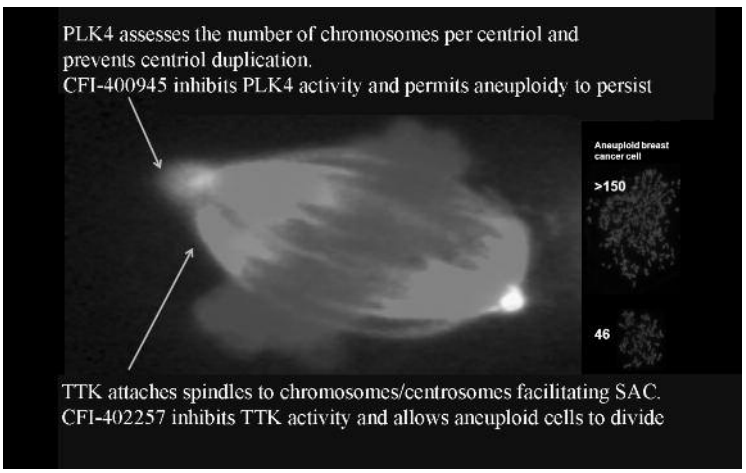

Figure 4 Image of a dividing cell showing where the indicated small-molecule inhibitors of mitosis could allow exploitation of cancer cell aneuploidy as therapy.

5. Conclusion

This presentation has briefly outlined three innovative anticancer approaches under investigation in our laboratory. By concentrating on unique aspects of tumor biology, we strive to identify strategies and targets that are applicable to a broad range of cancers and less likely to induce damaging side-effects in normal tissues. By looking back to Warburg's hypothesis, we hope to come closer to a future free of cancer.

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THE ROLE OF METABOLISM IN CANCER PROGRESSION

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Complex regulatory mechanisms enable cell metabolism to match physiological state[1]. Proliferating cells have metabolic requirements that differ from non-proliferating cells to allow production of biomass[2,3], and tissue specialization imposes further metabolic requirements. For cancer cells to proliferate and survive in a pathological tissue context they must adapt metabolism to meet the distinct requirements for cancer initiation and progression. Therefore, cancer cells likely have altered metabolism relative to most normal tissues in order to support the accumulation of biomass and to allow adaptation to cell stresses associated with the tumor environment[2,4-6]. Understanding how the nutrient requirements of cancer cells differ from that of normal tissues can define new cancer targets and suggest novel combinations of new and existing therapies[7-9].

Glucose metabolism is increased in tumors[2,4]. Most glucose consumed by cancer cells in culture is converted to lactate, while glutamine is the major source of tricarboxylic acid (TCA) cycle carbon[6,10] (Figure 1). However, nutrients levels and oxygen in cell culture differ from those found in tumors[6], and emerging data suggests that tissue of origin and environment can also have an impact on metabolic phenotypes[11-14]. To determine whether tumor tissue exhibits the same metabolic phenotype as cells in culture, and how the metabolism of tumors relates to that of normal tissues, we traced glucose and glutamine fate in tumor and normal tissue in mice[25]. Using several mouse cancer models, including lung and pancreatic cancer models driven by oncogenic *Kras* and loss of *Tp53*[15-17], we found that tumor metabolism in vivo differed from that observed in cell culture. Tumors exhibit increased lactate production from glucose, but the cancers analyzed unexpectedly used minimal glutamine. Instead glucose was a major source of TCA cycle carbon (Figure 2).

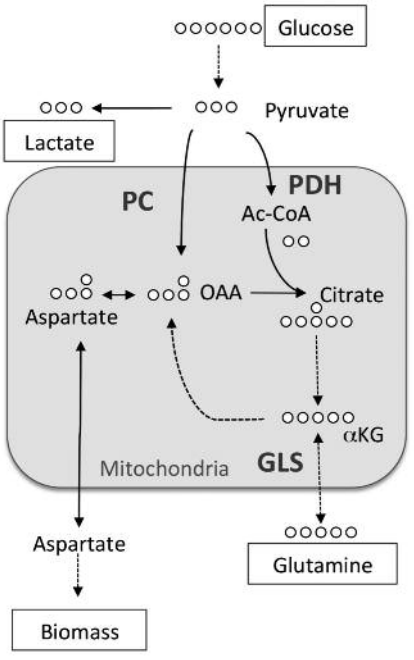

Figure 1 Glucose and glutamine in cancer cells.
A schematic showing the major fates of glucose and glutamine in central carbon metabolism, and how they can be used to generate lactate and aspartate. Most mammalian cells must synthesize aspartate, and this amino acid is required for de novo nucleotide and protein synthesis. Select enzymes considered in this study are included. (GLS, glutaminase; PC, pyruvate carboxylate; PDH, pyruvate dehydrogenase; OAA, oxaloacetate; αKG, α-ketoglutarate)

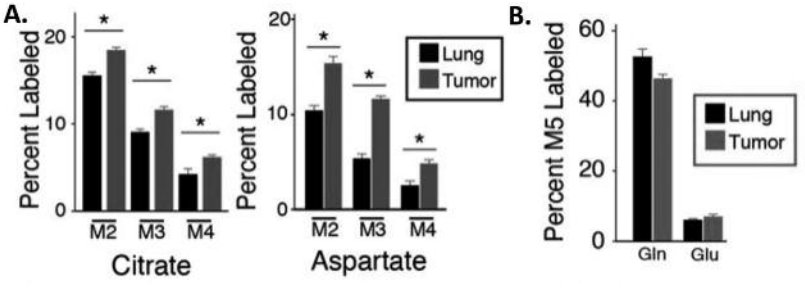

Figure 2 Nutrient use in tumors can differ from cancer cells in culture.
A. ^{13}C-glucose was infused into mice with *Kras^{G12D} Tp53$^{-/-}$* lung tumors, and the fate of glucose determined in the tumor and normal lung by mass spectrometry. Two carbon citrate and aspartate labeling (M2, M4) is indicative of glucose entry in to the TCA cycle via pyruvate dehydrogenase (PDH), while three carbon labeling of these metabolites (M3) suggests pyruvate carboxylase (PC) activity. Minimal labeling of TCA cycle intermediates from glucose is observed in lung cancer cells in culture (not shown) .
B. ^{13}C-glutamine use by lung tumors and normal lung tissue was also assessed. Unlike lung cancer cells in culture, minimal glutamate (glu) labeling (M5) is observed despite delivery of labeled glutamine (gln) . *$p<0.05$. Date adapted from Davidson et al., *submitted*.

Because the cancer cell lines generated from the autochthonous arising tumors in the mouse models exhibit a similar metabolic phenotype to other cultured cells, we questioned whether genetic adaptation to cell culture or tissue environment was responsible for the different metabolic phenotypes and re-implanted cancer cells to form tumors in syngeneic mice. The resulting tumors exhibited a similar metabolic phenotype to the autochthonous tumors, suggesting that tissue environment is an important determinant of how nutrients are used. This finding was confirmed by comparing the metabolism of human cancer cells lines and xenograft tumors derived from these cancer cells. The xenograft tumors also relied on glucose to support TCA cycle metaobolism, while the cells used to generate the tumors used glutamine in culture. This finding was evident in xenograft tumors derived from both *Kras*-mutant and *EGFR*-mutant cancers, arguing that the tissue environment is an important determinant of tumor metabolism.

Comparison of metabolism in mouse lung and pancreatic tumors that both arose as a result of $Kras^{G12D}$ expression and loss of *Tp53* show that these tumors share some common features, but also differ in the metabolism of amino acids. Pancreatic tumors rely heavily on catabolism of extracellular protein[18,19], while lung tumors appear to directly utilize some free amino acids. These findings suggest that tissue of origin can also contribute to the metabolic phenotype of cancer cells, and is consistent with previous analyses of metabolic gene expression demonstrating tumor metabolic networks are more similar to the tissue of origin than they are to tumors arising in a different organ[20].

To determine whether the differential use of glucose and glutamine in tumor and in cell culture represented context-specific metabolic dependencies, we utilized CRISPR/Cas9 to disrupt enzymes required for glutamine or glucose entry into the TCA cycle[25]. Deletion of pyruvate carboxylate (PC) had no effect on proliferation in culture, but completely blocked lung tumor formation in mice (Figure 3), findings consistent with other studies[21]. A similar dependency on pyruvate dehydrogenase (PDH) was observed in vivo, while loss of

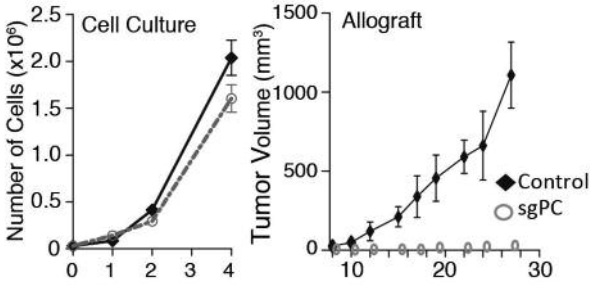

Figure 3 Understanding nutrient use in tumors can be predictive of metabolic dependencies.
Pyruvate carboxlase (PC) was delered in cancer cells derived from $Kras^{G12D}$ $Tp53^{-/-}$ lung tumors (sgPC). Proliferation of sgPC cells versus control cells was compared in culture (left) and after implantation to from allograft tumors (right).
Date adapted from Davidson et al., *submitted*.

glutaminase (GLS, required for glutamine metabolism) had no effect on tumor growth despite being required for cell proliferation in culture. These findings argue that understanding nutrient utilization can predict metabolic dependencies.

One major difference between tissues and cell culture is oxygen availability, as oxygen tensions are much lower in tissues, even when well perfused[6, 22]. A major role of respiration in cell proliferation is use of oxygen as an electron acceptor to generate aspartate[23,24]. Aspartate is more oxidized than the nutrients cells consume, so electron acceptors are needed to regenerate oxidized cofactors used for aspartate synthesis. Because aspartate is required for protein, purine and pyrimidine synthesis[3], and most mammalian cells lack the ability to acquire exogenous aspartate[23,24], the metabolism of tumors in vivo may be explained in part by differences in how tumors acquire aspartate in different environmental contexts.

The success of chemotherapies that target nucleotide metabolism demonstrates that altered cancer metabolism can be exploited to have an impact on patient outcomes[7]. In some cases drugs targeting metabolic pathways can contribute to patient cures, but because these drugs were developed and deployed based entirely on empiric evidence, how to identify new metabolic targets and rationally select patients is not known. The phenotypes we observe tracing metabolism in mouse tumor models align well with nutrient tracing studies in human tumors[14,21]. This may begin to explain why chemotherapies targeting metabolism have been successful despite selecting patients based on the cancer tissue of origin. Better defining the metabolic dependencies of tumors will uncover new metabolic enzyme targets, and could suggest how to combine new and existing drugs to improve cancer therapy.

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METABOLIC HETEROGENEITY IN CANCER CELLS AND TUMORS

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In cancer, metabolic reprogramming – the regulated alteration of metabolism in response to tumorigenic mutations and other factors – is viewed as an essential component of malignancy. Research in cancer metabolism is motivated by the hope that understanding the basis of metabolic reprogramming will stimulate the development of new approaches in cancer imaging and therapy, both of which have historically capitalized on altered metabolic states in tumors. Cancer cells use abundant nutrients like glucose and glutamine to feed metabolic pathways that promote energetics, biosynthesis, and redox homeostasis. There is now substantial information about how these pathways are regulated in conventional culture, but we know comparatively little about how metabolic phenotypes are established under more complex and stressful conditions, including intact human tumors *in vivo*. Closing this gap in knowledge should help us understand the relative roles of cell-intrinsic and -extrinsic influences on tumor cell metabolism.

I will discuss progress in understanding how metabolic phenotypes are specified in cancer cells. First, I will briefly discuss a large phenotyping effort to assess the breadth of cell-intrinsic metabolic diversity in non-small cell lung cancer (NSCLC) cell lines. By culturing over 80 cell lines under identical conditions and quantifying ~100 metabolic features in each, we generated a large, mineable database which can be used to assess metabolic diversity across the lines and identify novel correlations between metabolic and molecular features. We find that the metabolic effects of specific mutations/combinations of mutations can be observed when the data are viewed in aggregate. Examples involving carbon utilization in the TCA cycle will be discussed.

We are also making progress in analyzing patterns of nutrient utilization in living human tumors, where both cell-intrinsic and cell-extrinsic processes influence the metabolic phenotype. In humans, NSCLC is highly heterogeneous both in the genetic and environmental parameters that influence cell metabolism in culture. However, the impact of these factors on human NSCLC metabolism *in vivo* is unknown. We are conducting intra-operative infusions with ^{13}C-glucose in NSCLC patients to compare metabolism between tumors and benign lung. In addition, we perform extensive multi-parametric pre-surgical imaging to non-invasively assess areas of biological heterogeneity expected to influence the metabolic phenotype. Careful review of the imaging data prior to surgery then allows us to identify areas of heterogeneity to be sampled during the tumor resection. A fragment-by-fragment analysis of histopathology, gene expression and metabolism is then performed. We find that tumors of diverse grade, stage, histology and oncogenotype displayed enhanced glycolysis and glucose oxidation relative to the surrounding lung (Figure 1A, B). Importantly, although glycolysis appeared to be activated in the tumors,

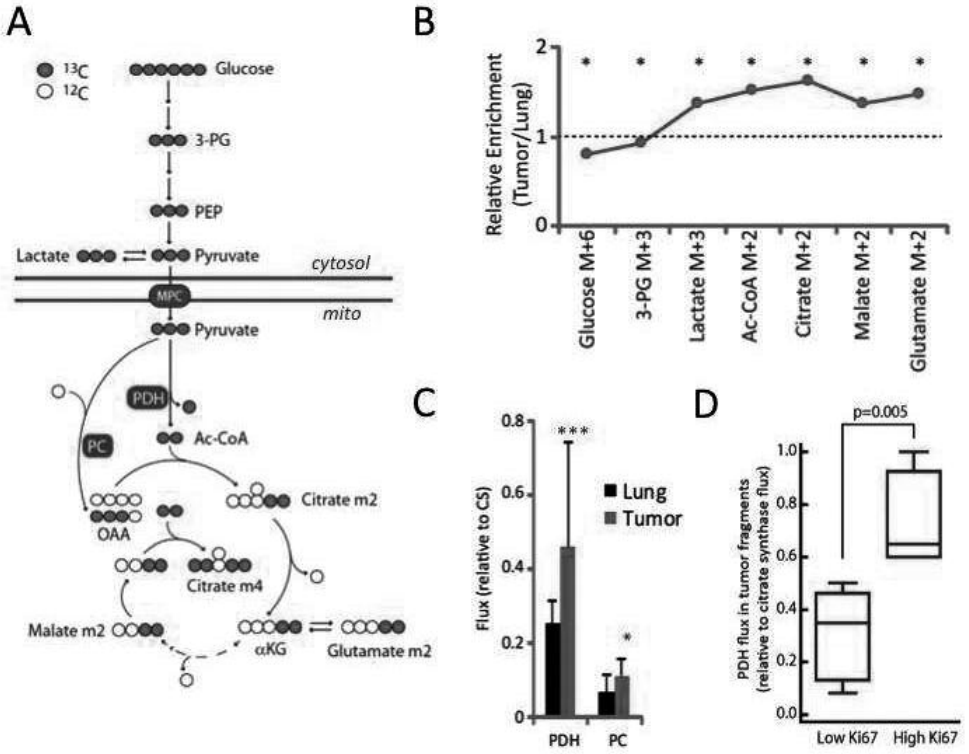


Figure 1

there was no evidence for systematic suppression of glucose oxidation, indicating that both aerobic and anaerobic glucose metabolism occur concomitantly in the same tissue *in vivo*. Metabolic flux analysis revealed that pyruvate dehydrogenase (PDH) was significantly more active in the tumors than surrounding lung (Figure 1C), and activity of this enzyme was positively correlated with the Ki67 fraction observed on quantitative histopathology (Figure 1D).

Interestingly, all tumors had evidence for oxidation of alternative nutrients in addition to glucose, as indicated by the relatively low ^{13}C enrichment in TCA cycle metabolites compared to glucose. We find evidence that both primary human NSCLC and xenografts derived from NSCLC cell lines take up lactate from the circulation and use it as a fuel for

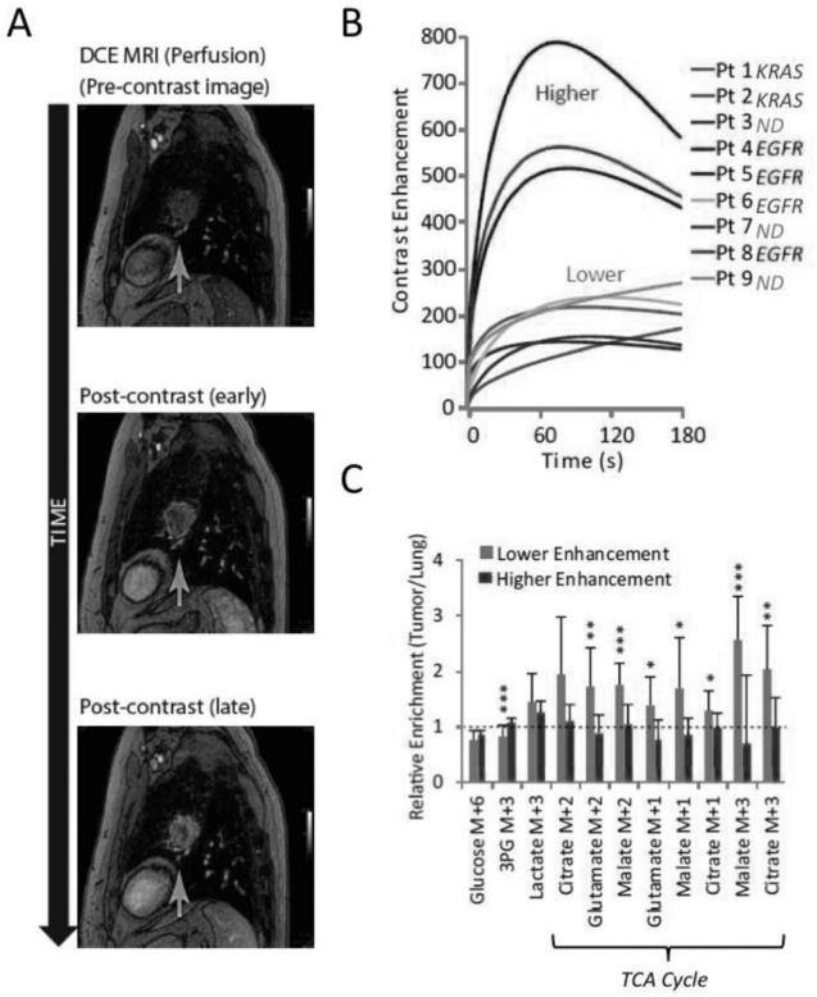


Figure 2

the tricarboxylic acid cycle. Additionally, metabolically heterogeneous regions were identified within and between tumors using dynamic gadolinium enhancement (DCE) as a surrogate for perfusion (Figure 2A, B). Regions of higher contrast enhancement (i.e. higher perfusion) demonstrated lower ^{13}C enrichment, likely reflecting contributions of unlabeled nutrients in well-perfused areas (Figure 2C). Consistent with this idea, RNA sequencing data from the same tumor fragments used for analysis of ^{13}C enrichment revealed alternative patterns of metabolic gene expression. Gene sets involving glycolysis, oxidative phosphorylation and pyruvate oxidation were enriched in tumor fragments with low DCE

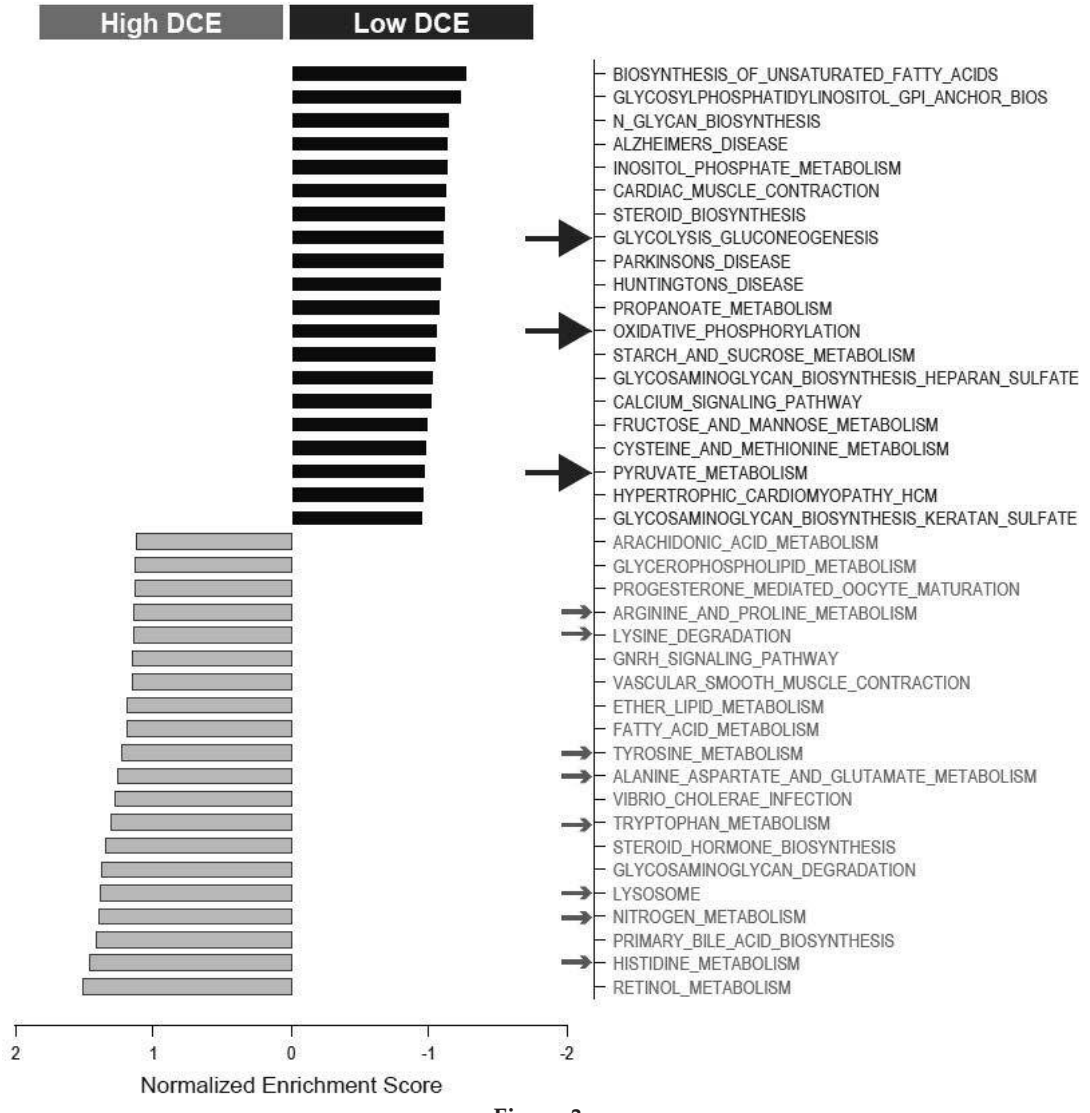
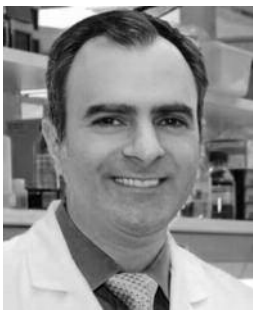


Figure 3

phenotypes (Figure 3, large arrowheads), consistent with the enhanced ^{13}C enrichment in metabolites from these tumors. In contrast, fragments with high DCE phenotypes were enriched for gene sets involving the metabolism of other nutrients, particularly nitrogenous metabolites including amino acids (Figure 3, small arrowheads). Altogether, the data suggest that heterogeneous metabolism of these NSCLC tumors is highly and predictably influenced by cell-extrinsic effects, including perfusion. They also emphasize the potential for existing and new imaging approaches to report alterations of metabolic flux *in vivo*.



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Specialty and Present Interest:
Cancer Metabolism, Metabolic Imaging, Inborn Errors of Metabolism

THE METABOLIC REGULATOR AMPK – FRIEND OR FOE IN CANCER

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The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status expressed ubiquitously in eukaryotic cells[1-3]. AMPK exists as heterotrimeric complexes comprising catalytic α subunits and regulatory β and γ subunits. In mammals, there are multiple genes encoding isoforms of each subunit (*PRKAA1/2*, encoding α1/α2, *PRKAB1/2*, encoding β1/β2, and *PRKAG1/2/3*, encoding γ1/γ2/γ3) - these combine to form up to twelve heterotrimeric combinations.

In the yeast *Saccharomyces cerevisiae*, the AMPK ortholog is required for the switch from rapid glycolysis (fermentation) and rapid growth that occurs when the glucose concentration in the medium is high, to the oxidative metabolism and slower growth that occurs when glucose runs low[4]. Fermentation is related to the Warburg effect that occurs in most rapidly proliferating tumor cells, with the exception that glycolysis in yeast results in production of ethanol rather than lactate.

AMPK heterotrimers can be activated >100-fold by phosphorylation at a conserved threonine residue (Thr172) within the α subunit kinase domain. The major upstream kinase phosphorylating Thr172 is a complex containing the protein kinase LKB1, although it can also be phosphorylated by the calmodulin-dependent kinase, CaMKKβ, in response to a rise in intracellular Ca^{2+}[2,5]. The findings that LKB1 was the principal upstream kinase provided the first links between AMPK and cancer[6,7], because *STK11* (encoding LKB1) had previously been shown to be the gene in which mutations cause an inherited susceptibility to cancer termed Peutz-Jeghers syndrome. Subjects with this syndrome carry monoallelic loss-of-function mutations in *STK11*, and develop numerous benign intestinal polyps, but also have an increased risk of developing malignant tumors at other sites[8]. Mutations in

the *STK11* gene are also quite frequent in spontaneous, non-inherited cancers, especially in non-small cell lung cancer where the frequency may be as high as 20-30%[9,10].

Unlike CaMKKβ which is Ca^{2+}-dependent, the LKB1 complex appears to be constitutively active, and modulation of the phosphorylation of Thr172 (and hence the activity) of AMPK is due instead to the fact that binding of AMP or ADP to the γ subunit of AMPK causes conformational changes that promote Thr172 phosphorylation by LKB1[11, 12], as well as inhibiting Thr172 dephosphorylation[13,14]. AMPK complexes containing γ1 or γ2 that are phosphorylated on Thr172 are also allosterically activated up to 10-fold by AMP, although not ADP[12]. All three effects of AMP binding (of which two are mimicked by ADP) are antagonized by ATP, making the AMPK system an exquisitely sensitive sensor of cellular energy status.

In 2001 it was reported that the biguanide drug metformin, now the front line agent for treatment of Type 2 diabetes, activated AMPK[15]. Metformin activates AMPK indirectly by inhibiting complex I of the respiratory chain, thus increasing cellular AMP:ATP and ADP:ATP ratios, a mechanism shared by other AMPK activators derived from traditional herbal medicines, such as berberine[16] or arctigenin[17]. Although some of the acute effects of metformin on hepatic glucose production are AMPK-independent[18], its longer-term insulin-sensitizing effects appear to be mediated by actions of AMPK on lipid metabolism[19]. Intriguingly, use of metformin to treat diabetes is associated with a reduced risk of cancer in humans[20].

Another AMPK-activating drug of interest with respect to cancer is the antifolate pemetrexed[21, 22], licensed for use in non-small cell lung cancer where AMPK may often be down-regulated due to loss of LKB1. Pemetrexed is unique among antifolates in inhibiting AICAR transformylase, the enzyme catalysing the first step in the conversion of the intermediate ZMP to purine nucleotides. It therefore activates AMPK by causing accumulation of ZMP, an AMP analog that mimics all three effects of AMP on AMPK. The primary target of pemetrexed is in fact thought to be thymidylate synthase, required for DNA synthesis. Interestingly, however, if thymidylate is provided in the medium of cells to circumvent inhibition of thymidylate synthesis, pemetrexed remains cytostatic due to AMPK activation, although it is no longer cytotoxic[22].

Once activated by energy stress in mammalian cells, AMPK acts to restore energy balance by switching on catabolic pathways that produce ATP, while switching off anabolic pathways that consume ATP[23]. Catabolic pathways switched on acutely include glucose uptake and glycolysis; while this would promote rapid glycolysis, i.e. the Warburg effect, in the longer term AMPK activation tends to promote instead the more energy-efficient and glucose-sparing oxidative metabolism, by increasing fatty acid oxidation, expression of oxidative enzymes, and mitochondrial biogenesis. Another critical catabolic pathway

switched on by AMPK is autophagy[24] which, like its upstream activator AMPK, can have both positive and negative effects in cancer[25]. AMPK also switches off most anabolic pathways including the synthesis of fatty acids, sterols, triglycerides, phospholipids, glycogen and rRNA[23], and inhibits protein synthesis by inactivating mTOR complex-1 (mTORC1)[26,27]. Finally, it causes cell cycle arrest in G1 phase[28,29].

AMPK would therefore be expected to limit cell proliferation, suggesting that it would suppress tumor growth. Is there any other evidence that AMPK mediates the tumor suppressive effects of LKB1? LKB1 also phosphorylates and activates 12 AMPK-related kinases (ARKs) in addition to AMPK[30], and it is possible that some of its tumor suppressor effects might be mediated by one or more of these (e.g.[31]). However, unlike AMPK, none of the ARKs are known to oppose the metabolic changes observed in tumor cells or cause cell cycle arrest, and none are activated by metformin. Finally, whole body knockout of AMPK-α1 (the sole α subunit isoform expressed in lymphocytes) accelerated the formation of B cell lymphomas caused by B cell-specific over-expression of Myc[32], while T cell-specific knockout of AMPK-α1 accelerated the development of T cell lymphomas triggered by knockout of the tumor suppressor PTEN in T cells (DGH/DVC, unpublished). In the former case, loss of AMPK was associated with a metabolic shift towards aerobic glycolysis (i.e. the Warburg effect), while in the latter case it was associated with up-regulation of the mTORC1 pathway and increased expression of HIF-1α, which would also be expected to promote the Warburg effect. These findings support the idea that AMPK-α1 is indeed acting as a tumor suppressor in these mouse models of lymphoma.

If AMPK does suppress tumor growth like its upstream kinase LKB1, you would expect there to be selection pressure for the pathway to be down-regulated in human cancers, which would reduce its restraining influence on tumor growth and proliferation. Clearly, this occurs in the 20-30% of non-small cell lung cancers that have lost expression of LKB1[9,10]. In addition, histochemical evidence suggests that the phosphorylated form of AMPK is reduced in tumor cells, compared with adjacent regions of normal epithelium, in up to 90% of biopsies taken for diagnosis of breast cancer[33]. Moreover, the α2 isoform of AMPK is down-regulated in many cases of hepatocellular carcinoma, and this is associated with poor prognosis[34]. Although the mechanisms underlying AMPK down-regulation in these cases are not known, one intriguing mechanism for down-regulation of AMPK-α1 has recently been reported[35]. MAGE-A3 and -A6 are closely related proteins encoded by neighbouring genes on the X chromosome. In common with most other MAGE proteins, they are normally only expressed in the testis, but become aberrantly re-expressed in many tumours (up to 25% of breast invasive carcinomas, 50% of colon adenocarcinomas and 75% of lung squamous cell carcinomas). MAGE-A3/-A6 bind the E3 ubiquitin ligase TRIM28, and this complex has recently been shown to target AMPK-α1 for proteasomal degradation

by promoting its poly-ubiquitylation[35]. A second mechanism for down-regulation of AMPK-α1 has recently been described by our laboratory, in tumor cells in which the Akt pathway is hyperactivated due to loss of the tumor suppressor PTEN[36]. Akt phosphorylates a conserved serine residue (Ser487) within a serine/threonine-rich loop of the C-terminal domain of AMPK (although not the equivalent residue of AMPK-α2, Ser491). Phosphorylation of Ser487 blocks the phosphorylation of Thr172 (and consequent activation) by the upstream kinases LKB1 or CaMKKβ, most likely due to a simple physical exclusion mechanism[36].

The evidence discussed above suggests that AMPK is a tumor suppressor that is often down-regulated in cancers. Paradoxically, however, a low level of AMPK may be beneficial to tumor cells to help them to survive the lack of oxygen and nutrients that often occurs while they are establishing a blood supply, or to withstand the stresses caused by cytotoxic anti-cancer treatments. AMPK may therefore be a "friend" or a "foe" in cancer, depending on the context. Consistent with the idea that AMPK may sometimes be a "foe", H-Ras mutant transformed mouse embryo fibroblasts (MEFs) that were deficient in both isoforms of AMPK (α1 and α2) grew normally in culture in vitro but very poorly as xenografts in immunodeficient mice in vivo, where they might be subjected to hypoxic and/or nutritional stresses[37]. By contrast, MEFs with knockout of α2 alone displayed increased susceptibility to H-Ras mutant transformation in vitro and tumorigenesis in vivo[38]. It has also been pointed out, following analysis of cancer genome databases, that the genes encoding the α1 and β2 isoforms of AMPK subunit are quite frequently *amplified*, rather than mutated or deleted, in human cancers[39] - this would be expected if they were oncogenes, rather than tumor suppressors. Since this behaviour was not observed with the genes encoding the other five subunits of AMPK, it suggests that the protective effects of AMPK in tumor cells might be confined to specific isoforms. A final example of AMPK acting as a "foe" in cancer comes from some of our own recent unpublished work, where we have shown that the anti-cancer drug etoposide activates AMPK-α1 in the cell nucleus by a mechanism involving phosphorylation of Thr172 by CaMKKβ. Etoposide causes double strand breaks during replication of DNA in S phase, and this can lead to apoptosis of rapidly proliferating cells while quiescent cells are less sensitive. We have shown that prior activation of AMPK by the Ca^{2+} ionophore A23187 can protect cells against the cytotoxic effects of etoposide. The likely mechanism is that AMPK causes a G1 cell cycle arrest and thus prevents the cells from entering S phase, where they are more vulnerable to etoposide. In this context, AMPK is clearly protecting the cells against the cytotoxic stresses caused by etoposide, and it is therefore possible that an AMPK inhibitor might be useful for cancer treatment, perhaps as an adjunct to etoposide.

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Speciality and Present Interest:
Cell Signalling Regulation, Physiological and Pathological Roles of AMP-activated Protein Kinase

MYC-MEDIATED PERTURBATION OF METABOLISM AND THERAPY

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The MYC oncogene belongs to the family of MYC genes, including MYCN (N-MYC) and MYCL (L-MYC), which are linked to human cancers such as Burkitt's lymphoma, neuroblastoma and lung cancer, respectively. MYC proteins belong to the MAX-MLX network of heterodimeric transcription factors that bind 'E-boxes' (5'-CACGTG-3') to regulate genes involved in cell proliferation, differentiation and metabolism. MYC is downstream of many signal transduction pathways in normal cells; when stimulated through pathways such as tyrosine receptor kinases, MYC is induced to produce the MYC protein that dimerizes with MAX to bind DNA and regulate a transcriptional program of metabolism, cell growth and proliferation[1]. MYC is under the control of growth factor signaling as well as nutrient availability, such that nutrient deprivation could result in diminished normal MYC expression and cell growth arrest. By contrast, deregulation of MYC in normal cells result in MYC binding not only to high affinity physiological binding sites but also invading into low-affinity binding sites and enhancers, resulting in an imbalance amplification of gene expression that triggers stress and cell death through activation of checkpoints such as p53. Elimination of p53, however, in tumorigenesis can unleash MYC's transcriptional power to drive deregulated cell growth that renders cells addicted to nutrients, such that deprivation of glucose or glutamine result in cell death. The conceptual framework has been exploited to target metabolism for therapy against MYC-driven cancers, particularly aiming at glycolysis and glutaminolysis – processes that are increased by MYC. While LDHA inhibition could curb MYC-mediated tumorigenesis[2], glutaminolysis remains an alternative survival pathway that could be targeted through inhibition of glutaminase[3-9]. Glutaminase is normally expressed from two different genes,

GLS that is normally expressed in kidney and brain, and GLS2 that is normally expressed in liver. Indeed, we have shown that knockdown of glutaminase (GLS) or inhibition with a small molecule (BPTES) diminishes the progression of a MYC-inducible human lymphoma xenograft model[3-7]. We further documented that glutaminase (Gls) is induced by MYC and is required for early tumor development in a MYC-inducible model of mouse liver cancer[8], which demonstrates a decrease in the expression of normal liver Gls2. In this regard, the isoform switch from Gls2 to Gls1 in mouse (and also human) liver cancer, renders tumors vulnerable to loss of one copy of Gls, which delayed tumorigenesis. We further showed that treatment with BPTES as a single agent was sufficient to prolong survival of mice bearing these MYC-induced liver cancers, providing proof-of-concept that targeting a single enzyme, in this case GLS, could change the course of the disease[8].

Targeting GLS, however, has limitations, since the oncogenotypes of cancers result in different re-wiring of metabolism that is best illustrated by metabolomics studies to different mouse models of liver cancer[10]. The MET oncogene-driven liver cancer model largely relies on glucose, whereas MYC oncogene-driven liver or lung cancers rely on both glutamine and glucose. As such, glutaminase inhibition should be considered in the context of the oncogenotype and metabolic profile of specific cancer types. Further, targeting metabolism could be constrained by the circadian regulation of cellular metabolism, rendering proliferating normal cells more sensitive to interference of metabolism at specific times of the day. In this regard, we tested the hypothesis that high oncogenic MYC would ectopically invade the circadian Clock regulated genes that are also driven by E-boxes[11]. The cell intrinsic clock machinery comprises of the central Clock-Bmal1 transcription factor, which induces Rev-erb's, Cry's, and Per's that in turn negatively regulated Bmal1 expression or Clock-Bmal1 levels, resulting in a circadian oscillation of Clock-Bmal1 function. Oscillation of the central clock transcription, which drive metabolic genes with E-boxes, results in oscillatory metabolism. Indeed, using an inducible MYC-ER system, we demonstrate that MYC could activate the negative clock regulators PER, CRY, and REV-ERBS and documented that the suppression of Bmal1 expression by MYC is mediated transcriptionally through REV-ERBs. Metabolic profiling in time-series experiments reveals oscillation of glucose and glutamine metabolic in the MYC-OFF state. In the MYC-ON state, intracellular glucose ceases to oscillate and when barely detectable by NMR and additional evidence suggest that it is converted toward lactate and biomass for cell growth. MYC, hence, can invade and disrupt the molecular circadian clock as well as metabolism, in favor of a growth metabolic program.

These observations have key implications for targeting metabolic enzymes that have been documented as being induced by Clock-Bmal1 and MYC. For example, ODC and NAMPT are both common targets for these transcription factors. As such, we hypothesize

that the dose-limiting toxicity of NAMPT inhibition, being thrombocytopenia, could be curbed in a lymphoma xenograft model by applying chronotherapy. In this regard, our preliminary studies illustrate that while NAMPT inhibition at two different times of the day resulted in similar efficacy in reducing lymphoma xenograft growth, one of administration time resulted in thrombocytopenia while the other time of drug administration was indistinguishable from the control treated animals (unpublished). These observations indicate that the combination of identifying metabolic vulnerabilities of MYC-driven cancer along with understanding circadian could strategically guide the use of metabolism-targeted drugs in the clinic through oncogenotyping and potentially chronotherapy.

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METABOLIC REGULATION BY RB TUMOR SUPPRESSOR GENE

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Control of biomass volume tightly synchronizes with cell cycle progression. This represents one of the mechanisms whereby cells maintain their size after rounds of cell division. Then, what is the mechanism by which biomass synthesis is coupled to cell cycle? Many of mitogenic signals mediated by Ras, AKT, Myc, etc., have been implicated in the regulation of metabolic pathways. In addition, tumor suppressing machineries mediated by p53 or PTEN appeared to direct rewiring in cellular metabolism. Then, what about pRB, which is the central molecule in the cell cycle control? pRB has been recently implicated in the control of glutaminolysis and OXPHOS. We, by using cell systems derived from various genetically engineered mice, discovered that pRB controls several other metabolic pathways. Determination of pRB targets in various genetic backgrounds significantly contributed to the following discoveries. pRB inactivation in thyroid C cells and MEFs induced N-Ras-dependent cellular senescence. This explained why C cell tumor developed in *Rb*-heterozygous mice stays in adenoma or low grade medullary carcinoma. The mechanism appeared to involve most of isoprenyltransferases, those are essential for the first step of Ras maturation. We further demonstrated that E2Fs, SREBPs and AKT were involved in this pathway (Ref #3, 5, *Muranaka et al*., in preparation, and *Sasaki et al*., in preparation) (Figure 1). Particularly in a p53-deficient background, RB status critically determines self-renewal activity without impact on cell proliferation (Ref#2). In ARF-deficient breast cancers, RB inactivation increased fatty acid oxidation (FAO). This via increase in mitochondrial superoxide production and Jun kinase (JNK) activity, triggers a feed forward loop between IL-6 and STAT3 leading to increase in self-renewal and drug resistance (*Kitajima et al*., submitted). The analysis of a srcoma system revealed that pRB

positively regulates transcription of phosphglycerate mutase (PGAM) 1 and 2, thereby directs rewiring in glycolysis and TCA activity (Ref#1, and *Kohno et al*., in preparation). This transcriptional regulation appeared to involve C/EBPβ and MEF2C,D. Thorough this functional interaction with PGAMs, pRB appeared to control the undifferentiated status of cancer stem-like cells. We will further demonstrate the evidence indicating that the RB-dependent myogenic differentiation in C2C12 cells depend on PGAM2. These findings indicated a case that pRB controls differentiation primarily by regulating cellular metabolism. I am going to introduce you to previously unexpected functions of pRB in cellular metabolism (Ref#4) unveiled by our unique experimental systems (Figure 2).

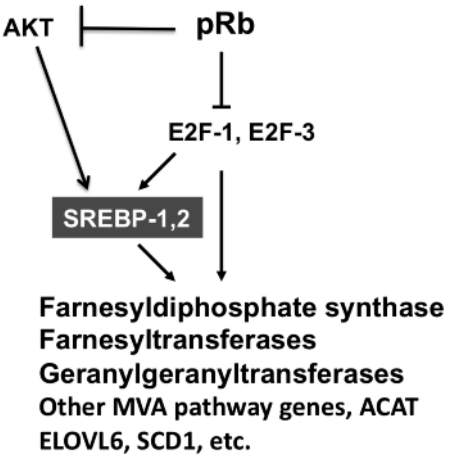

Figure 1 The mechanism whereby pRB regulates mevalonate (MVA) pathway

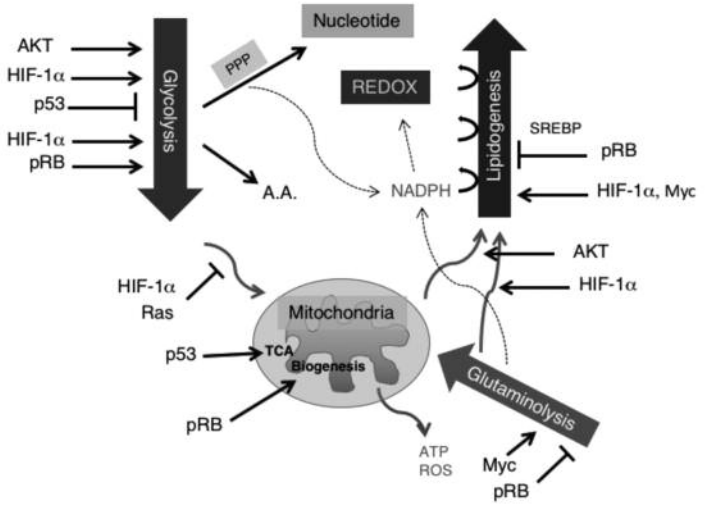

Figure 2 Possible roles of pRB in controlling central carbon metabolism and the relationship with other cancer-related genes.

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Specialty and Present Interest:
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FUMARATE METABOLISM IN CANCER AND TYPE 2 DIABETES

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* This abstract is dedicated to Dr. Patrick John Pollard who died unexpectedly on 10th June 2015. Patrick (Paddy) was an enthusiastic and exceptionally gifted scientist. We will remember him fondly as a friend and colleague (Figure 1).

Dr. Patrick John Pollard
2nd January 1974 – 10th June 2015

Figure 1

Altered intermediary metabolism is a hallmark of cancer and the abnormal accumulation of certain metabolites has linked dysregulated metabolism and cellular functions to tumorigenesis. Recently the abnormal accumulation of certain metabolites has been linked to dysregulated metabolism and cellular functions leading to tumorigenesis[1-3]. Such 'oncometabolites' are exemplified by fumarate, which accumulates as a consequence of loss of activity of the Krebs cycle enzyme Fumarate hydratase (FH). FH catalyzes the hydration of fumarate to malate and has been identified as a tumor suppressor; it is mutated in hereditary leiomyomatosis and renal cell cancer (HLRCC)[4]. Elevated fumarate has multiple cellular consequences including the stabilization of hypoxia-inducible factor (HIF1α) and activation of HIF dependent pathways such as angiogenesis, increased glycolysis and glucose metabolism[5,6]. In addition, metabolic pathways are dysregulated, the mitochondria of FH-deficient cells are dysfunctional, there is evidence of epigenetic deregulation, blocks in cell differentiation and the posttranslational modification of cysteine residues in proteins to form (*S*)-2-succinocysteine (2SC); an irreversible chemical process termed 'succination'[7] (Figure 2). Originally identified in adipocytes and gastrocnemius muscle in some rodent diabetic models, succination in these cells might be caused by glucotoxicity, but has functional consequences, including inactivation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase. Succination in FH-deficient cells and tissues is ubiquitous and occurs in multiple proteins involved in a variety of cellular processes, such as redox homeostasis, iron-sulphur cluster assembly[8], cell metabolism and the ubiquitin conjugation pathway. Previously we have shown that 2SC is a sensitive biomarker for HLRCC[9]. In FH-deficiency succination of the Kelch-like ECH-associated protein 1 (KEAP1), a key component of the ubiquitin-ligase complex responsible for degradation of transcriptional factor Nuclear factor (erythroid-derived 2)-like 2 (NRF2), abrogates its interaction with NRF2. This results in the constitutive

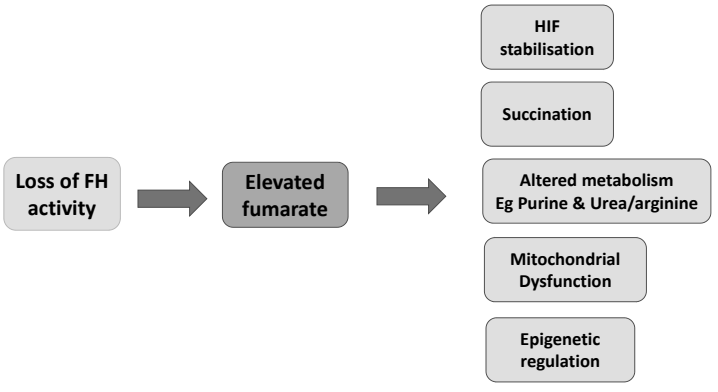

Figure 2 Multiple consequences of loss of Fumarate Hydratase activity

activation of the potentially oncogenic NRF2-mediated antioxidant defence pathway[10]. Also, mitochondrial aconitase is succinated on three critical cysteine residues in FH-deficient mouse embryonic fibroblasts (MEFs) and consequently displays impaired enzymatic activity[8] and succination of glutathione leads to an elevation of reactive oxygen species (ROS) signalling.

Previously we demonstrated that fumarate accumulation causes dysregulation of the urea cycle and some dependence on arginine, potentially highlighting an 'Achilles heel' for these tumours. Normalization of fumarate levels in the cytosol by re-expression of cytoplasmic-specific FH ameliorates the defects associated with renal specific FH deletion in mice[11] (Figure 3).

Building on our studies into the loss of FH (Fh1 in mice) associated with renal cancer, we have generated a novel murine diabetic model in which Fh1 is deleted in pancreatic beta cells (Fh1βKO) and in which glucose intolerance is related to dysregulated metabolism. This is particularly interesting given the additional links to HIF1α, glucose-induced insulin secretion (GIIS) and succination. These animals develop progressive diabetes and impaired GIIS. Analyses have shown that insulin secretion is reduced,

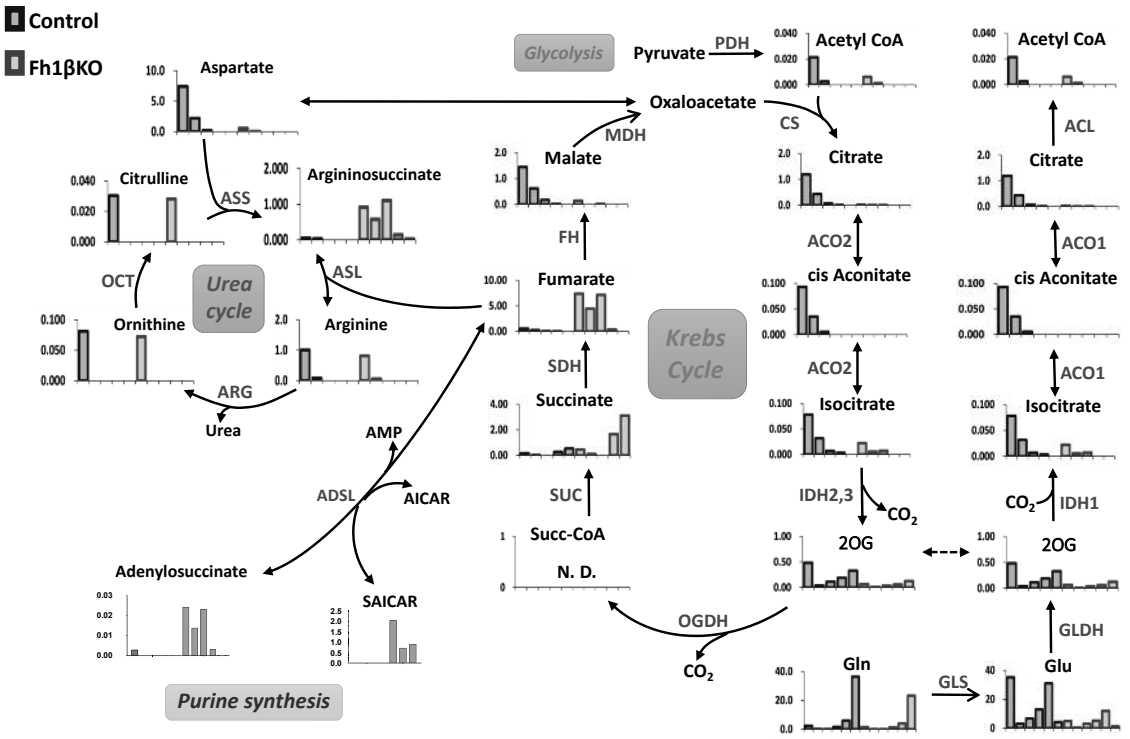

Figure 3 Fumarate accumulation alters metabolism in both the mitochondria and the cytosol

without significant differences in the K_{ATP} channel, calcium influx and exocytosis responses compared to control islets. Also, the pancreatic α-cells show significantly reduced glucagon secretion and increased glucagon content. These Fh1βKO islet cells exhibit dysregulated metabolism as determined by metabolite analysis, measurement of glucose oxidation and utilisation (measured by production of $^{14}CO_2$ and $^{3}H_20$ respectively) and accumulation of fumarate, particularly in high glucose concentrations (20mM). A screen of Fh1βKO islets revealed succination in a number of proteins, including Glyceraldehyde 3-phosphate dehydrogenase and other interesting targets, notably Park7 (also known as DJ1), which acts as a sensor for oxidative stress, a recognised driver of tumorigenesis and link to diabetes and to Parkinson's disease. Surprisingly glucose stimulated ATP: ADP ratio in Fh1βKO islets is normal in the early stages of disease (9-12 weeks); but drops significantly 4-6weeks later in very diabetic animals. This is mirrored in altered electrophysiological responses. Fh1βKO cells also show mitochondrial dysfunction exhibited by altered size and distribution.

Very significantly, analysis of human pancreatic islets has revealed evidence for succination of proteins in glucagon-secreting α-cells in Type 2 diabetes, indicating elevation of fumarate resulting from dysregulated cell metabolism. DJ1 was also identified as a succination target for human islets from both normal and type-2-diabetic donors.

Fumarate is elevated in both mouse and human islets exposed to hyperglycemic conditions for as little as 1 hour. Protein succination is increased in diabetic murine and human pancreatic islets and systemically; it is observed in the renal tubular cells and adipocytes of Fh1βKO mice. Interestingly there is evidence from matched, paired samples of increased succination in adenomas and colorectal cancers compared to normal mucosa of non-diabetic and diabetic individuals, none of whom have FH mutations. The most significant increased levels of succinaton are seen in the colorectal cancer samples of type-2 diabetic patients.

Globally we face the challenge of tackling diabetes, obesity and their consequences[12], such as predisposition to some cancers, there are compelling reasons to investigate links between these diseases. Currently, the connections between dysregulated metabolism and cancer are attracting significant interest fuelled in part by exploitation of mass spectrometry advances to facilitate high-resolution metabolic profiling of cells and tumours[13-15].

Our studies indicate that diabetes *via* hyperglycemia and elevated fumarate impairs α-cell function, and raise the exciting possibility that hyperglycemia-induced accumulation of fumarate links diabetes and co-morbidities including colorectal cancer and in the kidney. Here we propose that the insights into the myriad consequences of elevated fumarate conferred by our Fh1KO mouse models, linked to our studies on renal and colorectal cancer and diabetes will allow us to elucidate potentially common mechanisms evoked in cells as

a stress response (Figure 4). We hope that this work will not only provide novel insights into a serious global disease, diabetes, and a rare but aggressive disease, HLRCC; but also will increase our understanding of cellular metabolism and inform on potential targets for intervention.

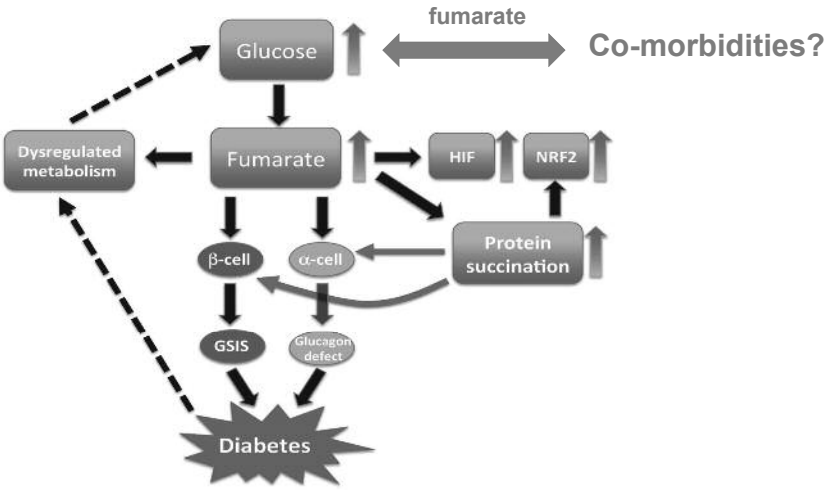

Figure 4 Is elevated fumarate a molecular link between hyperglycaemia, diabetes and co-morbidities?

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IDH MUTATIONS AND THERAPEUTIC STRATEGY IN CANCER

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Mutations in isocitrate dehydrogenase (IDH) 1 and 2 are frequently observed in acute myeloid leukemia (AML), glioma, and many other cancers. While wild-type IDHs convert isocitrate to α-ketoglutarate (α-KG), mutant IDHs convert α-KG to oncometabolite 2-hydroxyglutarate (2-HG), which dysregulates a set of α-KG-dependent dioxygenases, such as TETs, histone demethylases, EGLNs, and other enzymes. Because the role of mutant IDH is not necessary for normal cells, inhibitors directed against mutant IDH are expected to have minimum side effects as those of anti-cancer agents.

To determine whether mutant IDH enzymes are valid targets for cancer therapy, we created a mouse model of mutant IDH-dependent AML. Previously, the IDH mutation alone was shown to be insufficient for the induction of AML, and IDH mutations occur simultaneously with mutations in other genes such as *NPM*, *DNMT3A*, and *FLT3*. In accordance with these observations, we found that $NPM^{+/-}$ hematopoietic progenitor cells transduced with IDH2/R140Q, NPMc, DNMT3A/R882H, and FLT3/ITD cooperatively induced AML in a mouse model (Figure 1). However, when only three of these mutant genes were transduced, myeloproliferative neoplasms (MPNs) rather than AML was more frequently induced and their onset was delayed in any combinations of the mutant genes. These results clearly indicate that all four mutations are necessary for the efficient induction of AML. By using a combination of AML model mice with cre-loxp, we conditionally deleted IDH2/R140Q from AML mice, which blocked 2-HG production and resulted in the loss of leukemia stem cells. Accordingly, the progression of AML was significantly delayed (Figure 2). These results indicate that the function of IDH2 mutation is critical for the development and maintenance of AML stem cells, and that mutant IDHs are promising targets for anticancer therapy.

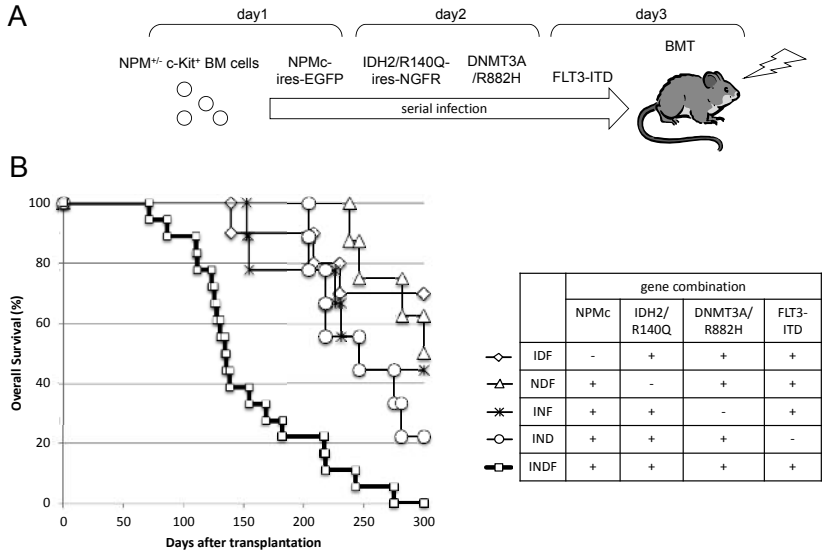

Figure 1 Establishment of an AML model harboring IDH2/R140Q.

A) Scheme of the experiments. c-Kit^{+} hematopoietic progenitor cells were isolated from *Npm1*$^{+/-}$ mouse bone marrow (BM) and serially infected with three or four of the following constructs: pMy-NPMc-ires-EGFP, pGCDN-IDH2/R140Q-ires-NGFR, pMSCV-DNMT3A/R882H-puro, and pMSCV-FLT3/ITD-neo. When cells were infected with three mutant genes, the infection of corresponding empty vector for the absent mutant gene was performed. The infectants were transplanted into irradiated mice.

B) NPMc, IDH2/R140Q, DNMT3A/R882H, and FLT3/ITD (NIDF) cooperatively induce AML in mice. The abbreviations for the gene combinations (IDF, NDF, NIF, NID, and NIDF) are indicated. IDF vs. NIDF, $P < 0.0001$; NDF vs. NIDF, $P < 0.0001$; NIF vs. NIDF, $P = 0.0009$; NID vs. NIDF, $P = 0.0006$; log-rank test.

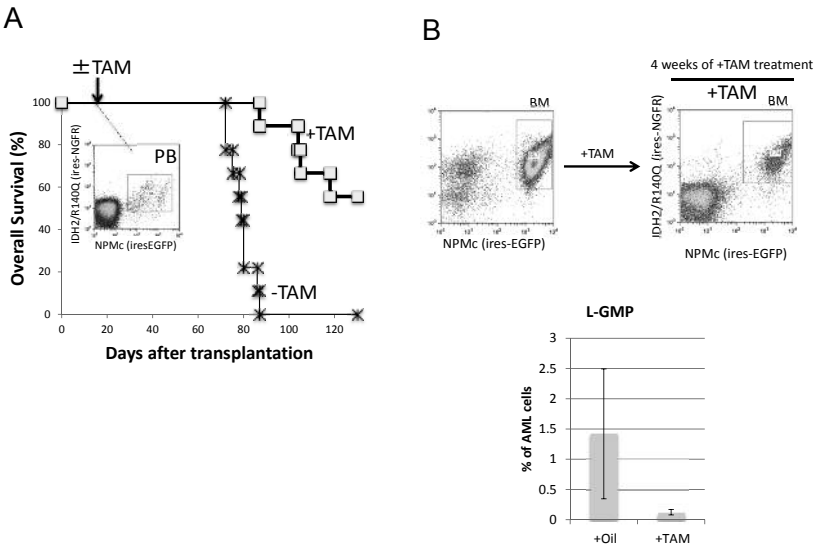

Figure 2 IDH2/R140Q is necessary for the maintenance of AML.

A) c-Kit$^+$ hematopoietic progenitor cells were isolated from ERT2-Cre$^+$ *Npm1*$^{+/-}$ mice, serially infected with pMy-NPMc-ires-EGFP, pGCDN-flox-IDH2/R140Q-ires-NGFR, pMSCV-DNMT3A/R882H-puro, and pMSCV-FLT3/ITD-neo [Nf(I)DF], and then transplanted into irradiated mice. The Nf(I)DF-induced AML cells were transplanted into secondary recipient mice. After 2 weeks, the secondary recipient mice were injected with corn oil or tamoxifen (TAM). The inset panels show the percentage of EGFP$^+$ leukemic cells in the PB at the start of tamoxifen treatment.

B) Deletion of IDH2/R140Q decreased the fraction of cells expressing LSC markers and exhausted EGFP$^+$ cells in BM. TAM treatment began 7 weeks after transplantation. BM cells were isolated from the mice and analyzed for NPMc, EGFP, and NGFR by flow cytometry. The mean percentages of EGFP$^+$ cells in the BM of three mice are shown. Bar graphs show the populations of L-GMP (Lin$^-$, Sca1$^-$, cKit$^+$, CD16/32$^+$, CD34$^+$) among EGFP$^+$ cells after TAM treatment. Genotyping of IDH2/R140Q was performed with BM cells at 2 weeks after TAM treatment.

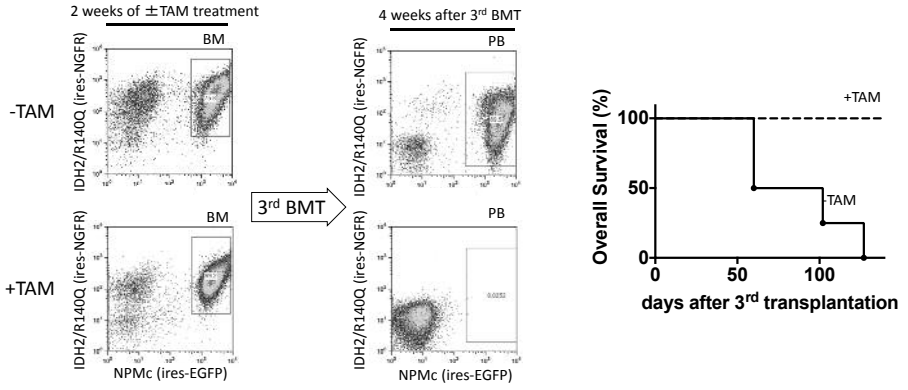

Figure 3 Deletion of IDH2/R140Q decreased the LSC population. BM cells treated or untreated with TAM for 2 weeks were isolated from the mice shown in Figure 3I. The third BMT was performed using these BM cells. The mice were not treated with tamoxifen after the third BMT. PB cells were isolated from mice 4 weeks after the third BMT and were analyzed for NPMc (EGFP) and IDH2/R140Q (NGFR) expression by flow cytometry. The number represents the mean percentage of EGFP$^+$ cells in the PB of 5 mice.

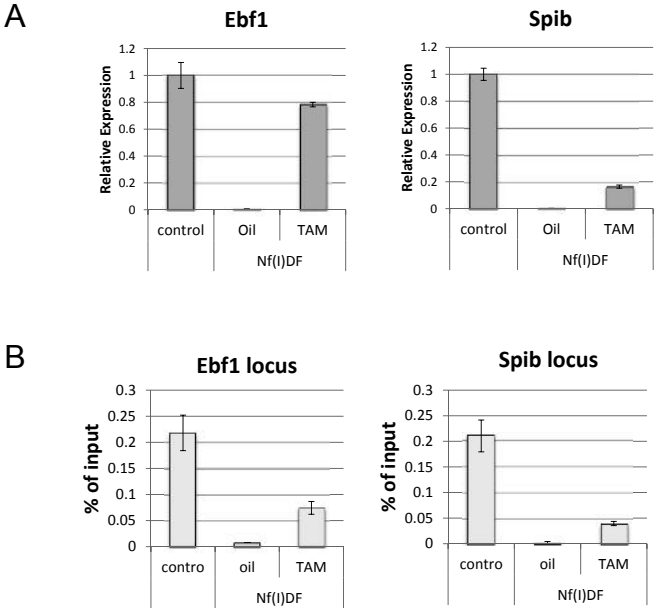

Figure 4 IDH2/R140Q negatively regulates the 5hmC modification and expression of differentiation-inducing factors.

A) Expression of *Spib* and *Ebf1* in NBM cells, untreated Nf(I)DF-AML cells, and TAM-treated Nf(I)DF-AML cells was analyzed by real-time PCR.

B) 5hmC modification of *Ebf1* and *Spib* loci in NBM cells, untreated Nf(I)DF-AML cells, and TAM-treated Nf(I)DF-AML cells was analyzed by real-time PCR. (n=3 per group)

To confirm the decrease in the number of LSCs, we performed a third transplantation using BM cells isolated from mice treated with or without tamoxifen for 2 weeks. When PB cells were analyzed 4 weeks later, the number of AML cells was increased in mice transplanted with untreated BM cells (Figure 3). Conversely, AML cells were almost undetectable in mice transplanted with tamoxifen-treated BM cells (Figure 3). All mice transplanted with tamoxifen-untreated BM cells died of AML by 127 days after the third BMT, whereas none of the mice transplanted with tamoxifen-treated BM cells died by 140 days (Figure 3. These results strongly indicate that IDH2/R140Q is necessary for the maintenance of the LSC population.

Based on these findings, we developed potent and specific inhibitors of mutant IDH1 and tested their effects on the mutant IDH1-dependent AML mouse model, created by introducing four mutant genes including mutant IDH1. The 2HG level was promptly and dramatically decreased in AML cells soon after treatment with the mutant IDH1 inhibitors, and the number of leukemia cells was reduced after a 4-week treatment. We also tested the effects of the inhibitors on patient-derived xenograft (PDX) models and cell lines of glioblastoma and other cancers with IDH1 mutations. The inhibitors decreased the 2HG levels in the tumors and inhibited the growth of the tumors. These results indicate that IDH1 mutant inhibitors are effective for the treatment for AML as well as glioblastoma with IDH1 mutations.

NPMc and IDH2/R140Q cooperatively activated the Hoxa9/Meis1 pathway, and IDH2/R140Q activated the hypoxia pathway. These two pathways are likely to be important for IDH2/R140Q-mediated engraftment/survival of $NPMc^{+}$ cells in mice. In addition to IDH2/R140Q and NPMc, expression of DNMT3A/R882H and FLT3/ITD is also required for efficient induction of AML. DNMT3A/R882H further upregulated the expression levels of Meis1. Furthermore, DNMT3A/R882H promoted the maintenance of cells in an undifferentiated state. Taken together, our results suggest that the activation of multiple signaling pathways is required for NIDF cells to induce AML.

Because IDH mutations and TET2 mutations are mutually exclusive in AML, the inhibition of TET-mediated conversion of 5mC to 5hmC is considered one of the main roles of mutant IDH. We found that levels of 5hmC on differentiation-inducing genes, such as Ebf1, Spib and Pax5 were decreased in AML cells with IDH2/R140Q and recovered by conditional deletion of IDH2/R140Q. In consistent with levels of 5hmC, expressions of these genes are upregulated in the AML cells and revreased by deletion of IDH2/R140Q.

Gene expression analysis revealed that IDH2/R140Q up-regulates a set of genes that is activated in response to hypoxia as well as Meis1. As 2HG inhibits EGLN that hydroxylates and marks HIF1α for ubiquitin-proteasomal degradation, it is probable that mutant IDH2-produced 2HG stabilizes HIF1α through inhibition of EGLN. Furthermore, it

was reported that Meis1 activates the transcription of HIF1α. In consistent with these informations, we showed that IDH2/R140Q increased the protein levels of HIF1α in cultured cells.

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Specialty and Present Interest:
Cancer Stem Cells, Hematological Malignancy, Cancer Epigenetics

EPIGENETIC MECHANISMS IN HEMATOLOGIC MALIGNANCIES LINKED TO METABOLIC PATHWAYS

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The instructions that endow cells with the capacity to manifest unique phenotypes reside in the chemical modifications, proteins, and RNA molecules that constitute the epigenome. It is the particular distribution of epigenetic marks that enables cells sharing the same genomic DNA sequence to differentiate into distinct cell lineages with specific functions. Just like in normal cells the epigenome also encodes the phenotype of tumor cells. Indeed, aberrant epigenetic patterning of the genome is emerging as a hallmark of acute myeloid leukemia, a malignancy characterized by a relative paucity of genetic lesions. AML can be classified into disease subtypes based on epigenetic signatures[1)]. These signatures were established by performing integrative epigenomic profiling of almost 800 patients enrolled in clinical trials in Europe and the US[1,2)]. More recently the nature of these signatures was further explored using next generation sequencing of the methylomes of these patients (3 and unpublished data) . Careful examination of these methylomes revealed that the most informative methylcytosine residues driving epigenetic clustering of AML patients are located in enhancer elements, positioned outside of CpG islands. Hence perturbation of enhancer function through aberrant cytosine methylation may play a key role in encoding the unique phenotypes and clinical outcomes of subsets of AML patients. Importantly, some of these signatures cannot be attributed to any particular somatic mutation suggesting that at least some epigenetic signatures arise due to other influences.

Based on these considerations we predicted that epigenetic signatures could be used as molecular blueprints that could point towards causative mechanisms that drive leukemia phenotypes. Along these lines we found that a subset of AMLs that share a particular

DNA hypermethylation signature exhibit somatic mutations of the IDH1 and IDH2 genes[2]. These enzymes normally catalyze the conversion of isocitrate to a-ketoglutarate (aKG). Various lines of investigation revealed that mutant IDH enzymes generate the oncometabolite 2-hydroxyglutarate, which can inhibit aKG dependent dioxygenases including the TET family of enzymes that convert 5-methylcytosine (5mC) into 5-hydroxymethlcytosine (5hmC)[4]. Indeed patients with TET2 mutations share the aberrant DNA methylation profile of IDH mutant AML patients[2]. TET2 is not a DNA binding proteins and must be recruited to DNA by other proteins to carry out its actions. We found that WT1, a transcriptional regulator often mutated in AML interacts with TET2[5]. Notably, somatic mutations in IDH1, IDH2, TET2 and WT1 are almost universally mutually exclusive of each other in AML. Patients with all four of these mutations exhibit reduced global levels of 5hmC and in genome wide profiling studies display overlapping 5hmC distribution profiles[5]. Whereas 5mC distribution shows weak inverse correlation with gene expression, 5hmC displays very strong positive correlation[5]. Collectively these data define a functional axis whereby WT1 mutation defines a subset of the effects emanating from TET2 loss of function, which in turn mediates a subset of the actions of 2HG generated by mutant IDH1 and IDH2[5,6]. The link between gene expression and 5hmC points towards perturbation of this mark as perhaps representing the dominant effect of this functional axis. Given that IDH mutants potentially affect many epigenetic enzymes including all the TET family proteins and jumanji histone demethylases[4], it would be informative to dissect out the contributions of TET2 to further distinguish its biological and epigenetic contribution to the IDH phenotype.

Further characterization of the epigenetic actions of TET2 was pursued by crossing TET2 deficient mice with $FLT3^{ITD}$ transgenic animals. The two mutations often occur together in AML patients and are associated with an unfavorable clinical outcome. While neither mutation alone causes leukemia, the combination of both together yielded a lethal phenotype[7]. Analysis of the methylomes of single vs. double mutant mice revealed that TET2 alone resulted in a small hypermethylated signature, and FLT3 in a small hypomethylated signature. However the combination of both together resulted in massive epigenetic dysregulation of genes distinct from those affected by either mutation alone[7]. Most of this perturbation consisted of DNA hypermethylation. Among the top methylated genes was GATA2, which was silenced in the double but not single mutant animals. Similar effects were observed in human AML patients with TET2 + $FLT3^{ITD}$ mutation[7]. Restoration of GATA2 rescued the TET2-$FLT3^{ITD}$ leukemia phenotype, demonstrating that loss of GATA2 plays a critical role in the biological effect of the combined mutations. To determine if epigenetic synergy is reversible we treated TET2-$FLT3^{ITD}$ leukemic mice with a specific FLT3 inhibitor (unpublished). This treatment only modestly affected the leukemia

phenotype and had little effect on epigenetic signatures. In contrast treatment of mice with DNA methyltransferase inhibitor drugs led to complete resolution of the leukemic phenotype. Leukemia blasts disappeared from blood and the mutant hematopoietic stem cells recovered the ability to differentiate normally and contributed to normal hematopoiesis. GATA2 methylation and gene expression normalized. However the mutant leukemia stem cells could not be eradicated. Hence purely reversing the DNA methylation effects reprogrammed leukemia stem cells to resume the hematopoietic program but could not kill them. Perhaps combination therapies would achieve this endpoint.

Finally, we wondered whether in addition to altering epigenetic programming, somatic mutations of IDH would affect AML epigenetic heterogeneity. We considered that epigenetic heterogeneity among populations of leukemia cells would be potentially result in biological actions such as improved population fitness and hence greater tendency to relapse. We developed an algorithm called MethClone that enables epigenetic allelic diversity to be measured[8]. We found that epigenetic allele burden is linked to unfavorable clinical outcome in AML, independent of somatic mutations or other biomarkers (unpublished). Genetic and epigenetic allelic diversity did not track together and appeared to be distinct biological phenomenon. AMLs could be classified into those with high epiallele burden with low somatic mutations, and a second group with low epiallele burden and higher somatic mutation burden. Surprisingly, IDH mutations were linked to the former, suggesting that IDH epigenetic effects are quite specific and direct, and do not display much random dispersion. The presence of epialleles was accompanied by transcriptional deregulation, as evidence from RNA-seq and single cell RNA-seq (unpublished). Perhaps shifting of epialleles thus enables AMLs to sample different transcriptional states. In summary, exploring AML epigenomes has provided the opportunity to discover novel disease mechanisms and develop new therapies, including the discovery of the IDH-TET2-WT1 axis and their downstream effects. Ongoing examination of the layers of the AML epigenome should continue to provide valuable insights into this mostly fatal disease.

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CARBON MONOXIDE-RESPONSIVE HEME PROTEINS REGULATE CANCER PROLIFERATION AND CHEMORESISTANCE

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Introduction

Gases constitute a group of smallest metabolites in biological systems. Among gases, carbon monoxide (CO) is generated by heme oxygenase that is inducible in cancer cells in response to exposure to stimuli such as radiation, hypoxia and anti-cancer reagents. Since gases are too small to handle for mining the specific receptors in biological systems, only little information has been available for CO-specific receptors. Previously, metabolome analyses allowed us to find lists of metabolites that are up- or down-regulated in response to CO-inducing conditions[1)], since CO has the ability to bind to proteins with metal-centered prosthetic groups, that is, enzymes. Of interest is observation that exogenous CO application to cultured cell lines acutely causes modest elevation of remethylation metabolites such as methionine and S-adenosylhomocysteine (SAH), a potent inhibitor of methyltransferation, and decreases in cystathionine and total amounts of glutathione in cells. These lines of observations led us to hypothesize that cystathionine β-synthase (CBS) serves as a CO-responsive target. Interestingly, this hypothesis was actually proven by observation that recombinant human CBS is inhibited by CO but not by NO[1)], suggesting that CBS accounts for a CO receptor.

CO regulates carbohydrate metabolism through CBS inhibition

Such an inhibitory action of CO on CBS causes alterations in methylation/demethylation of many proteins in cells. The surveillance of molecular targets affected by CO led us to determine that a glycolytic enzyme PFKFB3 that produces fructose 2,6-bisphosphate serves as a target molecule regulated be CO-sensitive methylation. In human

colon cancer HCT116 cells, CO induction or CBS knockdown turned out to down-regulate methylation of PFKFB3. This event might result from a metabolic response of SAH, an inhibitor of methyltransferase, by either CO-mediated CBS inhibition or CBS knockdown. Interestingly, PFKFB3 expressed in cancer cells is mostly arginine-methylated[2], and stress responses of CO causes demethylation of the enzyme. De-methylation of PFKFB3 reduces fructose 2,6-bisphosphate, a potent activator of PFK1, the rate-limiting enzyme for glycolysis, and resultantly up-regulates glucose bio-transformation towards pentose phosphate pathway (PPP) rather than glycolysis. Such responses to CO-mediated inhibition of CBS benefit increases in NADPH/GSH system to enhance cancer survival against anti-cancer reagents[2]. Simultaneously, the response of this kind might benefit synthesis of biomass such as nucletic acids through the PPP activation.

In a xenograft transplantation model using super-immunodeficient mice, cancer cells inplanted in spleen are metastasized in the liver. Under these circumstances, GSH was remarkably elevated in tumors to increase anti-oxidative properties, as judged by microscopic imaging microscopy[2,3]. To note is that CBS knockdown in the cancer cells accelerates cancer growth with increased GSH. Considering the inhibitory action of CO on CBS, GSH elevation in CBS-knockdown cancer cells appears to be inconsistent. However, this event might result from a back-up mechanism for supplying cystine/cysteine as a substrate of glutathione through CD44/xCT system that helps uptake of extracellular cystine in HCT116 cancer cells[3,4]. Although the relationship between increased amounts of GSH and cancer survival remains unknown, it is not unreasonable to speculate that increased GSH might benefit escaping from oxidative stress during cell division, so far as judged by increases in O_2 consumption and reactive oxygen species during G2/M phase in human-derived cancer cell lines[5]. The CO/CBS-dependent modulatory action on PFKFB3 methylation in cancer cells appears to determine directional glucose utilization to strengthen their anti-oxidative capacity for survival.

CO-responsive receptors besides CBS ?

Use of metabolome analyses for mining gas-responsive receptors allowed us to explore CBS as a CO-responsive switch in metabolic systems. However, technical limitation is the fact that putative CO-responsive macromolecules mined by this method might be enzymes. We are challenging to explore other candidates of macromolecules that directly capture gases like CO to regulate function of the target molecules using an approach of chemical biology. Among products of oncogenes with haem-binding motif, progesterone receptor membrane component 1 (PGRMC1/Sigma-2 receptor) has attracted great interests in cancer biology[6-8]. Suppression of the gene inhibits cancer growth, although the function of PGRMC1 has not fully been elucidated. Since CO has the ability to bind to the prosthetic

haem of PGRMC1, it is not unreasonable to speculate that this protein serves as a CO-responsive target that may explains anti-cancer effects of CO[9-11]. Further investigation is now underway in our laboratory.

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HEME OXYGENASE-1 AND METABOLIC REPROGRAMMING IN RESPONSE TO ISCHEMIA

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Heme oxygenase-1 (Hmox1) is an inducible stress protein that is increasingly recognized as an important mediator of cellular homeostasis. Hmox1 is a known downstream target of hypoxia-inducible factor 1 (HIF1), and it is induced by both hypoxia and glucose deprivation[1,2]. Here, we investigated a potential role of Hmox1 in metabolic reprogramming in ischemia after vascular occlusion, a key problem associated with cardiovascular diseases.

Initial studies using yeast as a model system revealed that the absence of the Hmox1 ortholog, Hmx1, results in altered gene expression, including those involved in cellular redox control (e.g., antioxidant enzymes), glucose transport (*HKT2*) and mitochondrial respiration (*COQ7*)[3]. Phenotypically, Hmx1-deficient yeast cells had decreased mitochondrial membrane potential and respiration that was rescued by overexpression of *HMX1*, *Hmox1*, and *COQ7*. Hmx1-deficiency also decreased mitochondrial coenzyme Q content that was similarly rescued by over-expression of *HMX1*, *Hmox1*, and *COQ7*. These studies suggested that Hmox1-deficiency affects cellular energy metabolism.

We next confirmed the putative role of Hmox1 in energy metabolism using skin fibroblasts obtained from littermate *Hmox1*$^{-/-}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{+/+}$ mice (Balb/c background). Moreover, compared with wild type cells, Hmox1-deficient fibroblasts showed abnormal metabolic adaptation in response to hypoxia (1% O_2), including attenuated HIF1α stabilization. Hypoxia resulted in rapid induction of Hmox1 protein in wild type cells that preceded stabilization of HIF1α, suggesting that Hmox1 may be upstream of HIF1.

A unilateral hind limb arterial resection model was used to examine the role of Hmox1 in ischemia-mediated energy adaptation *in vivo*. Compared with littermate wild type control animals, *Hmox1*$^{-/-}$ mice were observed to have impaired blood flow recovery (Figure 1) and neovascularization, and to suffer from auto-amputation in response to ischemia (Figure 2). Auto-amputation preceded blood flow recovery and was not rescued by bone marrow transfer experiments. We therefore explored a role for Hmox1 in the skeletal muscle response to ischemia. Compared with *Hmox1*$^{+/+}$ mice, skeletal muscle fibres of *Hmox1*$^{-/-}$ mice had decreased oxidative phosphorylation capacity and elevated steady state glucose uptake. Ischemia was associated with induction of Hmox1 transcript that preceded that of known HIF1 target genes, such as *glucose transporter-1* and *vascular*

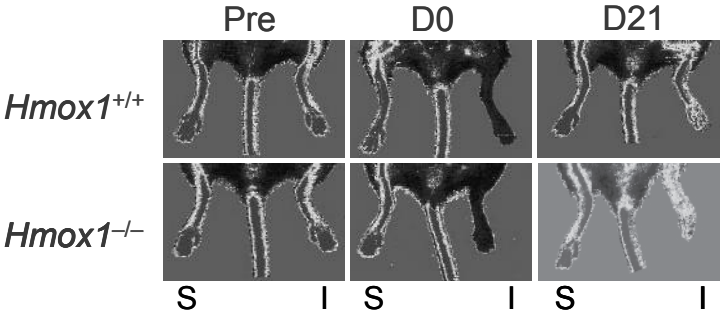

Figure 1 Laser Doppler images of sham (S) and ischemic (I) limbs of *Hmox1*$^{+/+}$ and *Hmox1*$^{-/-}$ mice before (Pre), immediately (D0) and 21 days (D21) after hind limb ischemia.

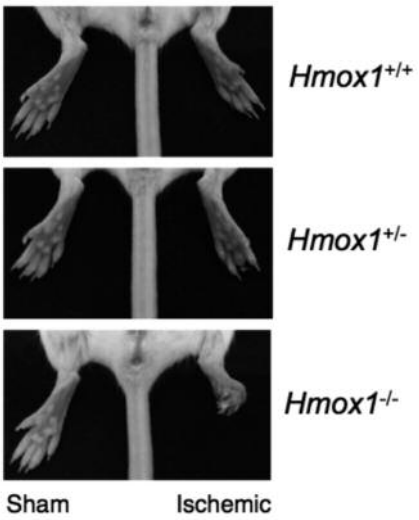

Figure 2 Images of sham and ischemic limbs of *Hmox1*$^{+/+}$, *Hmox1*$^{+/-}$ and *Hmox1*$^{-/-}$ mice 21 days after hind limb ischemia, illustrating tissue injury and auto-amputation in mice deficient in Hmox1.

endothelial growth factor. Reminiscent of the situation in fibroblasts, ischemia induced the expression of Hmox1 protein in skeletal muscle before HIF1α. Together, these data suggest that *Hmox1* is upstream of HIF1, in addition to being a known down-stream target of HIF1. This scenario is analogous to Hmox1 being up- and down-stream of the transcription factor YY1[4].

We propose a novel role for Hmox1 in cellular energy reprogramming in response to hypoxia, and a new paradigm for the interplay between Hmox1 and HIF1α in which Hmox1 is both downstream and upstream of HIF1. Insights obtained from our ischemia model may have important implications not only for cardiovascular diseases, but also other pathologies in which oxygen supply and energy metabolism are perturbed, such as cancer.

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PROTEIN-BOUND REACTIVE SULFUR SPECIES AND ITS TRANSLATIONAL BIOSYNTHESIS: POTENTIAL IMPLICATION FOR OXIDATIVE STRESS AND TUMOR BIOLOGY

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Many organisms utilize high-level energy metabolism that involves the chemical reactivity of molecular oxygen, i.e., redox activity. The oxygen-dependent redox reaction, mostly mediated by reactive oxygen species, then modulates and optimizes biological functions of various protein effector molecules. The energy metabolism of aerobic organisms makes use of the redox activity of molecular oxygen. Reactive oxygen species (e.g., superoxide anion [O_2^-] and hydrogen peroxide [H_2O_2]) are reduced metabolites derived from molecular oxygen that are produced during mitochondrial respiration or defense responses during infection of cells and tissues. Some reactive oxidants were thought to be harmful agents that mediate oxygen toxicity (oxidant toxicity theory). Indeed, these oxidant species may be involved in the pathogenesis of different diseases associated with oxidative stress[1-5]. These disorders include infections; inflammations; cancer; lifestyle-related diseases and metabolic diseases such as arteriosclerosis and diabetes mellitus; and neurological disorders such as Alzheimer's disease. The clinical application of antioxidant agents to prevent and treat these diseases has not yet achieved the hoped-for results, however.

Reactive sulfur species (RSS) like cysteine hydropersulfide (CysSSH) has been long time known to be physiologically formed in various organisms including prokaryotes and eukaryotes including mammals[6]. Abundant endogenous formation of CysSSH recently reported indicates pivotal physiological roles of these RSS derivatives[6,7]. In fact, our current study identified RSS as a major component of low-molecular-weight polysulfur compounds endogenously generated in cells. Its biosynthesis is effectively catalyzed mainly by two enzymes such as cystathionine γ-lyase (CSE) and cystathionine β-synthase

(CBS) by use of cystine (CysSSCys) as a substrate (Figure 1). We have unequivocally verified generation of appreciable amount of CysSSH, and its derivative glutathione hydropersulfide (GSSH) in cultured cells and tissues in vivo[7]. Cysteine persulfide thus formed is highly nucleophilic and a stronger antioxidant than is cysteine. This unique feature of reactive cysteine persulfide relies on adjacent electron pairs in what is called the α-effect (Figure 1)[6,8]. In fact, hydropersulfides, i.e., reduced forms of these RSS were potent scavengers for reactive oxygen species, especially for hydrogen peroxide. RSS also showed a strong redox signaling regulatory function via electrophile thiolation. A prime example of this is interaction of RSS with an endogenously generated electrophile 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP)[7, 9-11]. Here, we clarified that extremely high levels of protein-bound cysteinyl persulfide or RSS formation in most Cys-containing proteins expressed in cells and tissues, of which extension reaching almost 100% at particular Cys residues of many proteins. This fact suggested that CysSSH might be incorporated into nascent polypeptides and/or synthesized at a translational level and thus initially expressed to function as a natural amino acid residue in proteins. Surprisingly, a clear translation-coupled Cys polysulfuration (TCysPS) was revealed herein, directly involved in a remarkable translational CysSSH biosynthesis and its incorporation into

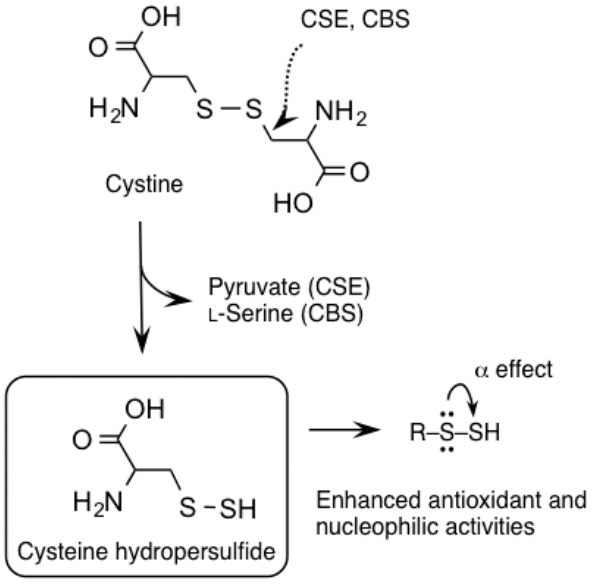

Figure 1 Cysteine persulfide formation mediated by CBS and CSE.
C-S bond cleavage in cystine by CBS and CSE results in formation of cysteine persulfide. Antioxidant and nucleophilic activities of terminal thiol residues are enhanced by an effect brought about by lone-pair electrons of adjacent sulfur atoms.

proteins ubiquitously occurring among different organisms. Exploring such a unique TCysPS pathway may advance a new paradigm of molecular biology and especially cellular translational mechanism, evolving an innovative era of the modern redox biology. Also, several recent reports suggest a potential involvement of CBS and CSE and their product hydrogen sulfide in tumor promotion and progression, and even in drug resistance[12-14]. These tumor-promoting effects of CSE/CBS may be caused via strong antioxidant activity of RSS. Therefore, TCysPS, if occurring extensively in situ in tumor tissues, may contribute to the tumor progression. This discovery of versatile physiological functions of the protein-bound RSS produced via TCysPS may warrant its potential medicinal and pharmaceutical application for many diseases including cancer[15].

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Specialty and Present Interest:
Chemical Biology, Oxidative Stress, Microbial Pathogenesis, NO & Redox Biology, Redox Signal Transduction, Polysulfur Chemistry & Biology

IDENTIFICATION OF A METABOLIC PATHWAY FOR ACTIVATION OF THE HYPOXIA GENE REGULATORY RESPONSE IN CANCER

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Cancer research has traditionally focused on the biology of transformed cells and the genetic changes that drive tumor malignancy. However, it has become increasingly clear that tumours develop within - and actively shape – specialized microenvironments that are typically characterized by low oxygen tension (hypoxia). The hypoxic microenvironment drives transcriptional responses mediated by hypoxia inducible transcription factors (HIFs) that enable oncogenic adaptation. Our laboratory is interested in elucidating the mode of action of HIFs, epigenetic mechanisms of gene regulation in hypoxia and how the integrated function of these mechanisms determines the cellular response to hypoxia in physiology and disease, notably cancer. The von Hippel-Lindau tumor suppressor protein (VHL) plays a critical role in regulating the cellular hypoxic response by functioning as an E3 ubiquitin ligase determining the rapid degradation of HIFs in normoxia. Thus, in hypoxia, the cellular hypoxic response is initiated by release of VHL and stabilization of the HIFs. Unexpectedly, we have discovered a new mechanism for activation and stabilization of HIFs that is VHL-dependent but independent of oxygen-sensing. This mechanism involves metabolically active enzyme complexes that are up-regulated in many cancers and that we have identified as hitherto unknown VHL-interacting proteins. Thus, these findings link the tumor metabolic phenotype to regulation of the activity and function of hypoxia-inducible transcription factors.

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CYTOPROTECTION AND METABOLIC REPROGRAMMING GOVERNED BY KEAP1-NRF2 SYSTEM

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NRF2 is a master transcriptional activator playing a critical role in the defense mechanism against oxidative insults[1-3] (Figure 1). NRF2 activates genes encoding cytoprotective enzymes and antioxidant proteins in response to electrophiles and reactive oxygen species (ROS) of exogenous and endogenous origins. Under unstressed conditions, NRF2 is constantly ubiquitinated by KEAP1 and degraded in the proteasome. During exposure to electrophiles or ROS, KEAP1 is inactivated, and NRF2 is stabilized. Consequently, NRF2 activates transcription, conferring resistance against xenobiotic and oxidative stress. NRF2 inducers have been found especially effective for improvement of diabetic nephropathy, multiple sclerosis, and ischemia reperfusion injury.

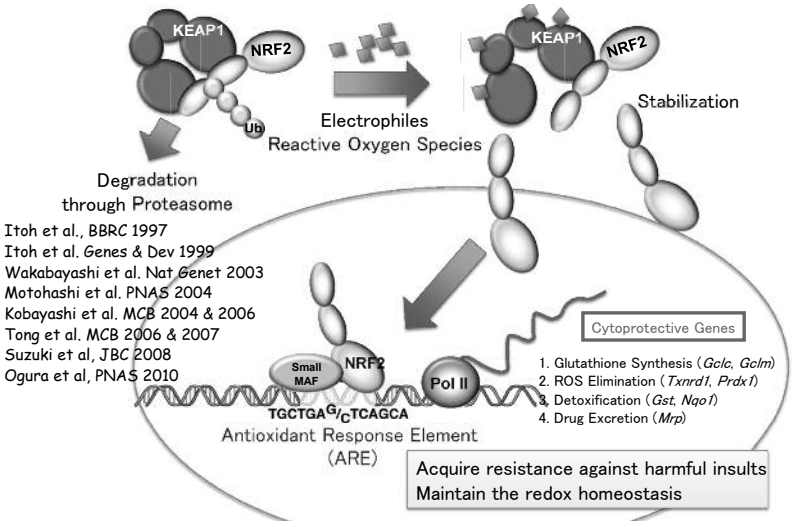

Figure 1 KEAP1-NRF2 System Plays a Central Role in the Maintenance of Redox Homeostasis

While NRF2 activation is beneficial to our health, NRF2 is responsible for the malignant progression of various human cancers. Recent cancer genome projects revealed that *KEAP1* gene and *NRF2* gene are often mutated in human cancers[4,5]. According to the COSMIC database, non-synonymous mutations of *KEAP1* gene are distributed widely in the coding region, while those of *NRF2* gene are clustered in the two motifs in the N-terminal region, which are necessary for the interaction between KEAP1 and NRF2. In either case, NRF2 cannot be ubiquitinated and stabilized, resulting in the constitutive activation of its target genes. Because NRF2 activates many cytoprotective genes, constitutive stabilization of NRF2 confers chemo- and radio-resistance and leads to poor prognosis[6].

We found that NRF2 not only enhances survival of cancers by activating cytoprotective genes but also redirects glucose and glutamine into anabolic pathways by activating metabolic genes, which are advantageous for cancer proliferation[7] (Figure 2). Enhanced activity of PI3K-AKT signaling enables NRF2 to induce the metabolic genes and modulate metabolism. We observed that NRF2 stabilization under the sustained activation of PI3K-AKT pathway in hepatocytes results in the liver enlargement[8]. Intriguingly, it was accompanied by the marked increase of bile duct-like structures, suggesting that the robust activation of NRF2 affects the cell type specification during the liver development in addition to cell proliferation. We also found that NRF2 activation promotes hepatocyte proliferation during compensatory liver hypertrophy after partial portal vein ligation, implying that NRF2 contributes to proliferation under the transient and physiological enhancement of proliferative signals[9].

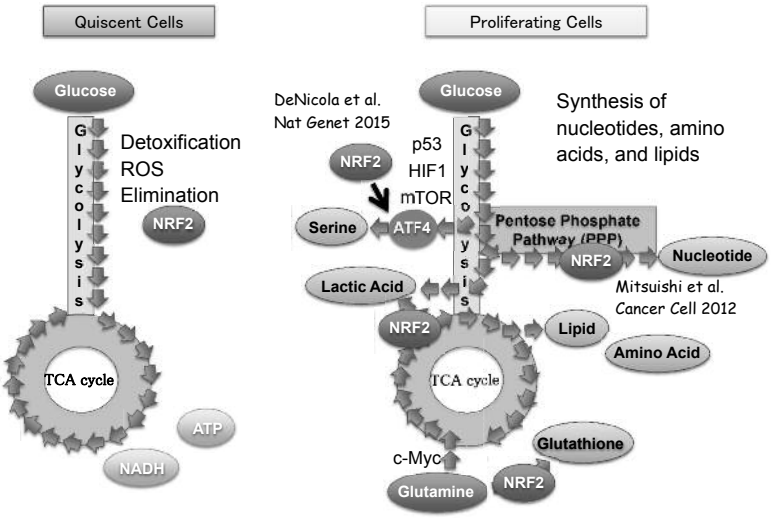

Figure 2 Contribution of Nrf2 in Metabolic Reprogramming

To understand the molecular basis of the potent transcriptional activation mediated by NRF2 under the sustained activation for PI3K-AKT pathway, we purified endogenous NRF2 complex from Keap1: Pten double deficient liver. NRF2 and its heterodimeric partner molecule, small MAF, were obtained. CBP and p300, which are already known coactivators of NRF2, were also obtained. In addition to these factors, many subunits of Mediator complex and SWI/SNF chromatin remodeling complex were obtained. Intriguingly, among the subunits of the Mediator complex, MED16 turned out to directly interact with NRF2 through its transactivation domains, Neh4 and Neh5[10]. Approximately three fourths of the NRF2 target genes exhibited blunted induction in response to electrophilic stimuli. Moreover, MED16 disruption significantly sensitized cells to oxidative stress. These results indicate that KEAP1-NRF2-MED16 axis is essential for the NRF2-mediated cell protection (Figure 3). Our on-going analysis of the endogenous NRF2 complex purified from Keap1 single deficient liver will give more clear answer to the mechanism how the sustained activation of PI3K-AKT signaling enhances the NRF2 activity.

From these results, we propose that NRF2 is a facultative accelerator of cell proliferation, activating cytoprotective genes for the maintenance of redox homeostasis and metabolic genes for the contribution to the metabolic reprogramming in cooperation with other oncogenic signals (Figure 4).

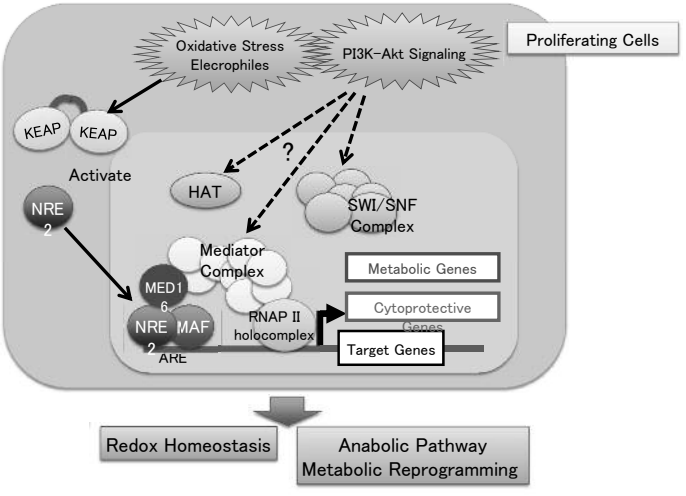

Figure 3 KEAP1-NRF2-MED16 Axis Transduces Oxidative Stress into Cytoprotective/Metabolic Gene Expression

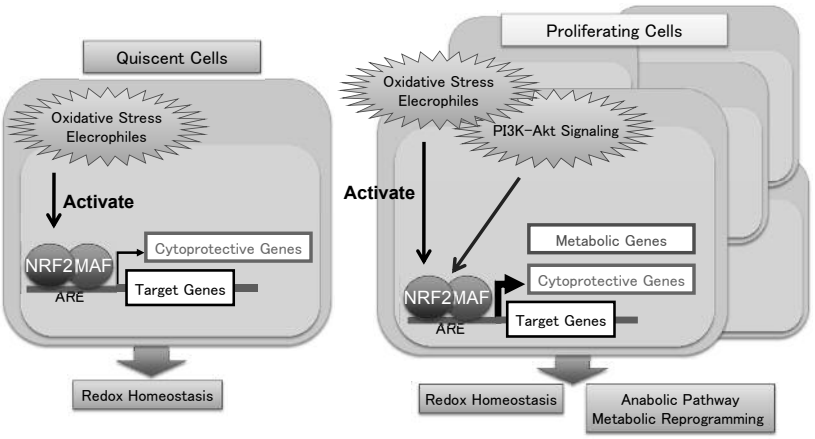

Figure 4 NRF2 Is a "Facultative Accelerator of Cell Proliferation " Maintaining Redox Homeostasis and Reprogramming Metabolism

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Specialty and Present Interest:
Transcriptional Regulation Mechanism for Antioxidant Response, Development of Anticancer Therapy for NRF2-addicted Tumors

MITOCHONDRIA REGULATE CANCER

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Historically, mitochondrial metabolism has been erroneously viewed as inconsequential for the metabolic demands of rapidly proliferating cells[1,2]. Furthermore, the mitochondria have been ascribed to produce copious amount of reactive oxygen species (ROS) that promote DNA damage and genetic instability[3]. This view mainly stems from two long-standing observations. The first observation, made in the 1920s, states that cancer cells take up glucose and produce large quantities of lactate in the presence of ample oxygen[4]. This is referred to as aerobic glycolysis or the Warburg effect. This observation led to the hypothesis that mitochondria are damaged in tumors; thus, cancer cells primarily utilize glycolysis as the major metabolic pathway for proliferation[2]. A second observation is that tumors frequently produce high levels of mitochondrial ROS in order to invoke genetic instability and ultimately tumorigenesis[5]. Consequently, mitochondrial dysfunction was designated as a metabolic hallmark of cancer cells.

The notion that mitochondria in tumor cells are dysfunctional was challenged in the 1950s[6] by the demonstration that like normal tissues such as the liver, tumor cells effectively oxidized the fatty acid palmitate[7]. Moreover, enzymes of the tricarboxylic acid (TCA) cycle had similar activities in the mitochondria of tumors as the mitochondria of normal tissues[8] and that the high rate of glycolysis is a hallmark of both normal and neoplastic cells[9]. Importantly, these observations and models have largely been validated over the past 50 years. Recent studies provided genetic evidence that mitochondrial metabolism is essential for tumorigenesis[10-12].

It is not surprising that mitochondrial metabolism would be necessary for tumorigenesis. Mitochondria are well appreciated as biosynthetic and bioenergetic

organelles for their role in producing metabolites and ATP, which are byproducts of the tricarboxylic acid (TCA) cycle and the mitochondrial membrane potential, respectively. The TCA cycle metabolites such as oxaloacetate and citrate generate cytosolic aspartate and acetyl-CoA that are required for pyrimidine and fatty acid synthesis, respectively. The TCA cycle produces reducing equivalents NADH and FADH2, which deliver their electrons to the electron transport chain (ETC) that ultimately utilizes oxygen as the final acceptor (respiration). An emerging idea is that mitochondria also function as signaling organelles. Two notable mitochondrial-dependent signaling mechanisms involve the release of reactive oxygen species (ROS) for protein thiol oxidation and the release of citrate, which generates acetyl-CoA used for histone acetylation, an important mechanism to regulate epigenetics in cancer cells. Mitochondrial ROS-dependent signaling controls numerous biological responses including proliferation, differentiation, and adaptation to stress as well as physiological and pathological outcomes such as immunity, cancer, and aging. For example, mitochondrial-derived ROS have been implicated in hypoxic signal transduction and activation of hypoxia-inducible transcription factor 1 (HIF-1), which is necessary for tumor cells to metabolically adapt to hypoxia. Thus, mitochondria provide ATP, building blocks for macromolecular synthesis and important signaling molecules for tumor cell proliferation, survival and metabolic adaptation[13].

The recognition that mitochondrial metabolism is essential for tumorigenesis has led to idea of targeting mitochondria for cancer therapy. Intriguingly, the anti-diabetic drug metformin has emerged as an anti-cancer agent. In diabetic patients, the therapeutic effect of metformin is a result of significantly decreasing hepatic gluconeogenesis to diminish circulating insulin levels[14]. Epidemiological studies have suggested that patients on metformin, in order to control their blood glucose levels, are less likely to develop cancer[15]. In addition, metformin increases the survival rate of patients that have already developed cancer[16]. Furthermore, multiple laboratory-based studies have also provided evidence that metformin may serve as an anti-cancer agent[17-19]. Due to the strong, retrospective clinical evidence and laboratory-based experiments there are more than 100 ongoing clinical trials assessing metformin's anti-cancer effects in combination with current standard treatments[20].

There are two not necessarily mutually exclusive mechanisms to explain the anti-tumor effects of metformin[21]. First, metformin decreases blood glucose and consequently circulating insulin levels. Insulin is a known mitogen for many cancer cells. Insulin and insulin growth factors (IGFs) stimulate the pro-tumorigenic PIK3 signaling pathway in tumor cells that are insulin and/or IGF receptor positive[22]. It is important to note that not all cancers are insulin sensitive therefore reduction of insulin levels would be irrelevant. A second possible mechanism by which metformin acts as an anti-cancer agent is through

inhibition of mitochondrial ETC complex I to diminish tumor growth. In 2000, two studies demonstrated that metformin *in vitro* inhibits mitochondrial complex I[23,24]. Recent work has provided mechanistic understanding of how metformin and related biguanides inhibit complex I[25]. Yet, it was only recently shown in experimental models of cancer that the *in vivo* anti-tumorigenic effects of metformin are also dependent on inhibiting mitochondrial complex I[26]. Specifically, metformin inhibits mitochondrial ATP production and induces cell death when glycolytic ATP diminishes due to limited glucose availability. In the presence of ample glucose, metformin decreases proliferation through mechanisms that are not fully understood. Metformin similar to other complex I inhibitors in prostate cancer cells does not limit the ability to generate TCA cycle intermediates due to induction of glutamine-dependent reductive carboxylation[27]. However, in breast cancer cells metformin through complex I inhibition diminishes TCA cycle intermediate production[28]. A leading mechanism to explain metformin's anti-proliferative effect is that metformin decreases mitochondrial ATP production, resulting in AMPK activation and diminished mTOR activity, a necessity for the anabolism of proliferating cells[29]. It is likely that this mechanism cannot solely explain the anti-proliferative effect of metformin since many cancers that are unable to activate AMPK are still susceptible to the drug[30].

An important concern is whether metformin, as an anti-cancer agent, has a favorable therapeutic index. Metformin requires organic cation transporters (OCTs) that are present in a few tissues such as liver and kidney[31]. Thus, the current dosing of metformin for diabetes has a remarkable safety profile. Tumor cells can also express OCTs to allow the uptake of metformin. However, not all tumor cells express OCTs; thus, metformin would not accumulate in these tumors and inhibit mitochondrial complex I. The uptake of the positively charged metformin at normal pH into the mitochondrial matrix to inhibit complex I requires a robust mitochondrial membrane potential[26]. In going forward, clinical trials using metformin as an anti-cancer agent should assess the expression levels of OCTs and mitochondrial genes in the tumors in order to identify those likely to be susceptible to metformin, those with highest expression. The combination therapy of metformin with PI3K inhibitors that reduce glucose uptake and glycolysis will also be more efficacious than metformin alone. Lastly, it is not clear whether the anti-diabetic dosing of metformin used in current clinical trials will allow for accumulation to levels necessary to inhibit mitochondrial complex I in tumors. It might be possible to escalate the dosing of metformin to higher levels than currently used for diabetes without causing toxicity.

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Specitaly and Present Interest:
Mitochondria as Signaling Organelles

mTOR GROWTH SIGNALING AND METABOLISM

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mTOR is the target of the immunosuppressive drug rapamycin and the central component of a nutrient- and hormone-sensitive signaling pathway that regulates cell growth and proliferation. We now appreciate that this pathway becomes deregulated in many human cancers and has an important role in the control of metabolism and aging. We have identified two distinct mTOR-containing proteins complexes, one of which regulates growth through S6K and another that regulates cell survival through Akt. These complexes, mTORC1 and mTORC2, define both rapamycin-sensitive and insensitive branches of the mTOR pathway. I will discuss new results from our lab on the regulation and functions of the mTORC1 and mTORC2 pathways.

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Specialty and Present Interest:
Physiological and Pathological mTOR Pathway Activation in Cellular and Whole-Organism Context

SERINE METABOLISM IN CANCER DEVELOPMENT

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Cancer cells show changes in metabolism that help to support enhanced proliferation and tumour growth in abnormal environments and under conditions of fluctuating nutrient availability. However, these metabolic alterations can also impose vulnerabilities that may be exploited for therapy. Recent studies have shown that many cancer cells have a high dependency on serine, which can be synthesized *de novo* or taken up from the medium. The serine synthesis pathway (SSP) enzymes are overexpressed in several tumour types[1,2] but those cancer cells that do not show up-regulation of this pathway are much more dependent on exogenous serine[3]. Following serine starvation, intracellular serine levels drop dramatically in these cells and a period of adaptation is required to allow the up-regulation of the *de novo* SSP. Intracellular serine is an allosteric activator of pyruvate kinase M2 (PKM2), the isoform of this glycolytic enzyme that is expressed in cancers and other proliferating cells. Following starvation, the reduction in serine levels results in lower PKM2 activity, a decreased flux through this step of glycolysis and the diversion of glucose derived carbons into the SSP[4]. This response is accompanied by a transcriptional activation of the key SSP enzymes, allowing the cell to switch from exogenous to *de novo* synthesized serine supply. However, the decrease in glycolytic rate that is a result of the reduced PKM2 activity results in less ATP production, and to compensate cells channel more pyruvate into mitochondria, allowing an increase OXPHOS for more efficient energy production. This increased mitochondrial respiration is accompanying increase in ROS that must be limited to prevent oxidative damage and cell death. Interestingly, we found that the tumour suppressor p53 can help to support the adaptation of cancer cells to serine starvation[3]. The activation of p53 in response to serine

depletion leads to a transient cell cycle arrest, allowing the cells to channel metabolites into glutathione synthesis to provide increased antioxidant defense. Cells lacking p53 are less able to survive the switch to *de novo* serine synthesis because they are unable to adequately limit ROS.

An appreciation of the importance of serine in cancer cell growth has prompted us to look more closely at the fate of serine in the cell. Several biosynthetic pathways utilise serine, including protein synthesis, the one carbon cycle, and the synthesis of other amino acids, sphingosine and phospholipids. Serine can be converted into glycine, which is also required for nucleotide and glutathione synthesis. Although serine and glycine can be readily interconverted so that either might be used for nucleotide synthesis and one-carbon metabolism, we found that exogenous glycine cannot replace serine to support tumour cell growth in culture, with higher concentrations of glycine inhibiting proliferation[5]. Further analysis revealed than in the absence of serine, glycine was converted to serine, a reaction that results in the depletion the one-carbon pool. Accordingly, glycine-only fed cells were rescued by addition of folate to replenish the one carbon pool.

Our observations that p53 can help limit redox stress and so support cell survival under serine starvation mirrors a general observation that excessive ROS can be detrimental to cell growth. The antioxidant activity of p53 is mediated through the transcriptional activation of a number of genes encoding proteins with ROS limiting activities. In human cells, one of these is TIGAR, an enzyme that regulates the level of fructose-2, 6-bisphosphate (F-2,6-BP) and helps to support antioxidant defense by increasing NADPH and GSH levels[6]. Although the ability of p53 to drive the expression of TIGAR suggests a role for TIGAR in limiting tumour development – possibly by preventing the accumulation of oxidative damage – studies have shown that oncogenic transformation promotes increases in oxidative stress that can be damaging to the cell, and that the ability to limit ROS can also be important for cancer cell survival[7]. Interestingly, although many tumours lack p53 activity, we detected high levels of TIGAR in many human cancers and cancer cell lines that lack wild-type p53, suggesting that TIGAR expression can be uncoupled from p53 and provide antioxidant functions that help to promote tumour development. p53-independent expression of TIGAR is also seen in In mouse models, where TIGAR expression is only weakly responsive to p53, but is induced in response to oncogenic signals such as loss of APC, Myc activation and increased ROS itself[8]. *In vivo*, loss of TIGAR leads to increased ROS levels that impede survival in regenerating epithelial tissue and inhibit tumour development in several cancer models[9].

Although ROS can be detrimental to cell growth, ROS are also required for mitogenic signaling and cell proliferation. This dual activity in both promoting proliferation and cell death makes prediction of the outcome of ROS limiting treatments in cells or *in vivo*

difficult[10]. Our results support a role for TIGAR in limiting damaging ROS in intestinal regeneration and proliferation induced by APC loss. However, previous studies have shown that loss of APC also leads to the increased expression of Rac1, which promotes proliferative ROS through NOX[11]. In these studies, loss of Rac1 led to decreased ROS and inhibited proliferation. Rac1 and TIGAR therefore have opposing effects on ROS, although both are important to support hyperproliferation resulting from APC deletion in intestinal crypts. To test whether TIGAR simply functions to limit ROS induced by Rac1, or whether TIGAR limits a pool of damaging ROS that is functionally distinct from the proliferative ROS induced by Rac1, we simultaneous deleted both Rac1 and TIGAR in APC null crypts. This resulted in a more profound inhibition of crypt proliferation than seen following loss of either Rac1 or TIGAR alone, suggesting that the pro- and anti-proliferative effects of ROS can be independently modulated in the same cell. Our results show that two key targets induced by the Wnt pathway function to integrate the different ROS signals for optimal cell proliferation.

As many cancer cells were found to be sensitive to serine depletion, we sought to develop a mechanism to deplete circulating serine levels, to assess whether this might have a selectively detrimental effect on tumour development *in vivo*. Dietary depletion of serine does not significantly impact the general health of mice, although a clear reduction in serum serine and glycine levels can be detected in these animals[3]. In xenograft models, tumours grew more slowly in animals maintained on a serine/glycine free diet, with an accompanying increase in survival. More recently we have been testing the effect of this diet on cancer progression in genetically engineered mouse models of cancer, including an EuMyc lymphoma model, an intestinal adenoma models based on APC deletion and a pancreatic cancer model driven by mutations in KRas and p53. To test the hypothesis that serine starvation will further enhance the vulnerability of cancer cells to increases in oxidative stress or depleted antioxidant functions, we are examining the effect of combining dietary serine/glycine starvation with TIGAR depletion on cancer development.

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TUMOR MICROENVIRONMENT AND CANCER METABOLISM

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Angiogenesis, formation of new blood vessels, is essential for tumor progression, invasion, and metastasis. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are primary regulators of angiogenesis. Anti-VEGF antibodies (e.g., bevacizumab) and small molecular inhibitors of VEGFRs (e.g., sunitinib and sorafenib) were validated as the first cancer therapeutic agents targeting the tumor microenvironment. However, the effectiveness of anti-angiogenic treatments is limited to certain types of cancer. In addition, they may not completely eradicate tumor growth and may elicit malignant progression by, for example, inducing resistance to chemotherapy. Thus, the molecular mechanism underlying the elimination of such resistant and refractory cancer cells needs to be elucidated. The adaptation of cancer cells to tumor microenvironments such as in response to hypoxia, nutrient deficiency, acidosis, and reactive oxygen species can be achieved via alteration of metabolic states to glycolysis, glutaminolysis, and other metabolic pathways, but metabolic pathways utilized under hypoxia and nutrient starvation is unknown.

We have previously reported that cancer cells resistant to hypoxia and nutrient starvation stimulated AKT phosphorylation, anchorage independent growth, cell migration and invasion in culture and increased angiogenesis and infiltration of macrophages into tumor tissues *in vivo*, partly under epigenetic regulations[1-5]. To elucidate metabolic pathways utilized in cancer cells under hypoxia and nutrient starvation, we conducted integrated analysis of epigenome, transcriptome and metabolome under control, hypoxia, nutrient starvation, and hypoxia and nutrient starvation *in vitro*. Glycolytic metabolites were accumulated under hypoxia, in contrast, accumulation of fatty acids and late

glycolytic metabolites were accumulated under nutrient starvation and hypoxia and nutrient starvation double stress (Figure 1). Interestingly, phosphoethanolamine was most accumulated in cancer cells under nutrient starvation and hypoxia and nutrient starvation by up-regulation of ethanolamine kinases (ETNK1/CHKA) and down-regulation of the following rate-limiting enzyme PCYT2 in the Kennedy pathway (Figure 2). Moreover,

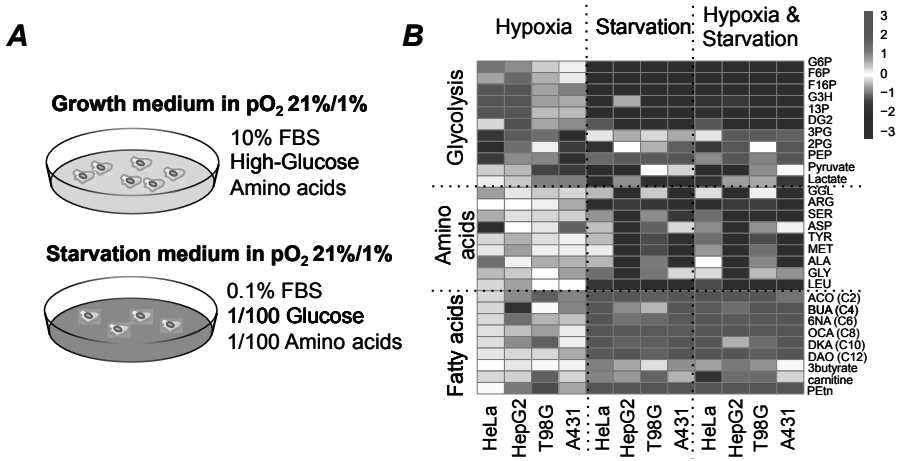

Figure 1 Accumulation of Fatty acids were observed under nutrient starvation, hypoxia and nutrient starvation in cancer cells in vitro. (A) Ingredients of growth rich- and nutrient starved-medium. (B) Metabolome analysis of cancer cells under hypxia, starvation, hypoxia and starvation.

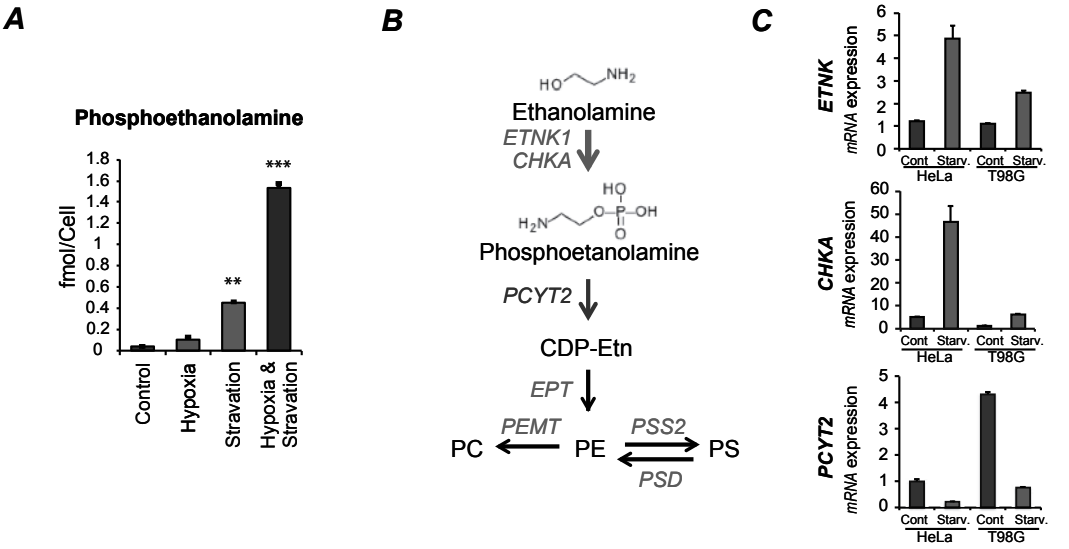

Figure 2 Phosphoethanolamine was accumulated in cancer cells under starvation.
(A) Accumulation of phosphoethanolamine (fmol/cell) in cancer cells (n=3).
(B) Biosynthesis of phosphoethanolamine. (C) Expression of ethanolamine kinases ETNK, CHKA, and rate limiting PCYT2 by real time PCR.

phosphoethanolamine (EAP) was accumulated in tumor tissue and stimulated tumor growth both in vivo (Figure 3), suggesting that phosphoethanolamine can play an important role in cancer cell survival under nutrient starvation.

Our results form the foundation of a strategy to attack hypoxia- and nutrient starvation-resistant cancer cells as an approach to leverage conventional chemotherapy and limit resistance to them.

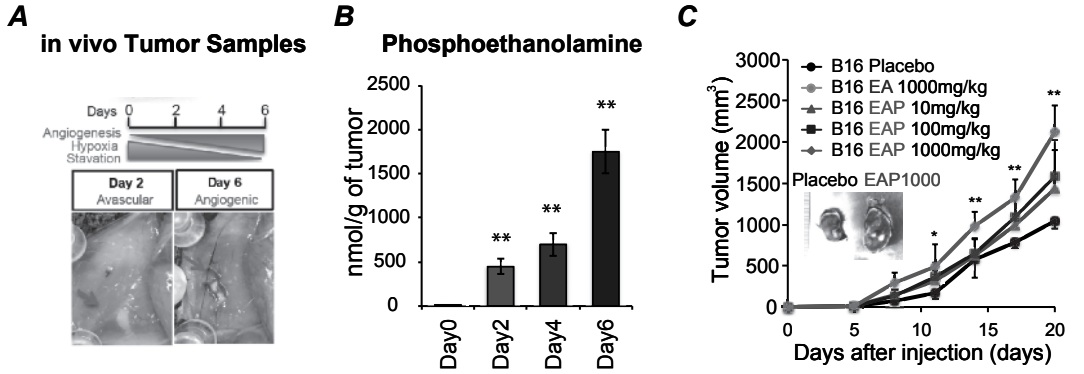

Figure 3 Phosphoetanolamine was accumulated and stimulated tumor growth in vivo.
(A) Preparation of in vivo tumor samples (n=4). (B) Phosphoethanolamine was accumulated in vivo tumor tissue. (C) Phosphoethanolamine stimulated in vivo tumor growth (n=4, B6 mice i.p. per every 2 days)

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DIALOGUES BETWEEN MACROPHAGE AND TUMOR CELLS PROMOTE MALIGNANCY

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The progression of tumors to malignancy is associated with a dynamically changing microenvironment that supports tumor growth and survival[1]. This microenvironment is populated with a wide variety of immune cells from both the innate and acquired system[2]. Indeed as soon as tumors emerge they engage the immune system suggesting that the tissue damage and possibly presentation of neo- or re-expressed foetal antigens results in immune cell activation[3]. However, in most cases this immune response does not result in the destruction of the tumor. Instead the data supports the contention that these immune cells are polarized to provide support to the tumor and thus perversely enhance its growth and progression[3,4]. Indeed a hallmark of cancer appears not only to be immune evasion but also an immune response that enhances malignancy.

Macrophages are one of the major immune cell types that are found in tumors often achieving surprising concentrations[1]. Early studies showed that their accumulation was usually associated with markers of poor prognosis[1]. Similarly the local tumor and circulating concentrations of macrophage growth factors such as colony stimulating factor-1 (CSF1) were also found to be associated with poor prognosis[5]. A good example of this was our perspective study in endometrial cancer which indicated that CSF1 was over-expressed in cancer and positively correlated with grade and stage and in a follow up study that tumor cell produced CSF1 was an independent predictor of survival[6,7]. Subsequently genetic removal of CSF1 and thereby tumor associated macrophages (TAM) in a mouse model of breast cancer caused by the mammary epithelial restricted expression of the Polyoma Middle T oncoprotein (PyMT) caused a reduction in tumor progression to malignancy and a dramatic decrease in metastasis[8]. Similar studies have been performed in

several models of cancer with comparable results[9,10]. These studies have given impetus to translation from mouse models into the human with the results that some reagents usually targeted to the CSF1 receptor (neutralizing antibodies and small molecule inhibitors) have been introduced into clinical trials with early efficacy reported[11].

Most studies of the functions of TAMs have been focused upon the primary tumor. In this context they have been shown to stimulate stimulating angiogenesis, induce tumor cell migration, invasion and intravasation and by suppress anti-tumor immunity[12]. In each case they are involved in reciprocal dialogues with the tumor cells adding support to the hypothesis that tumor cells develop mutations that result in enhancement of their malignancy through engagement of the immune system within the tumor microenvironment. A good example of this is in the TAM promotion of tumor cell invasion and intravasation. In several models from the PyMT one to patient derived xenografts (PDX) this activity involves a paracrine interaction between tumor cell synthesized CSF1 that induces macrophage expression of EGF that drives tumor cell migration, invasion and intravasation[13]. Blockage individually of either growth factors or their receptor signaling inhibits migration of both cell types[14]. Furthermore the macrophages act to open up vessels through the expression of VEGFa at a perivascular site[15]. This unique site of tumor cell escape into the circulation is characterized by a trio of cells, endothelial cells, macrophage and tumor cells expressing an isoform of Mena that can be identified histologically and that has been termed the "Tumor Microenvironment of Metastasis (TMEM)"[16,17]. This structure is predictive of the metastatic capacity of human breast cancers[18]. Interestingly and another example of the complex dynamics found in tumors, is that the ability of the TAMs to engage in this migration-promoting activity is dependent on IL4 that in the PyMT model is produced by CD4 positive T cells[19]. Thus a dynamic of cancer progression is immune cells engagement results in a TH2 skewed cytokine environment that promotes metastatic capacity[2].

Another prominent activity of TAMs is to induce angiogenesis[20] and in part the effects of their ablation causing a slower transition to malignancy in several mouse models of cancer is because they are involved in the angiogenic switch that is considered to be essential for the malignant state[21]. These TAMs in mouse models have been identified as being TIE2 ^{Hi,} a ligand that allows close apposition to vessels[22]. Thus ablation of Tie2 in the myeloid lineage results in the loss of this close association and a reduction in angiogenesis[23]. This angiogenic activity is in part mediate by VEGFa, a molecule highly expressed by macrophages. Indeed in human breast cancer macrophage VEGF expression is associated with markers of poor prognosis[24]. However, surprisingly myeloid cell ablation of VEGFa in the PyMT model although blocking the angiogenic switch, resulted in tumors progressing faster to more malignant state defined by histology[25]. This was

attributed to external selection pressure that selected for tumor cells less dependent on the tri-carboxylic acid cycle and more on glycolysis, that is a hallmark of advanced cancers. Recent studies in the PyMT model however have defined Wnt7b as an upstream macrophage regulator of VEGFa expression either in endothelial cells or TAMs[26]. Consequently, unlike the result obtained following genetic ablation of VEGF that accelerated tumour progression, in this case Wnt7b ablation inhibited the angiogenic switch but also slowed the progression to malignancy and inhibited metastasis[26]. These data suggest that Wnt7b is upstream of VEGFa but has more functions than simply stimulating the macrophage stimulation of angiogenesis. Wnt7b is highly unregulated in several human cancer types and is also expressed in macrophages at least in human breast cancer[26]. Thus these data also suggest given the relative failure of therapies based upon inhibition of VEGFa that the inhibition of WNT7b might be better strategy for control of primary tumors.

Many workers have contributed to understanding the activities of TAMs in tumors, not only through the biology described above but also through their production of a multitude of molecules such as proteases, chemokines and growth factors and immunosuppressive molecules[4,10,27,28]. The general consensus for these studies is that tumors recruit macrophage and polarize them to a state that reflects those seen during tissue repair or during development[29]. These two states may not be that different since macrophage play roles in development pertaining to branching complexity and the maintenance and function of stem cells and these roles may well be important to repair tissues to the normal state following injury[29]. However, to view macrophages as just two basic sub-types is a mistake that belies the complexity of these cells both in their origin and function. In fact most of the functions described above are performed by different sub-populations of macrophages that express canonical macrophage markers but display distinct expression of surface markers and unique transcriptomes[12]. Thus macrophages involved in promoting tumor cell invasion are different from those stimulating angiogenesis despite being within the same tumor. Nevertheless some rules do apply at least in mice that have led macrophages to be described as two polar opposites called M1, for those involved in pathogen and tumor rejection and M2, for those involved in tumor promotion. These states were originally defined by their arginine metabolism profile with the M1-type using arginine to generate the cytotoxic molecule inducible nitric oxide (iNOS) while the M2 macrophages metabolize arginine through the induction of arginase that has the consequence of suppressing cytotoxic T cell activity[30,31].

Insight into these differences in macrophage behavior and function have been obtained through viewing the tumor microenvironment using multiphoton microscopy[32]. This has led to observations of macrophage-tumor cell streaming to blood vessels, to the

visualization of tumor cell extravasation at the TMEN sites where macrophages cause pulsatile permeability events in blood vessels[15,33]. An additional characteristic of tumors as they grow is that they have significant areas of necrosis and hypoxia. These necrotic areas are highly populated by very motile macrophages that are engulfing dead cells but also probably attempting to vascularize these areas. While these macrophages have not been well characterized these observations mean that macrophage must function in areas that are highly hypoxic and suggest that they can readily switch to a glycolytic metabolism. Indeed these macrophages have high expression of HIF1a and HIF2. A characteristic of anaerobic metabolism is the generation of lactate. Tumor cell derived lactate drives macrophage polarization to a tumor trophic state through up-regulating the transcription factor HIF1a[34]. These HIF transcription factor in many contexts, controls a transcriptional programme that promotes angiogenesis and thus may play a role in the re-vascularization of necrotic area as tumors grow.

Many of these studies have been performed in mice and clinical translation has suggested similarities to human TAMs. However, there is a paucity of information on human macrophages from tumors. Indeed the canonical rules derived from the mouse studies immediately break down in humans as human macrophages do not express arginase and highly restricted mouse macrophage surface markers such as F4/80 are not found on human macrophages[29]. To address this issue we have profiled human breast cancer TAMs compared to normal resident macrophages and have defined new transcriptional signatures. Striking in these results is a significant shift in metabolic profiles particularly in lipolysis suggesting unique adaptation to the tumor microenvironment in humans. Such studies should enhance the ability to manipulate macrophages in humans from pro-tumoral to anti-tumoral functions as a potential therapeutic approach to enhance chemo-, radio- or immuno-therapy.

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Speciality and Present Interest:
Macrophages in Tumour Progression and Malignancy, Metastasis, Angiogenesis

CELL COMPETITION AND WARBURG EFFECT

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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[1,2]. 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)[3,4]. In addition, when tumor suppressor protein Scribble- or Mahjong-knockdown cells are surrounded by normal epithelial cells, the transformed cells undergo apoptosis and are removed from the epithelium (a summary of these processes are shown

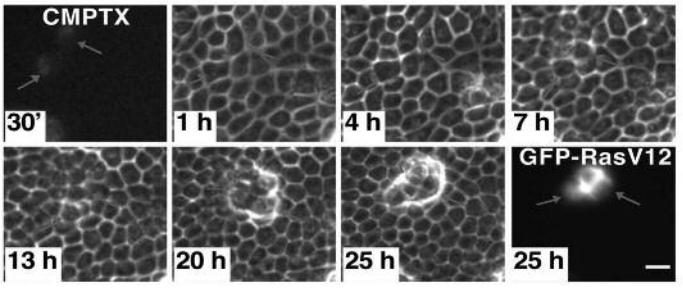

Figure 1 Apical extrusion of RasV12-transformed cells from a monolayer of normal epithelial cells. Arrows indicate RasV12-transformed cells that are apically extruded.

in Figure 2). 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 recently demonstrated that normal epithelial cells can recognize and actively eliminate neighboring transformed cells, implying a notion that the normal epithelium has anti-tumor activity that does not involve immune systems; thus we have named this process EDAC (Epithelial Defense Against Cancer) (Figure 3)[5]. Cell competition is a process by which different types of cells compete with each other for survival, and was originally identified in *Drosophila*. But, our data

Mutations	Phenomena	Reference
Ras	Apical extrusion or basal protrusion formation of Ras-transformed cells	Hogan et al. 2009 *Nature Cell Biology*
Src	Apical extrusion of Src-transformed cells	Kajita et al. 2010 *Journal of Cell Science*
Scribble	Apoptosis of Scribble-knockdown cells	Norman et al. 2012 *Journal of Cell Science*
Mahjong	Apoptosis of Mahjong-knockdown cells	Tamori et al. 2010 *PLoS Biology*

Figure 2 Cell competition between normal and transformed epithelial cells in mammals.
My lab has shown that various phenomena can occur at the interface between normal and transformed epithelial cells.
When these transformedcells alone are present, neither apical extrusion nor apoptosis occur, indicating that the presence of surrounding normal cells affects the signalling pathways and fate of neighbouring transformed cells. Then, what are the molecular mechanisms?

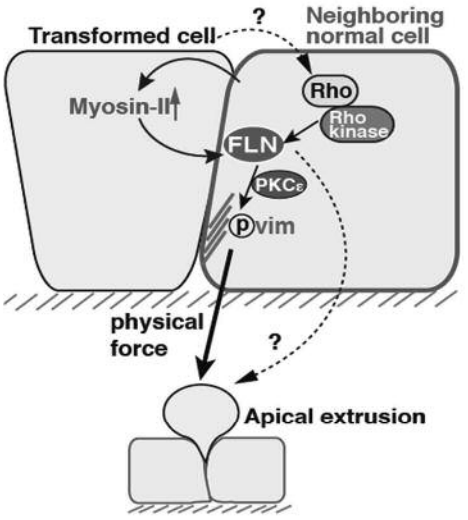

Figure 3 Schematics for EDAC (Epithelial Defense Against Cancer)

indicate that cell competition can also occur in mammals, though the molecular mechanism underlying this important biological process remains largely unknown.

Recently, we have found that mitochondrial activity is substantially decreased in RasV12-transformed cells when they are surrounded by normal epithelial cells. Increased expression of a member of PDK family is responsible for the down-regulation of mitochondrial activity. Addition of DCA (Dichloroacetate), an inhibitor of PDKs, significantly suppresses the apical extrusion of RasV12-transformed cells, suggesting that PDK-mediated mitochondrial down-regulation plays a positive role in the elimination of transformed cells. Furthermore, expression of LDH is enhanced in RasV12-transformed cells surrounded by normal cells, and suppression of LDH activity leads to formation of basal protrusions. These data suggest that the Warburg effect-like phenotype can occur at the initial stage of carcinogenesis, which plays a tumor-suppressive role by promoting elimination of transformed cells from epithelial tissues. By further developing this new research field, we would like to establish a novel type of cancer treatment: eradication of transformed cells by enhancing a defensive force of neighbouring normal epithelial cells.

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Specialty and Present Interest:
Cell Competition, Carcinogenesis

INNOVATIVE MEDICINE FOR METABOLIC REPROGRAMMING OF INTRACTABLE CANCER STEM CELLS

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The most important discovery in the field of cancer research in the past several decades was detecting cancer as a genetic disorder. Although cancer cures are earnestly needed, cancer deaths are increasing and were reported as the most common fatality in 2010, being only second to heart diseases. Cancer treatment remains challenging because of the diversity and heterogeneity of tumors. Cancer heterogeneity is reflected by the co-existence of cells with varying differentiation levels due to accumulation of genetic mutations and significant association with epigenetic alterations. Recent studies suggest that small cell populations, such as cancer stem cells, mimic normal adult stem cells in the dormant phase of the cell cycle and play a role in the biological behavior of tumors. Considering the importance of stem cell biology in cancer, we have recently studied the aforementioned issues and have discussed their possible rationale in this study (Figure 1). By utilizing small compounds, such as synthesized RNAs, including micro (mi) RNAs, we examined the effects of cellular reprogramming in epigenetics and metabolomics, which may be useful in suppressing and eradicating human malignancies, including gastrointestinal cancers (Figures 2-4). Our data indicated that it is possible to reprogram mouse and human cells to pluripotency by direct transfection of mature double-stranded miRNAs, which is similar to the introduction of induced pluripotent stem (iPS) genes. Because this reprogramming method does not require vector-based gene transfer, it holds significant potential for biomedical research and regenerative medicine. The efficiency of this reprogramming method needs to be improved. In this seminal study, we discuss the underlying mechanism of miRNA-based reprogramming and its possible application in cancer cell reprogramming as a novel therapeutic approach[1-3] (Figures 5,6). It is naturally

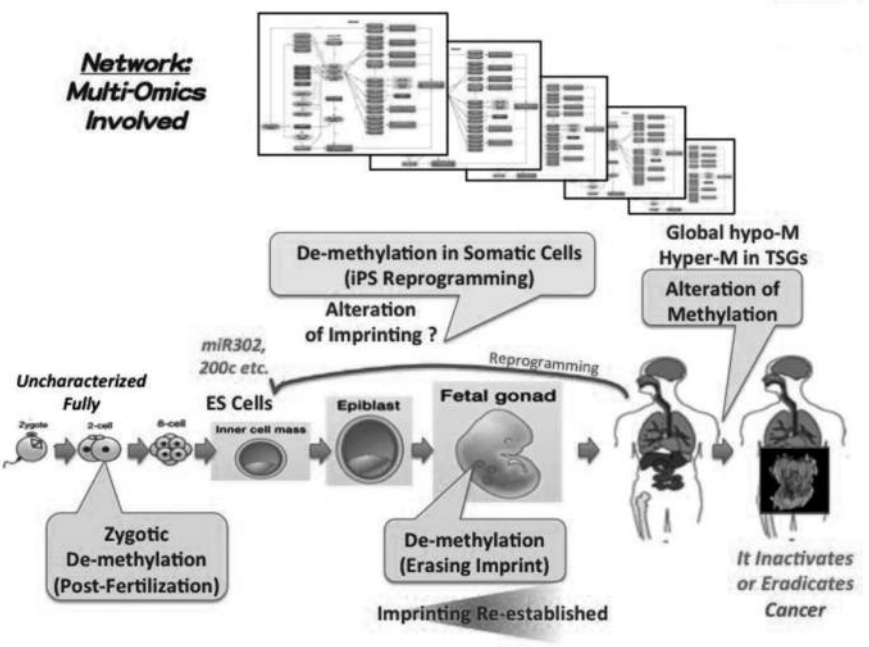

Figure 1 Whole schema of the application of regenerative medicine for cancer research.
In the process of research, the epigenetic and metabolic issues have emerged for the next-generation drug discovery. The scheme indicates that cancer is derived from normally differentiated cells, in which metabolic control plays a critical role under the epigenetic control for gene expression. Cancer exhibits phenotypic appearance largely, in which multi-omics, such as genomes, methylomes and transcriptomes, and metabolomes are involved.

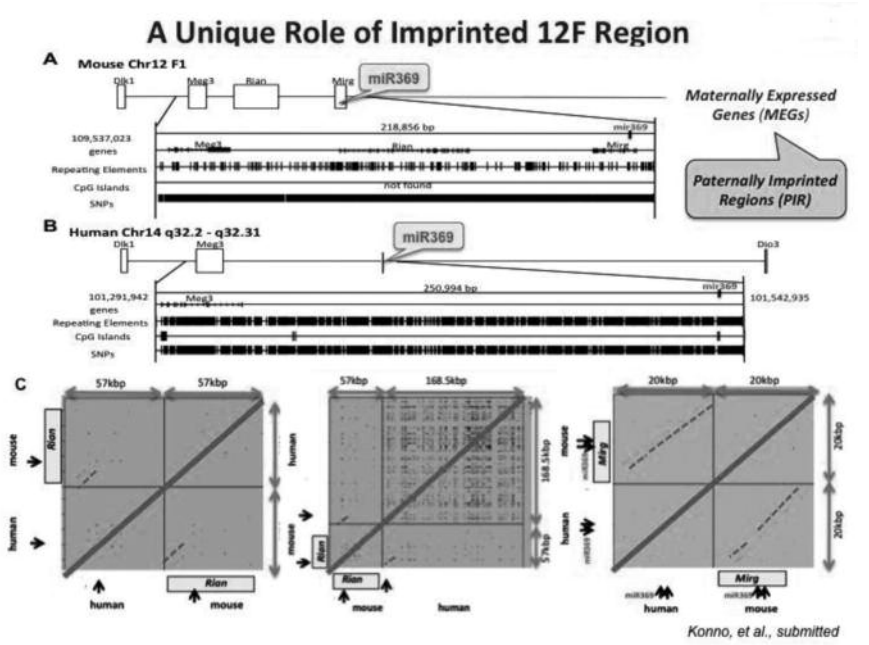

Figure 2 Computational analysis of mouse chromosome 12F, a critical genomic region, which determines the maintenance and susceptibility to cellular reprogramming.
The comparison between human and mouse cells indicated that micro (mi) RNA-369, a subclass of non-coding RNA, is conserved, whereas the other genes, such as mouse Rian and Mirg, are not. This suggests the unique role of miRNA-369[3].

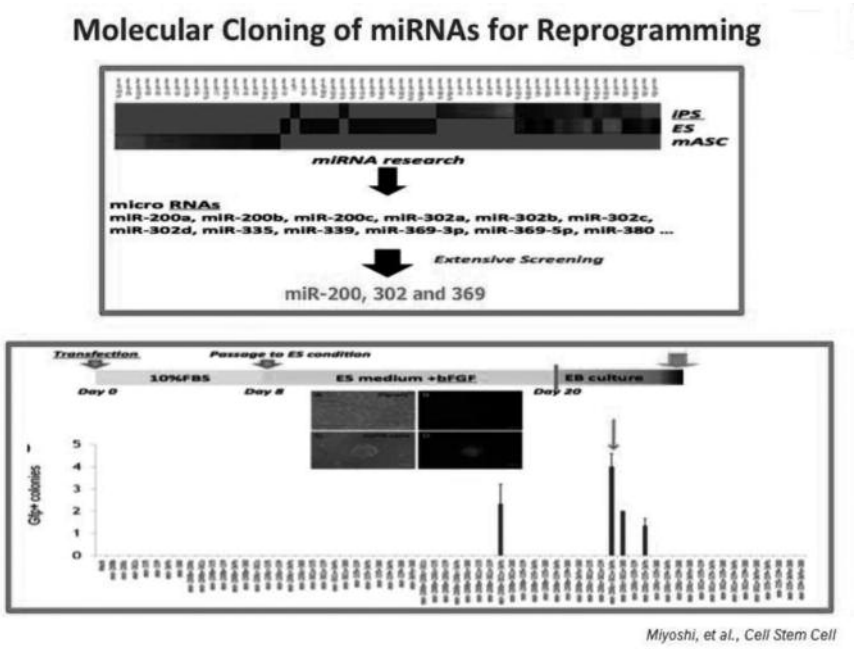

Figure 3 Screening for micro (mi) RNAs could induce cellular reprogramming.
NANOG-green fluorescent protein (GFP) reporter mouse cells, which were isolated from subcutaneous differentiated adipocytes in NANOG promoter driven GFP transgenic mice, were transfected by chemically synthesized miRNAs. These miRNAs were selected from an expression study by Array, which showed substantial expression in embryonic stem (ES) cells and induced pluripotent stem (iPS) cells but not differentiated adipocytes. The study allowed the identification of a set of miRNAs, composed of miR-200c, miR-302, and miR-369. These miRNAs are expressed exclusively in ES cells and iPS cells. The schema is modified (Cell Stem Cell 8(6):633-638, 2011).

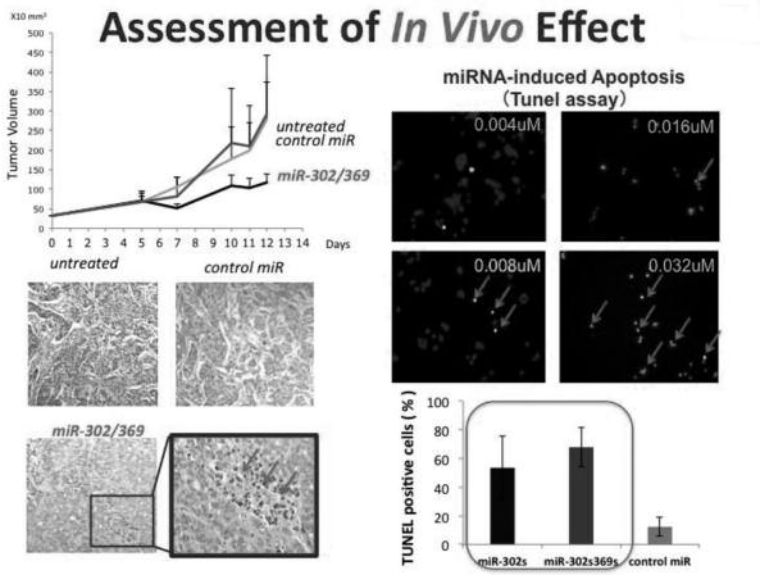

Figure 4 The effect of miRNAs on tumor cells, isolated from the expression study of embryonic stem cells and induced pluripotent stem cells.
The introduction of these miRNAs resulted in the inhibition of gastrointestinal cancer cell growth in vitro and the suppression of tumorigenicity in immune-deficient NOD-SCID mice in vivo. The mice experiment was performed by the intravenous administration of chemically synthesized miRNAs with a drug delivery system against pre-inoculated and formed tumors in NOD-SCID mice[4].

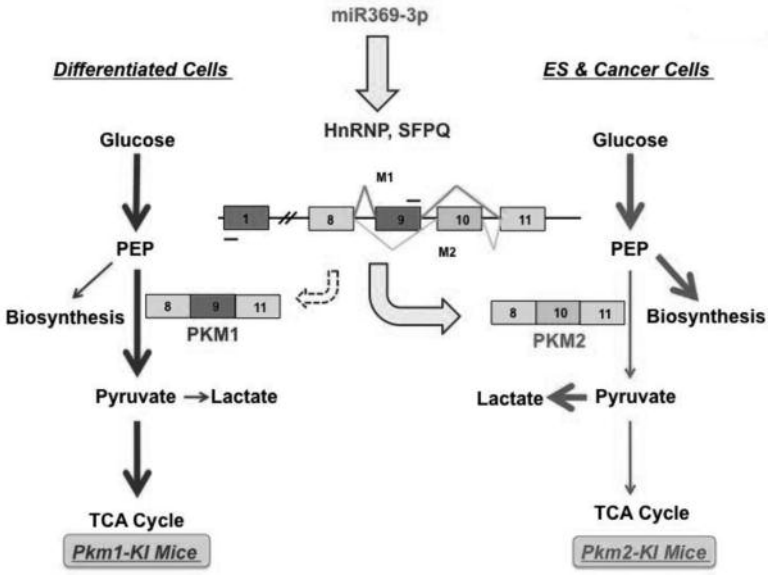

Figure 5 miR-369-dependent alternative splicing elicits the switching of pyruvate kinase (PK).
To end the biological effect of miR-369, we studied PK knock-in mice (Pkm1-KI and Pkm2-KI) in collaboration with Dr Numata and Dr Shima at the Miyagi Cancer Institute, Japan. The study of Pkm1-KI and Pkm2-KI provided information on the biological effect of miR-369, at least partially as a phenocopy[3].

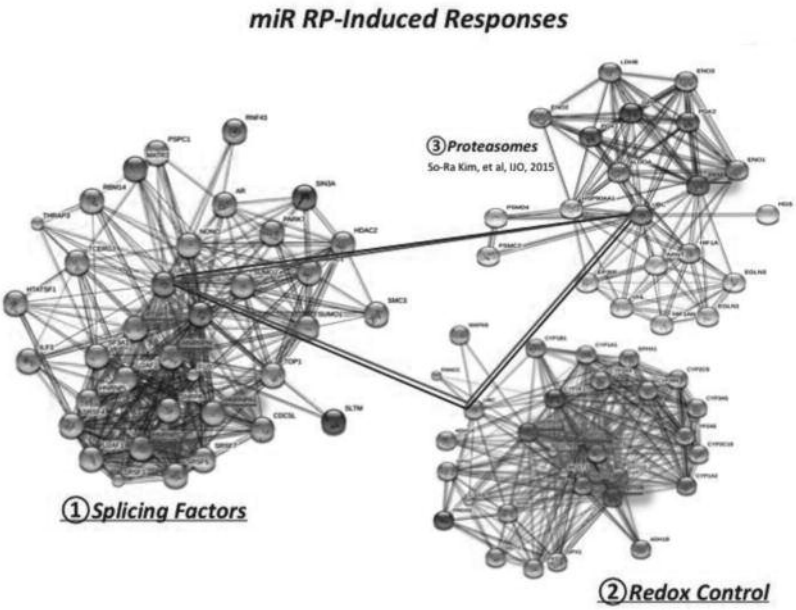

Figure 6 The protein-protein association analysis indicated three hab networks in a miR-369 dependent manner during cell reprograming.
Computational analysis revealed three networks: splicing factors (hnRNPa2b1, SFPQ etc.), redox control (GSTP, PKM, etc.), and proteasome group (UBC, etc.). The computationally predicted networks were confirmed by a wet experiment[3].

expected that the pharmacogenomics of targeting iPS genes may sensitize cancer cell populations that were largely refractory to conventional chemotherapy[2-4]. Taken together, these novel approaches are beneficial for cancer research and may provide an avenue to treat gastrointestinal cancers that are currently difficult to cure (Figure 7).

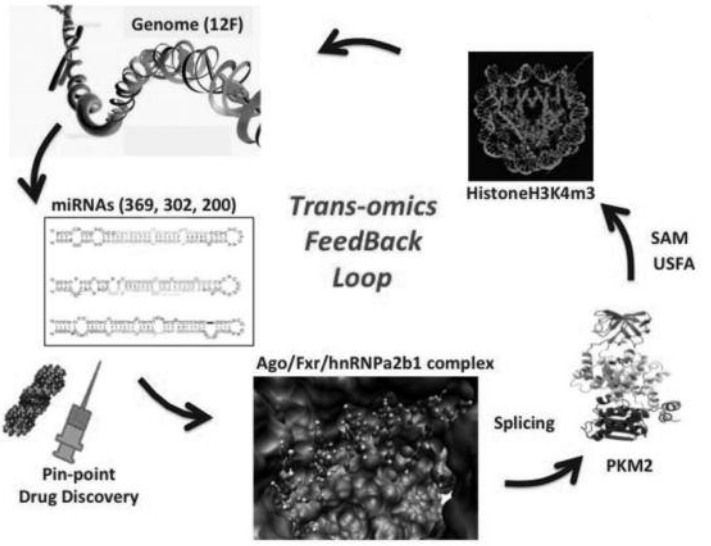

Figure 7 The innovative drug target of nucleotide medicine involves transomic feedback.
We propose a schema using an innovative drug target for cell-targeting therapy of cancer and regenerative medicine. Based on the study of mouse chromosome 12F, we identified several sets of reprogramming factors inducing micro (mi) RNAs, including miR-369. Such miRNAs work as a unique complex and they involve Ago, hnRNPa2b1 and Fxr. The hnRNPa2b1 exerts a unique function downstream, which dictates the metabolic process, where PKM is involved. The PKM regulates further epigenetic alterations of histone modifications via S-adenosylmethionine (SAM) and unsaturated fatty acids (USFA). In the reverse way, we showed that histone modifications control the epigenetics of miR-369 at chromosome 12F. Thus, the novel pin-point drug discovery targeting transomics (transcriptome, epigenome, and metabolome) would provide an avenue for innovative cancer medicine in the near future[1-4].

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Specialty and Present Interest:
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PI KINASES AND CANCER METABOLISM

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Phosphoinositide 3-Kinase (PI3K) is a central enzyme in a signaling pathway that mediates cellular responses to growth factors. This enzyme phosphorylates the 3 position of phosphatidylinositol-4,5-bisphosphate to produce phosphatidylinositol-3,4,5-trisphosphate (PIP_3) at the plasma membrane. A number of signaling proteins, including the Ser/Thr protein kinases, AKT and PDK1, contain pleckstrin homology domains that bind specifically to PIP_3. Thus, the generation of PIP_3 at the plasma membrane in response to activation of PI3K by growth factors results in the initiation of downstream Ser/Thr phosphorylation cascades that control a variety of cellular responses. The signaling pathway downstream of PI3K is highly conserved from worms and flies to humans and genetic analysis of the pathway has revealed a conserved role in regulating glucose metabolism and cell growth. Based on deletion of genes encoding the catalytic or regulatory subunits of PI3K in the mouse, PI3K mediates insulin dependent regulation of glucose metabolism, and defects in activation of this pathway result in insulin resistance. In contrast, mutational events that lead to hyperactivation of the PI3K pathway result in cancers. Activating mutations in PIK3CA, encoding the p110alpha catalytic subunit of PI3K or inactivating mutations in PTEN, a phosphoinositide 3-phosphatases that reverses the effects of PI3K, are among the most common events in solid tumors.

We have generated mouse models to interrogate the role of PI3Ks in metabolic control and in the generation of cancers. These studies indicate that inhibitors of PI3Ks could be effective in treating cancers in specific mutational backgrounds. Our first genetic knockout of the gene encoding the regulatory subunit of class Ia PI3K, PIK3R1 in hematopoietic cells revealed that class Ia PI3Ks are critical for activation of B cell lineage lymphocytes[1]. This

observation and subsequent studies revealing the importance of the class Ia catalytic subunit, PIK3CD for B cell activation led to exploration of inhibitors specific to the gene product of PIK3CD. One such inhibitor, idelalisib was approved for treating chronic lymphocytic leukemia, a B cell lymphoma, in 2014[2].

More than 20 PI3K inhibitors that target the gene product of PIK3CA have entered clinical trials for treating solid cancers. Although these inhibitors look promising, a variety of toxicities have created challenges. Some of the toxicities, such as hyperglycemia, are on target since inhibition of the PIK3CA gene product is expected to cause insulin resistance. However, other toxicities, such as severe rashes and diarrhea are unlikely to be a consequence of inhibition of PIK3CA. These results suggest that more specific inhibitors and inhibitors with specificity for the mutant form of PIK3CA could show better efficacy/toxicity profiles. In any event, there are ongoing approval trials for several PI3K inhibitors, including a pan-PI3K inhibitor from Novartis (BKM120) and an alpha-specific PI3K inhibitor from Novartis (BYL719) that will be discussed below.

Since the gene product of PIK3CA mediates glucose uptake and metabolism downstream of insulin and other growth factors, it is expected that PI3K inhibitors that target the PIK3CA gene product should cause inhibition of glucose uptake and impair anabolic processes downstream of glucose in tumors that have mutations in PIK3CA or where growth factor stimulation of PIK3CA is driving glycolysis. Consistent with this idea, lung adenocarcinomas that develop in the context of PIK3CA activating mutations have high rates of glucose uptake as judged by imaging of ^{18}F-flurodeoxyglucose positron emission tomography (FDG-PET)[3]. Importantly, inhibition of PI3K activity causes a dramatic decline in FDG-PET signal and causes tumor regression in this model.

In order to examine whether PI3K inhibitors affect FDG-PET in human patients and whether an acute decline in FDG-PET correlates with clinical responses, we performed quantitative FDG-PET measurements in ER-positive breast cancer patients prior to and two weeks after treatment with the PI3K inhibitor, BKM120. This study showed that BKM120 caused a decline in FDG-PET in a significant subset of patients. Importantly the subset of patients whose FDG-PET showed a decline were ultimately found to show clinical benefit as judged by stable disease or tumor shrinkage, while those patients whose tumors did not show an acute drop in FDG-PET also failed to show clinical benefit[4]. Interestingly the change in FDG-PET and the clinical benefit did not always correlate with the presence of PIK3CA mutations, suggesting that tumors without PIK3CA mutations may still be dependent on wild type PIK3CA.

In order to better understand how PI3K inhibitors affect glucose metabolism, we have recently evaluated the effect of these inhibitors on flux through glycolysis as well as on the steady state levels of intermediates in glycolysis[5]. These studies have revealed the

surprising results that PI3K inhibitors have a major effect on aldolase activity. This affect on aldolase is not a consequence of inhibition of AKT. Inhibition of AKT impairs glucose uptake and phosphorylation, as expected, however the PI3K-dependent activation of aldolase is mediated by Rac-dependent mobilization of actin. In quiescent cells, much of the aldolase is bound to F-actin in an inactive state and the mobilization of actin causes release and activation of aldolase.

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Specialty and Present Interest:
PI3K and Cancer Metabolism

TARGETING CANCER MICROENVIRONMENT-SPECIFIC METABOLISM REORGANIZES TUMOR VASCULATURE

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The heterogeneity present in tumor cells and in the tumor microenvironment is a hallmark of many advanced cancers. This hallmark is especially true for hypovascular tumors, such as pancreatic cancer. We have examined molecular and biochemical mechanism of cancer cells adapting to severe hypoxic and nutrient-deprived condition present in pancreatic cancer. Our results showed that tumor cells alter cell cycle regulation and cell death programs through both the PI3K-Akt pathway and AMPK and its downstream kinase pathways. We also employed biochemical analyses to study the mechanism of cancer cell survival under these conditions and found that the cells use anaerobic respiration and fumarate respiration[1-3].

Hypoxia and nutrient starvation affect the biological behavior of cancer cells and can alter the outcomes of most of chemotherapy treatments[4]. Thus, we investigated novel agents with cytotoxicity in hypoxia and nutrient-deprived conditions. We successfully identified several candidate compounds that reduced cancer cell tolerance to nutrient starvation. The candidate compounds include kigamicin D, pyrvinium pamoate, angelmarin, and arctigenin[5]. Several of these candidate compounds actually showed antitumor activity in xenograft models. Based on these results, we conducted an investigator-initiated clinical trial using arctigenin-rich extracts from fruite of *Arctinum lappa* Linne in patients with gemcitabine-refractory pancreatic cancer.

Arctigenin shows clear antitumor activity in various xenograft models. There is also an additive or synergistic effect with anticancer drugs including gemcitabine, irinotecan, cis-platinum, and nab-paclitaxel (our unpublished data). We hypothesized that arctigenin might remodel the tumor microenvironment and improve blood supply because there was

preferential arctigenin toxicity in poorly perfused hypoxic tissue. In order to examine this possibility, we utilized perfusion analysis on MRI with Gadolinium-DTPA as a contrasting material, bioluminescence analysis using a hypoxia-resposive luciferase construct integrated tumor cells and pimonidazole staining. Microvasculature was analyzed by immunohistochemical analysis using antibodies against CD31 and α-smooth muscle actin and electron microscopic analysis.

When a human pancreatic cancer cell line xenograft using Suite-2 integrating 5X HRE-Luc was examined MRI perfusion with and without arctigenin administration for 14 days, MRI signal in the tumor was markedly reduced in mice treated with arctigenin than in control mice. Furthermore, hypoxia imaging using HIF-driven luciferase illuminated on IVIS showed remarkable decrease in hypoxia-induced luminescence as shown in Fig. 1. These results clearly showed the increased tissue perfusion in tumor following arctigenin treatment. Same results were obtained with xenograft model using human pancreatic cancer cell MiaPaca-2 integrating the same hypoxia-inducible luciferase construct. Reduction of hypoxia in tumor tissue has a critically important impact on cancer cell behavior including sensitivity to treatment we further examined the degree of hypoxia in tumor tissue by pimonidazole staining. Pimonidazole was given to mice before sacrifice. A representative example is shown in Fig. 2. The results clearly confirmed the reduction of tumor hypoxic region by arctigenin treatment. We also conducted morphological analyses using CD31 staining and found that microvessel density in tumor in mice treated with arctigenin was almost same to that in control mice. However, pericyte coverage was analyzed by staining with α-sma antibody and analysis of co-localization of CD31 and α-sma showed pericyte coverage of tumor microvessel increased in tumors in mice treated with arctigenin than in control mice. Electron-microscopic analysis of tumor vasculature revealed that arctigenin treatment result in less branching and more ordered and straight network of vasculature. All these results clearly showed that arctigenin treatment remodeled and normalized tumor vasculature. Present "vascular normalization" described by bevacizumab treatment[6] may explain the mechanisms for the combinational effects of arctigenin and other anticancer drugs by improving drug delivery and sensitivity to drugs.

Arctigenin also shows preferential toxicity to the "cancer initiating cell population," which is similar to metformin and phenformin in vitro and in vivo (our unpublished data). Cumulatively, our data suggest that arctigenin, and potentially antiausterity agents, could be a novel class of anticancer treatments.

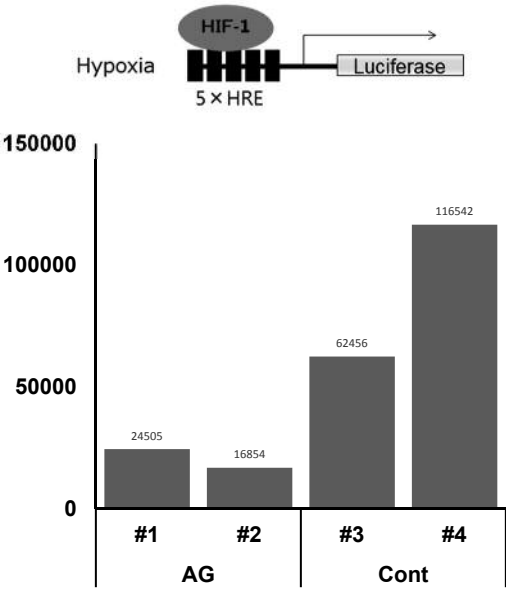

Figure 1 Suit2/5HRE-Luc cells,supplied by Harada, Kondoh, Hiraoka (2006) were transplanted into nude mice and luciferase activity was measured by IVIS after 14 days treatment with arctigenin or control diets.

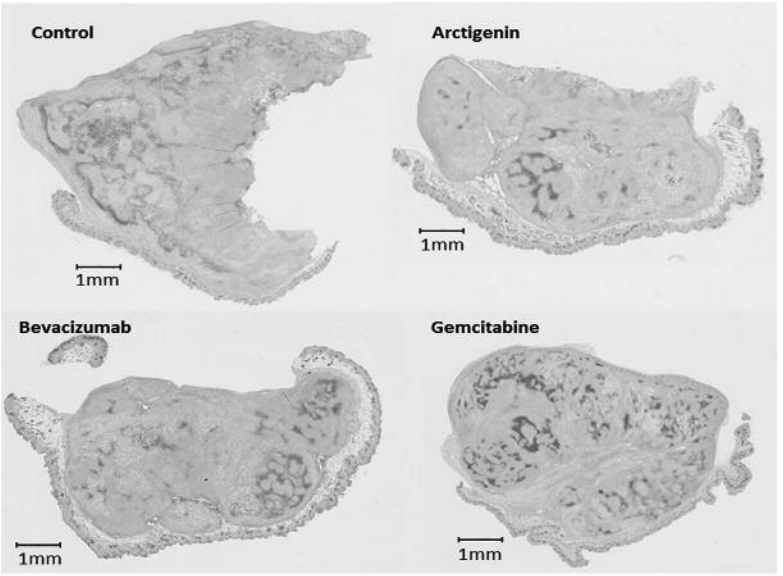

Figure 2 Pimonidazole staining of xenograft tissues of MiaPaca 2 cell containing 5X HRE-Luc construct after 4-week treatment as indicated.

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Specialty and Present Interest:
Biochemistry. Tumor Microenvironment and Cancer Cell Metabolism

INHIBITORS OF MUTATED ISOCITRATE DEHYDROGENASE AS A POTENTIAL NEW THERAPY FOR AML: FROM THE BENCH TO THE CLINIC

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Somatic mutations in isocitrate dehydrogenase 1 and 2 (IDH1/2) have been identified in a spectrum of solid and hematologic malignancies following their initial discovery in glioma[1,2] and acute myeloid leukemia (AML)[3]. Our understanding of IDH1/2 mutation prevalence continues to evolve with further genomic analysis of different tumors. Currently, 68-74% of low grade gliomas and secondary glioblastomas, 40-52% of chondrosarcomas, 11-24% of intrahepatic cholangiocarcinomas (IHCCs), 6-10% of AMLs, and 3% of myelodysplastic/myeloproliferative neoplasms (MDS/MPNs) are thought to have IDH1 mutations; 9-13% of AMLs, 3-6% of MDS/MPNs, and 2-6% of IHCCs are thought to have IDH2 mutations.

Wild-type IDH1 and IDH2 are metabolic enzymes found in the cytoplasm and mitochondria, respectively, which function as homodimers to convert isocitrate to alpha-ketoglutarate (α-KG) with the concomitant generation of NADPH. The recurrent mutations identified in cancer all map to one of three key residues in the active site (R132 in IDH1, and R172 and R140 in IDH2) (Figure 1). Since the cancer-associated mutant enzymes are deficient in α-KG production, an initial hypothesis suggested that IDH1/2 were tumor suppressors in which mutations led to a loss of function. However, in IDH1/2 mutant cancers the wild-type allele is still expressed, and the heterozygous nature of these mutations suggested a gain-of-function mechanism of oncogenesis. An Agios study combining non-targeted metabolomics and structural biology identified this gain of function, demonstrating that mutant IDH1 generates the oncometabolite, R-2-hydroxyglutarate (2-HG), from α-KG and NADPH[4]. This neomorphic enzymatic activity is shared by mutant IDH2[5]. 2-HG is usually produced at low levels (<300 μM) through errors

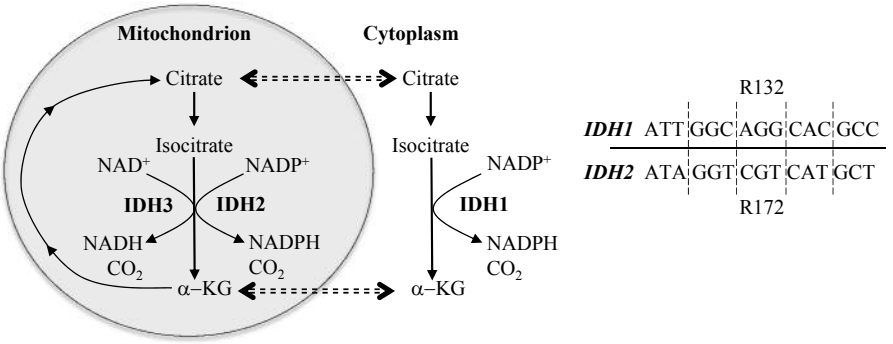

Figure 1 Isocitrate dehydrogenase (IDH) 1/2 normal enzyme function, and site of mutations found in cancer

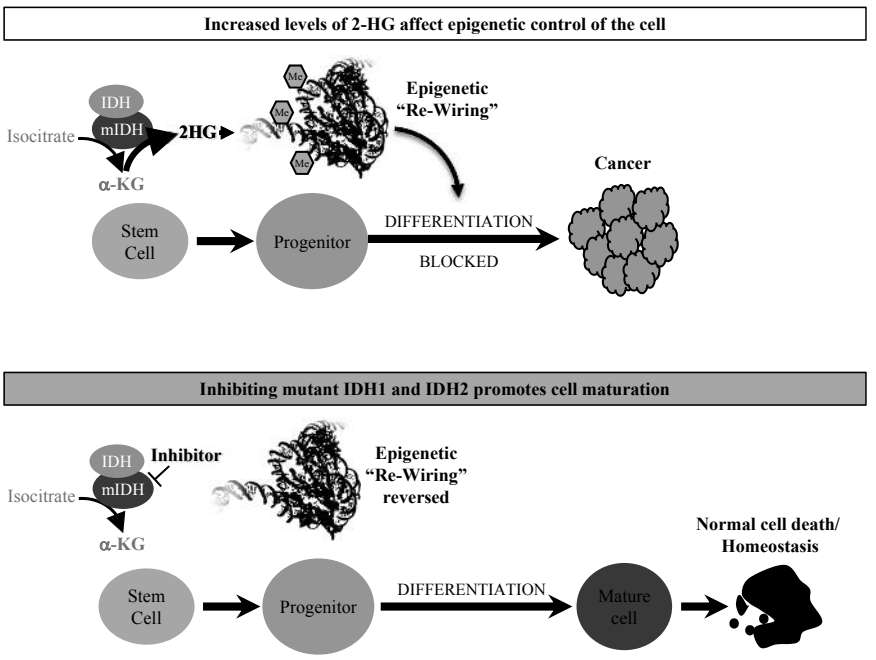

Figure 2 IDH mutations lead to a block in normal cellular differentiation

of metabolism, and is cleared by dehydrogenase housekeeping enzymes. The very high levels of 2-HG (in the mM range) produced by mutant IDH1/2 overwhelm the clearance mechanism, leading to the significant 2-HG accumulation seen in IDH1/2 mutant cells and tumor samples from patients with IDH1/2-mutant cancers[4,5]. 2-HG accumulation can also be measured in patient blood samples[6], and can be detected by imaging of inaccessible solid tumors[7], although further research is required to fully interpret its role as a pharmacodynamic marker of mutant IDH1/2 activity.

IDH1/2 mutations are thought to initiate and drive oncogenesis in cooperation with other mutations, and there is significant evidence to support the role of 2-HG as a functional oncometabolite. Due to its structural similarity to α-KG, 2-HG is a competitive inhibitor of several α-KG-dependent histone and DNA demethylases, and high levels of 2-HG thus result in DNA and histone hypermethylation[8]. These epigenetic changes lead to a block in cellular differentiation. Multiple pre-clinical studies have demonstrated that inhibition of mutant IDH1/2 reverses the hypermethylation and restores normal cellular differentiation[9-11] (Figure 2).

Such promising pre-clinical data provided the rationale for the clinical development of inhibitors of mutant IDH1/2. AG-221 and AG-120 (Agios) are first-in-class, oral, potent, reversible, selective, small molecule inhibitors of the IDH2 and IDH1 mutant enzymes, respectively. Both molecules can restore cellular differentiation, as demonstrated in IDH-mutant patient AML samples *ex vivo*[12] and in a mouse xenograft model[13]. In a primary human IDH2-mutant AML xenograft mouse model, AG-221 treatment reduced 2-HG levels and conferred a dose-dependent, statistically significant survival benefit versus vehicle treatment[14].

The clinical development of AG-221 and AG-120 encompasses both hematologic and solid IDH-mutant malignancies. Approximately 6-10% of AMLs have IDH1 mutations, and 9-13% have IDH2 mutations. AML has a poor prognosis, with a 5-year survival rate of 26%[15]. The median age at diagnosis is 67 years[15], consequently many patients cannot tolerate standard of care chemotherapy and transplant treatment strategies. There have been few significant advances in AML treatment for decades, the notable exception being for one particular AML subtype, acute promyelocytic leukemia (APL). Here, use of the differentiation agent, all-trans retinoic acid, in combination with arsenic trioxide has been effective in achieving high remission and overall survival rates, even without additional use of cytotoxic chemotherapy.

Phase 1/2 clinical trials for both AG-221 and AG-120 are ongoing in patients with IDH2 or IDH1 mutation-positive advanced hematologic malignancies, respectively (ClinicalTrials.gov NCT01915498 and NCT02074839). Both are single-arm, open-label, dose escalation and expansion studies, in which either AG-221 or AG-120 is given orally as a

single agent. Along with safety endpoints, objective responses are assessed by investigators using International Working Group (IWG) AML and MDS criteria. In both studies patients enrolled are heavily pre-treated, and the majority have relapsed/refractory AML. To date, both AG-221 and AG-120 have been well tolerated, and display favorable pharmacokinetic/pharmacodynamic profiles; for both drugs, after multiple doses, plasma 2-HG levels were reduced from the elevated baseline levels by up to 98%[16, 17]. Preliminary overall response rates (including complete response [CR], partial response [PR], and platelet and marrow responses) were ~30-40%. However, traditional IWG criteria may overlook certain aspects of clinical benefit in patients who do not achieve CR. For example, complete recovery of platelet and neutrophil counts is required to achieve a PR. In some patients, the PRs appear to be as durable as complete remissions. Some patients with stable disease are also demonstrating an improvement in neutrophil and/or platelet counts. Such PRs may convert to CRs over time, or remain as the best response but still confer significant clinical benefit for the patient. These observations might reflect the differentiation mechanism of action of mutant IDH1/2 inhibitors, which is fundamentally different to cytotoxic chemotherapy, and may occur over a longer timeframe. Evidence for differentiation of cancer cells has been demonstrated in patients receiving AG-221 or AG-120. Further data will be required to fully characterize the response patterns to, and clinical benefit of, targeted differentiation agents such as mutant IDH inhibitors. A phase 3, open-label, randomized trial comparing AG-221 with the standard of care in IDH2-mutant relapsed/refractory AML (IDHENTIFY, NCT02577406), and a phase 1, open-label trial of AG-881 (an inhibitor of both mutant IDH1 and IDH2) in advanced IDH1 or IDH2-mutant hematologic malignancies (NCT02492737), have recently opened. Further clinical studies combining AG-221 or AG-120 with standard chemotherapy are also planned (Figure 3).

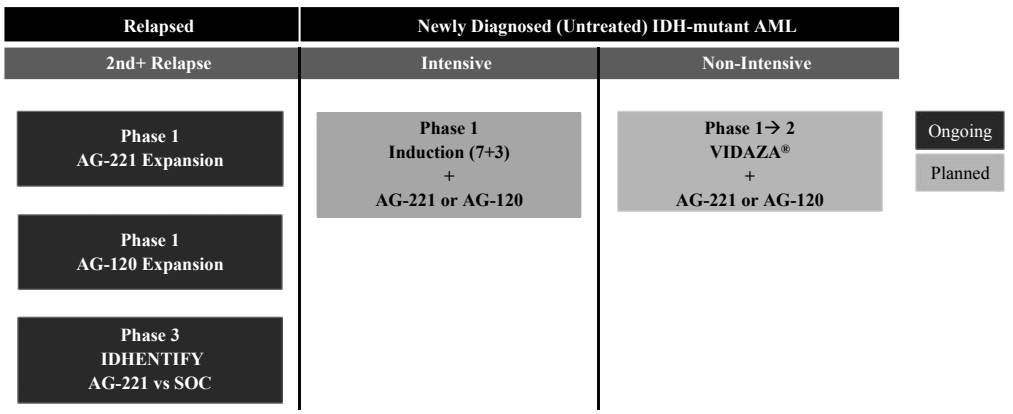

Figure 3 IDH development program targets multiple lines of treatment from relapsed/refractory to frontline AML

In IDH-mutant low-grade glioma, secondary glioblastomas, IHCCs and chondrosarcomas, IDH1 mutations are more frequently observed than IDH2 mutations. These tumors are currently difficult to treat and have low survival rates. A phase 1 dose escalation and expansion trial of AG-120 in patients with advanced IDH1 mutant solid malignancies, including glioma, chondrosarcoma and IHCC, is ongoing (NCT02073994), and preliminary results suggest that treatment is well tolerated, with treatment durations of up to 12 months[18]. Reductions in 2-HG levels were observed, and magnetic resonance imaging/spectroscopy is being utilized to non-invasively measure 2-HG levels in gliomas, along with tumor volume changes, pre- and during AG-120 therapy. A phase 1 study of AG-881, which has improved blood-brain barrier penetration, in patients with IDH1 or IDH2-mutant advanced solid tumors is also underway (NCT02481154). Results from these studies are awaited and will inform the continued development of mutant IDH inhibitors.

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Specialty and Present Interest:
Drug Development, Hematologic Malignancies

LARGE-SCALE POOLED SHRNA SCREENS AND THE DISCOVERY OF NOVEL CANCER TARGETS FOR DRUG DISCOVERY

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Cancer is driven by the accumulation of somatic genetic alterations that later the activity of tumor suppressor and oncogenes. Together these activities lead to the dysregulation of cell proliferation, cell survival and metabolic pathways, among many other, that culminate in the acquisition of the transformed phenotype characteristic of malignant tumors. Advances in Next-Generation Sequencing and projects like the Cancer Genome Anatomy project and the International Cancer Genome Consortium have given us a detailed picture of the genetic alterations across a broad spectrum of cancer. This however, does not immediately lead us to a detailed functional understanding of those genes whose protein products are necessary for the maintenance of cancer viability. In order to more deeply understand the genes and pathways critical to the survival of cancer cells we have taken on the functional annotation of the Cancer Cell Line Encyclopedia (*Nature 2012;483:603-7*) through the use of deep pooled shRNA libraries. The resulting effort termed Project DRIVE (Deep RNAi-screening for Viablity Effects in cancer) has led to the generation of drop-out shRNA data for 7,500 genes targeted by 20 shRNAs per gene across 300 cancer cell lines. The data from this effort are highly robust identifying all known oncogenes mutated in the set of interrogated cell lines, along with multiple new cancer dependent features not previously described. These data and several exemplary targets will be discussed in further detail in this lecture.

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Specialty and Present Interest:
Cancer Drug Discovery

WHAT CAUSES ALTERED METABOLISM IN COLON CANCER CELLS?

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Metabolic reprogramming is a common hallmark of cancer. Most cancer cells produce ATP and the precursors of biomass (e.g., nucleotides, amino acids and lipids) by shifting from oxidative phosphorylation to aerobic glycolysis, commonly known as the "Warburg effect". Recently these cancer specific metabolic pathways have been considered as attractive targets for cancer therapy. However, the mechanisms underlying the control of cancer cell metabolism is poorly understood.

To address underlying mechanisms that induce metabolic reprogramming of cancer metabolism, we applied capillary electrophoresis mass spectrometry (CE-MS) metabolic profiling[1-3] to paired normal tissues and tumor tissues obtained from 275 patients with colon cancer. Significant changes in the levels of many metabolites were observed between normal and tumor tissues. Unexpectedly metabolite levels altered quite early and remained unchanged among cancer stages. S-adenosylmethionine (SAM), was the most increased metabolite. Glucose was the second most decreased metabolites in tumor tissues, whereas lactate, the final product of glycolysis, was increased, which indicates activation of the glycolysis, termed the Warburg effect.

We then applied transcriptome analysis to paired normal-cancer colon tissues and revealed that alterations in mRNA levels also occurred at the adenoma stage. Regarding energy metabolism, expression of mRNAs encoding pentose phosphate pathway, some glycolysis and fatty acid synthesis were increased in tumor tissues. On the other hand, expression of mRNAs encoding most of gluconeogenesis, fatty acid oxidation and tricarboxylic acid (TCA) cycle were reduced.

Colon cancer is associated with mutations in oncogenes and tumor suppressor genes

such as *adenomatous polyposis coli* (*APC*), *Kirsten-ras* (*KRAS*) and *TP53*. We applied next generation sequencing technologies for the detection of somatic mutations to the tumor tissues and found mutation frequencies in *APC* of 78%, *TP53* of 68% and *KRAS* of 48%. Similarly to metabolome and transcriptome data, these mutations were umbiased among cancer stages. We investigated whether APC, KRAS and TP53 mutations affected metabolism. However, regardless of presence or absence of these mutations, the levels of most of metabolites were largely-unaltered in tumor tissues.

Next, we investigated the structural properties of normal and tumor tissues obtained from patients with various cancer stages using transmission electron microscopy and found mitochondrial swelling, cristae disappearance and matrix clearout in tumor tissues. These results indicate that mitochondrial abnormality may be associated with colon cancer development as well as metabolic reprogramming of colon cancer metabolism.

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Specialty and Present Interest:
Metabolomics, Cancer Metabolism, Cancer Epigenetics

NUTRIENT SENSING AND METABOLIC REPROGRAMING IN THE TUMOR STROMA

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Cancer therapeutic targets have been extensively and almost exclusively focused on oncogenes and tumor suppressor genes, as well as on their mechanisms of action and regulation. An emerging alternative strategy involves the identification of proteins that although not being encoded by oncogenes or tumor suppressors, however, are required to accommodate cancer-driven nutrient and metabolic stresses.

It is becoming more apparent that the ability of cells to respond to changes in nutrient availability is essential for an adequate control of metabolic homeostasis. In this regard, mTORC1 is central to these processes. The autophagy substrate and signaling adaptor p62 is located in the lysosomes and participates in both the activation of mTORC1 in response to amino acids and the shuttling of autophagy-targeted proteins to the autophagosome[1]. We have shown that p62 binds Raptor and the Rag proteins, as well as TRAF6, which is also required for mTORC1 translocation to the lysosome and its subsequent activation. This involves the TRAF6-catalyzed K63 ubiquitination of mTOR that is critical for its activation by amino acids. More recently we have identified new components of the p62-interacting complex that translate nutrient sensing events into mTORC1 activation[2]. More recently, studies in our laboratory have identified a new kinase cascade critically involved in the activation of the mTORC1 complex in response to the nutritional environment of the tumor cell, specifically the levels of amino acids[3]. That is, our new data demonstrate that p62 is phosphorylated at two specific residues T269/S272 and that this is mediated via a novel cascade that includes MEK3/6 and p38δ. This new pathway is orchestrated by the PB1-containing kinase MEKK3 through a PB1-PB1 selective interaction that links MEKK3 to p62 on the lysosome surface. This phosphorylation results in the recruitment of TRAF6 to

p62 on the lysosomes, which triggers the K63-mediated polyubiquitination. This event has recently been shown to be important for the full activation of mTORC1[2]. Importantly, we have also shown that both MEKK3 and p38δ, like p62, regulate autophagy through mTORC1 and are required for the proliferation of various types of cancer cells in vitro and in vivo. Furthermore, genetic inactivation of MEKK3 or p38δ mimics that of p62 in that it leads to inhibited growth of PTEN-deficient prostate organoids. Of great relevance from the point of view of human disease, analysis of human prostate cancer samples showed upregulation of these three components of the pathway (namely, p62, MEKK3, and p38δ), which correlated with enhanced mTORC1 activation in human cancer. In order to determine the in vivo relevance of this pathway in an endogenous model of hepatocellular carcinoma (HCC), in collaboration with Dr. Karin (UCSD) we have generated mice in which the tumor and mTORC1 suppressor, TSC1, was genetically inactivated in hepatocytes using an Alb-Cre mouse line. This was done in a wild-type background or in mice in which p62 has been knocked out specifically in the hepatocyte fraction. Interestingly, whereas the inactivation of TSC1 in hepatocytes (that results is sustained activation of mTORC1) results in HCC induction, the simultaneous inactivation of p62 severely abrogates tumorigenesis induced by TSC1 deficiency. Consistent with our model, mTORC1 activation by TSC1 deficiency was totally inhibited in a p62-deficient background. These results demonstrate that p62 is a novel and bona fide positive regulator of mTORC1, and is critically involved in various types of cancer and in the control of autophagy.

The role of the tumor stroma is also an important aspect of the carcinogenic process and its potential resistance to therapy. We have recently shown that at least one of the critical mechanisms by which the microenvironment influences the tumor epithelium involves p62-deficient stromal cells. That is, p62 levels are reduced in the stroma of several tumors and its loss in stromal fibroblasts resulted in increased tumorigenesis of epithelial prostate cancer cells. The mechanism involves the regulation of cellular redox through an mTORC1/c-Myc pathway of stromal amino acid metabolism, resulting in increased stromal IL-6 production[4]. Our newest data establishes how tumor-derived signals promote p62 downregulation in the stroma, as well as how the loss of p62 in that cell fraction contributes to resistance to anti-tumor therapy. To firmly establish this notion, we have generated a mouse line in which p62 was selectively inactivated in fibroblasts by crossing p62-floxed mice with FSP1-Cre mice. Surprisingly, these mice develop spontaneous prostate tumors, which even reach the high-grade PIN phenotype. Therefore, p62, although a tumor promoter in the epithelium, is a powerful tumor suppressor in the stroma through its ability to regulate mTORC1 in both cellular compartments. These results raised the question of whether mTORC1 inhibitors efficacy could be improved if mTORC1 is selectively targeted in the epithelium without affecting the stroma.

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Specialty and Present Interest:
Nutrient Sensing and Cancer Metabolism in the Tumor Microenvironment

THE MICROBIOME AND MICROBIAL-DERIVED METABOLITES IN CARCINOGENESIS: THE FIBER-MICROBIOTA-BUTYRATE AXIS IN HISTONE ACETYLATION AND TUMOR SUPPRESSION AS A CASE STUDY

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It is highly controversial whether dietary fiber protects against colorectal cancer because of conflicting results from human epidemiologic studies. These studies have been complicated by the participants' genetic heterogeneity as well as differences in the composition of their gut microbiota and the utilization of different fiber sources. To eliminate these confounding variables, we analyzed a gnotobiotic mouse model of colorectal cancer[1]. Our experiments investigated the function of butyrate because it is a short-chain fatty acid produced by bacterial fermentation of fiber in the colon at high (mM) levels and has potent energetic and epigenetic properties in colonocytes[2-4]. Here, we report that fiber does have a strong tumor-suppressive effect but in a butyrate-dependent manner that is mediated through the microbiota. The incidence, number, size, and histopathologic progression of AOM-induced colorectal tumors were significantly diminished in BALB/c mice provided a high-fiber diet only when they were colonized with defined microbiota that included a butyrate-producing bacterium. The tumor-suppressive effect was attenuated when mice were colonized with the same microbiota except with the wild-type butyrate producer replaced by a strain harboring a 0.8-kb deletion in the butyryl-CoA synthesis operon. To confirm that butyrate is a causal factor, the tumor-suppressive effect was recapitulated in mice lacking any butyrate-producing bacteria when they were provided a butyrate-fortified diet. Our data support a general mechanism that includes bacterial fermentation rather than fiber exclusively bulking luminal contents and speeding colonic transit to minimize exposure of colonocytes to ingested carcinogens. Our data also support a molecular mechanism that is metaboloepigenetic. Due to the Warburg effect[5], cancerous colonocytes rely on glucose so butyrate was metabolized less and accumulated

as an HDAC inhibitor that increased global histone acetylation levels, increased apoptosis, and decreased cell proliferation. *Fas* and *p16* are hyperacetylated and overexpressed target genes that may account for these effects. To support the relevance of this model to human cancer, we demonstrate that butyrate accumulates in human colorectal adenocarcinomas, and this is associated with higher global histone acetylation levels. These results link diet and microbiota to an abundant metabolite that influences epigenetics and cancer predisposition.

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Mouse Models of Disease, Epigenetics, Microbiome

BEYOND GLUCOSE AND GLUTAMINE: SOME CANCER CELLS UTILIZE EXTRACELLULAR MACROMOLECULES TO FUEL CELL GROWTH

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To grow and divide, cells require a continuous supply of nutrients that support energy production and macromolecular synthesis[1)]. Despite being surrounded by a diverse supply of bioenergetic substrates, mammalian cells preferentially metabolize low molecular weight nutrients such as glucose and amino acids. However, proteins are the most abundant organic constituents in body fluids and thus have the potential to function as important alternative nutrients, if accessible to cells. Consistent with this idea, it was recently shown that Ras-induced macropinocytosis could promote the uptake of extracellular proteins and lipids[2-4)].

Lysosomal degradation of extracellular macromolecules can sustain cell survival and induce activation of the amino acid sensor, mechanistic target of rapamycin complex 1 (mTORC1)[5)]. However, catabolism of endocytosed proteins as an essential amino acid source fails to elicit significant cell accumulation. Unlike its growth-promoting activity under amino acid-replete conditions, we discovered that mTORC1 activation suppresses proliferation when cells rely on extracellular proteins as an amino acid source (Figure 1). Inhibiting mTORC1 results in increased catabolism of endocytosed proteins and enhances cell proliferation during nutrient-depleted conditions in vitro and within vascularly compromised tumors in vivo. Thus, by preventing nutritional consumption of extracellular proteins, mTORC1 couples growth to availability of free amino acids. These results may have important implications for the use of mTOR inhibitors as therapeutics.

Besides mTORC1, mammalian cells possess a second amino acid-sensing kinase: general control non-derepressible 2 (GCN2). The combined effects of these two kinases allow cells to adapt to varying levels of essential and non-essential amino acids, but how their

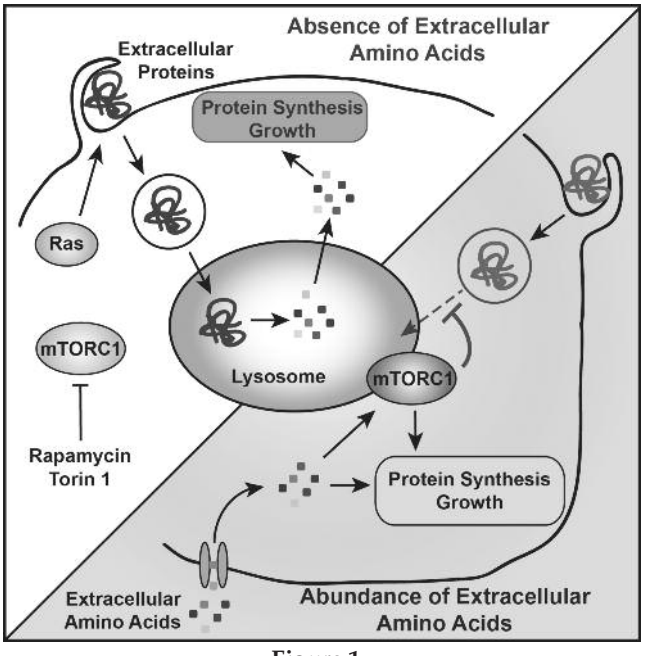

Figure 1

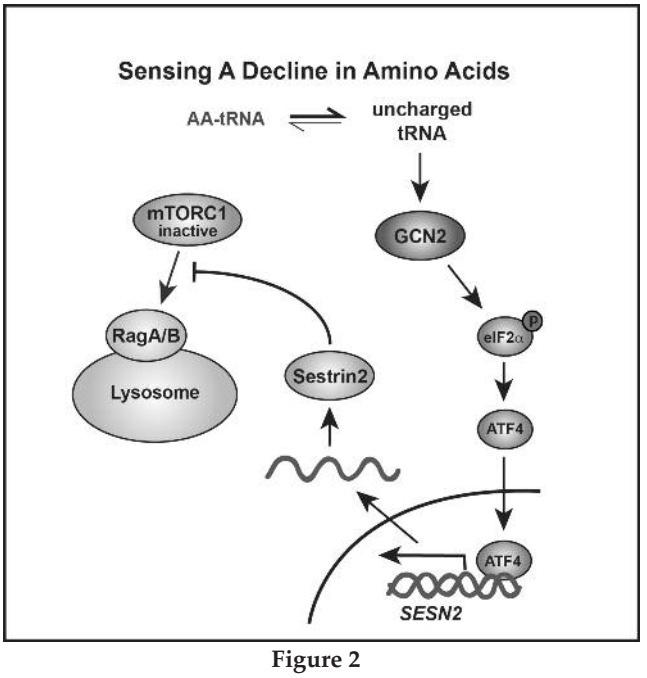

Figure 2

activities are coordinated remains poorly understood. We recently found an important link between GCN2 and mTORC1 signaling[6]. Upon deprivation of various amino acids, activated GCN2 up-regulates ATF4 to induce expression of the stress response protein Sestrin2. The upregulation of Sestrin2 is required to block lysosomal localization of mTORC1 and therefore to inhibit its kinase activity (Figure 2). The GCN2/ATF4-dependent induction of Sestrin2 occurs in response to deprivation of a variety of essential amino acids. In addition, Sestrin2 induction is necessary for cell survival during glutamine deprivation, indicating Sestrin2 is a critical effector of the GCN2-ATF4 pathway that regulates both essential and nonessential amino acid homeostasis through mTORC1 suppression.

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The Regulation of Cellular Metabolism During Cell Growth/Differentiation and on the Role that Metabolic Changes Play in the Origin and Progression of Cancer

CONCLUDING REMARKS

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The most remarkable characteristics of cancer is unregulated and disorganized unlimited proliferation and tremendous progress in understanding of underlying molecular mechanisms of this unlimited proliferation yielded many promising ways of diagnosis and treatment of cancer for last three decades. Molecular target therapy accomplished remarkable achievement in several types of cancers but unexpectedly high diversity of genetic mechanisms and unexpectedly early emergence of resistance to a specific inhibitor are somehow frustrating. Diversity of cancer and emergence of resistance might have a same root, plasticity and diversification occurs by intrinsic and microenvironment-induced mechanisms. Considering development of cancer treatment, this plasticity of cancer is an extremely difficult obstacle. A longstanding well accepted metabolic hallmark of cancer is Warburg effect. This metabolic characteristic has been mostly cancer specific and can be applicable for wide range of cancers. Much attention has been paid for cancer metabolism recently partly because widely applicable therapeutics might be possible when based on cancer metabolism. Another reason is discoveries in mechanistic links between genetic alteration of cancer and metabolic processes. Elucidation of link between p53 function and mitochondrial respiration and K-ras function and glucose and glutamine metabolism are representative ones. In addition, discovery in genetic alteration in genes coding for enzymes involved in TCA cycle and associated metabolism and discovery in the biological activity of the resultant metabolic intermediate and endproducts, large amount of 2-hydroxyglutarate and fumarate were another turning point.

The 46th symposium concentrated on the mechanisms of metabolic regulation of central carbon metabolism and amino acid metabolisms in particularly cancer specific context, epigenetic modulation by metabolic intermediates, microenvironment metabolic interactions, and diagnostic and therapeutic application of metabolism. Reactive oxygen,

nitrogen and sulfur species are another interesting topics discussed in this symposium, especially in connection to cancer cell biology. Through wide range of discussions and topics on cancer metabolism, cancer metabolism turned out to be not so simple as Warburg stated about 100 years ago. Oxygen supply to cancer tissue is quite limited. Therefore tumor tissues are mostly facing to severe hypoxia that simulates anaerobic glycolysis as an environmental factor through HIF-1 and HIF-2 pathways, which is often activated by genetic alteration in tumor cells or even by change in metabolism of cancer. But some genetic alterations in cancer cells sometimes more directly activates anaerobic glycolysis by inactivating oxidative phosphorylation machinery such as cytochrome C oxidase by p53 loss of function mutations. However, as discussed in the present symposium, to compensate loss of efficient ATP generation system oxidative phosphorylation, cancer cells utilize many ATP generation systems other than anaerobic glycolysis as pointed out by Warburg. Alteration in glutamine metabolism is a representative one but understanding of the role of glutaminolysis is changing from previously proposed one, anaplerosis. Some interlink between energy metabolism and nucleic acid precursor synthesis is critical for continuous cancer cells proliferation. Understanding of the importance of glycolysis in maintaining cancer cells metabolism is also changing. Glycolysis is not simply important for ATP generation but is important for synthesis of precursor of purine and reducing equivalent for regeneration of NADPH, which is important for lipid synthesis and recycling reducing equivalent that is critically important for defense mechanism against oxidative stress. Protection of cancer cells from oxy-stress through oxidation response pathway, sulfur containing compounds and even sulfur containing gas are critically important for cancer cells survival, therefore could be targets for anticancer strategy.

Cancer specific metabolites are critically important not only for biomarker of diagnosis but also for understanding cancer biology, which is directly related to cancer treatment. 2-hydroxyglutarate is the representative onco-metabolite which is generate in large amount by mutant isocitrate dehydrogenase I and II (IDH-I and II). The biological or pathological role of 2-hydroxyglutarate is found to be inhibition of demethylase. Specific inhibitor against mutant IDH I and II were invented and evaluated in preclinical and clinical studies. However, efficacy of this inhibitor was not perfect and some proportion of the patients respond well. If the role of the 2-hydroxyglutarate is modulation of epigenetic regulation in leukemogenesis or carcinogenesis by IDH-1 and 2 mutation, why the efficacy of the inhibitor is not 100%? Genetic and epigenetic background other than IDH mutation might influence the outcome and microenvironment might be another determinant.

As illustrated in the present symposium, cancer metabolism is obviously a new clue to understand cancer but obviously we need more extensive study on cancer metabolisms in order to understand cancer and to develop novel methods of treatment.