Pursuit - The Journal of Undergraduate Research at The University of Tennessee

Volume 4
Issue 2 *Spring 2013*

Article 3

March 2013

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Paolo Vignali
University of Tennessee, Knoxville, pvignali@utk.edu

Pursuit: The Journal of Undergraduate Research at the University of Tennessee

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Metabolic Rescue of "Glucose Addicted" Cancer Cells *In Vitro*

PAOLO DARIO ANGELO VIGNALI
Advisors: Dr. Ruoning Wang and Dr. Douglas Green

College Scholars Program, University of Tennessee, Knoxville

Transformations in the glycolytic metabolism of neoplasms modulate their robust cellular division. This characteristic leads to an "addiction" to glucose for continued proliferation and viability. This study investigated whether glucose metabolites could rescue cellular viability in glucose-starvation conditions, a model of the inter-tumoral nutrient-deficient environment. Findings illustrated potential cellular viability rescue with pyruvate addition in glucose-deprived conditions, yet the same potential was not observed with lactic acid, a metabolite that exists at characteristically high concentrations within the intertumoral microenvironment. These results could implicate a predominance of certain metabolic pathways in nutrient-starved cells. Molecular transport capacities across plasma membranes are tied closely to the effects of metabolites within the cell; therefore, the role of the MCT/CD147 transporter complex in lactic acid oxidation was investigated and was found to noticeably enhance this metabolic pathway.

Introduction

Cancer cells typically present with alterations in their cellular metabolism, most notably, a bias toward aerobic glycolysis (Warburg Effect) and subsequent lactic acid production[1,2]. This metabolic remodeling is far less efficient at generating ATP compared to oxidative glucose metabolism; therefore, cancer cells must instigate an abnormally high level of glucose and glutamine absorption to compensate. The increased uptake of glucose is accomplished by a cohort of factors, including HIF-1, Atk/mTOR, and c-Myc, which directly modulate metabolic gene expression and enzymatic activities[3]. These metabolic manipulations are positive adaptive effects for a variety of reasons, including the reduction of reactive oxygen species within the mitochondria and an increase in glycolic precursors for biosynthetic processes[4]. Both of these effects are critical for the continued robust cellular replication characteristic of aggressive cancers. This addiction to glycolysis has portrayed tumor metabolism as an attractive target for novel cancer therapeutics[5].

Pyruvate kinase isotype M2 (M2-PK) was discovered to be one of the key enzymes responsible for this enhanced aerobic glycolysis. Pyruvate kinase catalyses the final step of glycolysis, transferring a phosphate group from its substrate, phosphoenolpyruvic acid (PEP), to ADP, producing pyruvate and ATP. M2-PK, the dimeric form of pyruvate kinase, has reduced affinity for PEP which results in a predominance of biosynthetic precursor synthesis from glycolic intermediates instead of further oxidative metabolism. This shift is essential for accelerated proliferation as many amino acids, nucleotides, and phospholipids can be synthesized via these intermediates. This isotype is the predominant form associated with many cancers[6], further implicating aerobic glycolysis as a positive adaptation in tumorigenesis.

Similar metabolic reprogramming is observed in normal rapidly dividing cells in which growth factors initiate accelerated cellular division and differentiation[7]. In the case of cancer cells, however, metabolic transformation must be initiated in the metabolically stressful and dynamic microenvironment of a solid tumor, in which essential nutrient concentrations are in constant flux[8,9]. The hyperbolic presence of glucose through layers of the solid tumor leads to necrosis in the glucose-starved interior. In this study, the effects of glucose deprivation were examined in two cancerous cell lines – B16-OVA, a mouse melanoma line, and Jurkat, a human leukemia line – and attempt to tease out potential rescuing capabilities of glucose metabolites. Simulating conditions similar to the inter-tumoral nutrient-starved microenvironment can provide an understanding of how cancers maintain their robust proliferation in metabolically hostile environments.

Materials and Methods

Tissue Culture

The B16-OVA, MEF, HeLa, and Pheonix cell lines were cultured in DMEM medium plus 10% fetal calf serum (FCS) and 1% NEAA, pyruvate, and penicillin/streptomycin. The Jurkat cells were cultured in RPMI medium with identical additions. Pheonix cells were transfected in Opti-MEM media. The glucose starvation (G-) medium was DMEM medium without pyruvate and glucose plus 10% dialyzed FCS and 1% NEAA and penicillin/streptomycin. Cultures were grown for at least 5 days in G- medium supplemented with 11 mM glucose. Glucose was dissolved in Milli-Q water to 1.1 M then filtered to ensure sterility. Sodium pyruvate and sodium lactate stocks were prepared at 4 M concentrations. Transfections and transductions were performed with Lipofectamine 2000 [Invitrogen, New York] and Polybrene [Millipore, Massachusetts] respectively. Nocodazole dosing for cell cycle arrest was carried out under the following concentrations: 20 ng/mL for Jurkat, 25 ng/mL for HeLa, 50 ng/mL for MEF (SV40), and 75 ng/mL for B16-OVA.

Vectors

Vectors were provided by Dr. Ruoning Wang (St. Jude Children's Research Hospital, Memphis, Tennessee). They were constructed in the pCMV background with the following inserts: (1) pIRES-MCT1-2A-CD147, (2) pIRES-MCT4-2A-CD147, (3) pGFP-v-RS-mPDK1-shRNA, (4) pGFP-v-RS-LDHa-shRNA, (5) shRNAø. 2A linkages were designed according to previous literature[10,11].

Western Blot Analysis

Freshly isolated cells were spun down and the pellet was dissolved in 5 volumes of RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X

100) with 1:200 phenylmethanesulfonylfluoride (PMSF). After incubation on ice for 15 minutes, insoluble components were removed through centrifugation (10,000g, 10 minutes). Proteins in the supernatant were separated on Criterion XT [Bio-Rad, California] gels and transferred to Hybond-C Extra [Amersham Biosciences, Pennsylvania] membranes. Antibodies described below.

Flow Cytometry

All flow cytometry was performed using FACS Calibur [Becton Dickinson, New Jersey] equipment in combination with Cell Quest Pro [BD Biosciences, New Jersey]. Data was analyzed using FlowJo [Tree Star, Oregon].

Antibodies

Immune antibodies used as cell death markers include Annexin V [eBioscience, California] at 1:200 concentration and 7-AAD [eBioscience, California] at 1:20 concentration. Cell cycle analysis was completed with hypotonic PI buffer – 0.1% Triton-X100, 0.1% sodium citrate, 50 μg/mL PI – as well as αHistone H3 [BD Biosciences] at 1:100 concentration. CD98 and CD147 expression was determined using αHu CD147 and CD98 [eBioscience] and αMo CD147 and CD98 [eBioscience]. Western blot analysis was performed using αPDK and αLDH [Santa Cruz Biotechnology] at 1:1000 concentration and α2A described in previous literature[8,9] at 1:1000 concentrations.

Metabolic Assays

Glycolytic flux was determined by measuring the detritiation of [3-^{3}H]-glucose. Lactic Acid oxidation flux was determined by the rate of $^{14}CO_2$ released from [U-^{14}C]-lactic acid. Both metabolic flux assays were determined by scintillation counters.

Results

Pyruvate partially rescues cellular viability whereas lactic acid shows no significant effect: Rapid decline of cellular viability in B16-OVA and Jurkat lines under glucose starvation environments supported previous data on the nutrient dependence of glucose[8,9] [Fig. 1]. Upon investigation of the rescuing capabilities of glucose metabolites, pyruvate demonstrated modest cellular rescue at 10 mM concentrations [Fig. 2], whereas lactate displayed no sustainable positive effects at 10, 20, and 40 mM concentrations. Syncronization of the cell cycle in B16 and Jurkat cultures further supported evidence that pyruvate effectively rescued cell viability [Fig. 3,4]. Furthermore, the rescuing capacity of pyruvate was more impressive in syncronized cells. Syncronization was accomplished by treatment with nocodazole (NOC), an anti-neoplasm agent that prevents cellular division by inhibiting spindle fiber formation during mitosis. NOC induces a moderate degree of cell death in culture, therefore all results were normalized for the effect of NOC treatment alone.

Overexpression of MCT/CD147 transporter complex induces increased lactic acid oxidation: The ability of metabolites to benefit cellular survival depends greatly on their ability to be transported across the plasma membrane, suggesting an important role for MCT/CD147 transporter complex in lactic acid oxidation. To investigate the effects of MCT/CD147 transporter complex expression on metabolic events, stable MEF (SV40) and B16-OVA lines overexpressing murine MCT1 or human MCT4 were generated. The intent was to elucidate potential variables that could lead to increased cellular viability with lactic acid supplements in glucose starvation environments. Using radiolabeled lactic

acid, an obserable increase in lactic acid oxidation was observed in both MCT1 and MCT4 B16-OVA transfectant lines as well as in the MCT1 MEF transfectant line [Fig. 5]. Rates of oxidative glucose metabolism my also be increased, albeit much less dramatically. These results were anticipated however, as Glut-1 is the primary glucose transporter in mamalian cells and the MCT/CD147 transporter complex could also serve as a transport for glucose and its metabolites.

Discussion

Robust cell death in glucose starvation environments was anticipated [Fig 1], as the media was stripped of all glucose metabolites. Glutamine was still present in the media, yet it did not provide any long-term rescuing effects. Short-term rescuing capacity of this nutrient was not examined. Pyruvate showed the highest rescuing capabilities compared to lactic acid [Fig 2], potentially illustrating that MCT/CD147 transporter complex plays a large role in metabolite effects within the cell. This contradicted the initial hypothesis as it was anticipated that lactic acid would show rescuing effects due to its increased concentration in tumor mincroenvironments, a well documented phenomenon[1,2,3,8,9]. The role of MCT/CD147 transporter complex in metaolic events within the cell was analyzed by metabolic analysis of lactic acid oxidation in cells with MCT/CD147 overexpression [Fig. 5]. Significant increases in metabolic rates in the transporter overexpression lines was observed, thereby providing a possible cause for the lack of rescuing effects in previous experiments. Briefly, insufficient lactic acid absorption rates could be attibuted to low endogenous MCT/CD147 transporter complex expression in WT B16-OVA and Jurkat cell lines, thereby inhibiting the potential for metabolic rescue via lactic acid metabolism. Future studies will examine these MCT/CD147 transfectants in glucose deprivation environments to identify potential increases in glucose metabolite rescue.

Cellular replication is an energetically expensive event; therefore, efforts were made to synchronize the majority of the culture population into one particular phase of the cell cycle [Fig. 3]. Synchronizing cell cycles eliminates extraneous variables that could alter cellular viability results, as cells undergoing one particular phase of the cell cycle may be more susceptible to apoptosis in nutrient-deprived environments. Following M-phase arrest by nocodazole treatment, the cells were released into various starvation environments [Fig. 4]. These results provide a more accurate representation of the necessity of glucose in cellular replication as well as the rescuing capacity of pyruvate. Further studies will examine rescuing effects of glucose metabolites in the MCT/CD147 overexpression lines following cell cycle synchronization.

In attempts to direct cellular metabolism against certain pathways, MEF (SV40) and B16-OVA cells were transduced with either lactate dehydrogenase A (LDHa) or pyruvate dehydrogenase kinase type 1 (PDK1) shRNA constructs. Knocking down PDK1 or LDHa would create a bias towards oxidative metabolism by promoting acetyl-coenzyme A conversion or inhibiting lactic acid production, respectively. However, no obserable knockdown was achieved, suggesting either a misfunction of the shRNA construct, or potentially, the dependence on these metabolic enzymes in cellular viability and proliferation. These enzymes play crucial roles in metabolic reprogramming, disfavoring the oxidative metabolism of pyruvate, in a variety of cell types, including numerous cancer cell lines[9]. Conditional-expression transfectants will be generated to examine knockdown of these enzymes in future experiments.

From this study, the rescuing effects of glucose metabolites have illustrated how cancerous cells may adapt to the intertumoral nutrient-deprived microenvironment. Nutrient rescuing capacity may be related to the cell's ability to transport nutrients across the cellular membrane[12]. In examination of the effects of MCT/CD147 transporter complex overexpression, this tenant held true, as cellular viability was higher in overexpression lines. These experiments illustrate some of the mechanics of metabolic reprogramming within cancer cells, most notably, the metabolism of glucose metabolites in glucose-deprived environments as well as the importance of transporter expression in rescuing cellular viability.

In the future, the rescuing capacity of these glucose metabolites will be examined in more physiologically relevant conditions. This can be accomplished by developing 3D cell cultures in conditions mimicking the inter-tumoral environment, for example by reducing oxygen concentrations. The microenvironment surrounding the tumor plays a major role in its metabolic reprogramming events, therefore, to gauge a more accurate cellular response to nutrient starvation, these differences between *in vivo* and *in vitro* cultures must be accounted for. Glutamine dependence in cancer cells will also be investigated. Glutamine plays a pivitol role in replenishing citrate concentrations needed for lipidneogenesis, and starvation of glutamine has been shown to drastically affect cellular viability[13].

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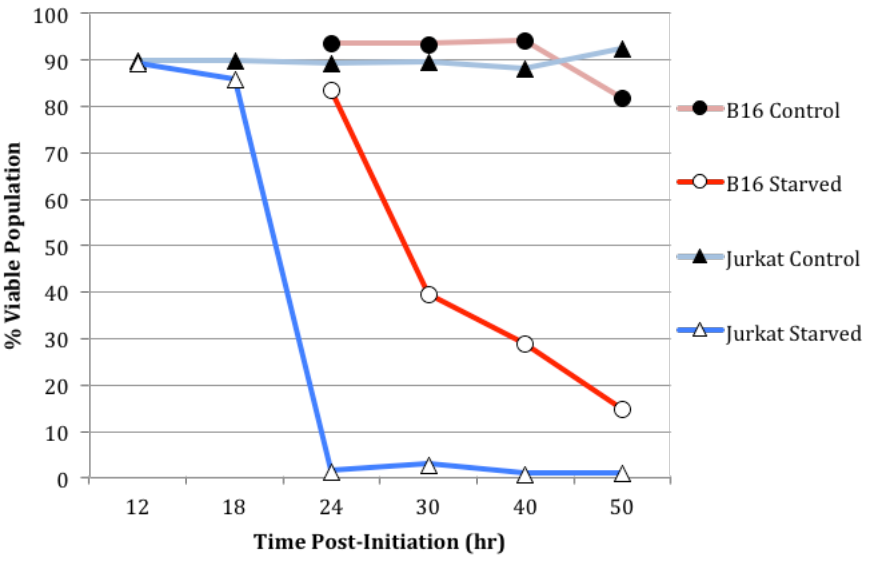

Figure 1: Glucose Starvation of Jurkat and B16 cells.
Cells were cultured in DMEM media lacking pyruvate with 10% dialysized FBS for a week. $5x10^5$ cells were then seeded into a 6-well plate and incubated for 5 hours to allow for adhesion of the B16 line. Cells were then washed twice with phosphate buffered saline and covered in G^- DMEM media. Cells were incubated for 12, 24, 30, 40 and 50 hours and then stained with Annex V and 7AAD and analyzed via flow cytometry.

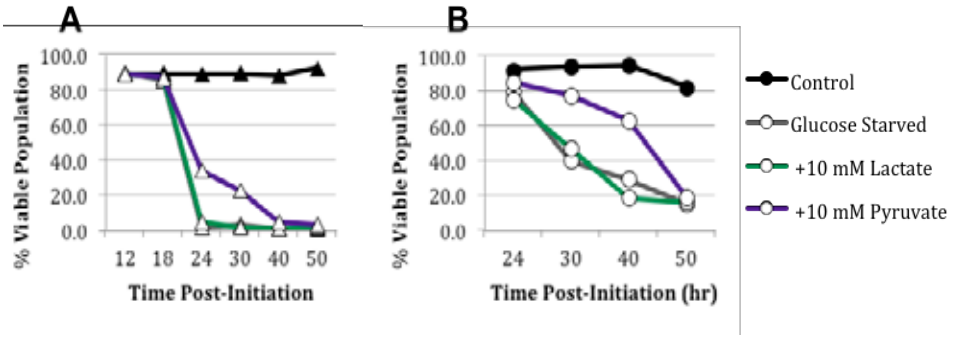

Figure 2: Metabolite Rescue of Jurkat (A) and B16-OVA (B).
Cells were cultured in DMEM media lacking pyruvate with 10% dialysized FBS for a week. $5x10^5$ cells were then seeded into a 6-well plate and incubated for 5 hours to allow for adhesion of the B16 line. Cells were then washed twice with phosphate buffered saline and covered in G^- DMEM media with the appropriate metabolite supplement. The control line was supplemented with 11 mM glucose. Cells were incubated for he indicated hours and then stained with Annexin V and 7AAD. Cell death was analyzed via flow cytometry.

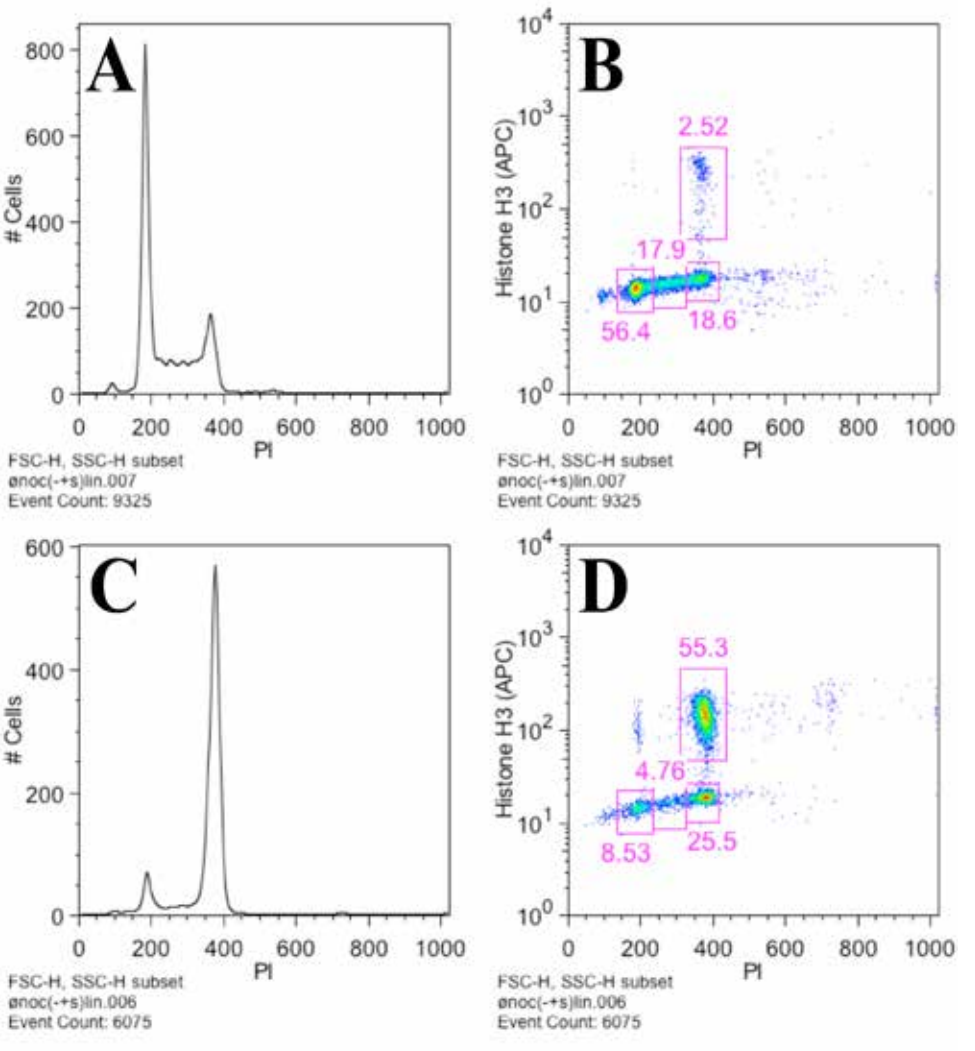

Figure 3: PI and Histone-H3 staining of Jurkat cell line with nocodazole treatment. Jurkat cells were cultured in standard DMEM media. $5x10^5$ Jurkat cells were seeded onto a 6 well plate with (C&D) and without (A&B) the addition of 20 ng/mL nocodazole. Following a 24 hour incubation, the cells were collected and fixed in a hypotonic PI solution with 1:100 αHistone-H3 (APC). Cell death was analyzed via flow cytometry.

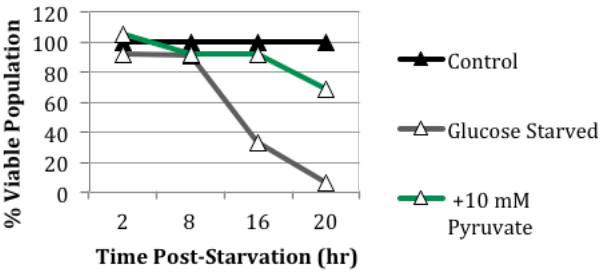

Figure 4: Pyruvate rescue of Jurkat cellular viability following cell cycle synchronization.

Jurkat cells were cultured in DMEM media lacking pyruvate with 10% dialysized FBS for a week. $5x10^5$ cells were then seeded into a 6-well plate. Cell cultures were subjected to 20 ng/mL nocodazole treatment and incubated for 24 hours for cell cycle arrest. Cultures were then washed twice with PBS and placed in fresh G- media with respective nutrient supplements. Cell death was analyzed via flow cytometry.

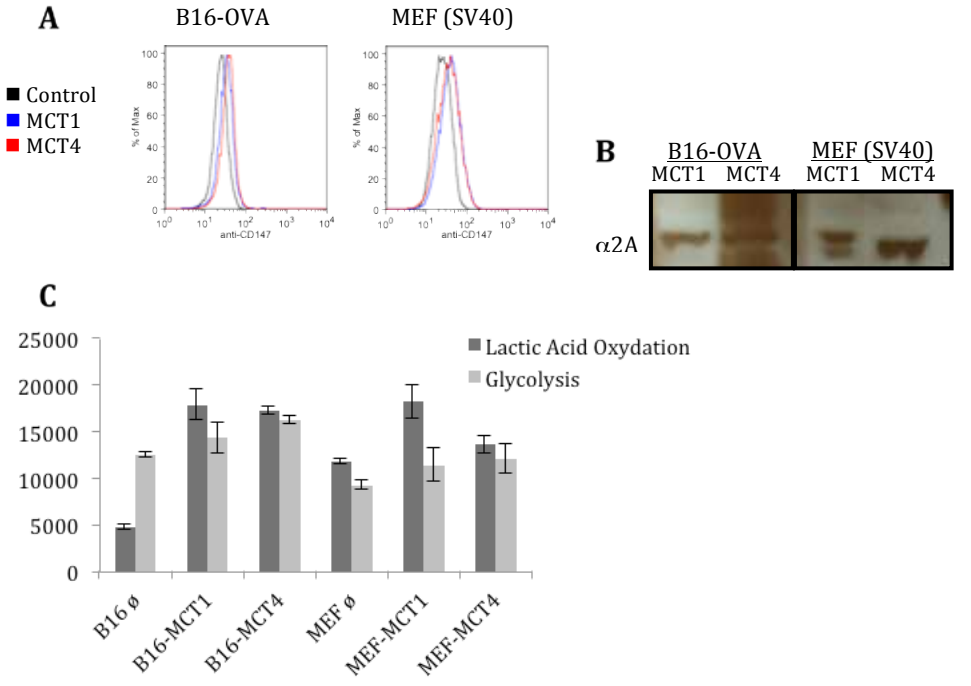

Figure 5: Lactic acid and glucose oxidation in B16-OVA and MEF (SV40) lines overexpressing MCT/CD147 transporter.

B16 and MEF cells were generated to stably overexpress MCT/CD147 transporter. Determination of expression was performed via flow cytometry (A) and Western Blot analysis (B). Metabolic rates were determined with radiolabeled lactic acid and glucose, respectively (C).

About the Author

Paolo Vignali is a member of the College Scholars program, studying oncology and immunology. This past summer, he participated in the Pediatric Oncology Education Program at St. Jude's Children Research Hospital in Memphis, Tennessee doing research. He also volunteers with Clinic Vols and is vice president of NEURO at the University of Tennessee. He hopes to go on to graduate school to pursue an M.D. Ph.D. degree.

About the Advisors

Dr. Douglas Green is the Chair of the Department of Immunology at St. Jude Children's Research Hospital in Memphis, Tennessee. He received his Ph.D. from Yale University. His research interest is the study of central mechanisms of apoptosis in cancer and the immune system.

Dr. Ruoning Wang is a postdoctorate at St. Jude Children's Research Hospital in Memphis, Tennessee. He received his Ph.D. from the University of Texas at Houston.