Establishment IROA methodology for comparative profiling of ccRCC-kidney pairs metabolome using *in vivo* produced heavy isotope labeled standards

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Introduction

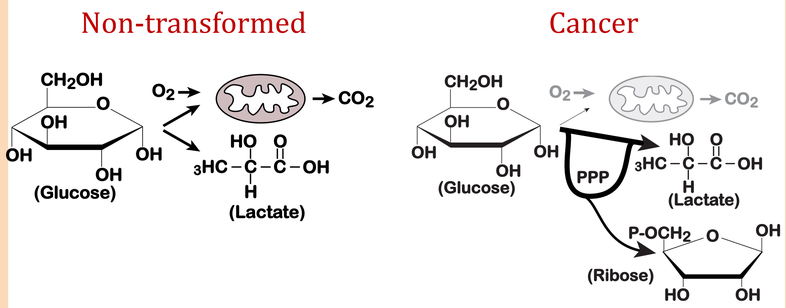
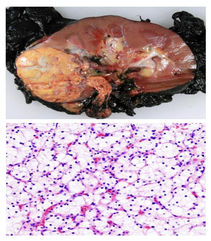


Fig 1. Cartoon illustrating metabolic reprograming known as the Warburg Effect.

Clear cell renal cell carcinoma (ccRCC) is enriched in lipids, particularly, cholesterol esters



TCGA data shows PPP enzymes correlate with poor patient survival

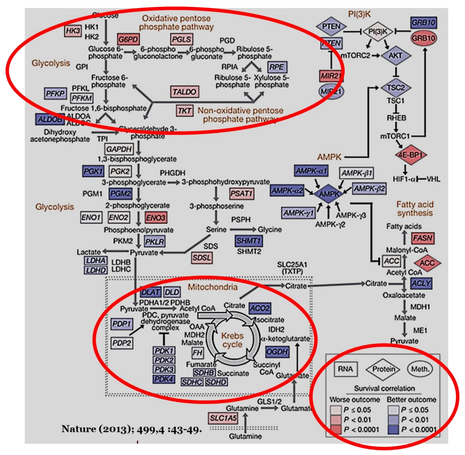
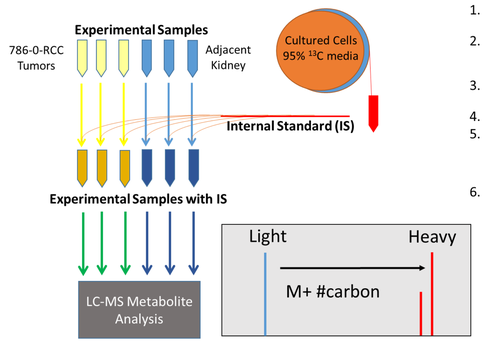


Fig 2. Overview of the many metabolic alterations associated with clear cell Renal Cell Carcinoma (ccRCC), as reported in The Cancer Genome Atlas.

Experimental Design



1. Grow cells in heavy labeled media
   a) 3 passages
2. Prepare extracts
   a) ccRCC $1e^6$ cells/mL
   b) Lymphocytes $7e^6$ cells/mL
3. Extract metabolites from solid tissue
   a) 10mg/mL
4. Mix heavy light extracts 1:1
5. Analyze via LC-MS
   a) HILIC (Luna NH2)
   b) Reverse Phase (Hydro-RP)
   c) Both positive and negative polarities
6. Process data
   a) Unbiased feature finding (Compound discoverer)
   b) Biological validation H:L pairs w/ ClusterFinder)
   c) Find unique features in smoker samples
   d) Further compound validation (ms/ms, standards)

IROA-based Phenotypic Workflow

Fig 3. Diagram depicting the workflow an Isotope Ratio Outlier Phenotyping experiment. Here we hope to compare metabolic profiles from different tissue types including patient derived tumors.

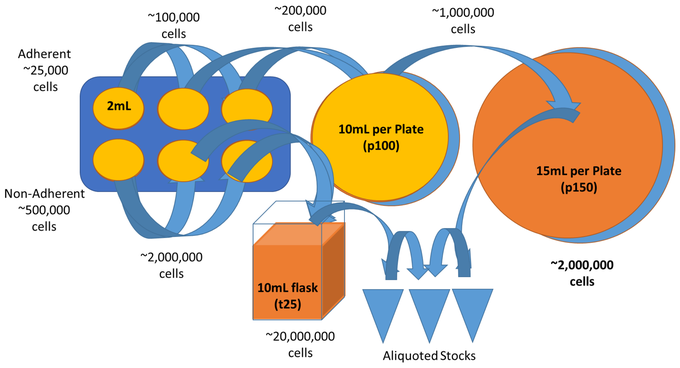


Fig 4. Basic growth strategy. To ensure a rich and diverse internal standard, a panel of cell lines were grown for a minimum of 5 passages in IROA-95-300 mammalian growth medium. Different culture conditions were tested to ensure detectable levels of isotope labeled biologically relevant metabolites.

Results

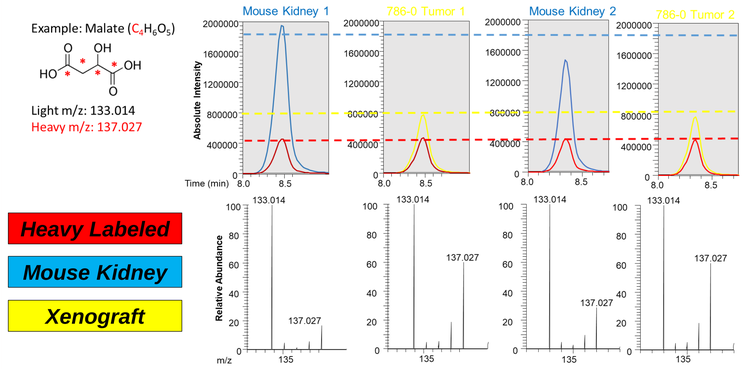


Fig 5: Isotope Ratio Outlier Analysis (IROA). Over laid XICs H:L pairs, detected in mouse tissue extract spiked with 95% IROA cultured cell line extract.

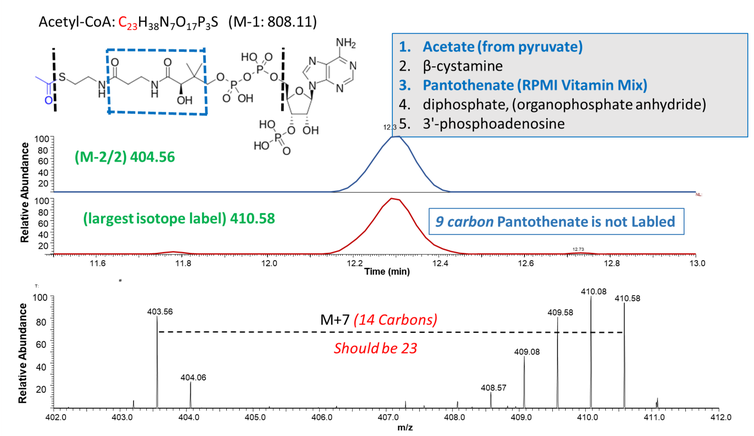


Fig. 6: Incomplete labeling observed, resulting from utilization of nutrients in RPMI vitamin mix. Interestingly, ClusterFinder still detected this H:L pair which can be annotated manually and compiled in curated library. While potentially perplexing, anomalies such as this can be predicted and may not be unavoidable due to some nutrients being essential to mammalian diets. Another example is NAD, where nicotinamide scavenged.

Table 1: Distribution of key biologically relevant classes of metabolites being used to benchmark labeled internal standard mixture. Data only represents various RCC models as well as treatments to ensure maximum coverage the metabolome with an emphasis on central carbon, specifically PPP intermediates.

Benchmarking Central Carbon Metabolites	Targeted compounds	Cultured 3 days	TKT-KD	OA Treated	Yeast Extract	Combined
	108	81	90	84	44	103
Glycolysis *	12	9	12	12	0	12
PPP	6	0	6	2	0	6
TCA	9	6	6	6	0	8
Amino Acids **	28	25	25	25	22	25
Nucleotides	13	13	13	13	0	13
Nucleosides	14	5	5	5	12	12
Glycosylation	8	8	8	8	0	8
Other ***	18	15	18	18	10	18

Table 2: Summary of qualitative differences observed in metabolite pools obtained from 786-0 cell line extract (condition 1) compared to 786-0 orthotopic xenografts.

Light / Heavy	Total	Up in Tissue	Up in Culture
	81	35	11
Glycolysis	9	4	0
PPP	0	0	0
TCA	8	4	1
Amino Acids	24	5	4
Nucleotides	13	4	5
Nucleobases	5	5	0
Glycosylation	8	5	0
other	14	7	3

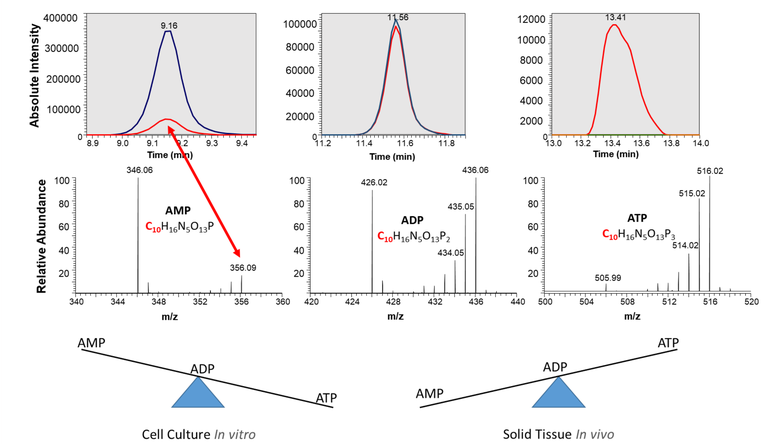


Fig 7: Abundances of nucleotide pools is different in cultured cells compared to tissue samples. This observation may indicated a different energy charge dependence for rapidly proliferating cells in a dish vs. solid masses such as tumors.

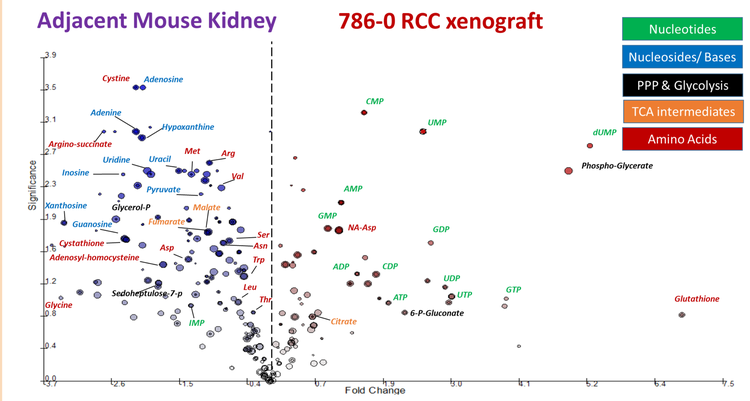
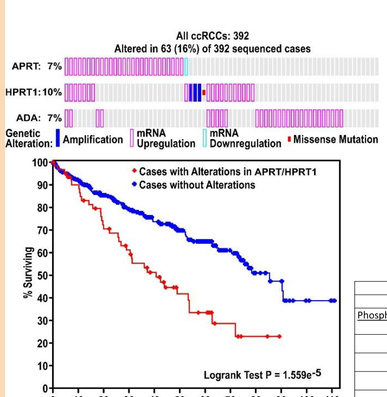
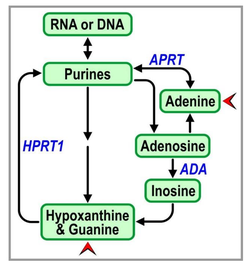


Fig 7: Statistics reveal an interesting difference in nucleic acid metabolism. Volcano plot illustrating difference in metabolite abundances observed when comparing extracts obtained from xenograft tumors and mouse kidney. Notably nucleosides in general are up in mouse kidneys while phosphorylated nucleotides are up in ccRCCs, possibly indicating a mechanism for RNA salvage facilitated by the micro environment.





PRPP Km (affinity) spreadsheet data from BRENDA database		
Phosphoribosyl transferase enzyme	Km range (for PRPP as substrate)	Notes
NAMPT/PBEF	0.00051-0.0232	low range is in presence of ATP, which stimulates enzyme activity, pyridine synthesis enzyme
APRT	0.006-0.0089	adenosine nucleotide salvage enzyme, mutations in this enzyme cause kidney failure
UMPS/OPRT	0.016	value is for mouse enzyme, not human (should be similar), pyrimidine synthesis enzyme
HPRT	0.0191-0.076	purine salvage enzyme
UPRT	0.026	value is for S. cerevisiae enzyme
PPAT	0.14-0.48	de novo purine synthesis enzyme

Fig. 9: RNA salvage is an interesting target for ccRCC therapeutics. Up regulation of RNA salvage enzymes correlates with poorer patient outcomes and may present an interesting therapeutic target.

Conclusions

- IROA Phenotyping procedure has been successfully implemented for the analysis of ~100 compounds found in mouse tumor-kidney pairs. And automated using ClusterFinder.
- In house internal standard development allows for the production a flexible and diverse mixture of labeled compounds depending on cell line and culture conditions.
- Nucleotide (NMP/NDP/NTP) balance might be extremely different in vivo
- Global RCC vs. Adjacent Kidney differences may indicate the stromal kidney micro environment is providing nucleosides/ bases to RCC tumors for RNA salvage
- IROA media is very rich, containing a variety of nucleosides and nucleobases not present in atypical medias which could be utilized to study RNA salvage

Further work will be dedicated to applying this method to analyze tissue lysate obtained from human patients to confirm observations from mouse models.

Acknowledgements

American Cancer Society

IROA TECHNOLOGIES ...making metabolic profiling easy®

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Supported by NCI, VA, DoD

NIH National Cancer Institute

University of Cincinnati