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Metabolic Analysis of Isogenic Cells with Altered Cancer-Related Genes

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Introduction

Abstract

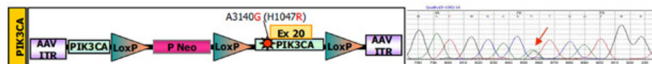
For this study we combined two new technologies to elucidate the changes in cellular energy metabolism engendered by a cancer-associated gain-of-function mutation in the PI3K gene. Horizon Discovery has developed GENESIS™, an endogenous-gene targeting platform that enables the creation of isogenic human cell lines to permit precise comparisons of human cells differing in a single gene or genetic mutation. Biolog, Inc. has developed Phenotype MicroArray™ (PM) Technology enabling the direct measurement of energy metabolic rates in cell lines with an array of substrate chemicals. We have compared an immortalized human mammary epithelial cell line that is wild-type for PI3Kα against two isogenic clonally derived cell lines (1A7 and 9B3) in which the endogenous PI3K gene was constitutively activated via the introduction of a kinase domain mutation (H1047R) by homologous recombination. Phenotype MicroArray PM-M1 with Biolog Redox Dye Mix MA was used to assess the generation of energy in these cells from 91 different biochemical substrates, of which ~17 appear to be metabolized to produce energy. Metabolic rates in the PI3K mutant cell lines were decreased for phosphorylated sugars (G-6-PO4, G-1-PO4, F-6-PO4, glycerol-PO4) and to a lesser degree for adenosine, propionic acid, and butyric acid. Rates for other substrates were variable depending on the clone tested.

Background

Oncogene addiction is a relatively recent hypothesis which postulates that cancer cells become 'hardwired' to depend on the elevated activity of a subset of pro-growth and survival pathways. This concept has close parallels to the Warburg Effect [1] in which cancer cells profoundly alter their energy metabolism compared to normal tissues. It is therefore plausible to postulate that cancer mutations, such as mutant PI3K, may manifest their oncogene addiction phenotypes at the level of cellular metabolism. Cancer cells typically consume glucose more rapidly compared to normal cells, producing energy inefficiently by glycolysis because they inactivate the more efficient mitochondrial energy production process of oxidizing pyruvate, and instead use the pyruvate to overproduce lactic acid. A group at Harvard has provided evidence that the activation of the enzyme pyruvate kinase M2 is involved in these profound metabolic changes [2]. A better understanding of the induction of the Warburg Effect and how defined cancer gene mutations shut-off or alter the utilization of specific energy sources could lead to more effective and safer treatments for many cancers given that normal tissues are more metabolically efficient and less dependent on specific energy sources.

In order to understand how metabolic pathways are specifically altered in genetically distinct and diagnosable patient cohorts, Horizon Discovery created isogenic 'X-MAN' cellular models of cancer induction that allow for precise and well controlled experiments to be performed on defined patient-relevant tumor vs normal genotypes *in vitro*. In this study we have used Biolog's PM Technology to fingerprint and compare the energy metabolism pathways in a reference 'normal' mammary cell line versus isogenic 'mutant' lines created using Horizon's GENESIS gene alteration technology. In these isogenic X-MAN (**M**utant **A**nd **N**ormal) mutant lines, one copy of the PI3K gene is activated, as it is in typical human cancers.

Figure 1.



Cell Line Construction and PM Assay Protocol

Methods

X-MAN cell-lines are created using a parvoviral-mediated homologous recombination technique, which provides orders of magnitude increases in gene-targeting frequency over older DNA plasmid-based techniques. There are 4 basic steps to the process:

1. The design and synthesis of a homology targeting vector, incorporating a selectable marker flanked by two regions of homology to the target gene locus and two internal tandem repeats used to package these vectors in rAAV particles. In one homology arm, a mutation is introduced that recapitulates a disease-causing mutation found in humans.
2. The creation of recombinant virus using standard 293T packaging systems, and the infection of target cells of interest.
3. Limited dilution cloning and selection based on neo drug resistance.
4. PCR screening to identify positive clones, followed by gDNA and cDNA sequencing to confirm the presence of the mutation in the endogenous target locus after homolgous recombination has occurred. A Southern blot is then performed to confirm that a single knock-in event has occurred.

For this study, the parental HME1 mammary cell line was engineered using a virally-packaged targeting vector (Figure 1) which introduces a point mutation (H1047R) in exon 20 (the kinase domain) of PI3K. Recombinant cell lines were identified using PCR-based screening on isolated clones. To examine clonal variation, two clones were isolated and tested.

Biolog Phenotype MicroArray PM-M1 is a 96-well panel preloaded with 91 different substrates that human cell lines may metabolize (Figure 2.a.). If cells added to the array panel can metabolize a substrate in the well, the cells generate energy and reduce a tetrazolium dye (Figure 2.b.), forming a purple color in the well (Figure 2.c.). Assays are started by inoculating wells with a cell suspension (Figure 3.a.). The cells are allowed to adapt and induce enzymes needed for substrate utilization. Then the Biolog redox dye is added to all wells and the PM panels are placed inside the OmniLog, an instrument which incubates and reads the color formation in all wells every 15 minutes (Figure 3.b.). The OmniLog with companion software can generate kinetic graphs indicating metabolic rates for each substrate (Figure 3.c.). Alternatively, the color formed in wells can be determined by reading OD-590 with a standard microplate reader, as was done in the experiments performed at Horizon.

Figure 2.a.

Figure 2.b.

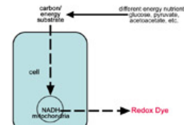


Figure 2.c.

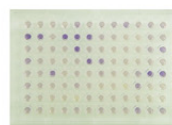
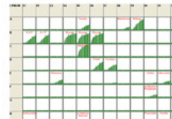


Figure 3.a.

Figure 3.b.

Figure 3.c.



The cell lines were cultured in DMEM/F12 media (Horizon) or RPMI 1640 media (Biolog) with 10% serum, 20 ng/ml EGF, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, harvested after trypsinization, washed twice with PBS, and resuspended in Biolog IF-M1 with 0.5% serum, 0.3 mM glutamine, and no glucose. 50 uL of this cell suspension was seeded into the wells of PM-M1, the cells were allowed 40 hours to adapt to the various substrates, and then the metabolic activity on the various substrates was determined by adding 10 uL of Biolog Redox Dye Mix MA to all wells. The purple color formed by reduction of a tetrazolium dye was determined by reading OD-590 in a microplate reader after 4 hours (Horizon) or by measuring rate slopes in the OmniLog instrument (Biolog). The metabolic activity values were then normalized by subtracting the average value of the three negative control wells in the PM-M1 panel.

Results and Conclusions

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The parental cell line metabolized ~17 of the 91 different biochemical substrates in the Biolog PM-M1 panel. Results, shown in Figure 4.a. (performed at Horizon) and 4.b. (performed at Biolog) indicate that the two clonal mutant cell lines with PI3K activated exhibit a consistent shift in cellular energy metabolism. Metabolic rates in the PI3K mutant cell lines were decreased for phosphorylated sugars (G-6-PO4, G-1-PO4, F-6-PO4, glycerol-PO4) and for adenosine, propionic, and butyric acid. Metabolic rate changes were variable and somewhat clone-dependent for other substrates.

These experiments reinforce the documented advantages of analyzing cancer with isogenic cell lines [3,4]. To examine metabolic shifts, we combined the scope and precision of PM metabolic assays with the defined cancer vs normal genotype specific readout of X-MAN isogenic human cells lines, engineered to contain selected patient-relevant cancer mutations. By testing a larger set of mutations, both singly and in combination, in various host cell lines that model different tumors, it should now be possible to gain a detailed understanding of the relationship of cancer genotype with altered energy metabolism.

Figure 4.a.

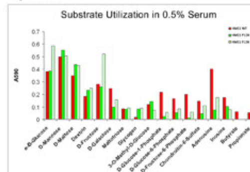
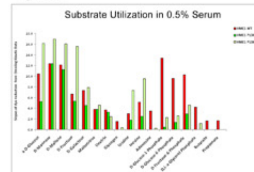


Figure 4.b.



References

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Acknowledgements

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