Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-231-2825.

1. **Grantee Institution:** University of Pittsburgh- of the Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 1/1/2011 - 12/31/2014

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Margaret C. McDonald, PhD

4. **Grant Contact Person's Telephone Number:** 412-383-7474

5. **Grant SAP Number:** 4100054875

6. **Project Number and Title of Research Project:** 03 - Breaking Metabolic Symbiosis in Tumors: A New Cancer Treatment Paradigm

7. **Start and End Date of Research Project:** 1/1/2011 - 12/31/2012

8. **Name of Principal Investigator for the Research Project:** Bennett Van Houten, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 540,253.18

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project	Cost
Van Houten	Principal Investigator	10% January 2011-December 2012	$79,402.59
Barbi De Moura	Postdoctoral Fellow	80% January 2011-March 2011; 30% April 2011-July 2011; 50% April 2012-July 2012; 100% August 2012-December 2012	$64,962.90
Qian	Postdoctoral Fellow	100% January 2011-December 2012	$163,241.10
Roginskaya	Lab Manager	100% January 2011-Dece,ber 2012	$207,730.15
Santos	Graduate Student	25% January 2011-August 2012	$24,916.45

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name, First Name	Position Title	% of Effort on Project
Graves, James	Co-Investigator	20%
Varum, Sandra	Postdoctoral Fellow	5%
Rodrigues, Ana	Postdoctoral Fellow	5%
Moschos, Stergios	Co-Investigator	20%
Goetzman, Eric	Assistant Professor	5%
Nakajima, Erica	Medical Student	100%
Furda, Amy	Graduate Student	20%
Wang, Jingnan	Graduate Student	50%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes____X_____ No_____

If yes, please indicate the source and amount of other funds:

1. Head and Neck Cancer Specialized Program of Research Excellence (SPORE) (NCI P50CA097190) Pilot Project
"Metabolic symbiosis: A new therapeutic target in head and neck tumors"
$60,643 direct costs

2. Skin Cancer SPORE (NCI P50CA121973) Pilot Project
"Bioenergetics: A new therapeutic target in metastatic melanoma"
$35,000 direct costs

3. Howard Hughes Medical Institute Predoctoral Fellowship
"Identifying the presence of metabolic symbiosis in head and neck squamous cell carcinoma xenografts and human tumor samples"
Predoctoral Fellow: Erica Nakajima, an MD student and Dr. Van Houten's mentee.
$19,500 direct costs

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes____X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds awarded:
Control of Mitochondrial Structure, Function and Energetics by c-Myc (PI: Ed Prochownik, a collaborator)	☒ NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	6/1/2012	$2,550,000 Total funds	$1,581,000 Total funds

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes__X_____ No_____

If yes, please describe your plans:

Several collaborators plan to submit grant applications to NIH and the U.S. Department of Defense.

12. Future of Research Project. What are the future plans for this research project?

We are working to determine whether metabolic inhibitors, when combined with traditional anticancer agents like cisplatin, show increased efficacy in killing tumor cells grown in culture. If these cell-culture models show promise, we will transition to animal studies.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male		1		1
Female			3	3
Unknown				
Total		**1**	**3**	**4**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				1
Non-Hispanic		1	3	3
Unknown				
Total		**1**	**3**	**4**

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	2
Black				
Asian			2	1
Other		1		1
Unknown				
Total		**1**	**3**	**4**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes____X_____ No_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

Purificación Estévez García, MD, PhD, Visiting Scientist
Medical Oncology Department Instituto de Biomedicina de Sevilla (IBIS)
Hospital Universitario Virgen del Rocio, Seville (Spain)

Jingnan Wang, PhD Student
Tsinghua University
Beijing, China

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes___X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

Dr. Van Houten's investigation into the role of metabolism in tumor cell growth under this project has fostered a collaborative research environment at the University of Pittsburgh Cancer Institute (UPCI). Within the Molecular and Cellular Cancer Biology Program that Dr. Van Houten co-leads at UPCI, a strong research group is focused on understanding the important role of metabolism and mitochondria in cancer and uncovering novel laboratory findings, with the aim of translating results into more effective therapies for patients.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes__X_____ No_____

If yes, please describe the collaborations:

Dr. Van Houten collaborated with Eugenia Dogliotti, DSc, of the Department of Environment and Primary Prevention, Istituto Superiore di Sanita, Rome, Italy. The team studied the bioenergetics of cells from patients with Cockayne syndrome, which resulted in the following publication: Pascucci B, Lemma T, Iorio E, Giovannini S, Vaz B, Iavarone I, Calcagnile A, Narciso L, Degan P, Podo F, Roginskya V, Janjic BM, Van

Houten B, Stefanini M, Dogliotti E, D'Errico M. An altered redox balance mediates the hypersensitivity of Cockayne syndrome primary fibroblasts to oxidative stress. Aging Cell. 2012 Jun;11(3):520-9. PMID: 22404840.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written

response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Cancer cells require nutrients to grow. Finding out what they ingest and how they grow remains one of the frontiers of cancer biology. More than 70 years ago, Otto Warburg found that tumor cells enjoyed consuming large amounts of glucose through glycolysis, even when supplied an abundance of oxygen. He and his team surmised that the mitochondria of tumor cells were broken and, therefore, could not perform oxidative phosphorylation (OXPHOS). This idea was later proven incorrect, as many tumor cells do show significant respiration and mitochondrial function is vital for their growth.

However, use of one of the most important technologies for identifying metastatic tumors, positron emission tomography (PET) imaging of F^{18}-labeled deoxyglucose, clearly indicates that metastatic tumor cells have an increased glucose uptake. Furthermore, the identification of mitochondrial DNA (mtDNA) mutation accumulation in many different types of tumors suggests that mitochondrial dysfunction does occur in tumor cells.

This project tested the hypothesis that tumor cells display remarkable metabolic flexibility and, depending on environmental cues, can evolve into one of two populations of tumor cells: those that are highly glycolytic at the hypoxic tumor core, or those with robust mitochondrial oxidative phosphorylation (if well oxygenated through a vascular bed). Viewing tumor development and metastasis in this manner demands a two-pronged approach to kill both populations of cells by altering metabolism. This project directly tested such a metabolic symbiotic model of tumor development through three interdependent aims:

Aim 1: Analysis of metabolism and bioenergetics in tumor cells grown in culture
Tumor cell lines will be examined for their ability to grow on 84 different carbon sources using a 96-well format Biolog™ plate assay. A subset of carbon sources that provides good cellular growth will be used to examine carbon source-dependent rates of oxygen consumption (a measure of OXPHOS) and glycolysis using a Seahorse XF24 Flux analyzer (Seahorse Biosciences, Billerica, Mass.). These carbon sources will also be used to examine their ability to promote the production of adenosine triphosphate (ATP) through OXPHOS or glycolysis. Specific inhibitors of carbon source utilization will be examined for inhibition of cell growth and effects on oxygen consumption and glycolysis. Finally, the role of estrogen on OXPHOS and glycolysis will be measured.

Aim 2: Analysis of metabolic enzymes in tumor cells
Western blot analysis of several different proteins involved in glucose utilization will be performed. Gene expression profiles of known tumor cell lines will be interrogated for expression of specific genes showing protein changes. Additional real-time polymerase chain reaction (RT-PCR) analysis of OXPHOS and glycolytic enzymes will also be performed.

Aim 3: Analysis of tumor xenografts and primary human tumor tissue for metabolic markers of oxidative phosphorylation and glycolysis

Melanoma cells taken directly from patients with malignant melanoma tumors will be assessed for levels of oxidative phosphorylation and glycolysis using the Seahorse XF24 Flux analyzer. In addition, tumor cells from a xenograft model will be assessed in a similar manner. Real-time fluorescence imaging of glucose utilization in cells grown in culture and from a melanoma xenograft tumor model will be used to assess levels of glucose uptake and glycolysis. Cell sorting experiments using validated biomarkers of glycolytic and OXPHOS proteins (from Aim 2) will be used on fresh tumor samples to determine the fraction of cells that are high in glycolysis or high in OXPHOS, or perhaps both. Finally, mtDNA mutations will be examined in subpopulations of tumor cells expressing high levels of glucose transporters and glycolytic enzymes in both the xenograft tumor models and patients' tumor biopsies.

Results

Work that was performed to address these aims has resulted in a number of publications, which are cited throughout this section and listed under item 20.

Stated project milestones are followed by a description of progress made for each:

(1) Analysis of carbon source preference using the Biolog assay (Aim 1.1)

Cancer cells use different carbon sources to grow. While many studies suggest that cancer cells use high amounts of glucose for growth, no one has systematically analyzed what other types of carbon sources may help to support cancer cell growth. Under Aim 1.1, we have analyzed the carbon preference of four breast cancer cell lines using a 96-well plate filled with 90 different carbon sources varying from glucose to inosine and lactate. Surprisingly, all cancer cell lines were able to grow in lactate (see blue box and arrow in Figure 1). BT-20, an aggressive, triple-negative cell line, was able to grow the best using lactose as a sole carbon source. These data suggest that breast cancer cells are metabolically flexible and are capable of converting lactate to pyruvate by the action of lactate dehydrogenase. Pyruvate is then converted to acetyl coenzyme A in the mitochondria and used in the tricarboxylic acid (TCA) cycle to generate ATP by oxidative phosphorylation.

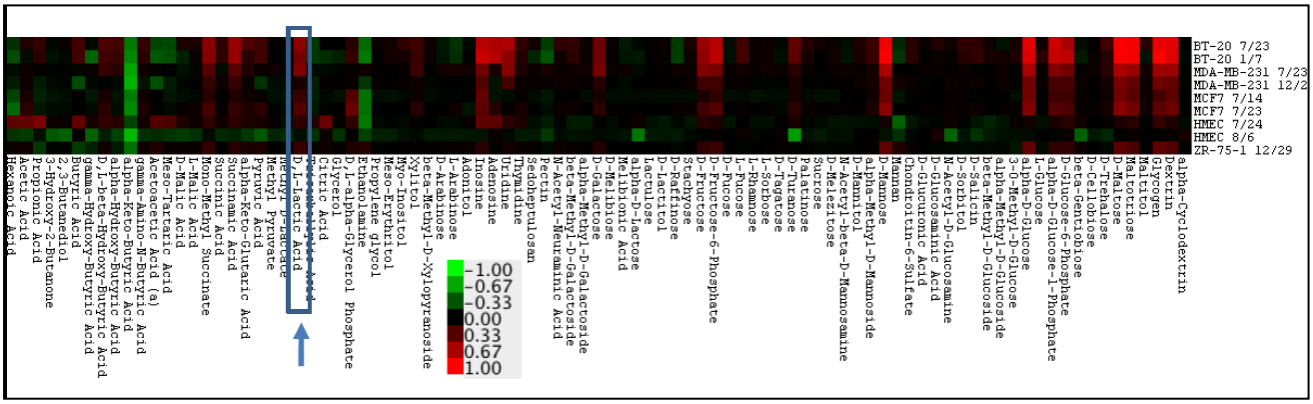

Figure 1. Carbon source utilization measured using a Biolog plate assay. Red indicates growth and green indicates growth inhibition.

(2) Analysis of inhibitor effects on glycolysis and OXPHOS, by cell growth, Seahorse XF24 Flux analyzer, and ATP (Aim 1.2)

Breast cancer cells were grown in the presence of the glycolysis inhibitor 2-deoxyglucose (2-DG) for 72 hours. Cytoquant was used to assess relative growth (Figure 2). There was a dose-dependent growth-inhibitory response to inhibitor for each cell line. 2-DG treatment (100 mM) also resulted in a large decrease (two-fold) in ATP production in most cells and a concomitant reduction in glycolysis and glucose utilization by OXPHOS, as determined using the Seahorse analyzer (data not shown). These results indicate that breast cancer cells are sensitive to growth inhibition by blocking glucose utilization. However, preclinical studies with 2-DG in xenograft animal models have shown that the inhibitor does not work efficiently *in vivo* without causing toxicity. Thus, we are currently examining other types of metabolic inhibitors.

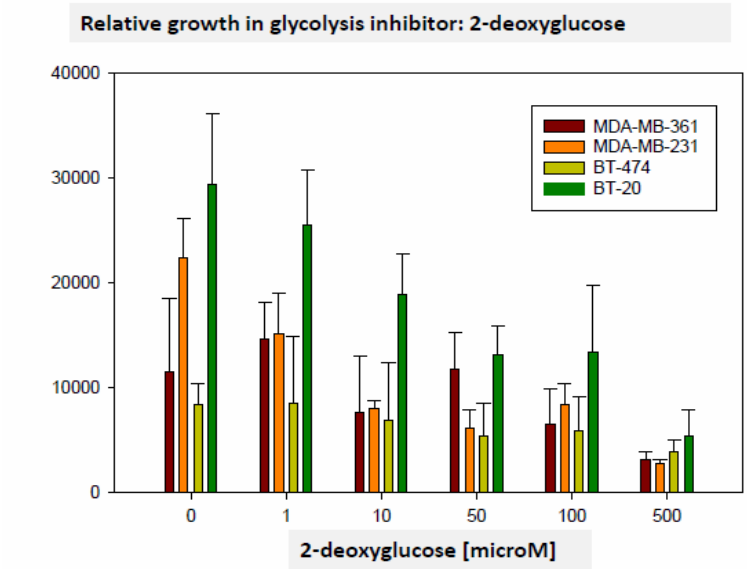

Figure 2. Effect of 2-deoxyglucose on breast cancer cell growth

(3) Effects of estrogen antagonists on OXPHOS and glycolysis, by the Seahorse XF24 Flux analyzer (Aim 1.3)

The Seahorse XF24 Flux analyzer (Seahorse Biosciences, Belerica, Mass.) makes real-time measurements of oxygen consumption (a measure of OXPHOS) and pH (a measure of the production of lactate by glycolysis). The instrument employs a dual-fluorescent probe, which simultaneously measures two indices of energy production (OXPHOS and glycolysis), including the oxygen consumption rate (OCR) and the rate of proton production, or extracellular acidification rate (ECAR). The former rate directly quantifies OXPHOS, which consumes oxygen to make water through a four-electron reduction at Complex IV (cytochrome c oxidase). The major source of proton production is lactate, made by glycolysis. In addition to the fluorescent probe, each well of cells can be treated with four different conditions. Experiments to provide a typical metabolic bioenergetic profile are performed by simultaneously measuring three replicates of four rates under five different conditions on each of 20 wells of cells.

First, basal levels of oxygen consumption and proton production are made. The next measurement condition (A) occurs during inhibition of ATP synthesis at Complex V (ATP synthase) by oligomycin. This process causes a build-up of protons across the inner matrix, with subsequent loss of electron flow, a decrease in oxygen consumption, and an ensuing rise in proton production because of the cell's sole dependence on glycolysis for ATP. The addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (B) causes the protons on the outside of the inner membrane to be carried across to the basic matrix, allowing the electron flow to proceed. In some cells, this process causes a large increase in oxygen consumption, which David Nichols has called "spare respiration capacity." The third injection (C) of 2-deoxy-D-glucose (2-DG) causes inhibition of glucose uptake into glycolysis and the TCA cycle. In some cells, this process is accompanied by an even further increase in oxygen consumption and loss of proton production due to glycolysis inhibition. The fourth and final injection (D) of rotenone, a Complex I (NADH:ubiquinone oxidoreductase) inhibitor, causes cessation of both electron flow and oxygen consumption.

Briefly, cells were grown in RPMI media containing serum and starved for 90 minutes in serum KHB media with no added carbon sources. Different carbon sources were injected into the plates, and real-time measurements of OXPHOS and glycolysis were determined. Since the cells were starved for carbon sources, glycolysis was suppressed, and we examined the effects of estrogen receptor changes to OXPHOS.

We found that estrogen stimulates both OXPHOS (Figure 3) and glycolysis (data not shown) in estrogen receptor positive (ER-positive) breast cancer cells. Antagonism of the estrogen receptor with either 4-hydroxy-tamoxifen (HT) or ICI caused a decline in OXPHOS (compared to estrogen-treated cells) in ER-positive (Figure 3), but not ER-negative (data not shown), breast cancer cells.

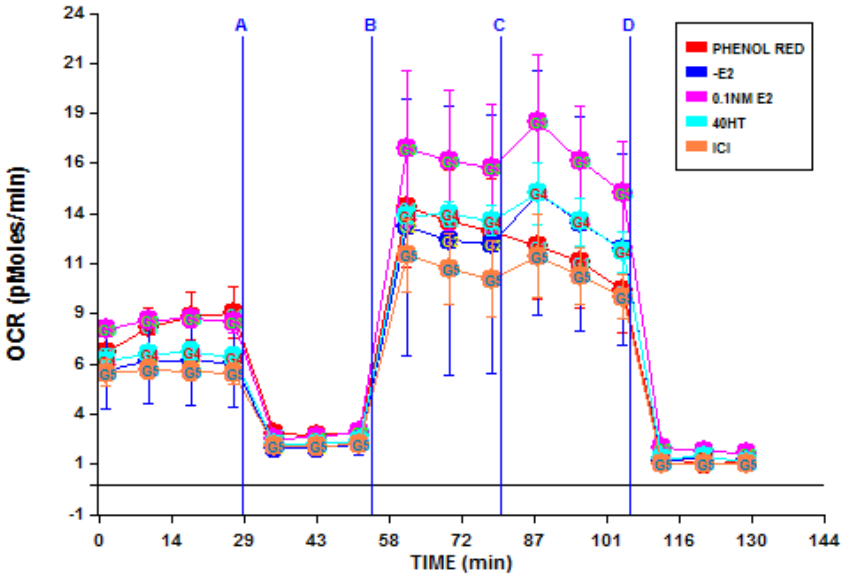

Figure 3. Estrogen (E2, pink) stimulates an increase in OXPHOS in ER-positive MCF7 cells.

(4) Western blot analysis of OXPHOS and glycolytic enzymes (Aim 2.1).

Various breast cancer cell lines were examined for expression of OXPHOS enzymes (Figure 4) or glycolytic enzymes (Figure 5) by western blot.

To test whether levels of OXPHOS and steady-state levels of ATP correlate with levels of complex V, we measured the protein expression levels of the subunits α and β of the ATP synthase catalytic core F1 by western blot. Figure 4 illustrates that both Complex V subunits were expressed at least two-fold higher in the luminal breast cancer subtype than in basal-like and claudin-low subtypes. High expression of Complex V subunits may contribute to an increase in ATP production and, so, may help to explain the higher steady-state ATP levels in the luminal cells. In contrast, the human epidermal growth factor receptor 2 (HER2)-enriched cells showed the highest expression levels of subunits α and β of Complex V.

In examining glycolytic enzyme expression (Figure 5), we found variance in the degree of lactate dehydrogenase (LDH) subunit expression in different types of breast cancer cell lines. Specifically, HER2-positive cell lines express high levels of both LDH alpha and beta, whereas triple-negative cells express slightly higher levels of LDH beta compared to the alpha isoform. Normal epithelial cell lines had relatively equivalent levels of the two. We also saw higher pyruvate dehydrogenase (PDH) levels in luminal compared to triple-negative basal or claudin low cell lines. Finally, despite the higher levels of OXPHOS and PDH in luminal and Her2-positive cell lines, we also saw higher levels of an inhibitor of PDH, called PDH kinase (PDK), which phosphorylates and inactivates PDH (pPDH) (Figure 5).

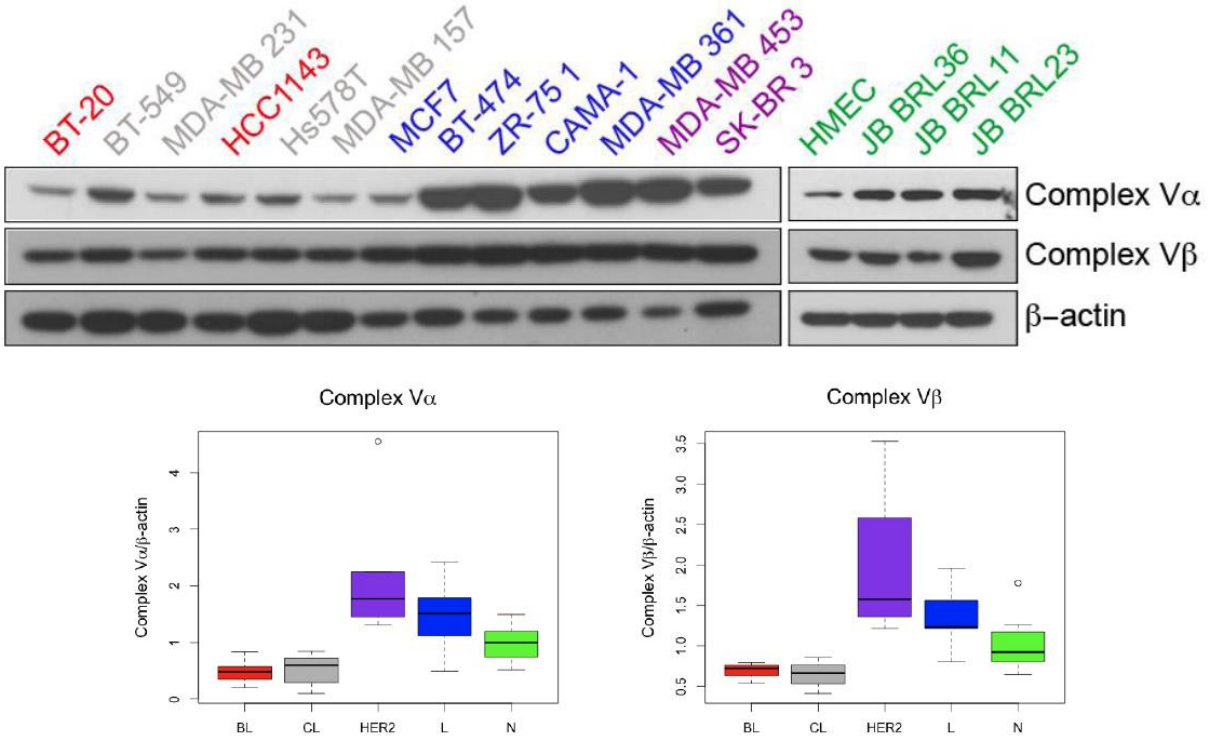

Figure 4. Expression of OXPHOS proteins, Complex V alpha and beta, in various breast cancer cell lines by western blot. BL, basal (red); CL, claudin low (grey); HER2, HER2 overexpressed (purple); L, luminal (blue); N, normal breast epithelial cells (green). Representative western blot analysis of Complex V subunits α and β in claudin-low (n = 4), basal-like (n = 2), HER2-enriched (n = 2) and luminal subtypes (n = 5), and normal-extended explants (n = 4). The bar graphs show protein quantification using the ImageJ program. Protein expression was calculated relative to the expression of β-actin. Results shown are mean ± SEM of at least three independent experiments.

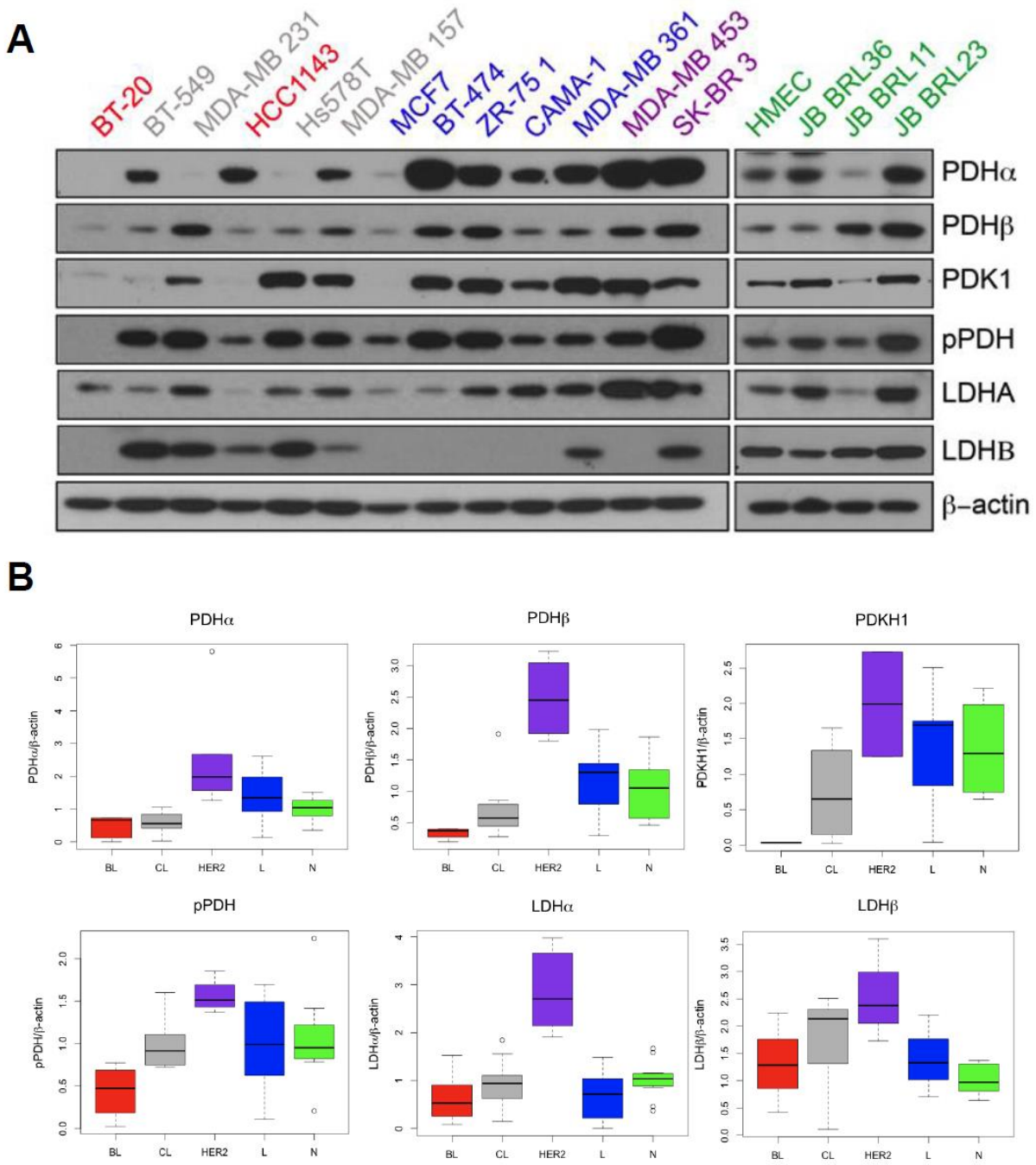

Figure 5. Expression of glycolytic proteins in various breast cancer cell lines by western blot. BL, basal (red); CL, claudin low (grey); HER2, HER2 overexpressed (purple); L, luminal (blue); N, normal breast epithelial cells (green). The bar graphs show protein quantification using the ImageJ program. Protein expression was calculated relative to the expression of β-actin. Results shown are mean ± SEM of at least three independent experiments.

(5) Analysis of published gene expression patterns in breast cancer cell lines for OXPHOS and glycolytic enzymes (Aim 2.2).

Data for this study were derived from Neve et al, 2006 and Hollestelle et al, 2010. Data were downloaded from Array Express (http://www.ebi.ac.uk/arrayexpress) and NCBI GEO (http://www.ncbi.nlm.nih.gov/geo) (accession codes E-TABM-157 and GSE16795, respectively).

Data normalization, summarization, and cancer subtype assignment: Raw data from both studies were normalized using the robust multi-array average (RMA) algorithm (Irizarry et al, 2003), and the mRNA expression datasets were reanalyzed as a single dataset. After normalization, probe sets that correspond to the same gene were combined and averaged. Genes with mean expression less than 6.0 and/or standard deviation less than 0.4 were considered noise and excluded from analysis. We chose to classify the data into basal-like/claudin-low and luminal/HER2-enriched due to the fact that claudin-low and basal-like cell lines have generally low OCR rates, whereas luminal and HER2-enriched cell lines have generally high OCR rates.

Gene expression data for 16 cell lines (BT20, BT549, MDA-MB 231, Hs578T, MDA-MB 157, MCF7, BT474, MDA-MB 361, MDA-MB 453, SK-BR 3, CAMA-1, ZR-75 1, UACC812, MDA-MB 134 VI, HCC1937, and MDA-MB 436) that had gene expression and Seahorse XF24 Extracellular Flux Analyzer data were used in feature selection. The HCC1143 cell line was not included in the analysis since no gene expression data were available in Hollestelle's dataset (Hollestelle et al, 2010). Genes that were more discriminating between basal-like/claudin-low and luminal/HER2-enriched general subtypes were selected using the HITON-MB algorithm (Aliferis et al, 2003). Briefly, this algorithm finds relevant variables by identifying "parents and children" of a variable in a Markov blanket.

Figure 6 depicts hierarchical clustering of the genes grouped by the Tight Cluster algorithm. The two heat maps correspond to the five different clusters identified around the genes selected by HITON-MB (gene names shown). Clusters 18, 35, and 36 contain genes that are generally upregulated in basal-like and claudin-low cell lines. The AKR1B1, CAPG, IGF2BP2, MSN, PDP1, and SGK1 found by HITON-MB are inside those clusters. Clusters 28 and 24 contain genes that are generally upregulated in luminal cell lines. Inside those clusters, we can find the genes AGR2 and XBP1 that were selected by HITON-MB.

In summary, the systems-level analysis we performed has expanded our knowledge about metabolic heterogeneity within breast cancer subtypes. We have developed a robust prediction algorithm that can predict levels of OXPHOS and have shown that breast cancer subtypes have distinct levels of basal OXPHOS. In addition, we have demonstrated that breast cancer subtypes are metabolically flexible—able to adapt their metabolism to environmental conditions and metabolite restrictions. These observations can be exploited in breast cancer therapy to develop a subtype-specific treatment. Furthermore, we have found evidence that breast cancer cells may use fatty acid beta-oxidation as a key metabolic pathway to generate energy.

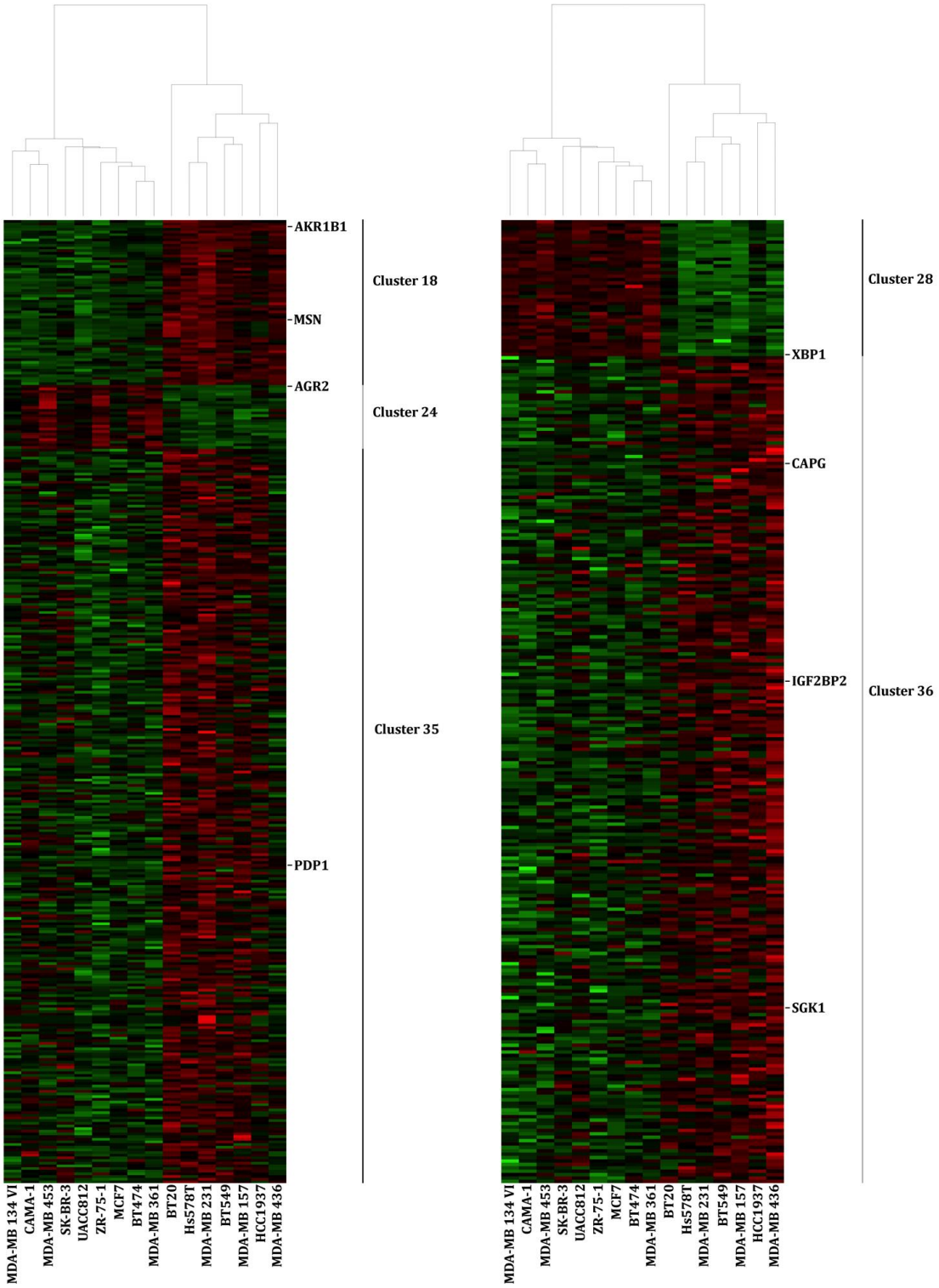

Figure 6. Hierarchical clustering of the genes grouped by the Tight Cluster algorithm.

(6) Analysis of OXPHOS and glycolytic protein expression in melanoma tissue microarrays (Aim 2.3)

This work was performed and published (see enclosed PDF):

- Ho J, de Moura MB, Lin Y, Vincent G, Thorne S, Duncan LM, Hui-Min L, Kirkwood JM, Becker D, Van Houten B, Moschos SJ. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. Mol Cancer. 2012 Oct 9;11:76. PMCID: PMC3537610

  Summary: Serum LDH is a prognostic factor for patients with stage IV melanoma. To gain insights into the biology underlying this prognostic factor, we analyzed total serum LDH, serum LDH isoenzymes, and serum lactate in 49 patients with metastatic melanoma. Our data demonstrate that high serum LDH is associated with a significant increase in LDH isoenzymes 3 and 4 and a decrease in LDH isoenzymes 1 and 2. Since LDH isoenzymes play a role in both glycolysis and OXPHOS, we subsequently determined (using tissue microarray [TMA] analysis) that the levels of proteins associated with mitochondrial function, lactate metabolism, and regulators of glycolysis were all elevated in advanced melanomas compared with nevic melanocytes. To investigate whether glycolysis and OXPHOS pathways might be linked in advanced melanoma, we determined expression of the monocarboxylate transporters (MCT) 1 and 4. Analysis of a nevus-to-melanoma progression TMA revealed that MCT4, and to a lesser extent MCT1, were elevated with progression to advanced melanoma. Further analysis of human melanoma specimens using the Seahorse XF24 extracellular flux analyzer indicated that metastatic melanoma tumors derived a large fraction of energy from OXPHOS. Taken together, these findings suggest that in stage IV melanomas with normal serum LDH, glycolysis, and OXPHOS may provide metabolic symbiosis within the same tumor, whereas in stage IV melanomas with high serum LDH, glycolysis is the principal source of energy.

(7) Analysis of OXPHOS and glycolysis in several melanoma cell lines and tumor biopsy material (Aims 3.1.1 and 3.1.2).

This work was developed and published in two peer-reviewed articles:

- Barbi de Moura M, Vincent G, Fayewicz SL, Bateman NW, Hood BL, Sun M, Suhan J, Duensing S, Yin Y, Sander C, Kirkwood JM, Becker D, Conrads TP, Van Houten B, Moschos SJ. (2012). Mitochondrial respiration—an important therapeutic target in melanoma. PLOS One 7(8):e40690. PMCID:PMC3422349

  Summary: The importance of mitochondria as oxygen sensors as well as producers of ATP and reactive oxygen species (ROS) has recently become a focal point of cancer research. However, in the case of melanoma, little information is available as to the extent to which cellular bioenergetics processes contribute to the progression of disease and (related to that) whether OXPHOS has a prominent role in advanced melanoma. In

this study, we demonstrate that, compared to melanocytes, metastatic melanoma cells have elevated OXPHOS levels. Furthermore, by treating metastatic melanoma cells with the drug Elesclomol, which induces cancer cell apoptosis through oxidative stress, we document (by way of stable isotope labeling with amino acids in cell culture [SILAC]) that proteins participating in OXPHOS are downregulated. We also provide evidence that melanoma cells with high levels of glycolysis are more resistant to Elesclomol. We further show that Elesclomol upregulates hypoxia inducible factor 1-α (HIF-1α), and that prolonged exposure of melanoma cells to this drug leads to selection of melanoma cells with high levels of glycolysis. Taken together, our findings suggest that molecular targeting of OXPHOS may have efficacy for advanced melanoma.

- Ho J, de Moura MB, Lin Y, Vincent G, Thorne S, Duncan LM, Hui-Min L, Kirkwood JM, Becker D, Van Houten B, Moschos SJ. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. Mol Cancer. 2012 Oct 9;11:76. PMCID: PMC3537610

  Summary: see item 6 above.

(8) Correlating hypoxic markers with glycolytic markers by flow cytometry in cell lines and human biopsy material (Aims 3.1.2 and 3.1.3).

Work performed under this aim examining hypoxic and glycolytic markers was published:

- Ho J, de Moura MB, Lin Y, Vincent G, Thorne S, Duncan LM, Hui-Min L, Kirkwood JM, Becker D, Van Houten B, Moschos SJ. Importance of glycolysis and oxidative phosphorylation in advanced melanoma. Mol Cancer. 2012 Oct 9;11:76. PMCID: PMC3537610

  Summary: see item 6 above.

We performed several pilot studies using flow cytometry and found that this approach was not appropriate for these analyses. We, therefore, did not pursue this line of experimentation further.

(9) Mitochondrial DNA sequencing (Aim 3.1.4).

These results were published:

- Furda AM, Marrangoni AM, Lokshin A, Van Houten B. Oxidants and not alkylating agents induce rapid mtDNA loss and mitochondrial dysfunction. DNA Repair (Amst). 2012 11(8):684-92. PMCID:PMC3878289.

We found no differences in mtDNA of tumor cells compared to normal human mtDNA, other than normal sequence variants. We addressed the question of whether persistent mtDNA damage causes a loss of OXPHOS by treating mouse embryonic fibroblasts with hydrogen peroxide

(H_2O_2) or the alkylating agent methyl methanesulfonate (MMS) and measuring several endpoints, including mtDNA damage and repair rates using qPCR, levels of mitochondrial- and nuclear-encoded proteins using western analysis, and a pharmacologic profile of mitochondria using the Seahorse Extracellular Flux Analyzer. We found that a 60-minute treatment with H_2O_2 causes persistent mtDNA lesions, mtDNA loss, decreased levels of a nuclear-encoded mitochondrial subunit, loss of ATP-linked oxidative phosphorylation, and loss of total reserve capacity. Conversely, a 60-minute treatment with 2 mM MMS causes persistent mtDNA lesions but no mtDNA loss, no decrease in levels of a nuclear-encoded mitochondrial subunit, and no mitochondrial dysfunction. These results suggest that persistent mtDNA damage is not sufficient to cause mitochondrial dysfunction.

(10) Visualize metabolic symbiosis *in vivo* (Aim 3.2).

This work was recently published:

- Nakajima EC, Laymon C, Oborski M, Hou W, Wang L, Grandis JR, Ferris RL, Mountz JM, Van Houten B. Quantifying metabolic heterogeneity in head and neck tumors in real time: 2-DG uptake is highest in hypoxic tumor regions. PLOS One. 2014 Aug 15;9(8):e102452. PMCID: PMC4134191.

  Summary:
  PURPOSE: Intratumoral metabolic heterogeneity may increase the likelihood of treatment failure due to the presence of a subset of resistant tumor cells. Using a head and neck squamous cell carcinoma (HNSCC) xenograft model and a real-time fluorescence imaging approach, we tested the hypothesis that tumors are metabolically heterogeneous and that tumor hypoxia alters patterns of glucose uptake within the tumor.
  EXPERIMENTAL DESIGN: Cal33 cells were grown as xenograft tumors (n = 16) in nude mice after identification of this cell line's metabolic response to hypoxia. Tumor uptake of fluorescent markers identifying hypoxia, glucose import, or vascularity was imaged simultaneously using fluorescent molecular tomography. The variability of intratumoral 2-deoxyglucose (IR800-2-DG) concentration was used to assess tumor metabolic heterogeneity, which was further investigated using immunohistochemistry for expression of key metabolic enzymes. HNSCC tumors in patients were assessed for intratumoral variability of (18)F-fluorodeoxyglucose (^{18}F-FDG) uptake in clinical positron emission tomography (PET) scans.
  RESULTS: IR800-2-DG uptake in hypoxic regions of Cal33 tumors was 2.04 times higher compared to the whole tumor ($p = 0.0001$). IR800-2-DG uptake in tumors containing hypoxic regions was more heterogeneous compared to tumors lacking a hypoxic signal. Immunohistochemistry staining for HIF-1α, carbonic anhydrase 9, and ATP synthase subunit 5β confirmed xenograft metabolic heterogeneity. We detected that heterogeneous (18)F-FDG uptake within patient HNSCC tumors and the degree of heterogeneity varied among tumors.
  CONCLUSION: Hypoxia is associated with increased intratumoral metabolic heterogeneity. (18)F-FDG PET scans may be used to stratify patients according to the metabolic heterogeneity within their tumors, which could be an indicator of prognosis.

(11) Targeting metabolic pathways in human melanoma xenografts (Aim 3.3).

This work was not pursued due to the loss of a key collaborator on this project, Dr. Stergios Moschos, who moved to the University of North Carolina, Chapel Hill.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X__ No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:

_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, and an abbreviated title of the publication. For example, if you submit two publications for Smith (PI for Project 01), one publication for Zhang (PI for Project 03), and one publication for Bates (PI for Project 04), the filenames would be:

Project 01 – Smith – Three cases of isolated
Project 01 – Smith – Investigation of NEB1 deletions
Project 03 – Zhang – Molecular profiling of aromatase
Project 04 – Bates – Neonatal intensive care

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Mitochondrial respiration—an important therapeutic target in melanoma	Barbi de Moura M, Vincent G, Fayewicz SL, Bateman NW, Hood BL, Sun M, Suhan J, Duensing S, Yin Y, Sander C, Kirkwood JM, Becker D, Conrads TP, Van Houten B, Moschos SJ	PLOS One. 2012;7(8):e40690	Feb. 2012	☐Submitted ☐Accepted ☑Published
2. Importance of glycolysis and oxidative phosphorylation in advanced melanoma	Ho J, de Moura MB, Lin Y, Vincent G, Thorne S, Duncan LM, Hui-Min L, Kirkwood JM, Becker D, Van Houten B, Moschos SJ	Mol Cancer. 2012 Oct 9;11:76	March 2012	☐Submitted ☐Accepted ☑Published
3. Metabolic symbiosis in cancer: refocusing	Nakajima EC, Van Houten B	Mol Carcinog.	August 2011	☐Submitted ☐Accepted

the Warburg lens		2013 May; 52(5):329-37		☑Published
4. Quantifying metabolic heterogeneity in head and neck tumors in real time: 2-DG uptake is highest in hypoxic tumor regions	Nakajima EC, Laymon C, Oborski M, Hou W, Wang L, Grandis JR, Ferris RL, Mountz JM, Van Houten B	PLOS One. 2014 Aug 15;9(8):e102 452	March 2014	☐Submitted ☐Accepted ☑Published
5. Overexpression of mitochondrial sirtuins alters glycolysis and mitochondrial function in HEK293 cells	de Moura MB, Uppala R, Zhang Y, Van Houten B, Goetzman ES	PLOS One. 2014 Aug 28;9(8):e106 028	April 2014	☐Submitted ☐Accepted ☑Published
6. Oxidants and not alkylating agents induce rapid mtDNA loss and mitochondrial dysfunction	Furda AM, Marrangoni AM, Lokshin A, Van Houten B	DNA Repair (Amst). 2012 Aug 1;11(8):684-92	Nov. 2011	☐Submitted ☐Accepted ☑Published
7. Impaired mitochondrial biogenesis contributes to depletion of functional mitochondria in chronic MPP(+) toxicity: dual roles for ERK1/2	Zhu JH, Gusdon AM, Cimen H, Van Houten B, Koc E, Chu CT	Cell Death Dis. 2012 May 24;3:e312	Oct. 2011	☐Submitted ☐Accepted ☑Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes___X_____ No_____

If yes, please describe your plans:

We have one large project that we are currently wrapping up for publication:

Moura MB, Santana-Santos L, Janjic BM, Latimer JJ, Benos PV, Van Houten B. (2014). Systems-level approach identifies oxidative phosphorylation as an important driver in breast cancer subtypes.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be

single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

While there has been no direct effect on cancer deaths or diagnosis, much of the work we have done has important implications in cancer research. Specifically, our work has shown that human tumors are metabolically flexible and can use a wide variety of carbon sources. Furthermore, tumor cells appear to use both oxidative phosphorylation and glycolysis to fuel their bioenergetic demands for rapid growth. Thus, metabolic inhibitors alone will not be sufficient to treat tumors; rather, these metabolic inhibitors will have to be combined strategically with current anticancer therapies.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes _____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?

Yes___ No____
If yes, indicate number of patent, title and date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Van Houten, Bennett	Professor of Pharmacology & Chemical Biology, University of Pittsburgh
eRA COMMONS USER NAME bvanhouten	

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(S)	FIELD OF STUDY
Clarion State College	PABSc	1976-80	Biology
Oak Ridge School of Biomedical Sciences	PhD	1980-84	Biomedical Sciences/Genetics

A. Personal Statement

I am the Richard M. Cyert Professor of Molecular Oncology in Pharmacology and Chemical Biology and Co-Leader of the Molecular and Cellular Cancer Biology Program at the University of Pittsburgh Cancer Institute. I am also the Associate Director of Basic Research for the Aging Institute at the University of Pittsburgh. Research in my laboratory is in two general areas: 1) mitochondrial function and disease; and 2) analysis of DNA nucleotide excision repair enzymes by single molecule techniques. With regard to mitochondrial function, my laboratory developed and patented an approach for measuring mitochondrial DNA damage using quantitative PCR (PNAS 1997). We have found that mitochondrial DNA damage is sufficient to decrease mitochondrial function and cause cell death (Nature, 2011; JBC, 2011). We also study cancer cell metabolism and the role of mitochondrial function using a Seahorse Bioanalyzer, which measures oxidative phosphorylation and glycolysis using oxygen and pH sensitive fluorophores in mammalian cells in real time. We are currently testing the hypothesis that aggressive tumor cells are metabolically heterogeneous, with sections of the tumor requiring mitochondrial function ATP production and cell growth (Mol. Cancer, 2012). Furthermore, we are examining tumor growth in a xenograft model using multi-modal imaging including 3D four red/IR channel fluorescence molecular tomography (FMT).

B. Positions and Honors

Positions and Employment

1979	Summer Research Student, Roswell Park Memorial Institute, Buffalo, NY (6/69-8/79)
1980-1984	Graduate Research Assistant, Oak Ridge Graduate School of Biomedical Sciences, University of Tennessee, Oak Ridge, TN
1984-1988	Postdoctoral Fellow, Department of Biochemistry, Lineberger Cancer Research Center University of North Carolina at Chapel Hill
1988-1990	Research Assistant Professor, Departments of Pathology and Biochemistry, University of Vermont
1990-1994	Assistant Professor, Departments of Pathology, Biochemistry and Microbiology and Molecular Genetics, University of Vermont
1994-1999	Senior Scientist, Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX
1994-1998	Associate Professor, Department of Human Biological Chemistry & Genetics, and Department of Pathology, UTMB

1998-1999 Professor, Department of Human Biological Chemistry & Genetics, and Department of Pathology, UTMB
1999-2008 Chief, Program Analysis Branch, DERT, NIEHS, NIH and Senior Investigator, Laboratory of Molecular Genetics, DIR, NIEHS, NIH.
2008- Richard M Cyert Professor of Molecular Oncology, Department of Pharmacology and Chemical Biology; Co-Leader, Molecular and Cellular Cancer Biology Program, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA
2009- Faculty member, Molecular Biophysics and Structural Biology Graduate Program, University of Pittsburgh and Carnegie Mellon University
2009- Faculty member, Graduate Program in Biochemistry and Molecular Genetics, University of Pittsburgh
2013- Associate Director for Basic Science, Aging Institute, University of Pittsburgh

Honors
2012 Provost Inaugural Lecture, Richard M. Cyert Chair in Molecular Oncology
2008 Medal of the Slovak Academy of Sciences for Support of Science
2005 NIH Award of Merit for evaluation activities in the Division of Extramural Research and Training
2003 NIH Award of Merit for exemplary performance in the management of the Toxicogenomics Research Consortium
2002 NIH Director's Award for outstanding effort conceptualizing, initiating, and implementing the NIEHS Toxicogenomics Research Consortium
2002 NIH Award of Merit for outstanding efforts in conceptualizing, initiating, and implementing the NIEHS Scientific Publications Information Retrieval System (SPIRES)
2001 NIH Award of Merit for outstanding effort conceptualizing, initiating, and implementing the NIEHS Toxicogenomics Research Consortium
2001 NIH Award of Merit for praiseworthy service in performing programmatic services, analyses, and evaluations to the benefit of NIEHS and the scientific community
9/96-8/31/99 Burroughs Wellcome Fund Scholar Award in Toxicology
1986-1988 Individual National Research Service Award

C. Selected Publications

1. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A. 1997 Jan 21;94(2):514-9. PMID: 9012815; PMCID: PMC19544
2. Simsek D, Furda A, Gao Y, Artus J, Brunet E, Hadjantonakis A, Van Houten B, Shuman S, McKinnon PJ, Jasin M. Crucial roles for DNA ligase III in mitochondria but not in XRCC1-dependent repair. Nature. 2011 Mar 10;471(7337):245-8. PMID: 21390132; PMCID: PMC3261757
3. Tann AW, Boldogh I, Meiss G, Qian W, Van Houten B, Mitra S, Szczesny B. Apoptosis induced by persistent single-strand breaks in the mitochondrial genome: critical role of EXOG (5' EXO/endonuclease) in their repair. J Biol Chem. 2011 Sep 16;286(37):31975-83. PMID: 21768646; PMCID: PMC3173182
4. Qian W, Choi S, Gibson GA, Watkins SC, Bakkenist CJ, Van Houten B. Mitochondrial hyperfusion induced by loss of the fission protein Drp1 causes ATM-dependent G2/M arrest and aneuploidy through DNA replication stress. J Cell Sci. 2012 Dec 1;125(Pt 23):5745-57. PMID: 23015593; PMCID: PMC4074216 (Cover and highlights).