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CONTRACTING ORGANIZATION: Cedars-Sinai Medical Center

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14. ABSTRACT Epidemiological data indicate that cholesterol-lowering pharmacotherapy, primarily HMG-CoA-reductase inhibitors ("statins"), reduce the risk Of aggressive prostate cancer PCs). The FDA-approved anti-obesity drug, Orlistat, which inhibits the enzyme fatty acid Synthase (FASN), has been shown to slow the growth of human prostate tumors in mice. Despite these advances, studies of lipid metabolism in PCa have lagged behind other areas of research on cell signaling, and limited information is available about how these promising preclinical and clinical data might be leveraged to improve patient outcomes. Our hypothesis is that PCa progression is dependent on a Palmitoyl-protein network regulated by FASN. We predict that the activity of this network can be suppressed by reducing levels of circulating cholesterol. Specific Aims: We will challenge this hypothesis with the following specific aims: Aim 1: Identify critical Palmitoyl-proteins in the FASN subnetwork. Test their functional roles. Aim 2: Determine whether the FASN-dependent Palmitoyl network can be suppressed in vivo by cholesterol reduction. This project is uncovering new information of potential relevance to (1) dietary influences on PCa risk, and (2) the role of cholesterol and other membrane lipids in PCa progression.					
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The Oncogenic Palmitoyl-Protein Network in Prostate Cancer

Grant number: PC093459

Year 3 progress report

INTRODUCTION

Alterations in lipid metabolism are now known to play a substantial role in prostate cancer (PCa) progression to end-stage disease, however the important molecular details of these changes in PCa are still poorly understood. Studies from our laboratory and others have hypothesized that (1) fatty acid synthase (FASN)-dependent palmitoylation (more accurately called S-acylation) and cholesterol-sensitive signaling mechanisms intersect during progression of PCa to castration resistant disease (CRPC); (2) FASN lies upstream from a largely unknown palmitoylated protein signaling network; and (3) this network might be sensitive to pharmacologic targeting of cholesterol using the cholesterol-lowering drug, ezetimibe. The overall objective of this study is to test the hypothesis that ***PCa progression is dependent on a palmitoyl-protein network regulated by FASN*** and that ***the activity of this network can be suppressed by pharmacologic reduction of circulating cholesterol level***.

The specific aims of the project are:

Aim 1: Identify critical palmitoyl-proteins in the FASN subnetwork. Test their functional roles.

Aim 2: Determine whether the FASN-palmitoyl network can be suppressed in vivo by cholesterol reduction.

BODY

Specific Aim 1: Identify critical palmitoyl-proteins in the FASN subnetwork. Test their functional roles.

Aim 1, Task 1. Identify the palmitoylation sites on the AR and palmitoylated proteins that associate with AR and FASN.

Proposed plan: We will use a novel proteomic strategy developed in our laboratory, termed PalmPISC, to verify the single known palmitoylation site on the AR, identify any other palmitoylation sites on the protein, and identify novel palmitoylation proteins and palmitoylated sites on proteins present in AR and FASN immune complexes.

Progress:

In year 3, we systematically optimized our Palmitoyl Protein Identification and Site Characterization (PalmPISC) method and dramatically increased the specificity of purifying palmitoylated proteins from PCa and other types of cells. Using the optimized PalmPISC method, we found that only less than 0.1% of total enriched proteins from LNCaP PCa cells were non-palmitoylated proteins (Fig. 1), indicating a high level of specificity of our improved purification method. In comparison, with our original PalmPISC method, about 10-20% of enriched proteins are non-palmitoylated proteins [1, 2]. Notably, most other published methods for the enrichment of palmitoylated proteins provide much less specificity than our original PalmPISC method — about 40-70% of enriched proteins were non-palmitoylated proteins [3, 4].

Moreover, we developed a rapid and deep proteomics profiling method by taking advantage of the latest proteomics instruments equipped at the Cedars-Sinai Biomarker Discovery Platform Core, including a very high-end mass spectrometer (LTQ Orbitrap Elite) and an ultrahigh performance liquid chromatography (UPLC) system (EASY-nLC 1000). Using this novel method, we were able to identify about 3,000 to 5,000 protein groups (with a false discovery rate of 0.01) from only 2 µg proteins extracted from LNCaP PCa cells in 2 to 5 h (Fig. 2). Compared with the previous generation of proteomics technology we were using at Harvard, our new method is at least 100 times more effective.

By integrating our optimized PalmPISC method, our deep proteomics profiling method, and a widely used quantitative proteomics method called stable isotope labeling by amino acids in cell culture (SILAC) [5], we identified 1137 putative S-acylated proteins, with a false discovery rate (FDR) of less than 0.01, from only 400 µg whole cell lysates extracted from LNCaP cells. This represents **the largest group of S-acylated proteins identified in a single study so far**.



Figure 1. Over 99.9% of non-S-acylated proteins can be depleted using our optimized PalmPISC method (unpublished data). The left two SDS gel lanes show proteins from 1% of whole cell lysates extracted from DU145 cells, while the right two lanes show total proteins purified from whole cell lysates using our optimized PalmPISC method. EXP indicates an experimental group, in which S-acylated proteins and contaminating proteins were purified. CON indicates a control group, in which only contaminating proteins were purified.

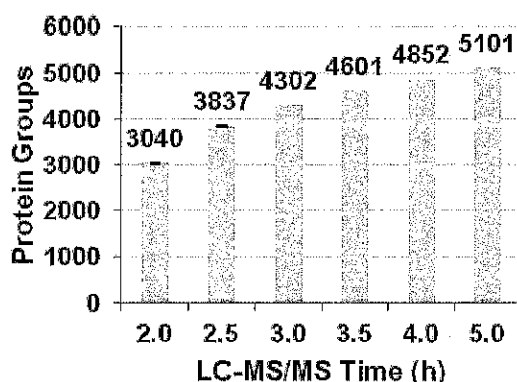


Figure 2. Our rapid and deep proteomics profiling method enables the identification of 3,000 to 5,000 protein groups from 2 µg whole cell lysates within 2 to 5 hours (unpublished data). Note that the method is highly reproducible so the standard deviations are very small.

Currently, we are applying our improved methods to identify the palmitoylation sites on the AR. We are also isolating AR and FASN complexes from PCa cells and identifying palmitoyl proteins from the immune complexes (which only contain a small amount of proteins) using our much more sensitive palmitoylproteomics methods.

Aim 1. Task 2. Identify palmitoyl-proteins downstream from FASN using a global proteomic strategy.

Proposed plan: We will use the PalmPISC method, in concert with the SILAC method of quantitative proteomics, to analyze the entire palmitoyl-proteome in PCa cells to identify proteins that are downstream from FASN.

Progress:

As we have already described, we coupled our original PalmPISC method with triplex SILAC and identified a number of palmitoylated proteins downstream of the epidermal growth factor receptor (EGFR), a potent oncoprotein regulating the expression and activity of FASN.

Aim 1. Task 3. Establish critical network relationships involving palmitoylated proteins.

Proposed plan: We will use bioinformatics tools and other information that arises from our proteomic studies of the palmitoyl-protein network to identify critical nodes and novel relationships between proteins within the network.

Progress:

As shown in a previous progress report, we coupled triplex SILAC with PalmPISC to identify palmitoylated proteins regulated by EGFR. We found that the translation machinery, which includes many ribosomal proteins and eukaryotic initiation factors, was extensively palmitoylated and that the palmitoylation level in the protein translation "interactome" was increased upon EGF stimulation. We also found that some cell adhesion molecules, including cadherins, catenins, and tight junction proteins were depalmitoylated upon EGF treatment. We are pursuing these interesting findings now.

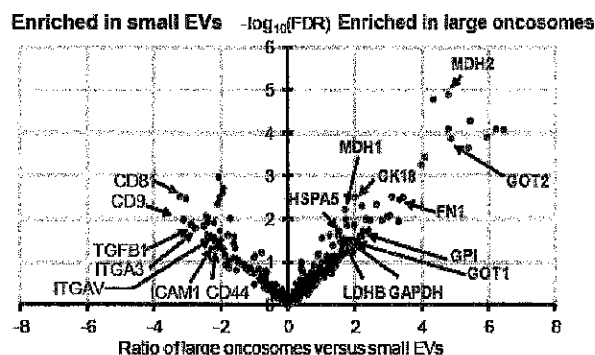
Aim 1. Task 4. Test potential biological functions of critical nodes.

Proposed plan: We will use a series of functional tests to determine whether perturbation of the proteins and relationships we identify in Tasks 1-3 evoke one or more biological responses in PCa cells relevant to tumor behavior in vivo. We will also look for opportunities within the data set to identify substrates for one or more palmitoyl transferases (PATs), which enzymatically modify proteins with palmitate residues. In addition, we will use a series of assays developed in our laboratory to assess whether elements of the palmitoyl-protein network can be implicated in the amoeboid tumor cell phenotype, as well as secretion and activity of oncosomes, membrane-bound particles with the capability of altering the tumor microenvironment that we recently linked to metastatic PCa.

Progress:

We profiled 529 candidate palmitoylated proteins from PC-3 PCa cells. Previous studies suggested that DHHC3 (a PAT) plays a critical role in mediating PCa metastasis, partly through the palmitoylation of integrin $\alpha 6 \beta 4$ [6]. We integrated DHHC3 stable knockdown, SILAC, and PalmPISC to comprehensively identify candidate DHHC3 substrates in PC-3 cells. As a result, we identified 30 DHHC3 substrate candidates, including the reported integrin $\alpha 6 \beta 4$ as well as unknown substrates such as tetraspanin-9 and CD44.

We have shown that EGF stimulation promotes the formation of large oncosomes, atypically large bioactive extracellular vesicles (EVs) associated with the amoeboid cancer cell phenotype [7]. It is likely that the palmitoylated translation machinery and palmitoylated cell adhesion protein network are critical palmitoyl-protein networks regulating the formation, secretion, and activity of oncosomes. We recently developed the technology to separate large oncosome EVs from small EVs (exosomes). In a recent quantitative proteomics comparison of large oncosomes and small extracellular vesicles, we found that some cell adhesion proteins (e.g., ITGA3, ITGAV, ICAM1, and CD44) and tetraspanins (e.g., CD9 and CD81) are relatively depleted in large oncosomes in comparison to small EVs (Fig. 3). We are currently



investigating whether elements of the palmitoylated cell adhesion protein network are implicated in the amoeboid tumor cell phenotype as well as secretion and bioactivity of oncosomes.

Figure 3. Volcano plots of the log₂-transformed SILAC ratios against the false discovery rate (FDR) (unpublished data). Red dots correspond to proteins enriched in large oncosomes; blue dots correspond to proteins enriched in small extracellular vesicles.

Specific Aim 2: Determine whether the FASN-palmitoyl network can be suppressed in vivo by cholesterol reduction.

High circulating cholesterol and androgen deprivation promote prostate cancer metastasis

In collaboration with Leland Chung's group at Cedars-Sinai, and using a novel model of PCa bone metastasis we developed and recently reported [8], we demonstrated for the first time that either high circulating cholesterol or androgen deprivation cooperates with activation of the RANK signaling axis to promote metastasis to bone and soft tissues. We also demonstrated that the number of circulating tumor cells, isolated by a NanoVelcro microfluidics system, was similarly increased in both the high cholesterol and androgen-suppressed conditions. These new data provide an exciting new in vivo model to examine the molecular mechanisms underlying the role of cholesterol as a mediator of PCa progression. For many years, our lab has been a leader in the study of the effects of high cholesterol on progression of PCa to advanced disease. We believe this is a major step forward in the field.

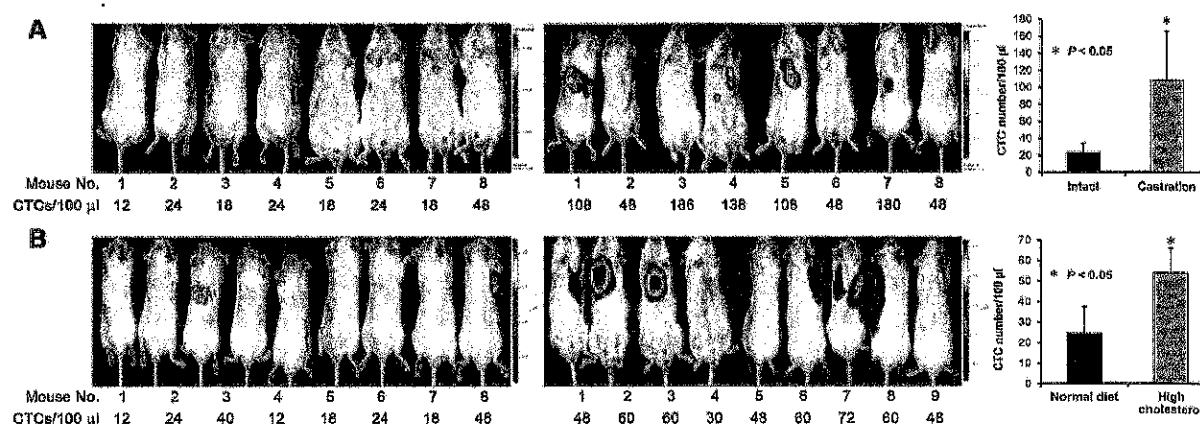


Figure 4. Androgen deprivation and high cholesterol diet enhanced PC bone and soft tissue metastases. A, Mice (N=8 per group) were either sham operated (Intact) or surgically castrated (Castrated) prior to i.c injection of LNRANKL-Luc-RFP cells. Mice were imaged every two weeks and their CTC counts were obtained on the cytopsin slides. Note higher number of metastasis, detected by bioluminescence, accompanied by higher number of CTCs in castrated than sham operated intact mice at 16 weeks. B, Similar study were conducted in mice fed with normal or high cholesterol diet. Note higher number of metastasis, detected by bioluminescence, accompanied by higher number of CTCs in high cholesterol diet fed than normal diet fed mice at 12 weeks.

KEY RESEARCH ACCOMPLISHMENTS

- Improved the specificity for the enrichment of palmitoylated proteins by 10-20 times.
- Improved the sensitivity for the identification of proteins by over 100 times.
- Identified new target proteins significantly enriched in large oncosomes in comparison to small EVs (exosomes).
- Demonstrated that large oncosomes were relatively depleted in some key palmitoyl-proteins.
- Found that high circulating cholesterol can enhance PCa bone and soft tissue metastases using a novel in vivo metastasis model we developed.

CONCLUSIONS

We have made substantial progress in year 3 in investigating the synergistic functions of protein palmitoylation, FASN, and cholesterol in PCa progression to metastasis. Studies addressing the

remaining tasks are ongoing and we anticipate their completion during the no-cost extension period. Additional funds from the PRCP will not be required.

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<u>BUDGET FOR ENTIRE PROPOSED PERIOD OF PERFORMANCE</u>						
BUDGET CATEGORY TOTALS*	BUDGET YEAR ONE	ADDITIONAL YEARS OF SUPPORT REQUESTED				TOTAL
		2nd	3rd	4th	5th	
A. PERSONNEL COSTS			\$107,629			
B. OTHER COSTS			\$8,957			
MAJOR EQUIPMENT						
TRAVEL COSTS						
MATERIALS, SUPPLIES, AND CONSUMABLES			\$6,000			
CONSULTANT COSTS						
SUBAWARD/ CONSORTIUM/ CONTRACTUAL COSTS						
RESEARCH- RELATED SUBJECT COSTS						
OTHER EXPENSES	2957		\$2,957			
C. TOTAL DIRECT COSTS	116586		\$116,586			
D. TOTAL INDIRECT COSTS	78113		\$78,113			
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT						\$116,586
TOTAL INDIRECT COSTS FOR ENTIRE PROPOSED PERIOD OF SUPPORT						\$78,113
TOTAL COSTS FOR THE ENTIRE PROPOSED PERIOD OF SUPPORT THIS AMOUNT MUST MATCH WITH THAT ENTERED ON THE SF 424, BLOCK 16A						\$194,699

* Itemize all budget categories for additional years on the Justification page that follows.