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PRINCIPAL INVESTIGATOR: Michael Freeman

CONTRACTING ORGANIZATION: Children's Hospital Corporation
Boston, MA 02115

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14. ABSTRACT Many proteins resident in tetraspanin-enriched microdomains, key structural and functional platforms in breast cancer, are regulated by post-translational palmitoylation. Our preliminary data suggests that DHHC3, a palmitoylating enzyme, plays a critical role in breast cancer cell growth, invasion, and /or metastasis. However, little is known about which proteins are palmitoylated by DHHC3 in breast cancer. Thus, we proposed to comprehensively identify DHHC3 substrates by integrating our palmitoyl protein identification and site characterization method with triplex SILAC. In year 1, we established this multiplexed quantitative palmitoyl-proteomics method in our lab. We tested and optimized the method during the analysis of cancer cells stimulated by epidermal growth factor. In this time- and membrane domain resolved proteomics experiment, we found that many translation factors are palmitoylated and targeted to membranes, while some cell adhesion molecules are depalmitoylated and dissociated from certain membrane microdomains, In response to epidermal growth factor simulation.					
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Introduction

Tetraspanin-enriched microdomains (TEMs) serve as key structural and functional platforms in breast cancer and many other types of cancers [1, 2]. TEMs comprise many laminin-binding integrins (*e.g.*, $\alpha 6\beta 4$, $\alpha 6\beta 1$, and $\alpha 3\beta 1$), tetraspanin proteins (*e.g.*, CD151, CD9, CD81, and CD82), and their partner proteins [3]. Many of these proteins are regulated by palmitoylation, which is critical for the regulation of protein localization, activity, and stability as well as multiprotein complex formation [4]. Protein palmitoylation in human is carried out by 23 palmitoyl acyltransferases (PATs) encoded by the *DHHC* gene family [5]. Recently, the lab headed by Dr. Martin Hemler, a co-PI of the funded project, showed that the ablation of *DHHC3* not only inhibits the palmitoylation of integrin $\alpha 6$ and $\beta 4$ subunits [6], but also disrupts TEMs and markedly alters cell morphology, invasion and signaling through focal adhesion kinase (FAK) in breast cancer cell lines. Altogether, these results strongly suggested that *DHHC3* plays a critical role during breast cancer cell growth, invasion, and/or metastasis. Since hundreds of cellular proteins are palmitoylated by only 23 human PATs, it is likely that *DHHC3* will target at least dozens of substrates. However, except integrin $\alpha 6$ and $\beta 4$ subunits, we know little about what else proteins are *DHHC3* substrates in breast cancer.

Previously, we developed a novel palmitoyl-proteomics method termed Palmitoyl Protein Identification and Site Characterization (PalmPISC) and applied it to characterize palmitoyl proteins enriched from nuclei-free membrane fractions of prostate cancer DU145 cells [7]. Interestingly, we found that the core protein network within TEMs is regulated by palmitoylation [7]. In this project, we proposed to comprehensively analyze *DHHC3* substrates in breast cancer (Aim 3). We planned to integrate our PalmPISC method with stable isotope labeling by amino acids in cell culture (SILAC) [8], a very accurate and robust quantitative proteomics method, to identify known and novel *DHHC3* substrates.

Body

In the first year, we tested and optimized the integration of PalmPISC with triplex SILAC labeling [9], by analyzing cells stimulated by epidermal growth factor (EGF). The workflow was shown in Figure 1. Three populations of DU145 cells were metabolically labeled with isotopically different SILAC amino acids in parallel. One group of cells were cultured in “light” SILAC medium containing natural lysine (Lys0) and Arginine (Arg0), another in “medium” SILAC medium containing $^2\text{H}_4$ -lysine (Lys4) and $^{13}\text{C}_6$ -arginine (Arg6), and the other in “heavy” SILAC medium containing $^{13}\text{C}_6$ - $^{15}\text{N}_2$ -lysine (Lys8) and $^{13}\text{C}_6$, $^{15}\text{N}_4$ -arginine (Arg10). After six doublings, when cellular proteins are at least 98% labeled with SILAC amino acids, the “light”, “medium”, and “heavy” DU145 cells were treated with 10 nM EGF for 0, 5, and 40 min, respectively. Cells were mixed at 1:1:1 ratio, membrane microdomains were separated, and palmitoyl proteins were isolated using our PalmPISC method. Proteins were separated by SDS-PAGE, digested in gel, and analyzed by liquid chromatography tandem mass spectrometry. MaxQuant was used for protein identification and quantitation [10]. Representative mass spectra were shown in Figure 2.

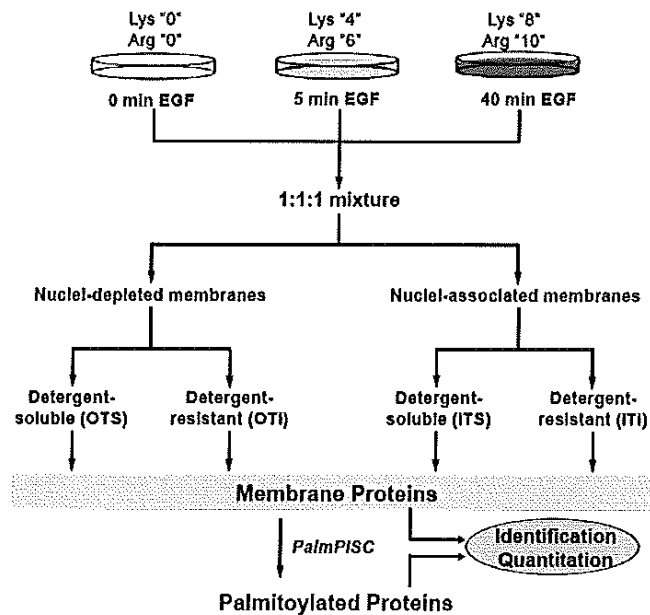


Figure 1. Workflow for identification of membrane and palmitoylated proteins that transmit EGFR signaling. Prostate cancer DU145 cells were metabolically labeled through growth in SILAC medium containing stable isotope-labeled lysine and arginine. Following stimulation with epidermal growth factor (EGF) for 0, 5, and 40 min, cells were harvested, mixed at 1:1:1 ratio, and disrupted by homogenization. Nuclei and associated organelles were pelleted by 500×g centrifugation and disrupted by sonication. Cellular membranes were precipitated by high-speed centrifugation, prior to the separation of detergent-resistant membranes from detergent-soluble membranes with Triton X-100. Membrane proteins were extracted from the four membrane domains (*i.e.*, OTS, OTI, ITS, and ITI) and subjected to PalmPISC to enrich palmitoylated proteins. Proteins were identified and quantitated by mass spectrometric analysis.

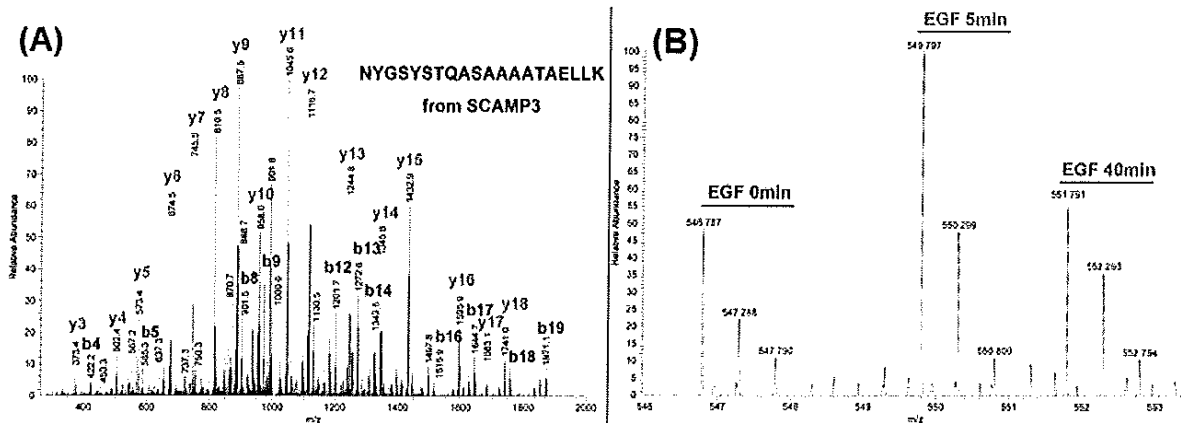


Figure 2. Representative mass spectra for protein identification and quantitation. (A) MS/MS sequencing of a doubly charged peptide with m/z 1009.50 led to the identification of a peptide NYGSYSTQASAAAATAELLK, which is derived from palmitoylated secretory carrier-associated membrane protein 3 (SCAMP3). (B) Quantitative analysis of a SILAC triplex derived from EGFR protein showed that the EGFR was about 2-fold upregulated after 5 min EGF stimulation and that the protein level dropped to about 1.2-fold after 40 min in the OTI membrane domain, which is enriched with lipid rafts. About 3000 membrane proteins were identified and quantified.

Intriguingly, the palmitoylation level of a variety of translation factors, including many cytosolic ribosomal proteins, was found to be about 30-50% increased after 5 or 40 min EGF stimulation (Fig. 3). In contrast, the palmitoylation level of mitochondrial ribosomal proteins was largely unchanged. The rapid increase of the palmitoylation level of those translation factors may significantly increase the level of membrane-associated translation machinery. Given that membrane-bound ribosomes usually produce plasma membrane-associated or secreted proteins, the upregulation of membrane-associated translation machinery may stimulate the synthesis of certain plasma membrane proteins or secreted proteins, some of which may be involved in cancer progression. In addition, it has been reported that mammalian target of rapamycin complex 2 (mTORC2), the S473-Akt kinase, is activated by association with the ribosome [11]. It is likely that the enzymes regulating the palmitoylation of ribosomal proteins are the missing link between EGF and ribosome-mTORC2.

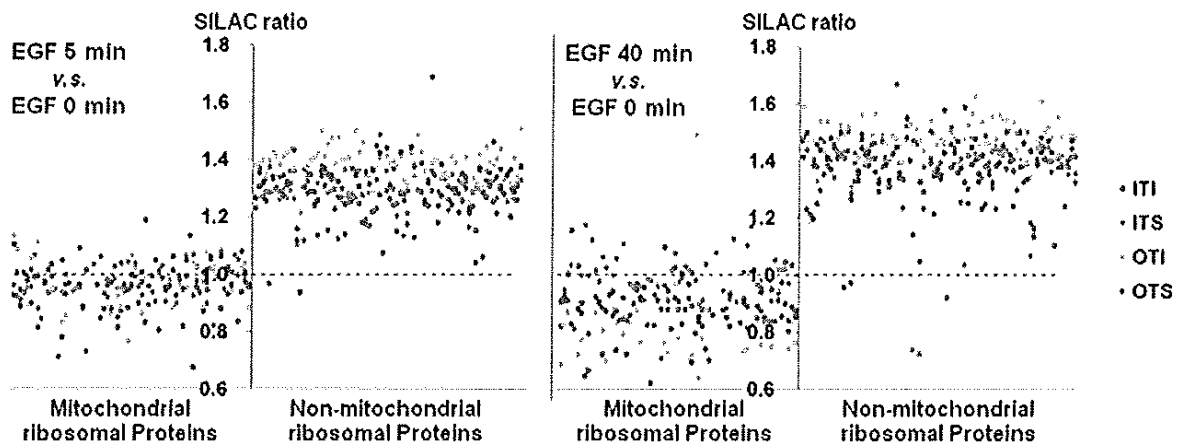


Figure 3. SILAC analysis shows that EGF stimulates the association of non-mitochondrial ribosomal proteins but not mitochondrial ribosomal proteins with membranes. 5 min EGF stimulation resulted in increased association of non-mitochondrial ribosomal proteins with different membrane domains, especially the lipid raft-enriched OTI domain (green dots). 40 min EGF stimulation further enhanced the association of non-mitochondrial ribosomal proteins with different membrane domains, but in contrast led to the dissociation of mitochondrial ribosomal proteins from membranes, especially the OTI fraction. Note that the vast majority of membrane proteins were not significantly changed after 5 or 40 min EGF stimulation (data not shown).

In addition to the translation factors, we found that many signaling molecules including Ras family proteins were palmitoylated and targeted to specific membrane domains after 5 min EGF stimulation, but their palmitoylation levels were restored after 40 min EGF stimulation. In contrast, a number of cell adhesion molecules including cadherins, catenins, and tight junction proteins were depalmitoylated, resulting in dissociation from specific membrane domains.

Key Research Accomplishments

1. Successful integration of our PalmPISC method with triplex SILAC.
2. Identification of about 3000 membrane proteins, including hundreds of palmitoyl proteins from different membrane domains.
3. Time- and membrane domain-resolved proteomic analysis showed that many translation factors are palmitoylated and targeted to membranes, while some cell adhesion molecules are depalmitoylated and dissociated from certain membrane microdomains, in response to EGF stimulation.

Reportable Outcome

Yang, W., Steen, H., Freeman, M. R., "Quantitative palmitoyl-proteomics analysis of epidermal growth factor receptor signaling in membrane microdomains" (The 2nd Innovative Minds in Prostate Cancer Today conference, Orlando, FL, USA, 2011)

Conclusion

In summary, our data suggest that protein palmitoylation plays a critical role in transmitting EGF signaling by regulating the association of a wide range of proteins with specific membrane domains. Targeting protein palmitoylation machinery may inhibit oncogenic signaling and thus providing a novel approach to treat cancer.

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