Analysis of Receptor Tyrosine Signaling in Stimulated Human Mammary Epithelial Cells

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Stimulation under multiple conditions with replicates increases both the confidence for relative quantitation and proteome coverage

Overview

- Our intent was to semi-quantitatively examine tyrosine signaling pathways in human mammary epithelial cells (HMECs) based on spectral counting.
- HMECs were treated with epidermal growth factor (EGF), 225 (a monoclonal antibody inhibitor for the EGF receptor), TCT (a constitutively expressed EGF ligand), or insulin-like growth factor-1 (IGF).
- Cell lysates were digested, enriched for phosphotyrosine (pTyr) with a peptide immunosupercipitation (IP) step, and analyzed by LC-MS on a metal-free, nano-flow 30 μm HPLC system.

531 unique tyrosine phosphorylation sites were identified with their phosphorylation levels characterized across all conditions (consisting of 2-4 biological replicates for each) with an FDR of < 1%. Of these, 48 sites have not been previously reported.

Introduction

- Phosphorylation is a well characterized cell signaling mechanism that is critical to normal and disease state functions.
- Phosphotyrosine (pTyr) is present at extremely low abundances compared to pSer and pThr and phosphotyrosine (pTyr) enrichment methods must be employed.
- Phosphopeptides are known to bind to metal surfaces. We use a metal-free, nano-flow HPLC system to decrease loss of peptide due to retention.
- Semi-quantitative measurements for phosphorylation levels can be based on spectral counting for the identified sites and by performing multiple biological replicates to increase the confidence of site quantitation.

Results

Replicate reproducibility

Protein abundance and phosphorylation level

Interaction network of detected and quantified phospotyrosine peptides

Methods

HMECs were grown in cell culture to ~60% confluence and treated with various growth factors and in the EGF antibody. Each condition replicates used about 2 x 10^8 cells (10–20 mg total protein).

Replicate reproducibility

Complete pTyr dataset

Selected spectral count ratios

Data Analysis

Complete dataset for five different conditions. All of these have replicate phosphotyrosine sites from a specific control (EGF 50 ng/ml) to the “Phosphopeptide” database. Each column represents the spectral counting for the phosphopeptide. Each row is a replicate sample for a specific condition. The ratios are used to compare the signal intensity. The peptides chosen are not only those with the highest expression but also those with the best mass overlap between technical replicates and the sum was adjusted for the number of technical replicates among conditions.

Conclusions

- Metal-free, nano-flow HPLC gives consistent, reproducible separations. Our LC platform can be modified to incorporate a TIO pre-column, which could further enrich pTyr peptides.
- A consistent and extensive set of pTyr sites were identified and semi-quantitatively measured. The dataset not only reflects the known biology, but also reveals 48 novel pTyr sites.
- Spectral counting yielded reliable reproducible comparisons for a large number of modification sites over several different stimulation conditions based upon use of biological replicates.
- The vast majority of observed pTyr sites were revealed to originate from lower-abundant proteins as compared to a global HMEC proteomic dataset.

Current work is focusing on increasing immunoprecipitation efficiency and incorporating isotopic labeling for improved quantitation.

References


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