PROTEIN EXPRESSION AND CELL SIGNALING QUANTIFIED IN RARE CELLS

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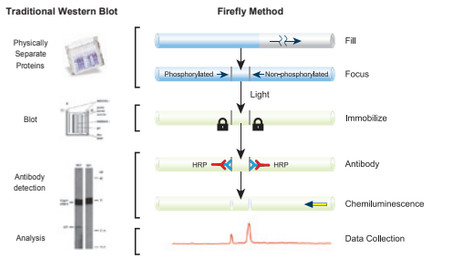
Abstract

The discovery of tumor stem cells in acute myeloid leukemia a decade ago initiated a field of research has accelerated growth in the past few years. Researchers are now describing tumor stem cells in a variety of hematopoietic and solid tumors. The impetus for much of this research is the desire to identify targets for drug intervention in these critical tumor populations. The molecular pathways that functionally define these cells are important therapeutic targets. Tumor stem cells are rare and with insufficient material to use standard assay methods. Although DNA microarray and/or qPCR are used to study tumor stem cells, their rare nature limits quantitative protein analysis. This creates a gap in our knowledge since many proteins, such as β-catenin or MAPK signaling proteins, are not regulated at the transcriptional level, but through post-translational modifications (phosphorylation, ubiquitination, etc..). Here we describe a technique utilizing a nanoimmunoassay platform (Firefly™) to measure tumor stem cell proteins. Transitional tumor stem cells (TCC+) were sorted from a patient tumor and lysed for analysis. A lysate of 400 cells was subjected to isoelectric focusing and immobilization. Immunodetection was performed and quantitation of signal was measured using HRP-labeled secondary chemiluminescence reagents. Here we report β-catenin protein concentrations of 192 ng/mg of total protein in the tumor stem cells, which was undetectable in 'non-stem' tumor cells. Comparisons of protein levels and the degree of phosphorylation are made between these samples, other tumor cell lines and hematopoetic stem cells.

The Firefly capillary nano-immunoassay



The Firefly™ Assay

Figure 1. The Firefly nano-immunoassay:

- **Fill:** A 5 cm capillary is filled with a separation buffer containing sample proteins and fluorescent peptide standards.
- **Focus:** Isoelectric Focusing (IEF) is used to focus the proteins.
- **Immobilize:** The focused proteins are immobilized by exposing the capillary to the UV light source.
- **Antibody:** Labeled antibodies are flowed through the capillary to bind to the immobilized proteins.
- **Detection:** Whole capillary imaging is performed using chemiluminescence.
- **Data Collection:** Capillary images are converted to signal vs. pI graphs for analysis.

Detection of phosphorylated and non-phosphorylated proteins

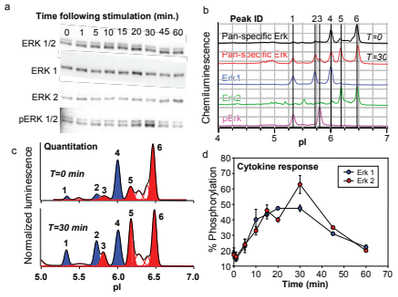
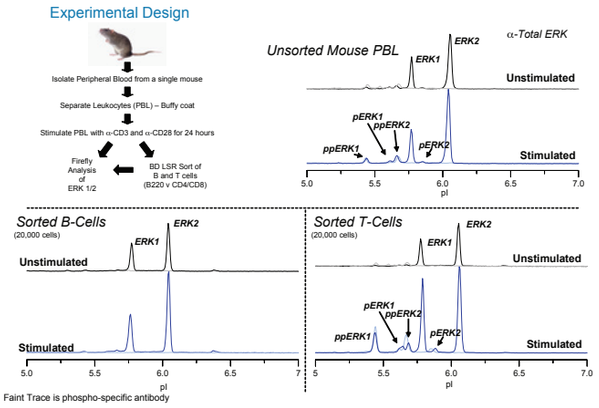
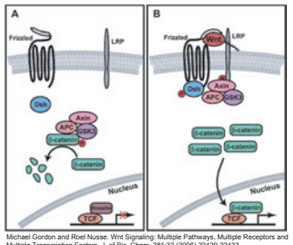


Figure 1. Analysis of phosphorylated forms and isoforms of ERK protein. Cultured HT-29 human colorectal adenocarcinoma cells were treated with the cytokines insulin (500 ng/ml) and TNF-alpha (100 ng/ml) for times from 0 to 60 min prior to preparation of cell lysates. (A) Conventional SDS-PAGE western blot of 0 to 60 min samples, probed with antibodies specific for ERK1, ERK2, ERK1&2 (pan-specific ERK antibody), and phosphorylated ERK1&2 (pERK1&2). (B) Firefly analysis of these same samples, probed with the same antibodies allows peak identification as follows: 1 ppERK1, 2 pERK1, 3 ppERK2, 4 ERK1, 5 pERK2, and 6 ERK2. (C) Differences in phosphorylation upon stimulation are evident in the pan-specific Ab profiles obtained for 0 and 30 min lysates. Integration of the peaks corresponding to each identified species allows percent phosphorylation of ERK1 and ERK2 to be calculated. (D) Using percent phosphorylation data generated as in panel C allowed phosphorylation vs. time of cytokine stimulation to be plotted for both ERK1 and ERK2. Percent phosphorylation shown is the sum of peak integrations for mono- and di-phosphorylated species.

Sample heterogenity masks specific response



Canonical β-catenin signaling pathway and model cell systems

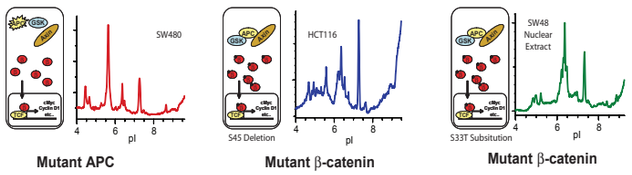


Michael Gordon and Roel Nusse. Wnt Signaling: Multiple Pathways, Multiple Receptors and Multiple Transcription Factors. J. of Bio. Chem. 281:32 (2006) 22429-22433.

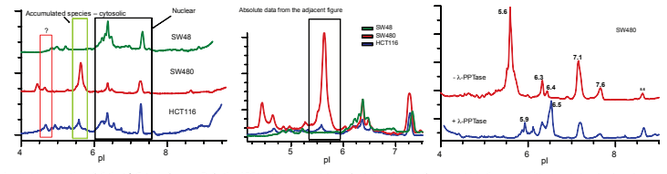
Table 1. List and *APC* gene status of cell lines examined

Cell line	APC status
Colo 201*	Mutant
Colo 205*	Mutant
Colo 320	Mutant
DLD 1	Mutant
SW 1222	Mutant
SW 832	Mutant
HT 29	Mutant
LS 174T	Wild type
SW 403	Mutant
CACO 2	Mutant
SW 480*	Mutant
SW 620*	Mutant
SW 948	Mutant
HCA 7	Wild type
HRA 19	Mutant
HCT 116	Wild type
LOVO	Mutant
JW	Mutant
HCA 46	Uncertain
SW 48	Wild type
CCO?	Mutant
SW 1417	Mutant
SKCO 1	Mutant

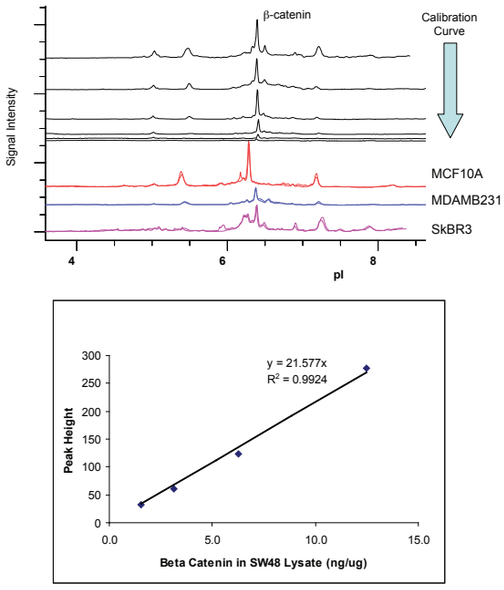
Defects in β-Catenin signaling pathway generate distinct IEF profiles



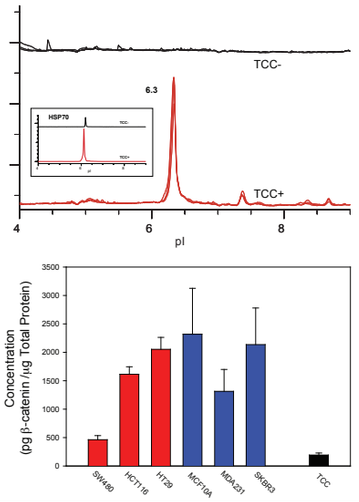
Phosphorylated β-Catenin is labile to phosphatase treatment



Cell lysate standard curve – β-Catenin



β-Catenin concentrations in various cell lines and 400 tumor stem cells



Advantages of Firefly nano-immunoassay platform

- Single antibody detection of multiple protein variants.
- Small sample size: One drop can potentially fill the entire 96 capillary array.
- Quantification of cell signaling pathways.
- Completely automated