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14. ABSTRACT
In this proposal, we explored the potential use of ubiquitin-dependent proteolysis as a "reverse genetics" tool in functional genomics studies. We developed a retroviral-based system that artificially targets random cellular proteins to the proteolytic machinery for degradation. To achieve this, a randomized peptide library was linked to a segment of the F-box motif of beta-TrCP, the F-box protein that mediates the ubiquitination of I(kappa)B(alpha) and (beta)-catenin via the multimeric SCF ubiquitin ligase. The resultant chimeric proteins were expected to direct any interacting proteins that are otherwise stable, to the SCF ligase for ubiquitination. As proof of principle, we used this system in a loss-of-function in vitro assay to identify putative genes involved in breast tumor invasion. MDA-MB-231 breast tumor-derived cells were tranduced with the retroviral chimera library and peptides that confer the ability to invade through an artificial extracellular matrix will be isolated using a modified Boyden chamber assay. Following multiple rounds of selection, the fusion proteins that provide invasion properties will be confirmed using tumorgenicity assays in nude mice. If successful, ubiquitination-based functional assays will undoubtedly contribute to the identification of potential protein targets for therapeutic intervention in breast cancer.

15. SUBJECT TERMS
Invasion, Ubiquitin, Proteolysis, SCF, F-box, Functional Genomics

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A. Introduction

The goal of this research project was to establish a functional genomics screen that utilizes a cell's own ubiquitin-dependent proteolysis system to screen for proteins involved in breast tumor invasion. The experimental design of the project was to express a randomized peptide library attached to a segment of the F-box domain of the protein β-TrCP in MDA-MB-231 breast tumor cells. The chimeric protein is expected to bind to and recruit otherwise stable cellular proteins to the SCF ubiquitin ligase for ubiquitin-dependent degradation. Cells that express this construct would then be selected for a gain of invasion using a Boyden chamber assay.

B. Body

We have asked for and were granted a one year extension for the completion of this research project from the DOD Breast Cancer Research Program. The extension was requested based on unexpected complications in constructing and amplifying the proteolysis targeting vector library of sufficient molecular complexity for functional genomics experiments. This problem was due to an inefficient transformation efficiency of *E. coli* cells which has since been overcome (described below).

We amplified a segment of the protein β-TrCP pertaining to the F-box domain from expression plasmid pFlag-CMV2-β-TrCP using the Advantage HF2 high fidelity polymerase (Clontech). We designed the oligonucleotides to include a 5' Flag epitope tag and 5' and 3' cloning sites for directional cloning. We sequenced the amplified DNA segment and found it to be free of mutations (completion of point #1 of SOW).

We then devised an experimental strategy to generate the DNA segment that encodes the random peptide library. This strategy was based on annealing a degenerate oligonucleotide with a complementary anchor oligonucleotide (both synthesized commercially). The oligonucleotides were designed to contain 5' and 3' restriction endonuclease sites for directional cloning, sequence which encodes a 5' poly-glycine linker sequence, and a degenerate $(N)^{21}$ DNA sequence that encodes peptides of random 7 amino acids. The sequence content of the degenerate oligonucleotide was designed to minimize the possibility of the introduction of a stop codon in the peptide library. We annealed the degenerate and anchor oligonucleotides and constructed the double-stranded DNA peptide library cassette by incubation with Klenow polymerase (Clontech). The efficiency of the annealing and synthesis reactions was evaluated by agarose gel electrophoresis (completion of SOW point #2).

We constructed the retroviral-based proteolysis targeting vector by first cloning the β-TrCP F-box segment synthesized above into the retroviral vector pLPC (a previous gift from S. Lowe, Cold Spring Harbor Laboratory). This vector was chosen due to its high level of expression from a CMV minimal promoter. We then directionally cloned the random peptide library cassette synthesized above in the cloning site 3' of the β-TrCP cassette. The efficiency of the ligation reaction was evaluated by agarose gel electrophoresis (completion of point #3 of SOW)

During the course of this study we encountered two serious problems. The first was a "bottle neck" effect at the step of the proteolysis library amplification in bacteria that severely limited its genetic complexity of our screen. In order to perform a comprehensive functional genetic screen for genes involved in breast tumor invasion, we estimated that a genetic complexity of $>10^{10}$ different proteolysis targeting plasmids would be required. However, using various commercially available strains of chemically competent *E. coli*, we could not achieve a transformation frequency $>10^{7}/\mu g$ DNA for the proteolysis targeting construct. We compared a wide variety of commercially available chemically and electrocompetent strains of *E. coli* to attempt to achieve the transformation efficiency to achieve the required complexity. We have now solved this problem by optimizing our

transformation procedure using a commercially available electrocompetent strain of *E. coli* that provides a transformation efficiency $>10^{10}/\mu g$ for our proteolysis targeting vector. The second problem we encountered was that the pLPC vector we obtained from Scott Lowe did not express the β-TrCP fusion protein when expressed in mammalian cells. We spent considerable time trying to decipher why the vector would not express, but ultimately resorted to re-cloning the entire proteolysis targeting library into the commercially available vector pLPX (Invitrogen). We have now achieved this goal and have amplified the library in bacteria and created a retrovirus pool that expresses our proteolysis targeting library.

Additional accomplishments pertaining to the research project include: obtaining and culturing of the MDA-MB-231 breast tumor-derived cell line, establishing puromycin killing curves for cell selections, production of high-titer retrovirus using Phoenix cells (provided by the Nolan lab)(completion of SOW#4), infection of cells with retroviruses and selection of infected cells using puromycin (SOW #6), and validation of a PCR based screen for library cassette amplification (SOW #8). Although our accomplishments have been many, the delays we encountered (outlined above) precluded us from achieving the selection of infected cells for the acquisition of invasion using the Boyden chamber assay (Table 1). We plan to complete these studies in the near future using funding obtained through the P.I.

SOW #	Status	Comments
1	Completed	
2	Completed	
3	Completed	Library made in retroviral vector pLPX
4	Completed	
5	Completed	
6	Completed	
7	Partially completed[1]	
8	Completed	
9	To be completed[2]	
10	To be completed[2]	
11	To be completed[2]	

[1]Boyden chamber assay has been established but cells not taken through multiple rounds of infection and selection.
[2]Delays in proteolysis library amplification and library construction (described above) have prevented the conclusion of these studies. They will be completed in their entirety at the expense of the P.I.

C. Key research Accomplishments

Construction of a retroviral-based proteolysis targeting vector containing a library of 7mer random peptides linked to a β-TrCP F-box domain. Overcoming the "bottle-neck" effect we encountered during library amplification which would have severely limited the complexity of the targeting library.

D. Reportable Outcomes

Abstract: Tan, YM, Spruck, C. Identification of genes involved in breast tumor invasion utilizing an ubiquitin-mediated proteolysis in vitro screen. Presented at the Era of Hope meeting 2005, Philadelphia, sponsored by the DOD Breast Cancer Research Program.

E. Conclusions

We have shown that a retroviral-based vector library containing a fusion protein containing an F-box domain linked to a random peptide sequence can be constructed and expressed in human cells with a genetic complexity sufficient for performing functional genomics-based screens. Unfortunately, due to several problems encountered during the course of this work, which we have since rectified, we were not able to test the library in an in vitro screen for invasion as initially proposed. We are confident that using this valuable research tool that we will be able to complete this analysis in the coming months.

G. References

None

H. Appendices

None

I. Supporting Data

1. **Construction of a chimeric ubiquitin-proteolysis targeting library.** We isolated the β-TrCP F-box motif by PCR. PCR primers were designed for amplification of a 410 bp segment corresponding to the F-box domain and using β-TrCP cDNA as a template. The β-TrCP F-box segment was then TA-cloned into pCR2.1 and positive clones verified by DNA sequencing (Fig. 1).

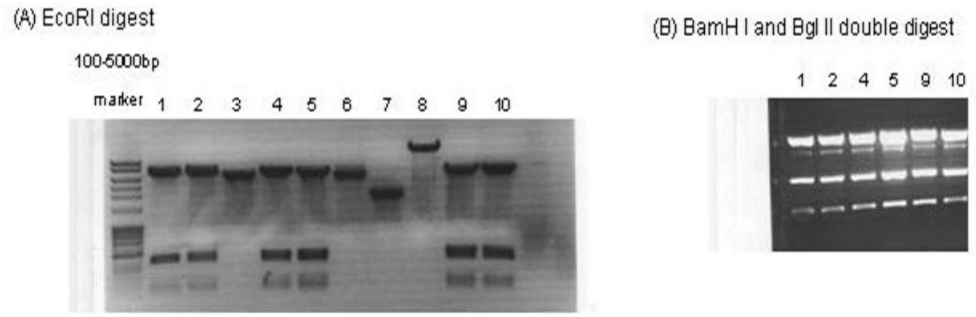

Figure 1. Cloning of the β-TrCP F-box domain. A, EcoRI digest, shows clones 1, 2, 4, 5, 9, and 10 contain the expected DNA band (350 bp). B, BamHI and BglII double digest confirms each positive clone. Clones were verified by DNA sequencing.

After confirmation that the β-TrCP F-box motif was correct, we next ligated the β-TrCP F-box motif into the retroviral vector pLPC. pLPC expresses inserts from a CMV-minimal promoter ensuring high level expression of our ubiquitin-proteolysis targeting construct. Several clones were checked using EcoRI and HindIII restriction enzymes, to ensure successful ligation and correct orientation of the β-TrCP F-box motif insert to the pLPC vector (Fig. 2).

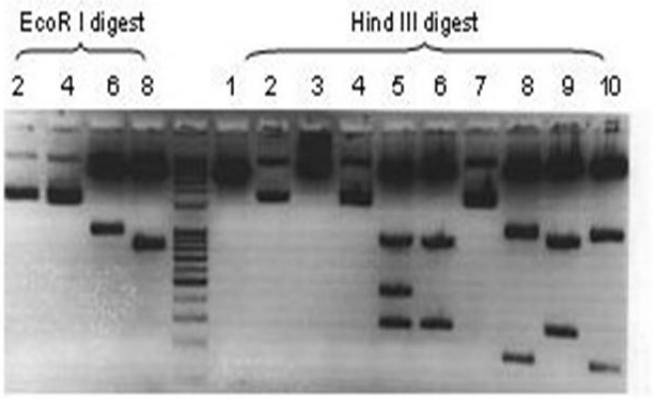

Figure 2. Preparation of pLPC-β-TrCP backbone. pLPC-β-TrCP clones (1-10) selected for Hind III and/or EcoR I restriction digest. Clones 8 and 10 match the expected band sizes (Hind III: 174, 1076, 5517 bp; EcoR I: 847 and 5920 bp) of β-TrCP being inserted in the correct orientation into pLPC vector.

A random 7-mer peptide library was created from degenerate oligonucleotides. Oligonucleotides were synthesized for a 5′ library primer (CGACCTCCTATTCTCACGGATCCGGTTC) and also for a 3′ library primer containing a XhoI restriction site to anneal to pLPC upstream of the stop codon (CCGCTCGAGCATGTT**CTA(**MNN)$_7$CGAACCGGATCCGTGAGAATAGA). These primers were then annealed and a library created by extension reaction with Klenow polymerase. The library "cassettes" were size fractionated on polyacrylamide gels, cut with BamHI and XhoI, and ligated into the pLPC-β-TrCP backbone**.**

The ligation product of the pLPC-β-TrCP backbone with the library cassette was transformed into electrocompetent *E coli* cells (Lucigen). This transformation step yielded few colonies so we re-attempted the procedure using ElectroMAX DH10B cells (Invitrogen). Unfortunately, we faced difficulties in obtaining the pLPC retroviral proteolysis targeting vector with both types of cells since low number of colonies was obtained.

2. Re-construction of backbone using a different retroviral vector, pLPCX.

In light of the failure to obtain colonies after the ligation step involving the pLPC-β-TrCP backbone and the library cassette, we decided to repeat the entire cloning procedure described above with a different retroviral vector, pLPCX. Employing the pLPCX vector required slight modifications in the cloning procedure with new primers for creation of different restriction sites.

APPENDICES

1. Meeting Abstract (attached)

2. Personnel receiving salaries from this grant:
   Charles H. Spruck – 5% effort
   YingMeei Tan – 95% effort

MEETING ABSTRACT

Identification of genes involved in breast tumor invasion utilizing an ubiquitin-mediated proteolysis in vitro screen

Authors Yingmeei Tan And Charles Spruck

Functional genomics approaches designed to identify and functionally characterize genes involved in breast tumorigenesis have been limited by the lack of sufficient molecular tools required for in vitro-based assays. In this proposal, we explore the potential use of ubiquitin-dependent proteolysis as a "reverse genetics" tool in functional genomics studies.

We will develop a retroviral-based system that artificially targets random cellular proteins to the proteolytic machinery for degradation. To achieve this, a randomized peptide library will be linked to a segment of the F-box motif of beta-TrCP, the F-box protein that mediates the ubiquitination of I(kappa)B(alpha) and (beta)-catenin via the multimeric SCF ubiquitin ligase. The resultant chimeric proteins are expected to direct any interacting proteins that are otherwise stable, to the SCF ligase for ubiquitination. As proof of principle, we will use this system in a loss-of-function in vitro assay to identify putative genes involved in breast tumor invasion. MDA-MB-231 breast tumor-derived cells will be tranduced with the retroviral chimera library and peptides that confer the ability to invade through an artificial extracellular matrix will be isolated using a modified Boyden chamber assay. Following multiple rounds of selection, the chimeras that provide invasion properties will be confirmed using tumorgenicity assays in nude mice.

Ubiquitin-dependent proteolysis could provide a powerful functional genomics tool to breast cancer researchers. If successful, ubiquitination-based functional assays will undoubtedly contribute to the identification of potential protein targets for therapeutic intervention in breast cancer.