

Breast Cancer Pathway Investigation Facilitated Using ZFN Technology

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Introduction

Immortalized and modified cell lines, as well as knockout animals are essential tools supporting the investigation of cancer ontogeny and the development of diagnostic and therapeutic approaches. While immortalized lines have yielded substantial information, they often differ significantly from wild type and do not provide a normal background against which to weigh results. Gene silencing using RNAi technology is becoming routine, but cannot be relied upon to yield 100% silencing of the gene of interest. Knockout animals provide a model system where lack of gene expression can be achieved in a near normal environment, but are labor intensive, expensive, and limited by having to interpret results in a nonhuman background.

We report here the generation of knockout and tagged human cell lines. Using zinc finger nuclease (ZFN) technology, precisely targeted gene disruptions and tagging were achieved in the near normal breast epithelial cell line MCF10A. As one example, we present data describing the knockout of the protein product of the phosphatase and tensin homolog (PTEN) gene. PTEN has been described as a tumor suppressor gene and has been associated with the development of breast cancer, as well as numerous other malignant diseases. Using ZFN technology we targeted the disruption of this gene and, as the result of nonhomologous recombination, identified and isolated stable clones carrying deletions of the PTEN gene which resulted in loss of expression of the protein. Additional knockout lines have been generated in the MCF10A background, including HER2, SYK, TP53, CDH1, and others.

In addition to generating knockout cell lines, we successfully labeled the MCF10A cell line by integrating a red fluorescent protein (RFP) in front of the first exon of the TUBA1B (NM-006082, alpha-tubulin isoform 1b) locus. The integration resulted in endogenous expression of the corresponding RFP-TUBA1B fusion that shows characteristic patterns of microtubules. The expression of RFP in this cell line can allow for easy tracking in even complex mixtures, potential monitoring of the effects of taxanes, and can serve as a background line upon which additional gene modifications can take place. Cell line growth characteristics of the RFP-tagged line, such as doubling time and phenotype, were indistinguishable from the unmodified wild type line.

Materials and Methods

Cell Culture

MCF10A Cells were initially acquired from ATCC (cat. CRL-10317) and maintained in DMEM/F12HAM media (Sigma 51448C) containing 5% v/v horse serum (Sigma H1270), hydrocortisone (Sigma H6909) at 10ng/ml, human insulin (Sigma I9278) at 0.5ug/ml, epidermal growth factor (Sigma E9644) at 10ng/ml, and cholera toxin (Sigma C8052) at 100 ng/ml. TALL104 cells were acquired from ATCC (cat. CRL-11386) and maintained in Iscoves (Sigma I3390) containing 20% fetal serum (Sigma F4135), 2.5ug/ml human albumin (Sigma A9731), 60units/ml IL2 (Sigma I2644), 0.5ug/ml D-mannitol (Sigma M1902), and 4mM L-glutamine (Sigma G7513).

Nucleofections

Nucleofections were performed with the Amaxa® Nucleofector® device (Cat. No. AAD-1001) and Nucleofector® Kit V (Cat. No. VCA-1003) from Lonza AG according to the product manual. Donor plasmids were designed and constructed in house. The fluorescent reporter gene was obtained from Evrogen (<http://evrogen.com/products/TagFPs.shtml>). CompoZr® ZFNs were designed and manufactured by Sigma-Aldrich. Fluorescent microscopy was performed with a Nikon Eclipse TE2000-E inverted research microscope and MetaMorph® software.

ELISA Assays

Cell lysates were prepared by bringing cell pellets to a concentration of 9x10⁷/ml using RIPA buffer (Sigma R0278), allowing 10 minutes at room temperature, then spinning to pellet debris. The supernatant was removed a protein concentration determined using the QuantiPro BCA assay kit (Sigma QBCA). Concentrations of PTEN were determined by ELISA (modified kit from R&D Systems – DYC847). Chemiluminescent signals were generated using peroxidase substrate (Sigma CPS2120) per instructions and read on a GloRunner Luminometer.

Cytolytic Assays

Cell viability in the cytolytic assay was determined using a standard chemiluminescent viability assay (Promega CellTiter-Glo, G7570).

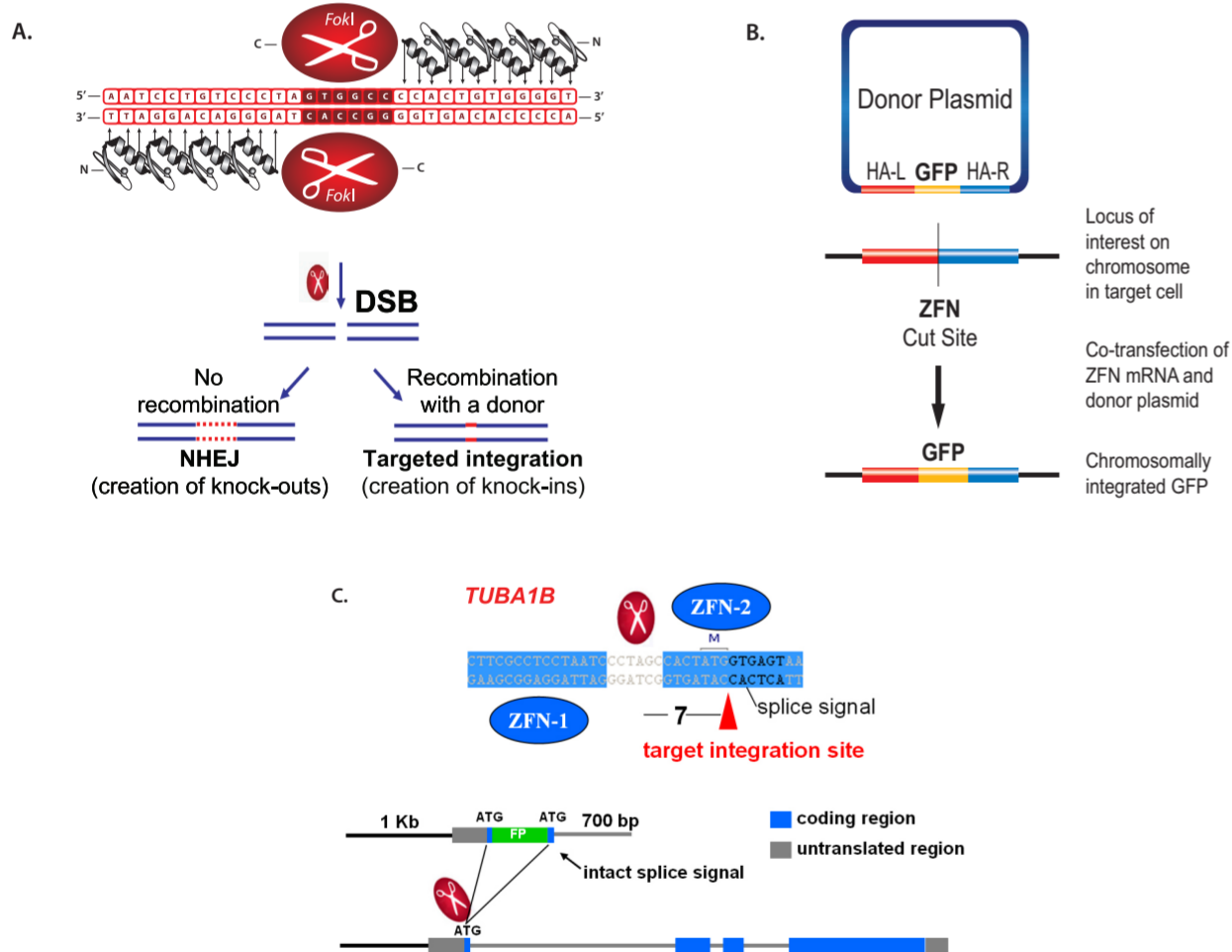


Figure 1: ZFN targeting mechanism and donor design.

- ZFNs bind to the target site. The FokI endonuclease domain dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either an error-prone NHEJ pathway or high-fidelity homologous recombination. NHEJ introduces deletions or insertions, which change the spacing between the binding sites so that ZFNs might still bind but dimerization or cleavage cannot occur. In the presence of a donor DNA carrying homology flanking the target site, homologous recombination can use the donor as template to repair a DSB, achieving targeted integration. Knockout lines were obtained by screening for NHEJ /indel events in the absence of a donor template. Tagged lines were generated by introducing a specific donor DNA template.
- Tagged Line Generic workflow: The donor plasmid consists of homologous arms (HA-L and HA-R) of the ZFN cut site flanking a fluorescent reporter molecule (RFP).
- Example demonstrating schematic of CompoZr™ ZFN binding sites/ZFN cut site with respect to the targeted integration site for TUBA1B loci.

MCF10A - PTEN (-/-), clone A2A9:

Primers Bolded and Underlined
ZFN Binding site in upper case RED
ZFN cut site in lowercase red

CCTGTTAAAGTTTGTATGCAACATTCTAAAGTTACCTACTTGTAAATAAAAATCAAGAGTTTTTTTTTCTTATTCTGAGGTT
ATCTTTTACCACAGTTGCACAATATCTTTTGAAGACCAATACCACCCACAGCTAGAACTTATCAAAACCCCTttgtGAAGATC
TTGACCAATGGCTTAAGTGAAGATGACAATCATTTTGACCAATTCACCTGTAAGCTGAAAGGGACGAACTGGTGAATGATAT
GTGCATATTTTATACATCGGGCAAATTTTT**TAAAGGCACAAGAGGCCCTA**

Genotype: del 19 / del 2

CCATAACCCACCCAGCTAGAA**CTTATCAAAACCCCTttgtGAAGATC**TTGACCAATGGCTAAGTGAAGATGACAATCATG **wt**
CCATAACCCACCCAGCTAGAA**CTTATCAAAACCCCTttgtGAAGATC**TTGACCAATGGCTAAGTGAAGATGACAATCATG **-19**
CCATAACCCACCCAGCTAGAA**CTTATCAAAACCCCTttgtGAAGATC**TTGACCAATGGCTAAGTGAAGATGACAATCATG **-2**

Figure 2: Gene sequence of selected PTEN knockout clone. Following nucleofection, cells were allowed three days to recover before single-cell sorting using a flow cytometer. After sorting, clones were allowed to expand for 4-6 weeks prior to extracting DNA for PCR amplification, topo cloning and sequencing. Clones yielding out of frame insertions/deletions and no wild type sequence were selected for further expansion and confirmation of sequence.

Figure 3: ELISA Assay of PTEN Knockout Cells.

Cell lysates were prepared from MCF10A near normal cells and from MCF10A treated to delete the expression of the PTEN gene, clone A2A9. The lysates were examined using a standard sandwich ELISA assay specific for PTEN. Signals were developed using a chemiluminescent peroxidase substrate. The PTEN lysates did not generate a signal above background, where background was determined by titrating a PTEN standard and applying a 95% confidence interval to the curve.

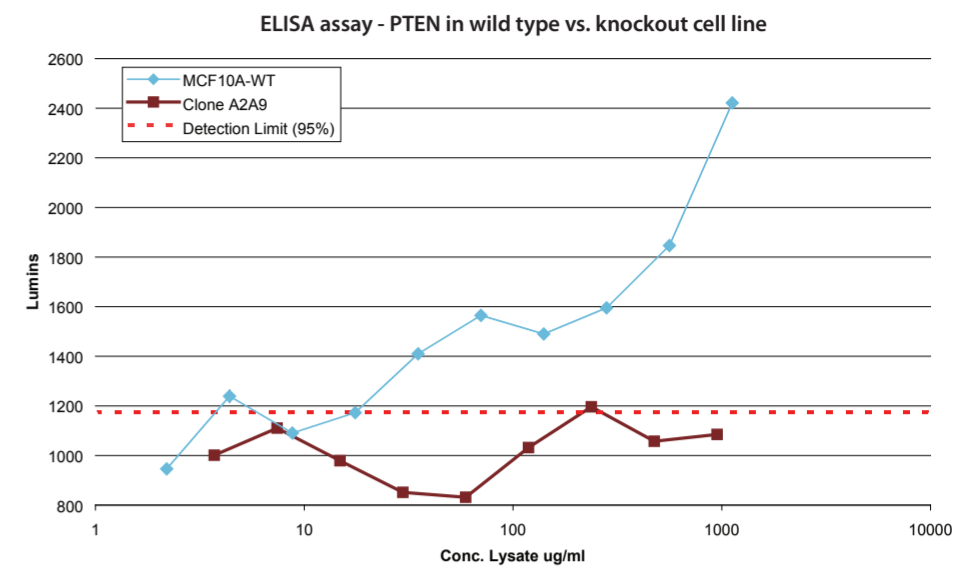
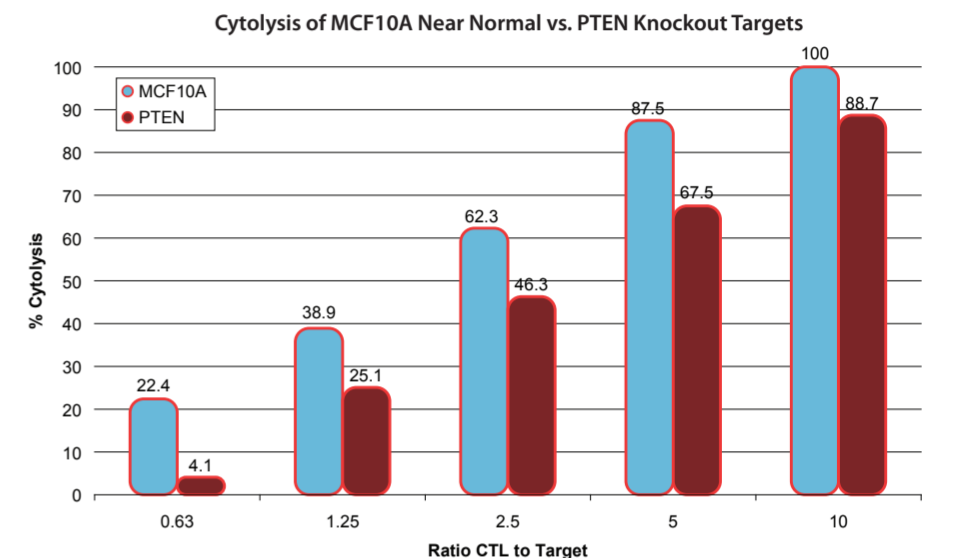


Figure 4: Cytolytic Assay of PTEN Knockout Cells vs Near Normal Cells.

Near normal MCF10A breast epithelial cells and PTEN-knockout cells were harvested at approximately 70% confluency by exposing to trypsin/EDTA for 15 minutes at 37C. Cells were washed twice then aliquoted, 20,000 cells were deposited per well, in a 96-well round-bottom plate. Cytolytic T cells (TALL1041) were added at various ratios. The plate was spun gently and incubated for four hours at 37C. Following incubation, cells were pelleted and washed twice. Remaining cells were examined for viability using a chemiluminescent assay. Percent cytotoxicity was determined by comparison to target cells receiving no cytolytic cells. Reflective of reported results, PTEN knockout cells demonstrated increase resistance to cytotoxicity.



Treatment of RFP-tagged TUBA1B MCF10A with paclitaxel

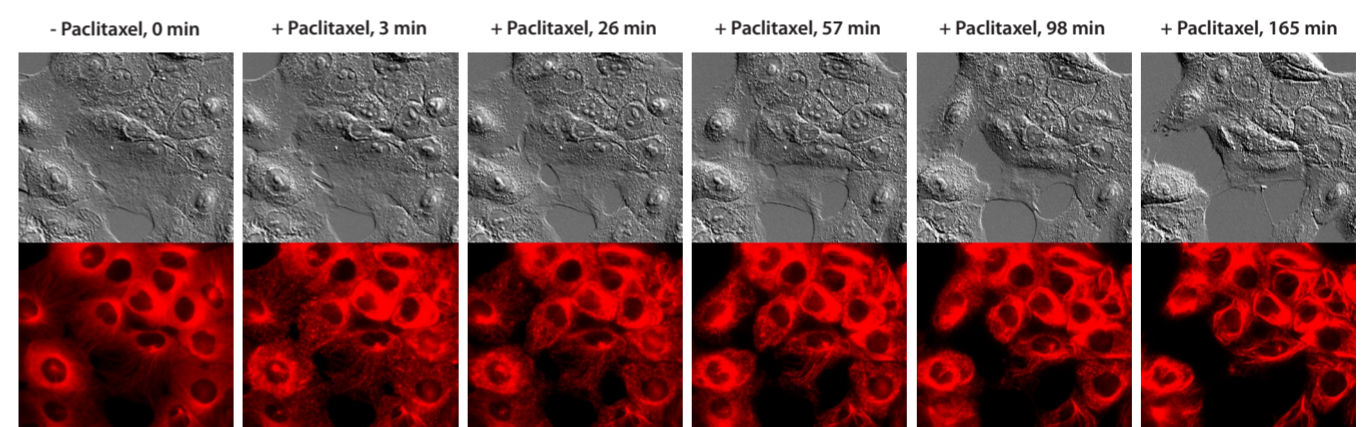
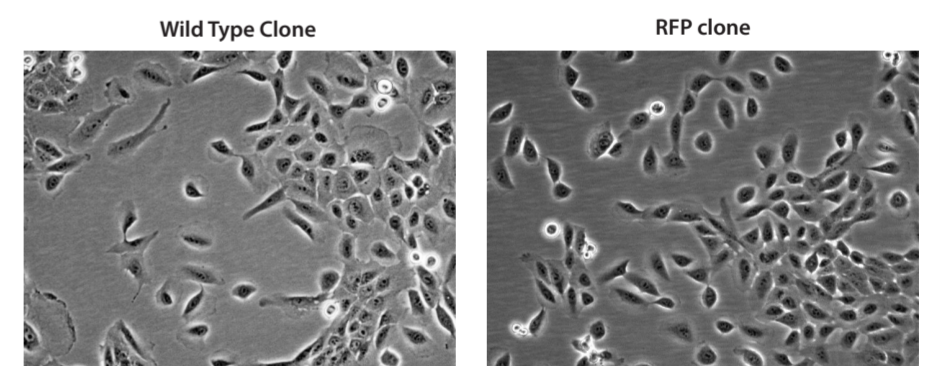


Figure 5: Paclitaxel effect on microtubules. Paclitaxel is a mitotic inhibitor used in cancer chemotherapy. Paclitaxel is thought to stabilize microtubules and as a result, interfere with the normal breakdown of microtubules during cell division. RFP tagged TUBA1B MCF10A cells were imaged by Differential interference contrast (DIC) and fluorescence microscopy while applying 20 μM Paclitaxel. As time progressed, typical tubulin bundles are formed. The doubling time of the RFP-tagged lines was determined to be 22.1 +/- 3.73hrs (n=17). Effectively, this is not significantly different from the reported doubling time of the MCF10A at 24hrs.³

Figure 6: Wild type and RFP-tagged lines are morphologically similar



Discussion/Conclusion

Immortalized cell lines are used extensively in investigative work and diagnostic screens. We report here the development of near normal cell lines with specifically targeted gene knockout. In the example presented, PTEN expression was eliminated following a 19 base pair and a 2 base pair deletion in each allele of the targeted exon of the PTEN gene. Loss of PTEN expression was confirmed by ELISA and qRT (not shown). The alterations are permanent and stable as cell lines have exceeded twenty passages with no change in expression and cellular characteristics. Using a similar approach, knockout lines have been generated for numerous breast cancer-relevant gene targets including HER2, SYK, TP53, CDH1, GSK3B, Rictor and more.

The targeted gene approach with Zinc Finger Nucleases can also be used to introduce genetic elements. Donor sequences can include tags, as demonstrated, or can include donor oligo sequences with as little as a single nucleotide difference (such as single nucleotide polymorphisms - SNP's). Work is ongoing to generate both additional gene knockout cell lines as well as cell lines containing SNP sequences.

This work demonstrates successful tagging of TUBA1B (α-tubulin 1b, microtubule) in the near normal MCF10A breast epithelial cell line. These cells, by all indications, retain normal expression of tubulin with no demonstrated affect on cell replication or morphology, while expression of the RFP tag remains under control of the endogenous tubulin promoter. The impact of taxane treatment (paclitaxel) can be easily visualized. These cells can also serve as a "foundation" line within which other gene alterations can be made allowing the advantage of easily following the modified cell line.

While future work includes generating additional modified cell lines, we will use these lines to examine the affect on both upstream and downstream pathway members regarding the targeted genes through the use of antibody-based arrays and comparable approaches. These lines and pathway analysis will also allow the examination of small molecule panels such as libraries of pharmacologically active compounds.

References

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