An Application of Zinc Finger Nuclease Technology to Create Multiple Complete Gene Knockouts in Polyploid Cancer Lines

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Overview

Oncogenes and cellular pathways represent potentially promising targets for therapeutic compounds. Among these, apoptotic pathways are differentially expressed in different patients and cancer lines. An individual patient's response to chemotherapy tends to differ depending on their unique genotype with regard to the apoptotic pathway proteins. These differences have been hard to model in isogenic settings, until the advent of the Zinc Finger Nuclease (ZFN) technology. Traditionally, cell line engineering techniques to disrupt specific genes were limited to time consuming homologous recombination and screening approaches in a limited number of cell lines. ZFN technology allows rapid and permanent disruption of specific gene loci in any cell line of choice. Sequence specific nuclease cleavage, followed by imperfect DNA repair, gives rise to permanent gene disruptions. As an application of the ZFN technology we demonstrate the construction of genetically defined cell lines. Two pro-apoptotic regulators, Bcl2–associated X protein, BAX, and Bcl2-like protein, BAK, genes were knocked out using ZFNs in three different cell lines, A549 (human lung carcinoma), DLD-1 and SW48 (human colorectal cancer). Complete knockouts were achieved, including in A549 where the BAX gene is tetraploid. DNA sequence alterations were verified by sequencing, and the absence of BAX and BAK proteins was confirmed by enzyme immunometric assay and Western blot. Cells lacking both BAX and BAK are impaired in the intrinsic induction of apoptosis as demonstrated by cell survival when treated with the apoptotic effector staurosporine, compared to the wild-type counterpart. The straightforward approach coupled with the power of ZFN technology to address both diploid as well as polyploid targets, makes this an ideal tool for the generation of genetically modified cell lines with their isogenic controls. Targeted gene editing provides an important tool for genetic manipulation that enables the study of cancer genes and creates isogenic screening tools to test the sensitivity of endogenous gene modifications to therapeutics.

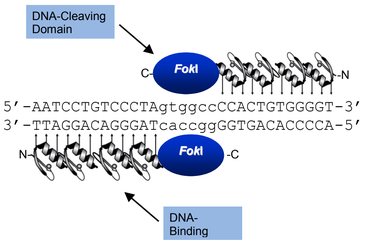

Figure 1: Zinc Finger Nucleases: Highly Specific Genomic Scissors Each Zinc Finger Nuclease (ZFN) consists of two functional domains. A DNA-binding domain comprised of a chain of two-finger modules, each recognizing a unique hexamer (6 bp) sequence of DNA. Two-finger modules are stitched together to form a Zinc Finger Protein, each with specificity of ≥ 24 bp. A DNA-cleaving domain is comprised of the nuclease domain of Fok I. When the DNA-binding and DNA-cleaving domains are fused together, a highly-specific pair of 'genomic scissors' are created.

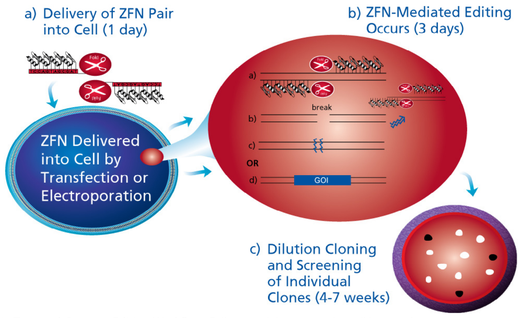

Figure 2: Targeted Genome Editing Workflow Following delivery into the cell by transfection or electroporation, the ZFN pair recognizes and heterodimerizes around the target site. The ZFN pair makes a double strand break and dissociates from the DNA. By non-homologous end joining, or homologous recombination repair mechanisms, 1-20% of cells are mis-repaired resulting in a gene deletion. Alternatively, a repair template may be transfected with the ZFN pair. 1-20% of cells contain gene integration in the target site. Dilution cloning or single cell sorting is used to generate single cell clones and clones are screened for presence of deletion or insertion.

Advantages of ZFN-mediated Gene Knockout Technology

- Rapid and permanent targeted disruption of endogenous loci
- Monoallelic or biallelic disruption (even tetraploid)
- ZFNs are expressed transiently
- No selection required
- Minimal screening effort
- Trait stacking is achievable
- Broad utility for animal models

Results

A549 with tetraploid BAX knockout

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TGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTTC  wt
TGCACCAAGGTGCCGGAACTGATCA--ACCATCATGGGCTGGACATTGGACTT  -2
TGCACCAAGGTGCCGGAACTGA----AACCATCATGGGCTGGACATTGGACTT  -4
TGCACCAAGGTGCCGGAACTGA-------CATCATGGGCTGGACATTGGACTT  -7
TGCACCAAGGTGCCGGA----------------------CTGGACATTGGACTT -21
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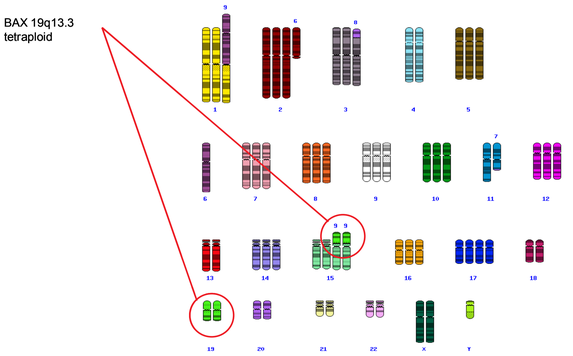

Figure 3: A549 with tetraploid BAX knockout Many cell lines are aneuploid and may contain multiple (>2) copies of the target gene. This is not a barrier for ZFN technology. The spectral karyotyping of A549 cell lines is shown. The BAX gene is tetraploid in A549. Following treatment with ZFN specific for BAX, a clone containing a unique disruption in all four alleles was isolated, see DNA sequences above.

Genotypes of BAX BAK double knockout cell lines

SW48 BAX (-/-) BAK (-/-)

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BAX locus
CAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTTCC wt
CAAGGTGCCGGAACTGATCAGA----TCATGGGCTGGACATTGGACTTCC -4
CAAGGTGCCGGAA------------------------------GACTTCC -30
BAK locus (homologous deletion 7bp)
CTCCTGCTCCTACAGCACCATGGGGCAGGTGGGACGGCAGCTCGCCATCA wt
CTCCTGCTCCTACAGCACCA-------GGTGGGACGGCAGCTCGCCATCA -7
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DLD1 BAX (-/-) BAK (-/-)

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BAX locus (homologous deletion 63bp)
GTGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTTCCTCCGGGA wt
GT-------------------------------------------------------------A -63
BAK locus (homologous deletion 38bp)
TGCATGCCTCCTGCTCCTACAGCACCATGGGGCAGGTGGGACGGCAGCTCGCCATCATCGGGG wt
TGCATGCCTCCTGC--------------------------------------CATCATCGGGG -38
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A549 BAX (-/-/-/-) BAK (-/-)

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BAX locus
TGCACCAAGGTGCCGGAACTGATCAGAACCATCATGGGCTGGACATTGGACTT wt
TGCACCAAGGTGCCGGAACTGATCA--ACCATCATGGGCTGGACATTGGACTT -2
TGCACCAAGGTGCCGGAACTGA----AACCATCATGGGCTGGACATTGGACTT -4
TGCACCAAGGTGCCGGAACTGA-------CATCATGGGCTGGACATTGGACTT -7
TGCACCAAGGTGCCGGA----------------------CTGGACATTGGACTT -21
BAK locus
TCCTGCTCCTACAGCACCATGGGGCAGGTGGGACGGCAGCTCGCCATCATCGG wt
TCCTGCTCCTACAGCACCATGGGGCAGG----ACGGCAGCTCGCCATCATCGG -4
TCCTGCTCCTAC----------------------GGCAGCTCGCCATCATCGG -22
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Figure 4: BAX BAK double knockout cell lines DNA sequences

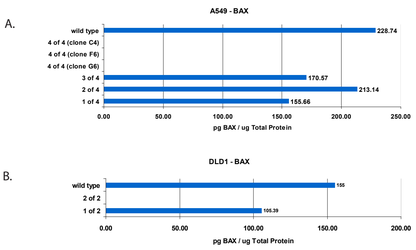

Figure 5: BAX Protein Determination BAX protein concentration was measured in wild-type and knockout cell lines using an enzyme immunometric assay specific for BAX. Calculations were derived by comparing BAX protein concentration in cell lysates to a standard curve of recombinant BAX protein. In the A549 (A), approximately 229 pg of BAX per ug of total protein was observed. Clones having 1, 2, 3, out of 4 alleles disrupted produce less BAX protein. For three unique clones, each having 4 out of 4 alleles disrupted, the measurement was below the lower limit of detection. In DLD-1 (B), the measurement for the BAX protein exceeded the limit of the assay and is greater than 155pg/ug total protein. In the clonal cell line having 1 out of 2 alleles disrupted, 105 pg of BAX per ug of total protein was observed, while in the clonal cell line with both alleles disrupted the measurement was below the level of detection of the assay. In Western blot analysis, BAX protein was detected in the wild-type, but not the knockout cell lines. Similar experiments specific to BAK protein are currently underway..

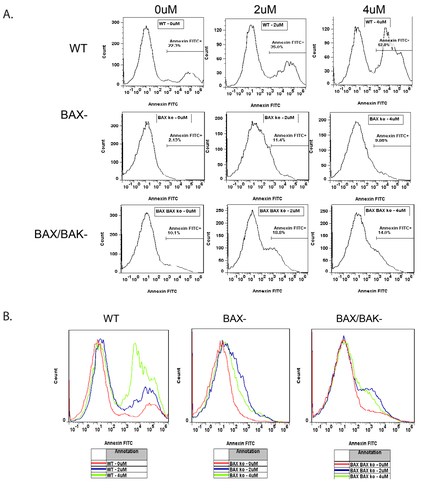

Figure 6: Induction of apoptosis and Annexin V cell staining DLD1 cells wt, BAX knockout, or BAX BAK double knockout were treated with the apoptosis effector staurosporine at 0mM, 2mM, or 4mM concentration. Cell survival was determined by staining with Annexin V-FITC and analysis by flow cytometry. The results are shown for the individual populations (A) and overlaid (B). The wild-type cells exhibit a significant shift (B-WT) in Annexin V staining with the addition of staurosporine indicating entry into apoptosis. The BAX knockout cell population exhibits a lesser shift (B-BAX-) in Annexin V staining when 2mM or 4mM staurosporine is introduced. The bulk of the BAX BAK double knockout population does not shift with the addition of staurosporine indicating resistance to entry into apoptosis (B-BAX/BAK-). The observed increase in Annexin V staining in the BAX BAK knockout (A-BAX/BAK-) is likely to be a function of alternate pathways independent of BAX and BAK (see references).

Methods

Human Cell Culture A549: human lung carcinoma; media: F12 Ham (Sigma N4888) containing 10% v/v fetal calf serum (Sigma F4135) and 2mM L-glutamine (Sigma G7513) (F12 complete media). DLD-1: human colorectal carcinoma cell line; media: RPMI (Sigma R5886) containing 10% v/v fetal calf serum (Sigma F4135), 1mM sodium pyruvate (Sigma 8636), and 2mM L-glutamine (Sigma G7513). SW48: human colorectal carcinoma cell line; media: Dulbecco's Modified Eagle's Medium (Sigma D5671) containing 10% v/v fetal calf serum (Sigma F4135), 1mM sodium pyruvate (Sigma 8636), and 2mM L-glutamine (Sigma G7513) (DMEM complete media).

Zinc-Finger Nuclease Design and Nucleofection Sigma-Aldrich uses a proprietary algorithm to design and assemble ZFN molecules. The assembled ZFNs are tested for DNA binding specificity and for ability to cleave at the target site on the chromosome. A pair of ZFNs is required as the endonuclease FokI must dimerize in order to catalize cleavage of double-stranded DNA. The ZFNs were nucleofected into the cells as plasmids (Lonza, Nucleofection Kit V or R).

Analysis of ZFN Activity The mismatch-specific nuclease assay, CEL-I (Transgenomics Surveyor Nuclease Kit), was used to confirm ZFN activity. PCR was used to amplify wild-type and mutant DNA in the region of ZFN binding. The DNA was denatured and re-annealed such that the annealing of wild-type DNA strand to a mutant strand will create a mismatch "bubble" that will be recognized and cleaved by CEL-I. The cleavage products were detected by acrylamide gel electrophoresis.

Generation and Genotyping of Clones Cells were plated using a limiting dilution protocol, such that statistically one third of a cell was deposited per well in 96-well plate. The resulting clonal cell lines were perpetuated in complete media and genomic DNA isolated to perform CEL-I assay as above. Wild-type PCR amplicon was mixed with clonal amplicon to ensure heteroduplex formation in the presence of a complete homologous knockout. PCR product from CEL-I positive clones was sequenced (Sigma Analytical Lab) to confirm deletion and determine number of affected alleles.

BAX Protein Determination The concentration of BAX protein in wild-type and knockout lines was determined by BAX-specific immunometric assay (Assay Designs). BAX protein concentration in cell lysates was compared to a standard curve of recombinant BAX protein.

Induction of apoptosis and Annexin V cell staining DLD1 cells wt, BAX knockout, or BAX BAK double knockout cells were treated with staurosporine (Sigma 55921) at 0mM, 2mM, or 4mM concentration for 2 hours. Annexin V-FITC Apoptosis Detection Kit (Sigma APOAF) was used to stain the cells prior to flow cytometry. The flow cytometry analysis software, FlowjoTM, was used to plot results.

Summary

In this communication, we demonstrate the rapid construction of genetically defined cell lines.

Two pro-apoptotic regulators, Bcl2–associated X protein, BAX, and Bcl2-like protein, BAK, genes were knocked out using ZFNs in three different cell lines, A549 (human lung carcinoma), DLD-1 and SW48 (human colorectal cancer). In all cases, including A549 where BAX is tetraploid, knockout lines were successfully generated. An immunometric assay was used to confirm that BAX protein is not produced in the complete knockout cell lines, and protein production was decreased in partial knockout cell lines. BAK protein analysis is ongoing. Cell lines were treated with the apoptotic effector staurosporine, stained with Annexin V and analyzed by flow cytometry. The knockout cell lines were impaired in the induction of apoptosis compared to the wild type cell lines.

Previously mutagenesis methods relied on random and non-targeted events to induce changes to the genome. Recently, Sigma-Aldrich formed an exclusive partnership with Sangamo BioSciences to make Zinc Finger Nuclease (ZFN) technology easily available to research scientists. A sophisticated design algorithm is used to determine an optimal recognition site and ensure specificity. After delivery of the transiently expressed ZFN pair, the target DNA is cleaved and naturally occurring DNA repair mechanisms repair the DNA imperfectly. We are able to take advantage of the inaccurate nature of DNA repair and isolate single cells having one or more altered alleles. This technique is targeted and specific, with permanent affects.

References

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