An Application of Zinc Finger Nuclease Technology to Create Knockout Cancer Lines

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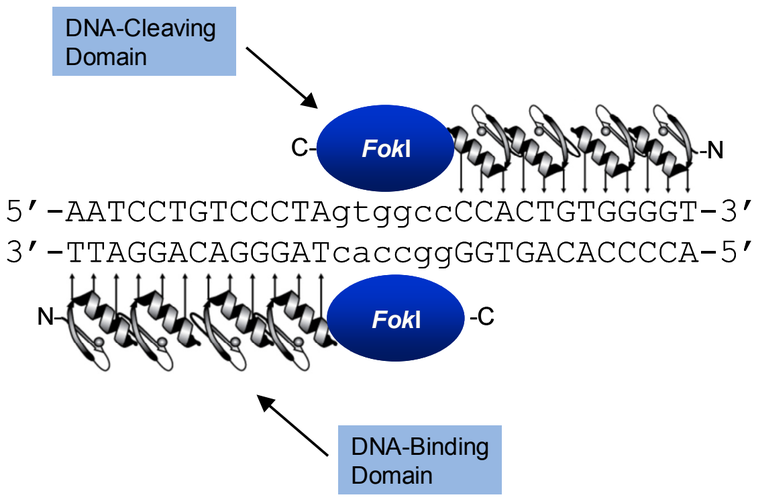
Overview

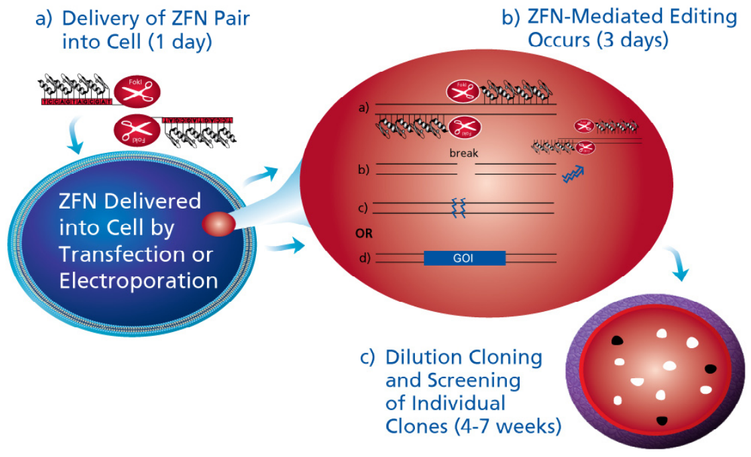
Oncogenes and cellular pathways represent potentially promising targets for therapeutic compounds in the treatment of cancer. Among these, apoptotic pathways have been observed to be differentially expressed in several cancer lines. Additionally, an individual patient's response to chemotherapy may differ depending on their unique genotype. As an application of zinc finger technology we demonstrate here the construction of genetically defined cell lines, which could be applied to high-throughput compound screening. The pro-apoptotic Bcl-2–associated X protein, BAX, gene was disrupted using zinc finger nuclease. Previously, cell line engineering techniques to disrupt specific genes was limited to time consuming homologous recombination and screening approaches. Sigma-Aldrich CompoZr™ ZFN technology allows rapid and permanent disruption of specific gene loci. Sequence specific nuclease cleavage, followed by imperfect DNA repair, gives rise to permanent gene mutations. Three cancer cell lines, A549 (human lung carcinoma), DLD-1 and SW48 (human colorectal cancer), each isogenic except for BAX gene knockout, were prepared using this technology. In addition to multi-allelic disruptions, cell lines with monoallelic disruptions were also isolated. DNA sequence alterations were verified by sequencing, and the absence of BAX protein was confirmed by enzyme immunometric assay. The straightforward approach coupled with the power of ZFN technology to address both diploid as well as polyploid targets, makes this an ideal tool in the generation of these cell lines. Targeted gene editing provides an in vitro cell based tool enabling study of cancer genes and screening cells for sensitivity to potential chemotherapeutics.

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



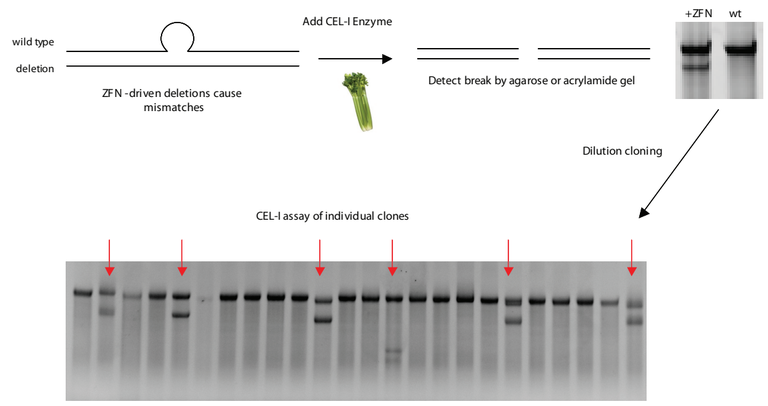
Following delivery into the cell by transfection or electroporation, the ZFN pair recognizes and heterodimerizes around the target site. The ZFN pair makes a double strand break and dissociates from the DNA. By non-homologous end joining, or homologous recombination repair mechanisms, 1-20% of cells 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 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**

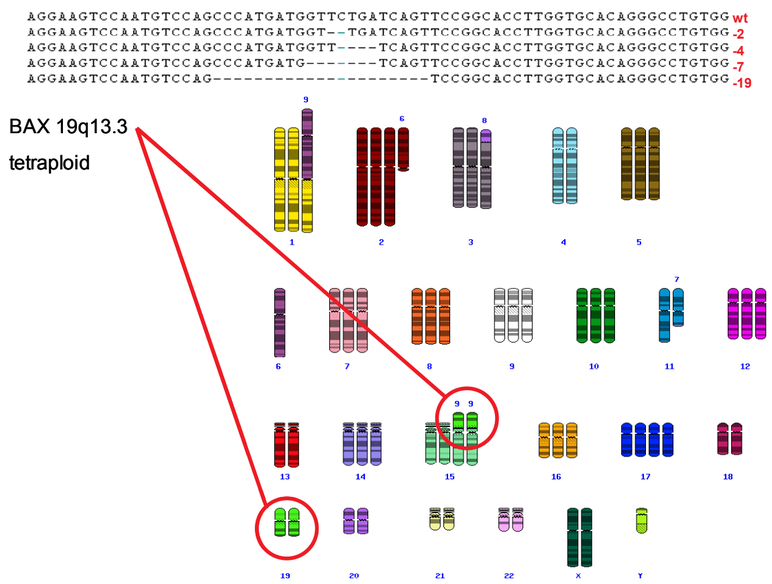
Results

Figure 3. BAX ZFN Delivery and Clone Screening



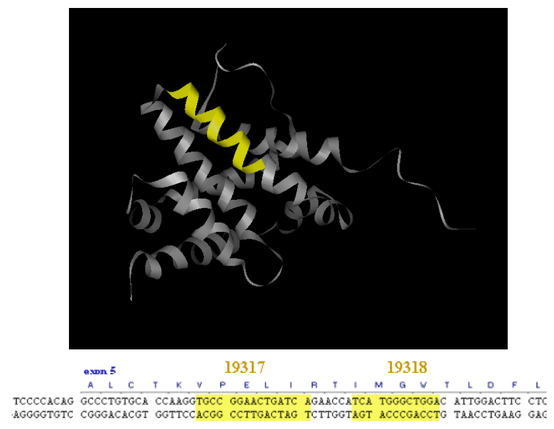
CEL-I enzyme, isolated from celery, is a nuclease that cleaves DNA at nucleotide mismatches to create a double strand break. PCR primers were used to amplify across the ZFN cleavage site in the genomic DNA extracted from ZFN-treated cells. The resulting PCR products, a mixture of wild-type and mutated amplicons, are denatured and re-annealed, such that wild-type strands may anneal to mutated strands. The resulting mismatch is recognized by the nuclease CEL-I. The digest products were observed by acrylamide gel electrophoresis. Shown here, ZFN's specific for BAX gene were nucleofected into target cells. CEL-I assay is used to verify presence of mutations. Single cells were isolated by dilution cloning and CEL-I assay was used to screen clones and identify those having mutations (indicated by red arrows). PCR amplicons from the positive clones were sequenced to confirm deletion and determine number of affected alleles.

Figure 4. A549 with tetraploid BAX knockout



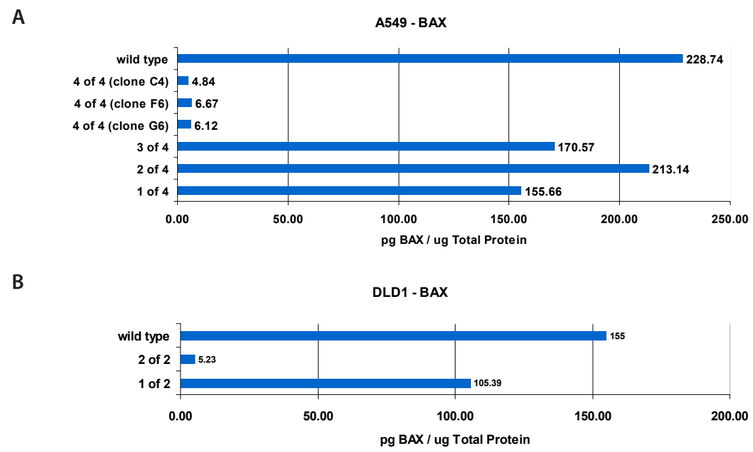
Many cell lines are aneuploid and may contain multiple (>2) copies of the target gene. This is not a barrier for ZFN technology. The spectral karyotyping of A549 cell lines is shown. The BAX gene is tetraploid in A549. Following treatment with ZFN specific for BAX, a clone containing a unique disruption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

Figure 5. ZFN Binding Site Location



The binding site for the ZFN pair is indicated by yellow highlighting. The translation is shown above the DNA sequence and in the ribbon structure of BAX the corresponding residues are highlighted yellow (residues 129 – 141). Potential results are that the deletion of base pairs cause a frameshift, resulting in no protein produced, or protein produced is destabilized or non-functional.

Figure 6. 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. It is expected that signal detected at this level is due to crossreactivity of the antibody, and no BAX protein is produced when 4 out of 4 alleles are disrupted. 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.

Table 1. BAX Clones Mutation Frequency

Cell Line	Gene	# Mutant Clones	Total # Clones Screened	Mutation Frequency
A549	BAX	9	115	8%
DLD1	BAX	16	144	11%
SW48	BAX	11	89	12%

In summary 348 clonal cell lines were screened by Cel-I assay. Of those 36 cell lines were genotyped and found to have 1, 2, 3, or 4 affected alleles depending on cell line ploidy. The mutation frequency was about 10%.

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). 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.

Summary

In this communication, we demonstrate the rapid construction of genetically defined cell lines. The pro-apoptotic Bcl-2–associated X protein, BAX, was targeted for deletion in three human cancer cell lines: A549, DLD-1, and SW48. 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.

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, specific, and permanent.

References

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