Selectable marker lines elucidate design rules for oligonucleotide gene targeting in mouse stem cells.

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Introduction

Gene targeting is one of the most powerful tools in genetic engineering. The ability to make specific mutations to genomic DNA has changed the way we research protein function, provided insight into gene regulation, and enabled the creation of mouse models of human disease. While not currently possible, one can imagine future applications in *ex vivo* gene therapy or other gene repair applications. Although it is a powerful and increasingly common tool, gene targeting requires sophisticated techniques, especially for knock-in type mutations. Significant expertise is required to carry out gene targeting techniques and problems can still emerge in the cloning, expansion, or transfection of large vectors. Thus, there is motivation to look for alternative methods of gene targeting.

One such method uses oligonucleotides to induce point mutations or small deletions or inserts. The oligonucleotide is homologous to the target gene, except for few bases site of mutation. The major barrier to using this technology is that the induced mutation occurs at a very low frequency. The mechanism of gene correction has not been demonstrated, but significant advances have been made in improving yields thorough protective chemistries and in gene delivery methods. Correction efficiencies for single-stranded deoxyribonucleotides (ssODN) have been reported as high as 1:1000 in CHO cells, but the best efficiencies to date in ES cells have been an order of magnitude lower. Efficiencies of 1:1000 in ES cells would be sufficient to allow PCR screening of clones in a few weeks.

Further research is required to understand which barriers are most prohibitive of gene correction: cellular uptake, nuclear import, availability of endogenous repair factors, or the rarity of the correction event. The focus of this paper is on the development of tools to systematically examine the variables involved in correction. The different parameters that affect gene targeting are important to identify. Such conditions present important design rules, highlight the mechanism of repair, and suggest rational designs.

Materials and Methods

-Oligonucleotides

Oligonucleotides were synthesized by the Nucleic Acids Facility at the University of Pennsylvania as described previously.

Mutant NeoS1 sequence

5'- GCCGCTTGGGTGGAGAGGCTATTCGGCTA**G**GACTGGGCACAACAGACAATCGGCTGCTCT -3'
3'- CGGCGAACCCACCTCTCCGATAAGCCGAT**C**CTGACCCGTGTTGTCTGTTAGCCGACGAGA- 5'

Neo1Cor 3'- cgaaCCCACCTCTCCGATAAGCCGAT**G**CTGACCCGTGTTGTCTGTTAGCcgac - 5'

Neo1WT 3'- cgaaCCCACCTCTCCGATAAGCCGAT**A**CTGACCCGTGTTGTCTGTTAGCcgac - 5'

-Construction of pNeoS1Pur cell line

Plasmid pNeoS1Pur was formed by cutting the puromycin cassette from pPur (*Clontech*) and inserting it in the multiple cloning site of pcDNA3.1+ (*Invitrogen*) at the HindIII and XbaI sites. Site directed mutagenesis (Stratagene) was used to mutate the 22nd codon of the neomycin resistance gene from TAT to TAG. Plasmid pNeoS1Pur was linearized at the MfeI site, purified by phenol chloroform extraction, and electroporated into AB2.2 mES cells. The electroporated cells were plated on multiple dishes for selection. After selection with 2 ug/mL puromycin, clones were picked into 24 well plates grown and frozen.

-Transfections

One million cells were plated on feeder cells on a 100mm dish the day before transfection. The cells were changed to non-selective media 3-5 hours before transfection. Lipoplex was formed by mixing 110 ug of ssODN and 55 uL Lipofectamine2000 (Invitrogen) in 2.75 mL of Optimem (Gibco). (Some transfections were conducted with two million cells and 2x each of the reagents.) The lipoplex formed at room temperature for 30 minutes and was then diluted with Optimem to a total volume of 7.75mL. The cells were aspirated, rinsed with Optimem, treated with lipoplex, and incubated. After 2 hours 10 mL of non-selective media was added to the dish. The following day the transfection solution was replaced by non-selective media. At 24, 36, or 48 hours post-transfection (see table 1) the media was replaced with media containing 300ug/mL G418. Selection was continued for another 6-10 days and clones were picked.

Results

We created a Neo susceptible cell line that can be corrected to neomycin resistance with either Neo1Cor or Neo1WT. Corrected cells display the desired phenotype (survival), and sequenced clones generally show the desired genotype (Table 1).

We found clonal variations, and also variation between the two oligonucleotides.

TABLE1: Preliminary Functional Testing Results

Clone	Cells plated	Selection Begins	ssODN	Colonies Picked	Sequenced G→C	Sequenced G→T
3	1000000	36 hr	Neo1Cor	0	0	0
4	1000000	36 hr	Neo1Cor	3	3/3	0
6	1000000	24 hr	Neo1Cor	6	4/5	1/5
7	1000000	36 hr	Neo1Cor	0	0	0
8	1000000	36 hr	Neo1Cor	0	0	0
8	1000000	24 hr	Neo1Cor	0	0	0
9	1000000	36 hr	Neo1Cor	5	4/5	1/5
9	1000000	24 hr	Neo1Cor	1	1/1	0

10	1000000	36 hr	Neo1Cor	2	2/2	0
6	2000000	48 hr	Neo1WT	16	0	12/12
6	2000000	48 hr	Neo1WT	14	NA	NA
9	2000000	48 hr	Neo1WT	44	NA	NA
9	2000000	48 hr	Neo1WT	67	NA	NA

Using the best clone (#9) and Neo1Cor, we looked at how reagent dosages affect delivery. Reagents are given in ug/cm^2 and data is in number of positives per 300,000 cells plated.

Table 2: Dose Testing with pNeoS1Pur clone 9 and Neo1WT

DNA	Lipid	replicate1	replicate2	replicate3
2	2	**3**	**6**	**10**
2	4	**13**	**10**	**16**
4	1	**1**	**3**	**2**
4	2	**6**	**11**	**11**
4	4	**8**	**8**	**10**
6	1	**1**	**0**	**1**
6	2	**6**	**4**	**6**
6	3	**12**	**21**	**15**

Discussion

Our results demonstrate successful ssODN targeting of a new mutant construct with two different oligonucleotides. The transfection efficiencies vary between different clones, implying a locus dependence in gene correction efficiency. Clonal variations could be due to differential transcription rates, chromatin binding dynamics, direction of replication, sequence dependant recruitment of repair elements, or serendipitous knockout of antagonistic genes due to the plasmid integration.

There is enough variation in dose testing to imply that delivery is still a critical issue, but even optimized delivery in an ideal clone with a preferred oligonucleotide is an order of magnitude from the desired level.

In the two clones tested, both showed over 5 times as many positives with Neo1WT correction oligonucleotide instead of Neo1Cor. Since the change of a single base is unlikely to affect delivery characteristics, basepairing must have a mechanistic effect on correction of endogenous targets. Basepairing could be dependent on the surrounding sequence or the repair mechanisms available to a particular cell line. Understanding these rules is an essential part of designing oligonucleotide targets. This cell line and others like it can be used for novel oligonucleotide, basepairing, gene silencing or overexpression, and microinjection studies. With about 100x increase in efficiency, gene targeting could one day be as easy as ordering materials, transfecting cells, and screening clones.