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This exploration grant was to test if it is possible to achieve efficient homologous recombination and gene targeting in immortalized but otherwise normal human breast epithelial cells. Although gene targeting has been achieved in somatic human cells using transfected targeting vectors, the efficiency of this process is very low necessitating the isolation and analysis of many thousands of cell clones before a targeted allele can be identified. Such approaches are impractical with normal human breast cells. We tested if the adoption of a different delivery method, needle microinjection, resulted in more efficient homologous recombination such that targeted clones could be identified after screening fewer colonies. Although we obtained antibiotic resistant clones, we have been unable to identify any clones with targeted alleles. We conclude that our idea was incorrect and that this approach will not be practically feasible without further modification.

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Introduction.

Gene targeting by homologous recombination to introduce defined mutations (knockouts and knock-ins) has been widely used in mice and there are now several examples where gene targeting has been used in human colon cancer cells and normal fibroblasts. The ability to perform gene targeting in normal human breast epithelial cells would allow the generation of genetically-defined human breast cells that would have wide applicability in breast cancer research. The approach used in colon cells and fibroblasts (electroporation of targeting vectors followed by the DNA analysis of many thousands of clones) cannot be used in human breast cells because normal breast epithelial cells cannot be transfected using standard methods such as electroporation and cannot be grown long enough to isolate enough clones. There are therefore no examples of successful gene targeting in normal human epithelial cells. In this Exploration Award proposal we suggested it may be possible to achieve more efficient gene targeting (necessitating the isolation and analysis of only a few hundred clones) using a different delivery method to introduce the targeting vector

Body.

Our hypothesis was that needle microinjection of targeting vectors into normal human breast epithelial cells will result in efficient homologous recombination and allow the isolation of cell lines containing defined mutations in endogenous genes. We had two aims. Aim 1. Use microinjection to make stable Neo-resistant normal breast cells. Aim 2. Determine if the p53 gene can be targeted in normal human breast epithelial cells.

Aim 1 was successfully completed. We optimized conditions for neomycin selection after injection and showed that when neomycin-resistance plasmids were injected we could select for resistant clones using 700μg/ml G418. However, after injecting the p53 gene targeting vector provided by our collaborator, Dr. Sedivy, and screening 94 resistant clones, we were unable to identify any genes where the endogenous p53 gene was targeted (Table 1) suggesting that we would be unable to successfully achieve our goals for Aim 2.

This result could indicate that the use of microinjection as a delivery method is simply not significantly better than electroporation (i.e. our original hypothesis was incorrect) or that there was a specific problem with breast epithelial cells (i.e. injection is in fact a better delivery method but there is something wrong with our normal human breast cells that make the p53 gene unable to be targeted). To discriminate between these possibilities we attempted to target the Myc gene in rat fibroblasts. We chose these cells because Dr. Sedivy has already targeted them using electroporation to deliver the targeting vector (Hanson and Sedivy, 1995). Thus we know that it is possible to target the Myc gene in these cells. Dr. Sedivy sent us his targeting vector, his parental (i.e. untargeted) cells (TGR1) and the cells that he successfully targeted using electroporation (HET cells). For both experiments in human breast cells and in rat fibroblasts, cells injected by both researchers (J. Thorburn and A. C. Lowe, nee Raukauskas) who were listed in the original grant application were combined to ensure that variation in injection technique between individuals was not responsible for the effects observed. We

optimized DNA purification and PCR conditions so that we could successfully isolate and amplify genomic DNA from small numbers of cells growing as isolated clones. Table 1 shows the combined results obtained from multiple injection experiments by both investigators.

Number of normal human breast cells successfully injected with p53 targeting vector	~1000
Number of G418-resistant clones	94 (~9%)
Number of clones with successfully targeted p53 genes	0
Number of rat fibroblasts successfully injected with Myc targeting vector	~1500
Number of G418-resistant clones	70 (~5%)
Number of clones successfully targeted Myc genes	0

Table 1.Results of injection experiments. While it was possible to obtain neomycin-resistant clones, the targeting vectors were not integrated into the homologous genes. This was the case for both the p53 gene in human breast cells and for the Myc gene in rat fibroblasts. Because the same rat fibroblasts were successfully targeted previously using electroporation of the same Myc-targeting vector, we conclude that our injection approach is not better enough to justify further pursuit of this approach at this time.

These results are disappointing because although we were able to obtain G418-resistant clones at the anticipated rate (we predicted in our original application that is we were to inject about 1000 cells, we might obtain 50-100 resistant clones), we did not obtain any homologously targeted clones. We predicted that if our hypothesis was correct (i.e. that injection results in much more efficient homologous recombination than other delivery methods) we would need to test no more than 50-100 clones in order to obtain successfully targeted genes. Since we were able to test 94 clones in the first series of experiments in breast cells and a further 70 clones in fibroblasts in the second series of experiments, we would have expected to find a few clones with targeted alleles if our hypothesis was correct. Because we were unsuccessful even with the fibroblast line that was previously targeted using the same vector that we used, we suspect that this result is not specific to breast epithelial cells but instead indicates that our original hypothesis (i.e. that injection would be a significantly more efficient delivery method than electroporation) is not correct.

Key research accomplishments.
We were unable to isolate targeted clones. The antibiotic-resistant clones that we could isolate after microinjection of were presumably integrated at other sites in the genome. We conclude that needle microinjection is not a practical method to deliver targeting vectors to breast epithelial cells or probably other mammalian cells.

Reportable outcomes.
None.

Conclusions.
This project was very high risk because although the rationale for using injection as a delivery method was reasonable, there had never been an example normal human cells were targeted at specific loci using injected vectors. The potential benefits if we had been able to isolate human breast cells with specific targeted alleles was however very significant. Thus the project was a good candidate for the Exploration Award mechanism. Unfortunately, our approach did not work and we have been unable to isolate breast cells with specific targeted alleles using this approach. Other approaches such as the use of engineered nucleases that cut genomic DNA at specific sites and allow more efficient homologous recombination which was recently described (Bibikova et al., 2003) may prove more effective and could be combined with the injection delivery method. Modifications to this project along these lines would still be worth testing. However, we think it would be worthwhile to attempt this approach only when there is an efficient engineered nuclease to work with.

Although we were able to achieve the experiments proposed in the original application in a timely manner, given the lack of progress, and our current belief that this approach will not be feasible, we wish to terminate this grant early to avoid continued use of resources on a project that seems unlikely to work.

References.

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Hanson, K.D., and J.M. Sedivy. 1995. Analysis of biological selections for high-efficiency gene targeting. *Mol Cell Biol.* 15:45-51.