TRANSGENIC TECHNOLOGY

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The Transgenic Technology Laboratory makes use of molecular genetic approaches to help understand gene function in the emergence and progression of cancers. We can accurately model changes in endogenous genes observed in human cancers by making use of technologies such as gene targeting or genome editing. By bringing together combinations of precise gene alterations we can generate sophisticated models of human cancers and help to understand how the combinations genetic changes contribute to the progression of the disease.

Embryonic stem (ES) cells have been valuable tools in helping with generating new models of cancer, as they have two key properties which facilitate this. Firstly, ES cells exhibit high levels of homologous recombination (HR), which allows us to precisely modify endogenous genes and insert mutations identical to those found in human cancers. Secondly ES cells will differentiate into a wide variety of cell types from different tissues. So once we have generated ES cells with the appropriate genetic alterations, we can then differentiate the ES cells into cells from the tissue of interest.

During the year, we have collaborated with other groups at the Institute to generate a wide variety of different types of genetically altered alleles. These included conditional knockouts, point mutations and inducible transgenes. Using this approach, we introduce mutations directly in the appropriate context of the endogenous gene, and this ensures that the changes we make directly imitate the mutations discovered in human cancers. Furthermore, we have been able to use these types of analyses to identify genetic modifiers that can play a role in the progression of the disease. Identifying such genetic modifiers not only enhances our understanding of the basic biology of the disease but in some cases can be potential therapeutic targets allowing the development of candidate disease treatments.

Making genome editing more effective for new allele generation

Increasingly, the adoption of genome editing by CRISPR-Cas9 technology has provided an attractive alternative to using ES cells as described above. Previously nucleases, such as zinc finger nucleases and TALENS were capable of accurate DNA modification, but were awkward to design and generate. As CRISPR-Cas9 is targeted by a short guide RNA (sgRNA), the design and application of this nuclease is much more straightforward. The application of the CRISPR-Cas9 system was further enhanced by the development of electroporation to deliver the reagents into zygotes for efficient genome modification. This allows direct introduction of CRISPR components along with donor template to initiate homology-directed repair (HDR) and directly produce correctly edited alleles.

Although these electroporation-based methods are very effective in introducing small deletions, insertions or substitutions at the site of DNA cleavage determined by the sgRNA, this method is restricted to short DNA modifications (typically <200 nucleotides) as the process relies on using single-stranded oligodeoxynucleotides (ssODNs) as repair templates. Microinjection of CRISPR-Cas9 reagents is an alternative to electroporation which can be used to generate knock-out mutations or in combination with ssODN or long single-stranded DNA (lssDNA) templates for the production of knock-in mutations. However pronuclear microinjection is technically challenging and is still limited by the synthesis limits of the lssDNA templates, as well as low efficiencies of accurate insertion of larger ssDNAs. So introducing larger and more complex DNA alterations, which can be accomplished using ES cells, cannot reliably be achieved by CRISPR-Cas9 methods

An alternative approach of HDR template delivery is to use recombinant adeno-associated virus (rAAV), which has emerged as a safe and effective gene delivery system with the inherent ability to transduce mammalian cells. Of note, rAAVs can also can stimulate gene targeting through promotion of homologous recombination. Naturally occurring serotypes of AAV can transduce cells and preimplantation embryos, and deliver the ssDNA genome to the nucleus with high efficiency. This approach was further improved by combining electroporation of CRISPR-Cas9 reagents as ribonucleoprotein with subsequent transduction using an rAAV encoding a donor template molecule which can achieve high targeting efficiencies.

In order to test if this approach would prove effective, we initially identified a suitable target locus. A fluorescent marker gene trap measure 4.4Kb of sequence was designed to target into exon 1 of a gene involved in human hepatocellular carcinoma (HCC; Fig. 1a). Prior to this experiment the introduction of sequence larger than 1kb by gene editing had been extremely challenging. An AAV1 virus was generated carrying the insertion sequence in two forms with the fluorescent marker in either the C-terminal or N-terminal orientation and was used in conjunction with four CRISPR guides located in the intronic sequence (Fig, 1b). Mouse embryos were generated by IVF and infected with either the C-terminal or the N-terminal rAAV for four hours prior to electroporation with the guides and Cas9 protein.

A total of 13 samples were obtained and screened for this project and, of these, seven were positive for insertion of the reporter gene, with six of these shown to be correctly inserted with the genome (Fig. 1c). Sequencing analysis of all six of these mice confirmed correct targeting of the entire transgene. Overall this experiment was shown to generate targeted alleles with nearly 50% knock-in efficiency. These data suggest that using an rAAV can work very efficiently for gene editing, and this method will allow larger and more complex genetic alterations to introduced into the genome.

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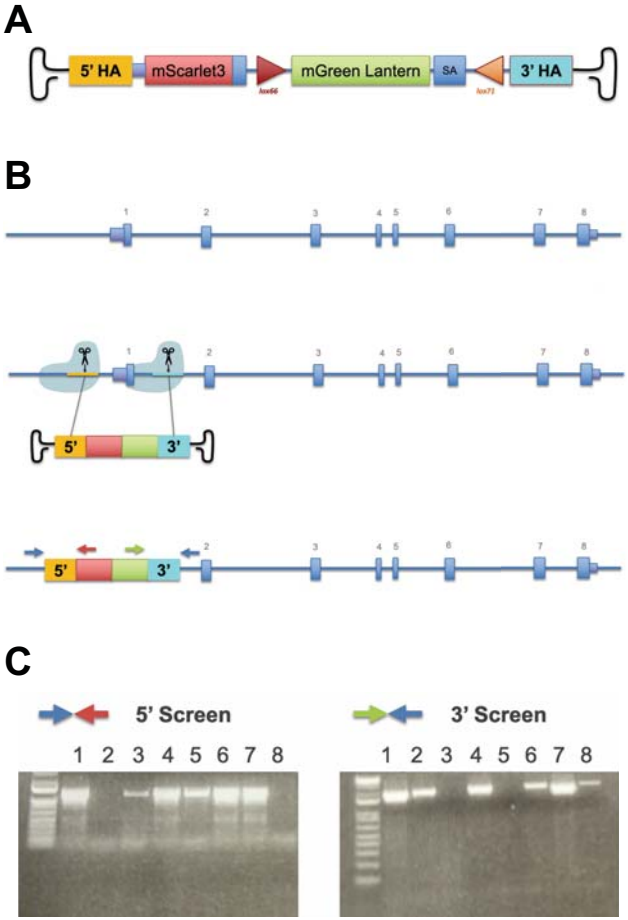

Figure 1. (A) Design of the rAAV1 used in a gene editing experiment. The AAV includes 5' and 3' homology arms along with mScarlet3 and mGreen Lantern fluorescent proteins (B) Inserting an rAAV1 into the target locus. Following cleavage of the DNA by the Cas9 RNP the rAAV genome is used as an HDR template inserting the fluorescent protein sequences into the gene of interest. Position of the screening primers is indicated on the modified allele. (C) Screening on the 5' (blue and red arrows) and 3'(green and blue arrows) sides of the homology arms. PCR screening from the endogenous gene sequence across the homology arms demonstrates that the rAAV1 sequences are inserted at the correct site.