Humanization of a large genome fragment using CRISPR/Cas9 and *recombineering*

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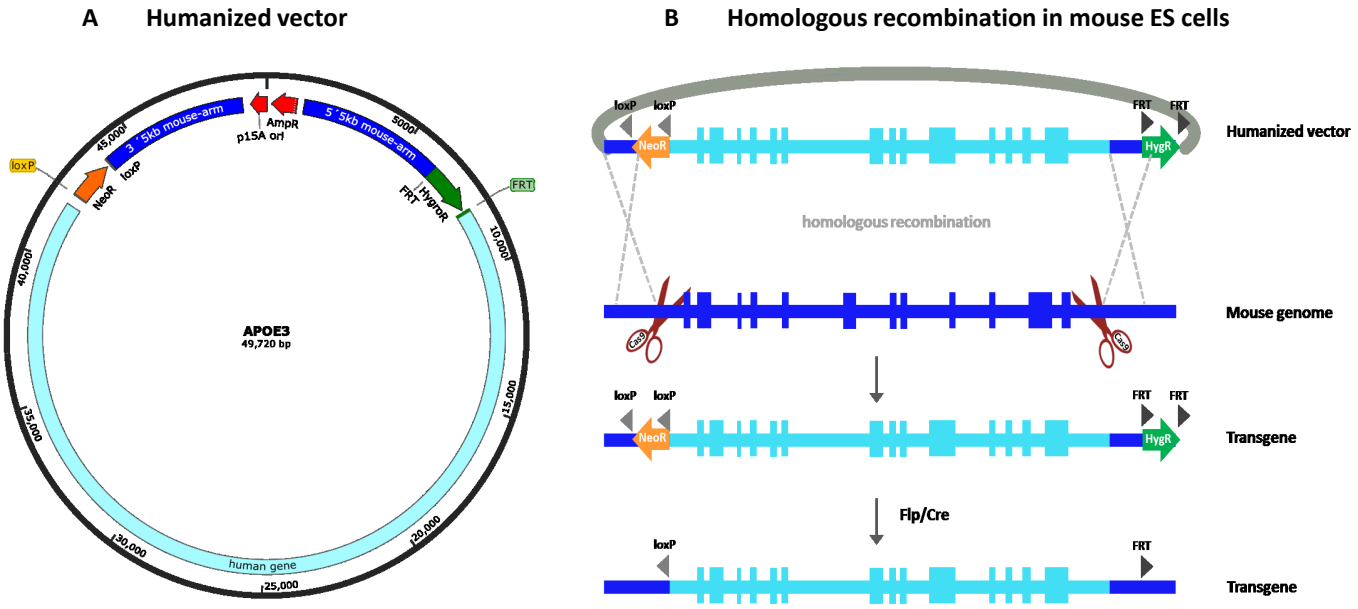
Animal models, including transgenic mice in which an endogenous gene is replaced by a human disease-associated gene variant are essential tools for biomedical research. While CRISPR/Cas9 has successfully been used to integrate small DNA sequences into a target locus, such complex genome engineering tasks remain challenging. In this study we combined very large targeting constructs with the potential of CRISPR/Cas9-mediated double-strand breaks to humanize a 33 kb locus in the mouse.

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation in the human genome. The 1000 Genomes Project established a worldwide reference for human genetic variations and serves as a genetic database of human diseases. Some SNPs are strongly correlated with major diseases. Appropriate genetically engineered mouse (GEM) models are essential to elucidate the significance of a specific SNP for a particular disease. Such animal models are challenging to set up, since large genomic fragments need to be replaced by their human counterparts.

As part of the EU funded project *Aged BrainSysbio* (www.agedbrainsysbio.eu) several humanized mouse models were developed for the gene *ApoE*. There are three common isoforms of the protein found in human populations: *ApoE2*, *ApoE3* and *ApoE4*, which differ structurally by two amino acid substitutions. *ApoE3* is the most common isoform and neutral with respect to Alzheimer´s Disease, whereas *ApoE4* constitutes the most important genetic risk factor and *ApoE2* may provide some protection against this disease.



▲ **Fig. 1:** Humanization of a gene in mouse ES cells.

A: Schematic overview of the ApoE3 targeting construct. The human part of the gene is flanked by selection markers HygroR and NeoR with 5kb mouse homology arms which allows for ES cell selection. The targeting construct bears an origin of replication (p15A) and an ampicillin resistance marker (AmpR). **B**: Homologous recombination of a humanized targeting construct in mouse ES cells. The double-strand breaks by CRISPR/Cas9 occur 5´and 3´ of the insert. The selection markers are flanked by the recognition sites loxP and FRT, respectively and can be removed by Cre or Flp recombinase, respectively.

Generation of a human transgene

Humanization of whole genomic fragments in the mouse genome is still challenging with all currently available technologies including CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9. CRISPR/Cas9 mediated gene editing is successfully used to generate targeted gene deletions, point mutations or insertions of small fragments. However, despite some advances, the efficiency of CRISPR/Cas9 based gene targeting for the integration of larger inserts remains consistently low. GeneBridges GmbH has been heavily involved in designing targeting constructs for homologous recombination in ES cells for over 10 years. Its patented Red/ET mediated recombination ("recombineering") system allows genetic engineering of tailor-made targeting constructs and is particularly well-suited for the precise assembly of large DNA molecules in E. coli. Targeting constructs are usually prepared with 5kb long homology arms to provide high recombination efficiency rate in ES cells. The size of the homology arms is chosen to allow convenient screening of the cells by PCR or Southern Blotting. However, the efficiency of homologous recombination decreases with the size of the intended insertion.

In order to humanize the *ApoE* genomic locus we therefore decided to use CRISPR/Cas9 to boost the recombination efficiency for the integration of a 50kb targeting construct [1]. A 33kb human fragment flanked on both ends with an antibiotic resistance marker either for neomycin (NeoR) or for hygromycin (HygroR) was assembled for positive selection in ES cells. Next, 5kb equivalent mouse homology arms were attached on both ends for later integration into the corresponding mouse locus. Our collaborator within the *AgedBrainSysbio* consortium ICS (Institut Clinique de la Souris, Illkirch, France), accomplished the downstream work and performed integration of a 50kb hybrid targeting construct into ES cells in combination with CRISPR/Cas9.

Integration of a point mutation into the humanized targeting construct

A humanized targeting construct for *ApoE3* was constructed first (Fig. 1A). Additional point mutations were then inserted using the Red/ET recombination system in E. coli and three additional constructs were generated using this method. The advantage of the well-established Red/ET technology is that several point mutations can be inserted simultaneously by vector-mediated homologous recombination. Multiple point mutations can also be achieved by CRISPR/Cas9 RNA injections directly into mouse zygotes, skipping the screening of ES cells. Although the direct CRISPR/Cas9 injection approach can save time and costs at the beginning of the project, a greater number of founders have to be screened later as a result of somatic mosaicism and allele complexity.

ApoE Haplotype	SNP	Selection	Nr. of clones screened	Nr. of positive clones	% positive	Germline
ApoE3	-	Neo + Hygro	93	6	6,5%	confirmed
ApoE2-5	snp-rs7412	Neo + Hygro	186	8	4,3%	confirmed
ApoE4-3	snp-rs429358	Neo + Hygro	372	15	4,0%	confirmed
ApoE2-5/6	snp-rs7412; snp-rs72654473	Neo + Hygro	ND	ND	ND	ND

▲ **Tab. 1:** Targeting-efficiency by combination of CRISPR/Cas9 and HR in mouse ES cells

CRISPR/Cas9 & homologous recombination for ES cell integration

The endogenous murine *ApoE* region was deleted by two double-strand breaks (Fig. 1B). Clones undergo homologous recombination repair, following introduction of the targeting construct bearing the human orthologue with 5kb mouse homology arms and two antibiotic selection marker cassettes into ES cells. In this way we have been able to humanize a 33kb region in mouse ES cells with an insertion efficiency of about 5% (Tab. 1). Without CRISPR/Cas9 the frequency of these HR events would have been very low or even zero.

Germline transmission was successfully confirmed for the first three models and phenotypical analysis of the animals could now be accomplished.

Summary

SNPs represent more than 90% of the genetic variation between individuals. The current challenge is to select relevant SNPs to study their function in human diseases. The humanization of larger genomic regions is almost impossible due to technical reasons.

As part of the EU funded FP7-project *AgedBrainSysbio* we were able to humanize the mouse ApoE locus by a combination of using Red/ET recombination to prepare large hybrid targeting constructs with point mutations and CRISPR/Cas9 to facilitate integration of a 33kb human fragment into the mouse genome. This approach can be easily adapted for the humanization of other mouse loci and will allow fundamental medical questions in human disease to be answered. ■

Literature

[1] Gennequin B, Otte DM, Zimmer A (2013) CRISPR/Cas-induced double-stand breaks boost the frequency of gene replacements for humanizing the mouse Cnr2 gene. Biochem Biophys Res Commun 441:815-819

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