Meeting Report 9th Workshop on Innovative Mouse Models

June 15-16, 2017, Leiden, the Netherlands

Topics:

- CRISPR/Cas9: basic biology, germ-line and somatic applications
- Humanized mouse models
- Embryo manipulation
- Imaging
- iPS and ES cells

On June 15 and 16, 2017, the 9th Workshop on Innovative Mouse Models was held at the Leiden University Medical Center, Leiden, the Netherlands. The primary goal of this two-day workshop has always been to bring together a diverse group of scientists interested in advanced genome alteration approaches in the mouse, including key developers of emerging technologies as well as researchers who wish to apply and assess these new approaches. The workshop encourages an in-depth and unvarnished discussion of these technologies and novel developments in a very open and informal way, easy accessible for young and senior researchers. Since this philosophy is also shared by the ISTT, the ISTT again co-sponsored this event. This year the workshop sessions consisted of:

- ✓ CRISPR/Cas9: mechanisms
- ✓ CRISPR/Cas9: chromosomal engineering
- ✓ CRISPR/Cas9: enhancing HDR
- ✓ Generation of Conditional alleles
- ✓ Humanized mice
- ✓ Genome Editing Methods
- ✓ Imaging and Somatic Gene Editing
- ✓ Forum discussion

Every session consisted of keynote lectures by invited speakers and some sessions also contained short presentations selected from the abstracts. In this way all participants were able to learn the principles, get an overview, and hear about the newest developments from senior professionals and junior, hands-on, experts.

Of course this edition was dominated by CRISPR/Cas9-assisted gene editing technology, whether or not in mouse models, and kicked off by **Jean-Paul Concordet**. Jean-Paul presented work of his group on evaluation of off-target and on-target scoring algorithms, published by Haeussler et al. in Genome Biology (2016) https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-1012-2. We learned that both off-target and on-target sites for gene editing with CRISPR/Cas9 can be predicted by popular algorithms, but that not all guidelines are equally good. Concordet and his team developed a new website (http://crispor.org) which predicts with better efficiency than the often used http://crispr.mit.edu/about. During this first mechanistic

session we learned about the different DNA repair mechanisms, the most common ones being Non Homologous End Joining (NHEJ) and Homologous Recombination (HR), but also about Microhomology-Mediated End Joining (MMEJ) and Homology Independent Targeted Integration (HITI), all active in their own time frame of the cell cycle and therefore used in dividing and/or non-dividing cells. **Jacob Corn** stressed the fact that we have to learn *all* the details of the CRISPR/Cas9 mechanism before we will be able to increase the frequency of homology-directed repair (HDR). He unveiled some mechanistic details which you can find on his blog https://cornlab.com/blog/cas9flap/ and he showed some of his results on the correction of the underlying gene defect in sickle cell disease. Additional to the story of Corn were the studies of **Vikram Pattanayak** from Addgene who initially hypothesized that off-target effects of *Streptococcus pyogenes* (Sp) Cas9 might be minimized by decreasing non-specific interactions with its target DNA site. SpCas9-short guide (sg) RNA complexes cleave target sites composed of an NGG PAM sequence (recognized by SpCas9) and an adjacent 20 bp protospacer sequence (which is complementary to the 5' end of the sgRNA). They previously theorized that the SpCas9-sgRNA complex might possess more energy than is needed for optimal recognition of its intended target DNA site, thereby enabling cleavage of mismatched off-target sites. Structural studies have suggested that the SpCas9-sgRNA-target DNA complex includes several SpCas9-DNA contacts, including direct hydrogen bonds made by four SpCas9 residues (N497, R661, Q695, Q926) to the phosphate backbone of the target DNA strand. They envisioned that disruption of one or more of these contacts might alter the energetics of the SpCas9-sgRNA complex so that it might retain enough capacity for robust on-target cleavage but have a diminished ability to cleave mismatched off-target sites. This resulted in a high fidelity Cas9, SpCas9-HF1.

The third session of the workshop focused on enhancing Homology Directed Repair and/or inhibiting Non Homologous End Joining. **Fengfeng Zhuang** introduced their very smart idea of using the high affinity binding of Avidin to Biotin by coupling ~~the~~ avidin to the C-terminus of Cas9 and biotin to the single-stranded donor DNA template, yielding the so-called CAB complex, in order to recruit the donor DNA tot the DSB site. They indeed show higher efficiencies of precise and imprecise editing. **Ralf Kühn** gave us an update on their search for proteins that might drive HDR or inhibit NHEJ. On an earlier occasion he showed the positive effect of SRC7 on HDR in HEK293 cells, but the protein showed to be detrimental to iPS cells. This time he showed results of two proteins: i53, a potent inhibitor of the key NHEJ protein 53BP1 and CTiP, an end resection stimulating protein. When DSBs occur, a process termed DNA end resection is activated, which involves the nucleolytic degradation of the broken ends in the 5′ to the 3′ direction. The resulting 3′ single-stranded DNA (ssDNA) ends then provide a platform for the recruitment of proteins that effectuate HR repair. Interestingly, DNA end resection inhibits NHEJ and triggers homology-directed DSB repair. Combined expression of i53 and CTiP works synergistically in promoting HDR in HEK293 cells. i53 also works in human iPS cells, but is not yet tested in combination with CTiP. In mouse zygotes i53 did not enhance HDR efficiency (however, this was based on 1 experiment!).

While synthetic single-stranded DNA olionucleotides can effectively be used to introduce small genomic alterations (single base-pair substitutions), their size limitation hampers the effective introduction of larger changes, such as the generation of knockin or conditional alleles. In session 4 **Tomoji Mashimo** therefore proposed the use of up to 3 kb long single-stranded DNA (lssDNA) as a targeting donor, which can be cleaved off from a double-stranded plasmid using nicking endonucleases and purified by agarose gelelectrophoresis. The generation of *GFP* knock-in and conditional alleles (the latter termed CLICK: CRISPR with lssDNA inducing conditional knockout alleles) was achieved in zygotes at a frequency of 10-20% (These results were obtained using zygote electroporation instead of zygote injection, equipment for electroporation was demonstrated by Sonidel Limited). On the other hand, **Ivo Huijbers** demonstrated that small single-stranded DNA oligonucleotide templates can be used to introduce *loxP* sites, but this requires two independent gene editing events. Two gRNAs to introduce DSBs flanking essential exons and two template oligonucleotides encoding *loxP* sites generated correctly targeted founder animals, *i.e.*, carrying the two *loxP* sites on the same allele, at an average frequency of 5,1%. CRISPR/Cas9 technology can also be used to create very large genomic alterations. In session 5 **Wojtek Auerbach** presented the exchange of ±250 kb in mouse ES cells for the corresponding human sequence.

A potential problem of the use of vector-encoded Cas9 protein is the uncertainty of the timing of DSB formation: breaks generated in G1 phase are less likely to be repaired by HR, which predominantly occurs in S/G2 phase. **Tomomi Aida** (session 6) therefore investigated the use of "cloning-free CRISPR", *i.e.*, the direct nuclear delivery of a Cas9 ribonucleoprotein (RNP) consisting of purified Cas9 protein in complex with chemically synthesized crRNA and tracRNA. Knockin of CreERT2 into the *Dct* gene was obtained with 30% efficiency (of which 60% in both alleles) upon zygote injection of Cas9-RNP, which was superior to injection of Cas9 mRNA. Optimal results were obtained with donor DNA carrying 1.5-2.0 kb homology arms that were closely located to the DSB site and injection of the Cas9-RNP and donor DNA 9-11 h after *in vitro* fertilization. However, smaller homology arms of ± 40 bp can be effective as well in gene modification in zygotes, possibly by a MMEJ-mediated mechanism and stimulated by overexpression of the endonuclease EXO1, yielding efficiencies of ±35%. Also **Kyoungmi Kim** presented data on injection of ribonucleoprotein, in particular of Cpf1-RNP that uses TTN or TTTN PAMs, requires only crRNA and introduces a staggered DNA double-stranded break. Furthermore, she demonstrated that the cytidine deaminase APOBEC1 fused to Cas9 nickase (nCas9) or catalytically deficient Cas9 (dCas9) can effectively substitute C for T at a specific position in yeast, mammalian cells and plant cells. *E.g.* delivery of such 'base editor' (BE) targeting the *Dmd* or *Tyr* gene, either as mRNA or RNP into mouse zygotes by electroporation or microinjection yielded site-specific single base-pair substitutions at frequencies of 44-57%.

Finally, in session 7 **Jos Jonkers** gave a broad overview of the use of gene modification technology in studying the development and behavior of breast cancer in mouse models. In particular, he demonstrated the possibility of *somatic* gene modification by CRISPR/Cas9: a novel model for invasive lobular breast carcinoma (ILC) was created by intraductal lentiviral delivery of CRISPR/Cas9 components designed to induce specific gene disruptions. This method allows rapid disruption of putative tumor suppressor genes to assess its *in vivo* effect on breast tumor development.

Traditionally, the workshop was concluded with a forum discussion, this time addressing the value and validity of mouse models for scientific and medical research. Despite the impressive advancements of massive parallel sequencing technology, improved *in vitro* tissue culture techniques (iPS cells, organoids) and improved methods of genetic modification, the audience concluded that mouse models remain irreplaceable for studying the immune system and other types of cell-cell interaction as well as cell-environment interaction. The audience highly appreciated IMM 2017, which had a strong focus on novel gene modification technology. Many participants expressed their interest in how this novel technology will be further implemented and developed within the transgenics facilities represented at the meeting. Their activities and the experiences of their clients will be the focus of IMM2019.

The organizers like to express their gratitude to the ISTT for their kind cooperation and sponsoring of the workshop. We hope to meet you all again during the IMM2019 edition!

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