national scientists attended by almost four hundred registered participants. The program covered the latest developments in the field as well as some of the most exciting applications with a focus on its possibilities in establishing model systems to study diseases that currently have no cure. The program included talks from technology development to CRISPR genome editing in high throughput screening-based approaches, in demanding cellular studies and in vivo experiments. The complete program can be seen on http://www.biokemi.org/meetings/118. In addition to talks, the meeting included a poster session of 30 high quality presentations as well as exhibition stands from nine companies providing reagents for CRISPR-based studies.

CRISPR opens doors in many research fields

The clear message of the meeting was that today’s winners of the CRISPR technology are the “ordinary” scientists and research groups in universities and research institutes. CRISPR gene editing technology makes it easier, faster and cheaper than ever before to permanently modify genes in tissue culture settings. Thus far complex patent issues are slowing down its usability beyond basic science, but the genetic revolution it has started is surely going to escalate bringing promises for cure for many incurable diseases.

Looking forward and using our experiences

For us as a science society this meeting was also an experiment to find out, if organizing big scientific events would still be the best way for the DSBMB board to serve our members. Would this be a good way to utilize our expertise, would there be need for it and if so, how to finance such events in the future? While we as a society will continue planning our future activities, one thing is sure: CRISPR technology as a meeting topic turned out to be a very successful and useful choice. Surely, we all learned a lot of modern gene editing and the CRISPR technology and many of us returned to our laboratories inspired to utilize what we learned.

Genome editing by, clustered regularly interspersed short palindromic repeats, (CRISPR) has made its entry in biotechnology and biomedical research worldwide and is with unprecedented speed moving into the clinic. CRISPR-edited cells were injected into a person for the first time just recently and several clinical trials, mostly focusing on use of CRISPR for cancer immunotherapy and stem cell gene therapy are on the way. Although transplantation of gene-edited cells (e.g. T-cells or hematopoietic stem cells) are likely to show clinical efficacy first, hopes are that genome editing directly in patients may one day become an effective and safe alternative to conventional gene addition therapies. In this review, I present preclinical examples of in vivo genome editing using CRISPR, and briefly discuss some of the main challenges toward genetic therapies based on genomic surgery.

I cannot say for sure, but my guess is that the molecular details of the adaptive immune system in bacteria are rarely discussed in the corridors of Christiansborg, home of the Danish Parliament. But on a grey and wet day in November 2016 Danish scientists with expertise in genetic engineering met with Danish politicians in a hearing organized by the Danish Council on Ethics to discuss just that. Like in biotechnology worldwide, the center of the discussion on that day in November was CRISPR, this array of repetitive sequences in bacterial genomes that contains genetic information captured from previous viral intruders and serves as a memory allowing bacteria to fight back attacking viruses (1).

Endonucleases in bacterial warfare

It is old news by now that such bacterial warfare is executed by endonucleases (e.g. Cas9), which are directed toward the incoming foreign DNA by a complex of two short RNA molecules, one of which is encoded by the CRISPR array (crRNA). Specific recognition of DNA is facilitated by base pairing between one of the DNA strands and the crRNA, allowing the endonuclease to perform its scissor function and cut both DNA strands. The unique DNA:RNA duplex formation is the heart of the CRISPR system, which can easily be adapted to target complexes of Cas9 protein (often derived from Streptococcus pyogenes, then referred to as spCas9) and an engineered single guide RNA to pre-
determined loci in the human genome (2).

CRISPR gene editing of human cells

After the first report describing the use of CRISPR in human cells (1), CRISPR-based technologies quickly spread to laboratories worldwide, but did not for real hit the eye of the public (and the press) until evidence of CRISPR-directed manipulation of early embryos was reported in 2015 (3). Together with the innumerable possible uses of the CRISPR/Cas9 system in design of transgenic animals and development of crops with new properties, the controversial and heavily discussed uses of the CRISPR/Cas9 system in in vitro-fertilized eggs and as a ‘gene drive’ facilitating effective spreading of transgenes in natural populations have attracted attention and made headlines.

It is crucial that the capacities of the CRISPR system are known and discussed by the public and among lawmakers, along with the more controversial applications. However, the enormous impact of CRISPR on modern genetics research and attempts to define causal linkages between genetic variation and biological phenotypes cannot be overrated.

Moreover, with unprecedented pace CRISPR tools are moving toward clinical translation in genetic therapies. Such use of the system targets somatic cells in patients without touching the germ line and, thus, does not possess an inherent risk of introducing genetic alterations in future generations. Several examples of preclinical studies showing proof-of-principle promise that genome editing therapies using CRISPR in human stem cells will soon be tested in patients. In fact, in an attempt to treat metastatic non-small-cell lung cancer with T cells harboring knockout mutations in the PD-1 gene CRISPR-edited cells were earlier this fall, for the first time, injected into a person (4), and similar trials for treatment of cancer are on the way in the US.

Although ex vivo genome editing trials are certainly likely to show efficacy first, hopes are that genome editing directly in the patient may one day become an effective and safe alternative to conventional gene addition therapies. Here, by discussing a few prominent examples of CRISPR-based in vivo gene therapy, I hope to give an impression of the current status of in vivo editing as therapy.

Delivering the tools – can we learn from conventional gene therapy?

Despite much frustration over the years, clinical gene therapies are currently showing great promise worldwide. Effective therapies of serious genetic conditions, such as immunodeficiency syndromes (5-7), lipoprotein lipase deficiency (8), hemophilia (9), and blindness caused by inherited retinal disorders (10,11) are recent successes that raise hopes for conventional gene therapies. Optimism in the field is further boosted by the growing involvement of the pharmaceutical industry (big pharma like GlaxoSmithKline and others) (12), which will further accelerate clinical translation of genetic medicines.

One of the great challenges and goals of gene therapy is to achieve persistent, preferably lifelong, expression of a therapeutic gene in a certain organ or cell type. Although nonviral gene delivery systems are showing some promise, gene transfer strategies based on viruses are currently more potent and, thus, often preferred. Virus-based transgene delivery exploits the natural capacity of viruses to carry and transfer genetic information, and viruses are capable of crossing barriers transporting genetic cargo from the blood stream (or from the growth medium in case of cultured cells) across cell and nuclear membranes and into the nuclei of transduced cells.

For delivery of CRISPR tools using viral vectors, a lot can be learned from virus-based delivery systems that have been successfully exploited in clinical gene therapy trials. But it is important to stress that the required properties of delivery strategies used for conventional gene therapy and for CRISPR-based genome editing are fundamentally different. Hence, whereas a primary goal in classical gene therapy is long-term therapeutic gene expression, short-term and potentially regulated intracellular production of Cas9 and sgRNA is likely to support sufficient endonuclease activity. With this in mind, it may not even be necessary to produce the CRISPR components inside the cells. In fact, very promising studies now support the administration of preassembled ribonucleoprotein complexes consisting of recombinant Cas9 protein and synthetic, chemically modified sgRNA (13,14). This strategy is useful for ex vivo editing of genomes in patient-derived stem cells and cultured cells in general, but is not likely to be feasible for in vivo editing directly in the patient.

CRISPR-directed knockout and correction of genes in preclinical gene therapy models

In a perfect world, correction of a disease-causing mutation is achieved in vivo by co-delivery of Cas9, sgRNA, and a donor DNA sequence, the latter which serves as the template for repair by homologous recombination. However, although efficient co-delivery is feasible in relevant tissues, e.g. mouse liver, the overall efficacy of the repair process can be challenged by low levels of homology-directed repair. This would argue that chances for success are highest in diseases in which positive selection of corrected cells occurs. In hereditary tyrosinemia type (HT1), a fatal liver disease caused by mutations in the gene encoding fumarylacetoacetate (FAH), hepatocytes with a reverted or repaired fah gene have a selective growth advantage. Not surprisingly, the first attempts to apply CRISPR for in vivo gene correction were made in a mouse model of HT1, which is homozygous for a fah allele harboring a splice site mutation causing the disease in humans (15). In this study, authors employed hydrodynamic co-injection of Cas9/sgRNA-expressing plasmid DNA and a single-stranded DNA (ssDNA) donor for repair and documented repair in approximately 1 of every 250 hepatocytes. Clonal expansion of repaired liver cells led to rescue of the phenotype, providing an example of in vivo correction of a mutation causing disease in humans.

In a later study, the same authors corrected the same causative splice mutation by co-delivering spCas9-encoding mRNA packaged in lipid nanoparticles with adenovirus-associated virus (AAV)-based vectors carrying a sgRNA expression cassette and the donor sequence for homology-directed repair (16). In this case, correction of the mutation, leading to expression of FAH, was evident in 1 of every 16 hepatocytes after a single injection of the editing cocktail. Repair rates in the same range were observed in mouse studies using AAV-di-
rected delivery of zinc-finger nucleases and the donor template for correction of mutations in the F9 gene causing hemophilia B (17).

As an alternative approach, Pankowicz and coworkers treated HT1 by hydrodynamic delivery of Cas9-encoding plasmid together with plasmid DNA encoding sgRNAs targeting the hydroxysterol 7-steroid 26 hydroxylase (Hpd) gene, which is involved in early steps of tyrosine metabolism (18). By introducing a deletion in the Hpd gene using CRISPR, it was possible to reduce the accumulation of toxic metabolites due to FAH deficiency and thus convert HT1 into a more benign variant of HT. This strategy, referred to as metabolic pathway reprogramming, has the advantage that treatment requires only introduction of knockout mutations rather than precise editing of the disease-causing mutation.

The liver is also the target organ in a trilogy of studies investigating CRISPR-directed knockout of the pcsk9 gene encoding proprotein convertase subtilisin/kexin type 9. The PCSK9 protein binds to the LDL receptor and instigates degradation of the receptor. Individuals with loss-of-function mutations in the pcsk9 gene therefore present with low levels of cholesterol and are less likely to suffer from atherosclerosis late in life.

First, adenovirus-based delivery of the spCas9 and sgRNA genes resulted in >50% pcsk9 gene disruption rate in mouse liver and markedly reduced levels of cholesterol (19). Using a similar delivery approach, the same level of disruption in the human pcsk9 gene was achieved in primary human hepatocytes engrafted in livers of fh−/− knockout mice (20). Essentially the same result (indel frequency >40%) was obtained in studies exploiting AAV as a delivery vehicle of Staphylococcus aureus Cas9 (saCas9) and pcsk9-targeting sgRNAs (21). Importantly, this study also showed that a single AAV vector could accommodate expression cassettes for both saCas9 (shorter than spCas9) and sgRNA.

Three back-to-back reports showing CRISPR-directed restoration of the dystrophin-encoding DMD gene recently caused a stir in the gene therapy community (22-24). In the mdx DMD mouse model carrying a nonsense mutation in exon 23, co-administration of AAVs carrying a saCas9 expression cassette and two sgRNA expression cassettes, respectively, resulted in deletion of exon 23 (23, 24). In muscles receiving the CRISPR components, the deletion rate was as high as 39% (24), leading to expression of a modified dystrophin gene (due to restoration of the open reading frame) and amelioration of dystrophin function in skeletal muscle after a single intramuscular injection. Similar rescue rates and partially restored phenotypes were reported in mdx mice treated with spCas9-encoding AAVs (22).

Challenges ahead – concluding remarks

Although the above-described examples of in vivo editing of genomes only give a little taste of a rapidly involving gene therapy strategy, they serve to illustrate not only the potential but also some of the challenges ahead. Knocking-out genes using CRISPR has therapeutic capacity, but repair processes based on homology-directed repair are still relatively inefficient, and many correction therapies will suffer from too low repair rates. Therefore, efforts to increase or bypass the need of homologous recombination should be prioritized. A recent study documented correction of visual function in a rat model of retinitis pigmentosa exploiting effective DNA knock-in strategies based on homology-independent targeted integration (25), thus bypassing the need for homology-based correction.

Another crucial aspect, which is well illustrated by the above-mentioned examples, is that efficacy seems to depend on potent delivery, which is easiest obtained through virus-based delivery of Cas9- and sgRNA expression cassettes. Gene therapy using CRISPR is going viral. However, this will inevitably result in long-term production of the Cas9-sgRNA complex, in mouse liver probably for months or even years, potentially resulting in off-target activities over time. Packaging of Cas9-sgRNA complexes in bioreducible lipid nanoparticles is an option that may show in vivo applicability in the future (26).

In our own work, we have shown that spCas9 and other endonucleases can be packaged in lentivirus-derived nanoparticles along with the donor template for homology-directed repair (27-30). As such virus-derived protein transporters do not carry the CRISPR genes but just the protein, they will only support short-lived endonuclease activity and may, thus, allow safer genome editing.

Just a few years ago, I would tell the students in my medical genetics classes that gene therapy strategies based on mutation repair were science fiction and far from reality. Well, times are changing, and CRISPR may eventually make it into in vivo genome editing therapies. We are not there yet, but as CRISPR seems to show up in every corner of the biomedical research landscape, focus on clinical translation will intensify in the next few years. The word is out – even in the corridors of Christiansborg.

The full article including acknowledgements and references can be found online on http://www.biokemi.org/biozoom.