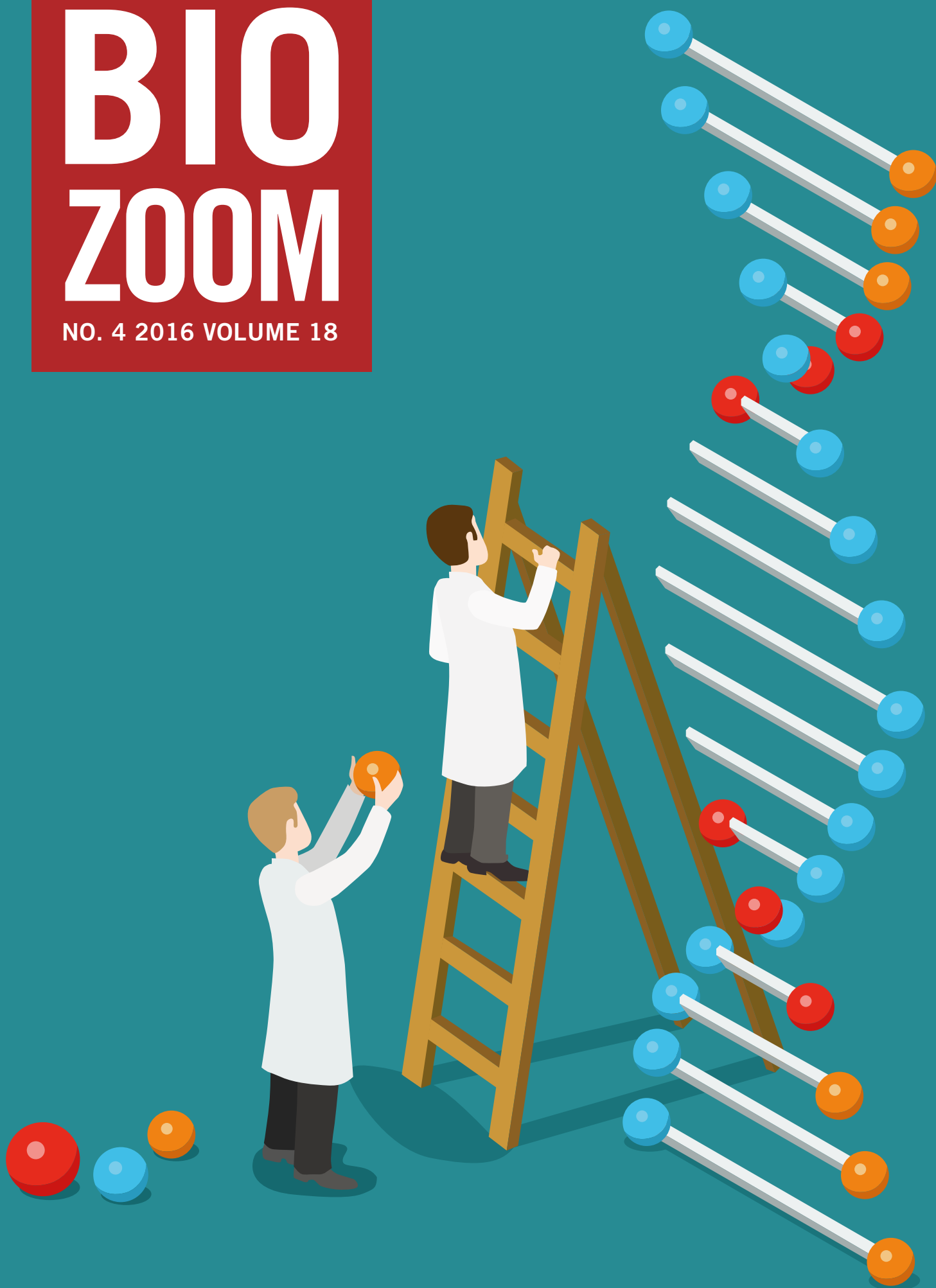


BIO ZOOM

NO. 4 2016 VOLUME 18



BioZoom

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TEMA: CRISPR genome editing**Learning to edit genes in a smart way**

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CRISPR goes viral – *in vivo* gene therapy based on genome editing

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Solving an unmet need in genome targeting – fast, precise and efficient indel detection by amplicon analysis

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Targeting the genome opens doors in plant biology

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Det Etske Råds udtalelse om genetisk modifikation af kommende mennesker

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FORSIDE:

Gene engineering illustration. Genetic lab scientists editing DNA chain. Creative people collection. www.sellingpix.com. Picture: www.shutterstock.com

LEARNING TO EDIT GENES IN A SMART WAY

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The CRISPR/Cas9 gene editing is a fast developing technology in biochemical research fields. The technology was the topic of the succesful 41th Annual Meeting in Danish Society for Biochemistry and Molecular Biology (DSBMB) <http://www.biokemi.org/meetings/118>.

DSBMB/ Biokemisk Forening organized a two day annual meeting entitled "CRISPR genome editing: From high-throughput screening to disease models" together with Yonglun Luo, Associate Professor at Aarhus University, Department of Biomedicine. The meeting was held 22-23 September, 2016 in the Lundsgaard Auditorium at the Panum Building at the premises of the Faculty of Health and Medical Sciences, Copenhagen University. The top-notch scientific meeting on the efficient and

rapid CRISPR gene editing technology gave what it promised: delivered latest information about this powerful method to edit any gene or genome in a basic laboratory setting.

An extensive and comprehensive program

The meeting that was opened by the Dean of Health and Medical Sciences, Professor Ulla Wewer, followed by lectures of eleven international and five

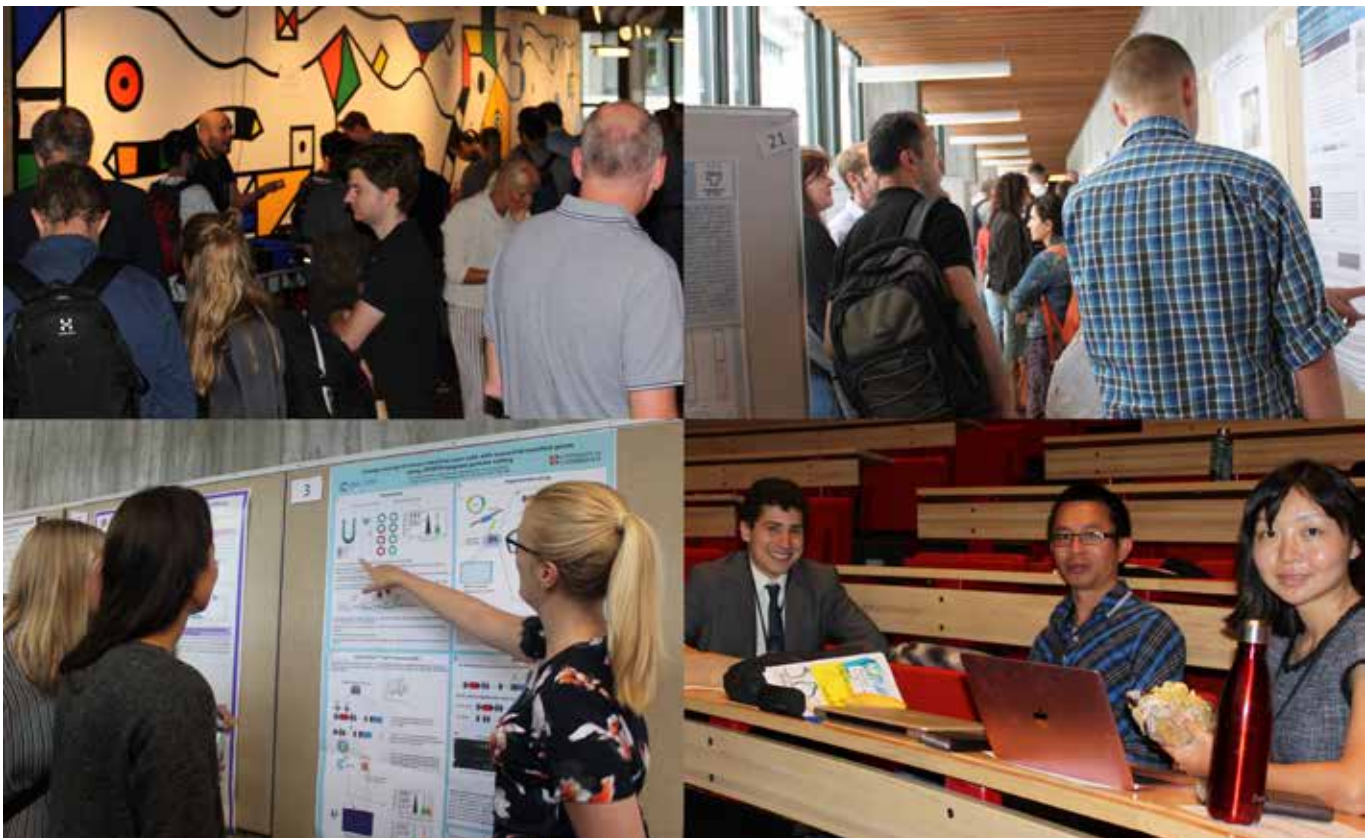


Figure 1: Highlights from the DSBMB annual meeting about CRISPR. Top left: Networking and interactions with exhibitors. Top right and bottom left: Scientific discussions during the poster sessions. Bottom right: Speakers Jonathan Gootenberg, Yonglun Luo, and Luhan Yang.

national scientists attended by almost four hundred registered participants. The program covered the latest development 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.

CRISPR GOES VIRAL – *IN VIVO* GENE THERAPY BASED ON GENOME EDITING

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*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 ribonu-

cleoprotein 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 adeno-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 hydroxyphenylpyruvate dioxygenase (*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 *fah*-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>.

PRECISION EPIGENOME EDITING – PROMISE AND CHALLENGE

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Epigenetic alterations are associated with the pathogenesis of many human diseases such as cancers, diabetes, and neurodegenerative diseases. Chemical treatments have been applied to modify the epigenome as ways of diminishing disease symptoms. However, these approaches are unspecific and cause various unwanted side effects. Recent developments of artificial, programmable epigenetic modifiers based on CRISPR-associated proteins provide new hopes for efficient and precise control, modification of the epigenome, and potentially human disease therapy.

The human epigenome is a highly organized and complex system composed of hierarchical modifications of DNA and histones. These modifications are known as epigenetic marks. Tight temporal and spatial control of these epigenetic marks ensure the proper gene expression and cellular functions. Epigenetic modifications include DNA methylation of cytosines predominantly in CpG dinucleotides, acetylation or methylation of the histone tails, and nucleosome positioning. The modifications play a decisive role in the regulation of gene expression and are linked to the pathogenesis of many human diseases (1).

The molecular mechanisms involved in the dynamic regulation of epigenetic modifications are complicated and are associated with many regulatory proteins and enzymes. However, there are still some so-called sole catalytic enzymes carrying out the key-processing steps of epigenetic modifications. For example, *de novo* DNA methylation is controlled by the DNA methyltransferases (e.g. *DNMT3A*, *DNMT3B*, *DNMT3L*).

The ten-eleven translocation (TET) family of proteins such as TET1 is essential for the initial activation of methylated cytosine (5mC) demethylation. Histone acetyltransferases (HAT) such as P300/CBP family enzymes can acetylate lysine at the histone tails. Histone methylation is catalyzed by a class of enzymes called histone methyltransferase (HMT), which transfer one to three methyl groups to lysine or arginine residues of histone proteins.

Epigenetic-modifying drugs are available, but unspecific

The fundamental correlation between epigenetic modifications and their consequences on gene expression and/or

the pathogenesis of human diseases are based on association studies or the application of epigenetic modifying drugs such DNA methyltransferase inhibitor 5-azacytidine and histone deacetylase inhibitor valproic acid. Several of these epigenetic-modifying drugs are making their way into therapeutics. Valproic acid has been used to treat epilepsy, migraines and bipolar disease for many years. But there are new epigenetic-modifying drugs in the pipeline as well such as *Chidamide* and *Belinostat* which are being approved for treatment of peripheral T-cell lymphoma.

However, most of these drugs generally cause a global epigenome remodeling. For example, treatment of cells with valproic acid results in global histone acetylation, which might have some unprecedented effects on cellular functions. To investigate the biological consequences resulting from specific epigenetic modifications at a certain genomic locus, great efforts have been spent during the last decade to develop molecular tools that can be programmed to specifically modify user-designed genomic loci, also known as "precision epigenome editing tools".

Zink-finger proteins, TALE proteins and the CRISPR-Cas9 are precise epigenome editing tools

To date, there are three classes of such tools available in the precision epigenome editing toolbox. All these tools share one fundamental molecular principle: They are made by fusing epigenome-modifying enzymatic proteins or their catalytic domains to programmable DNA binding proteins. These programmable DNA binding proteins include the zinc-finger proteins (ZFs), the transcriptional activator-like effector proteins (TALE), and the nuclease deficient clus-

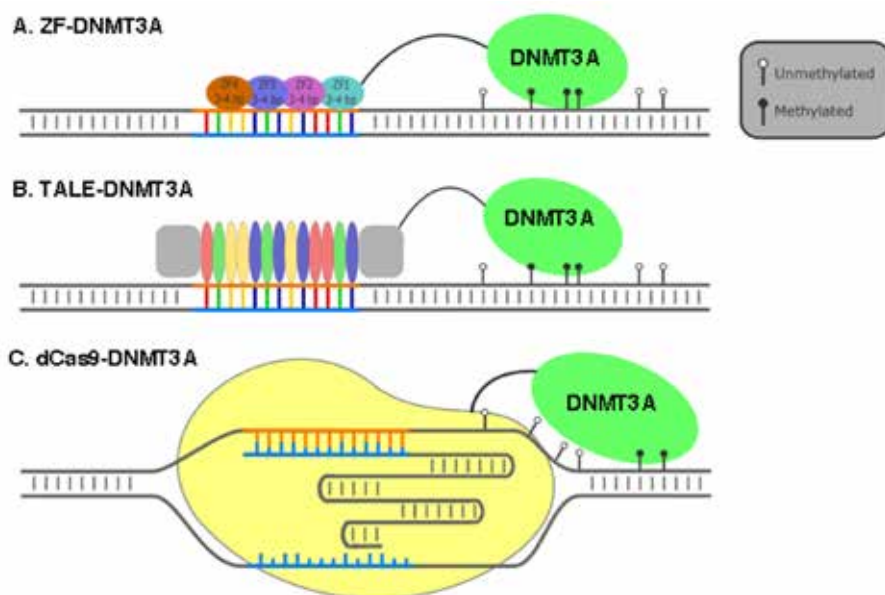


Figure 1. Schematic Illustration of Programmable Epigenetic Editing tools for targeted DNA methylation.

- A) Zinc finger proteins are constructed from a number of zinc fingers bound together each recognizing 3-4 bp in the DNA sequence. The DNA binding domain is fused to e.g. the catalytic domain of DNMT3A with a flexible linker and causes de novo methylation of the flanking CpGs.
- B) The TALE protein consists of a center repeated domain. Each repeat contains usually 34 residues in length. Residues at the 12th and 13th positions are hyper-variable and define the specific binding between TALE protein and the target DNA. Fusion of TALE protein to DNMT3A can achieve target DNA methylation of the adjacent CpGs that the TALE protein binds to.
- C) The CRISPR-dCas9 based epigenome editing tool is based on fusion of dCas9 to DNMT3A. The binding of dCas9-DNMT3A to specific genomic loci is guided by a programmable small gRNA.

tered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (dCas9).

The newly developed CRISPR-Cas9 system was originally discovered as an innate immune system in bacteria and archaea. Through molecular engineering, the bacterial CRISPR-Cas9 system has been successfully harnessed for genome editing in almost all kinds of cells and organisms (2). As illustrated in Figure 1, both ZF- and TALE-based epigenome editing tools are based on the dogma of specific protein-DNA recognition, whereas dCas9-based epigenome editing tools include complementary base pairing between a small, programmable guide RNA (gRNA) and the DNA. Thus, the same dCas9-based fusion proteins with epigenome editing characteristics can be programmed to almost any genomic locus simply by using another gRNA.

On the contrary, the ZF- and TALE-based epigenome editing fusion proteins have to be re-engineered for each specific locus, which is time-consuming, laborious and expensive. This makes harnessing the CRISPR-dCas9 system for epigenome editing the most prom-

ising and attractive tool for studying epigenetics, as well as providing a potential strategy for targeted epigenome therapy.

A promising tool for efficient and precise editing

Precision epigenome editing by CRISPR-dCas9 has been proven promising. This technology provides an alternative, robust approach to modulate gene expression and cell fate. Several studies have shown that targeted epigenetic modifications of specific genomic loci can be achieved through the RNA-guided dCas9-based artificial epigenetic enzymes. For example, dCas9 fused to the N-terminus of the catalytic core of the human acetyltransferase p300, the tetrameric VP16 transcription activator domain (VP64), the krüppel-associated box repressor domain (KRAB), the histone demethylase LSD1, or the DNA methyltransferase domain of DNMT3A have been used for either targeted histone acetylation, histone methylation or DNA methylations (3-5).

All these studies have consistently shown that programmable and tar-

geted epigenetic modifications can be achieved in cells through co-delivery of dCas9-based artificial enzymes and gRNAs. Subsequent effects on alteration of gene expression and cell fate have been achieved, if a critical epigenetic regulatory region is modified.

Still a problem with off-target recognition

However, the applications of CRISPR-dCas9-based precision epigenome editing tools are still facing one critical challenge: Off-targets. Off-targets can result from a combination of dCas9-dependent, gRNA-dependent, or catalytic enzyme-dependent factors. Epigenome editing by dCas9-based epigenetic-modifying enzymes is simply based on the binding of dCas9 to a specific DNA locus. One unique feature of dCas9 is that this protein can rapidly interrogate with DNA through interaction with a protospacer adjacent motif (PAM) across the genome. The PAM sequences are unique for each Cas9 ortholog, e.g. 5'-NGG for the Cas9 protein

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SOLVING AN UNMET NEED IN GENOME TARGETING – FAST, PRECISE AND EFFICIENT INDEL DETECTION BY AMPLICON ANALYSIS

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The development of efficient and precise genome editing tools like CRISPR/Cas9 call for improved methods for fast, precise and in-depth dissection of the insertion or deletion (indels) of bases in the DNA sequence. The traditional methodologies include enzyme mismatch cleavage assay (EMC) and DNA sequencing. EMC lacks sensitivity, resolution and high-throughput capability, and the sequencing of DNA is expensive and time-consuming. The development of the indel detection by amplicon analysis (IDAA) methodology may meet the requirements for fast, precise, sensitive and cost effective indel profiling in genome editing applications.

Figure 1. CRISPR/Cas9 gene editing. Workflow from CRISPR/Cas9 cell delivery, DNA extraction, and indel profiling to insight. Notably, the time in days from CRISPR/Cas9 delivery to DNA extraction normally lasts for 2-3 days, while time from indel analysis to insight depends on the indel detection methodology used and may take from 1 day to more than 5 days.

Gene editing tools

The nuclease-based gene editing tools such as meganucleases, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 are rapidly transforming capabilities for altering the genome of cells and organisms with great precision and in high throughput studies. In Denmark, we were among the first to embark on these technologies in mammalian cells. We used ZFN gene targeting in detailed analysis of glycoproteomes to resolve the extensive heterogeneities in glycan structures and attachment sites. ZFN targeting has been applied to truncate the O-glycan elongation pathways in human cells, generating stable 'SimpleCell' lines with homogenous O-glycosylation (1).

The introduction of CRISPR/Cas9 gene editing is a breakthrough, which has great potential in basic and biomedical research. Clearly, the simplicity and general access to CRISPR/Cas9 reagents has democratized the conduct of precise genome targeting in cells, tissues, organs, and whole animals.

Lack of efficient evaluation methods

A major limitation in application of precise gene editing lies in the lack of sensitive and fast methods to detect and characterize the DNA changes intro-

duced by CRISPR/Cas9. Precise gene editing induces double-stranded DNA breaks that are repaired by non-homologous end joining, which is error-prone, leading to introduction of indels at the target site. These indels are often small, difficult, and laborious to detect by traditional methods (2). This limits screening, detection and selection of the desired targeting events in cells, tissues and whole animals. Furthermore, the detection of indels induced by CRISPR/Cas9 should be accomplished by an automated high-throughput methodology that easily integrates into routine genome editing workflows. Ideally, the time required for indel profiling of a CRISPR/Cas9 targeted DNA sample should be as short as possible (Figure 1).

Current methods for accurate identification of the genetic modifications are few, laborious and require extensive screening of clones in order to identify correctly edited bi-, tri- or multi allelic genes. Moreover, the indel detection methods including next generation sequencing (NGS), Sanger sequencing and EMC lack sufficient speed and sensitivity (Figure 2).

In order to meet this challenge we have developed methods and protocols for fast, sensitive and cost-efficient detection of indels induced by precise genome targeting (2-4). Through the Consortium for Designer Organisms at the University of Copenhagen (<http://>



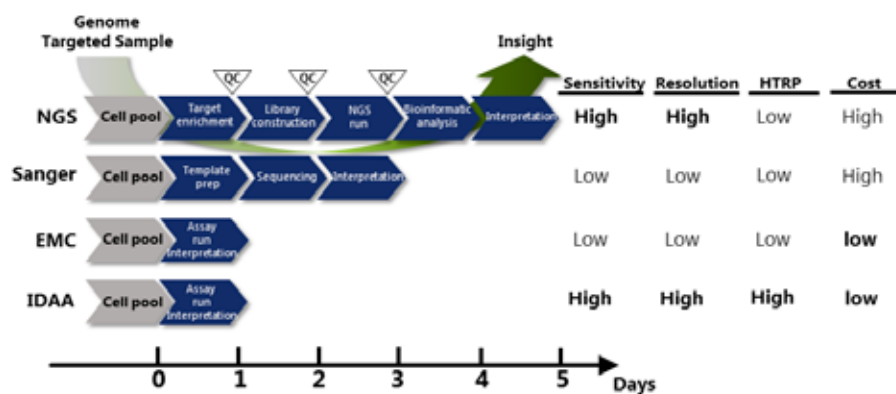


Figure 2. Indel detection analysis in genome targeting. Assay time and performance for four indel detection methods: NGS, Sanger sequencing, EMC and IDAA. Time in days on axis shown below for the required steps (blue hexagons) for each method from genome targeted cell pool (grey hexagon) to insight. QC (white triangle) represents necessary quality control steps. The table shown to the right displays the performance (high or low) of the respective methods with regard to sensitivity, resolution, high through-put amenability (HTRP) and cost. Preferred choices are shown in bold font.

www.cdo.ku.dk/), these methods have successfully been made available to national and international academic and biopharmaceutical groups working in the field of genome targeting.

Indel detection methodologies

The choice of indel detection methodology to be used is primarily determined by the scope of the experiment and available analytical resources. Currently, only a few methods enable assessment of the full spectrum of repair products in a cell population following CRISPR/Cas9 cutting. Although evaluation of indel sequences by NGS methods may be the best route to assess the full spectrum of CRISPR/Cas9 induced repair events, the time consumption and high cost constrain widespread adoption of this method. Therefore, the most commonly used indel detection method is the EMC assay (5).

Enzyme Mismatch Cleavage assay

EMC assays are based on selective endonuclease recognition and restriction/cleavage of heteroduplex DNA (not homoduplex DNA) formed after reannealing of heterogeneous amplicons possessing different nucleotide variations, including indels (5). Thus, the readouts of EMC assays are the cleaved amplicon fragments detected following endonuclease treatment of samples. EMC assays only require gel based electrophoresis and commercially available endonucleases such as T4 endonuclease VII (T4E7), endonuclease V (EndoV), T7 endonuclease I (T7EI), CELI or Surveyor nuclease.

However, the efficiency of EMC has been shown to depend on the proper-

ties of the mismatch-cleavage endonuclease. In particular T7EI and Surveyor possess a strong nonspecific activity and display a preference for heteroduplex DNA formed by deletions rather than single point mutations (4). Furthermore, CRISPR/Cas9 genome targeting predominantly generate minor indels (4,6), and endonuclease T7EI poorly discriminates the presence of single base deletion events (2). Therefore, EMC assays underestimates the editing efficiency in cells following CRISPR/Cas9 genome editing experiments.

DNA Sequencing

Optimal discrimination of DNA fragments down to single base differences has until recently only been possible by Sanger sequencing and NGS. By these approaches, target derived amplicons are cloned into plasmids or sequenced directly followed by bioinformatic deconvolution of the individual sequences and indels identified. Procedures such as tracking of indels by decomposition (TIDE) (<https://tide.nki.nl/>) have streamlined the workflow of amplicon cloning and sequencing. However, indel profiling is limited by indel size, lack of sensitivity and resolution for indel profiling of samples with high indel complexity (such as indels in cell pools) and is dependent on pairs of high quality Sanger sequencing data.

NGS is a powerful and widely accepted method for in-depth indel identification of a targeted genomic locus, but requires laborious, multi-step, skillful preparation of the amplicon libraries to be sequenced, and complex bioinformatics data analysis (Figure 2). Several online resources have been developed for indel quantification

and characterization of NGS data, including CRISPR Genome Analyzer (<http://54.80.152.219/>), CRISPResso (<http://crispresso.rocks/>) and CRISP-R (<https://bioconductor.org/packages/release/bioc/html/CrisprVariants.html>), that all provide a complete reports containing all NGS information.

Indel detection by amplicon analysis methodology

To meet the requirement for fast, precise, cost-efficient indel detection with down to single base discrimination, we turned to a sequenator based application based on fragment analysis (FA) commonly applied to linkage, microsatellite instability and loss of heterozygosity mapping and SNP genotyping. We have recently shown that by combining FA with a simple amplicon labelling strategy for unbiased and simple homogeneous fluorophore labelling of amplicons, we enabled fast, sensitive and robust profiling of indels induced by CRISPR/Cas9 in cell clones, cell pools and whole animals (2-4). We showed in a "head to head" comparison of IDAA vs. NGS that the sensitivity and resolution of IDAA is comparable to NGS with an indel detection sensitivity $\approx 0.1\%$ (4). Furthermore, IDAA is robust generating near identical profiles on independently repeated experiments. Finally, IDAA can be used for indel profiling regardless of the nature of the tools used to induce the indels analyzed, i.e. ZFNs, TALENs or CRISPR/Cas9.

An example of a typical CRISPR/Cas9 experiment analyzed by IDAA is shown in Figure 3. As can be seen, individual indels are clearly identified as single peaks within the chromatogram. Of notice, the most frequently occurring indel

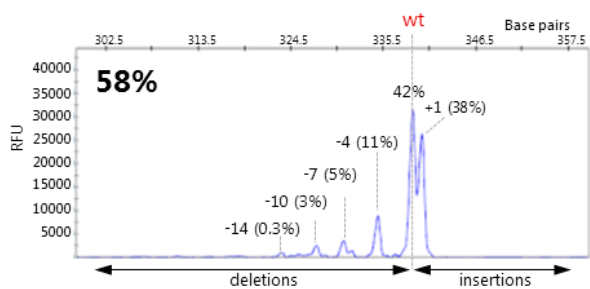


Figure 3. IDAA profile. The profile was generated from CRISPR/Cas9 targeting of the *cosmc* gene using our recently published protocol (4). Lipofectamine delivered CRISPR/Cas9 plasmids of CHO cells was followed by; 1) DNA extraction 2 days post transfection, 2) tri-primer fluorophore PCR amplification and 3) IDAA analysis. The turnaround time for full IDAA analysis from sample to insight is 5 hours, see Figure 2. Individual indels can be quantified, with individual indel frequencies shown at the peaks. A wild-type peak is marked by wt (red). Furthermore, the validated cutting efficiency of the CRISPR/Cas9 design used (58%) in this example is shown in bold in the upper left corner.

is a one base insertion, observed with 38% frequency. Rare deletion indels are found at lower frequencies. The total number of indels is estimated to 58% and thus, the CRISPR/Cas9 genome targeting efficiency (i.e. cutting efficiency) is 58%. Furthermore, 42% of the cellular targets (alleles) remain intact in size, which may be an acceptable level of un-targeted alleles depending on the aim of the genome targeting experiment conducted. Lastly, the 96-plate format of IDAA makes the assay ideal for high through-put genome targeting applications.

The chemistry of the assay is cost-efficient, requiring cheap and commercially available reagents in the form of primers available in a kit format (<http://tagc.dk/>). Downstream analysis requires commonly available DNA

sequenator instrumentation, often not used for Sanger sequencing anymore, but stashed and hidden away in laboratory store rooms. Alternatively, the latter analytical part can be handled by custom service providers of IDAA analysis (<https://www.eurofinsgenomics.eu/5746.aspx>), by shipment of the fluorophore labelled amplicons for analysis and return of data profiles similar to the profile shown in Figure 3. Raw data files can easily be reanalyzed using freely available software, as described in ref. 4.

The recent development of highly efficient precise genome editing tools, such as ZFN's, TALEN's and CRISPR/Cas9, call for improved methods for fast and in-depth dissection of the changes in DNA sequence. EMC lacks sufficient sensitivity, resolution and

high through-put capability and DNA sequencing is prohibitively expensive and time consuming for routine use in research laboratories. The development of the IDAA methodology may meet the requirements for fast, precise, sensitive and cost-effective indel profiling in basic research and more challenging genome editing applications such as therapeutic indel profiling.

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TARGETING THE GENOME OPENS DOORS IN PLANT BIOLOGY

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CRISPR/Cas9 is the new rising star among methods in molecular biology. The technique is a nuclease that is pre-programmed to identify a specific DNA sequence and cleave it. This ability to cut DNA in a selected location enables the molecular biologist to make use of the DNA repair mechanisms native to all cellular life. CRISPR gene editing hit plant biology three years ago, and since then the tool has been promising for application in plant research including basic science and targeted plant breeding in agriculture.

DNA repair mechanisms

When genomic DNA is broken in a living cell, mechanisms will activate to repair the break. These mechanisms are broadly labeled non-homologous end joining (NHEJ) and homology directed repair (HDR). Non-matching ends of broken DNA strands are joined, and the breaks are repaired by copying from any similar DNA that might be nearby – most often the complementary strand on the second copy of the same chromosome. In common for these repair mechanisms is that they can be used for editing the cleaved sequence.

In the case of NHEJ, the joining of non-homologous strands is error prone, and often leads to insertions, deletions, or substitutions of DNA in the programmed cut site. These are fairly random, although they most often tend to be insertions or deletions of 1 to 20 base pairs. HDR is used to deliver a partially complementary DNA strand to the cut site, enabling the cellular repair mechanisms to repair the Cas9 induced cut, by copying off the delivered DNA strand. The sequence around the programmed cut site is altered by altering the delivered DNA strand *i.e.* genome editing.

CRISPR revolutionizes programmable nucleases

Programmable nucleases have been examined since the 1980s, and include systems such as the zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). ZFNs and TALENs are able to target and

cleave double stranded DNA. However, they are more demanding to program, as the DNA recognizing entity consists of subsets of nucleotide-recognizing peptide sequences. Each of such newly programmed nucleases requires the *de novo* construction of a specific peptide.

The CRISPR system was a revelation when it was published in 2012 (1,2). Programming of CRISPR/Cas9 requires only the fusion to a RNA strand complementary to the required cleavage site. The Cas (CRISPR associated) protein remains the same. This reduces the cost of programming for the endonuclease, going from a series of cloning events to construct a new protein, to simply design and order an oligonucleotide and clone it into a predesigned plasmid.

The CRISPR/Cas9 programmable nuclease system is derived from the prokaryotic adaptive type II immune system. It uses the ability of subtypes of the Cas protein family to function as RNA guided endonucleases, when merged with a specific type of guide RNA. In the bacterial immune system, the Cas systems are preprogrammed with virus DNA recognizing RNA from previous viral infections, and render immunity against previously experienced virus by recognizing and cleaving viral DNA as soon as it enters the cell. In 2013 the first applications of targeted mutagenesis in model and crop plants using the CRISPR/Cas system in plant genome editing were published (e.g. 3). Already in this first wave of publications, plants were transformed with the CRISPR/Cas systems using both NHEJ and HDR.

Plant CRISPR in practice

Practical usage of the CRISPR/Cas system in plant genome editing has identified two hurdles. First, NHEJ is much more feasible than HDR since it is easier to use CRISPR as a hammer on a gene by destroying it than to do text editing by introducing single point mutations. Furthermore, introducing recessive mutations that confer loss-of-function by deleting the gene is easier than introducing dominant mutations leading to gain-of-function genes with new abilities. Second, when introducing the CRISPR/Cas9 machinery into plants by transformation, the resulting plant often contains a mixture of transformed and non-transformed plant cells. When mosaic plants develop it is not certain that the germ cells contain the construct and that the introduced changes are inheritable.

Use of knockout plants for basic research

Homologous recombination into plant genomes has historically had low success rates. Accordingly, knockout plants, where specific genes have been turned off, are investigated by using plants from large gene banks of randomly generated mutant plants. Such libraries of plant mutants where the affected gene has been identified serve as a biological resource center for researchers studying the function of specific plant genes. However, aside from well-established model plants such as *Arabidopsis thaliana* (Figure 1), where mutant libraries are well established, the access to mutant libraries of other plant species is limited.

The ability of CRISPR-Cas9 gene editing to knockout genes directly, has the potential to replace the construction of new plant mutant libraries in basic plant research. An example is the use of CRISPR-Cas9 in the examination of symbiotic nitrogen fixation related genes in *Lotus japonicus*, a group of plants where only a few mutant lines exist (4).

Another inherent issue in plant research using knockout mutants is the complex ploidy level of several main crop plants. Bread wheat (*Triticum aestivum*) is hexaploid and has several copies of the same genes. Investigation of the role of specific genes using gene inactivation often requires knockout of all gene copies to see an effect. The task

of generating plants with knockouts of all copies of a gene is an arduous task. In contrast, the design of programmable nucleases enables to target DNA sequences that are common for all copies of the same gene in order to acquire a full knockout plant.

Use of CRISPR in agriculture

Although the field of modern plant breeding encompasses a large array of methods to achieve crops of interest, it is in part based on the examination of randomly mutagenized mutant plants and the focused breeding of desired qualities that emerge in these mutants. Random mutations have for a long period of time been uncharted and solely selected based on the resulting traits. Genetic analysis has led to understanding of the underlying genetic traits that cause mutations we deem desirable in crops, and which we in part determine as domestication traits. It also stems from the knowledge accumulating from research of knockout plants, especially in the major model crop plants such as rice, maize and wheat. This information enables plant breeders to target genes for knockout by genetic screening of mutant libraries.

Use of the CRISPR/Cas9 gene editing holds the potential to speed up plant breeding efforts significantly. The use of this information has already been used to abolish mildew infection on hexaploid wheat, by knock out of the host recognition factors that enable the mildew fungus to recognize wheat as its host. Furthermore, targeting genes responsible for production or transport of toxic plant compounds can be used to create less toxic versions of crops like quinoa and cassava that today require lengthy processing before they are ready for human consumption.

As the CRISPR technology matures in plant biology, it holds potential to perform feats of genetic engineering that will likely enable breeders to create desirable traits from ancient crop types, such as fertilization requirements or disease resistance (5). CRISPR gene editing is still young in the plant field, but the coming decade will likely yield a plethora of applications that might change both the way crop plants are made and which crops we grow.



Figure 1: Seeding *Arabidopsis thaliana*. *A. thaliana* is most widely used model species for plants. Photo: Jeppe Thulin Østerberg.

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DET ETISKE RÅDS UDTALELSE OM GENETISK MODIFIKATION AF KOMMENDENDE MENNESKER

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Med fremkomsten af CRISPR har de etiske diskussioner om at genmodificere mennesker fået nyt liv. Hvis man sammenligner med debatten i 1990'erne, hvor verdens befolkninger først fik øjnene op for genteknologiens muligheder og faldgrubber, er dagens debat både mere ydmyg (i forhold til genetikens kompleksitet) og mere nuanceret (i forhold til muligheder og risici ved teknikken). I Det Etiske Råds udtalelse fra 2016 åbner et mindretal for, at sygdomme bør kunne fjernes fra kommende menneskers arveanlæg, hvis det bliver teknisk muligt

Udtalelsen kom i april 2016 (1) efter omtalen af det første kinesiske forsøg med at modificere menneskelige embryoner ved hjælp af CRISPR/Cas9 med det formål at ændre det gen, som er ansvarligt for den arvelige blodsygdom talassæmi (2). Som bekendt vakte forskningen international opmærksomhed ikke mindst på grund af de etiske implikationer ved at forskere for første gang havde modificeret menneskelige embryoner – ikke som led i grundforskning, men med det langsigtede mål at ændre kommende menneskers sygdomsdispositioner.

Dermed kunne forskningen ses som det første skridt i retning af at gøre op med den konsensus, der indtil nu har været i forskningsmiljøer, i etiske råd og blandt lovgivere, og som senest blev udtrykt af International Society for Stem Cell Research (ISSCR) i deres Guidelines for Stem Cell Research and Clinical Translation: *“Until further clarity emerges on both scientific and ethical fronts, the ISSCR holds that any attempt to modify the nuclear genome of human embryos for the purpose of human reproduction is premature and should be prohibited at this time”* (3).

Et mindretal åbner døren på klem for ændringer af humane gener

Rådets anbefalinger kunne også ses som et begyndende opgør med denne konsensus, men det er dog vigtigt at være opmærksom på, at det kun er et mindretal på 6 ud af Rådets 17 medlemmer, som mener, at der ikke vil være principielle problemer i at fjerne sygdomsanlæg før fremfor efter fødslen, hvis det på et tidspunkt skulle blive muligt. Samt

at dette mindretal *ikke* anbefaler at gå i gang med sådanne modifikationer på nuværende tidspunkt, men først når der udvikles sikre metoder til at fjerne sygdomsanlæg fra kønsceller eller befrugtede æg.

Alligevel kan udmeldingen opfattes som kontroversiel, det er til vores kendskab første gang, medlemmer af et etisk råd har anbefalet at foretage – meget snævert definerede – ændringer, som vil gå i arv til kommende generationer.

Hele Rådet er enigt om at lægge stor vægt på de risici, som manipulation med kommende mennesker vil indebære, blandt andet som følge af den mangelfulde viden om *off-target* effekter og ukendte langtidsvirkninger ved at ændre på arveanlæggene. Men mindretallet mener, at såfremt disse risici kan bringes ned på niveau med, hvad der gælder for andre behandlinger, så bør man ændre loven, så det bliver lovligt at fjerne sygdomsdispositioner på kønsceller og embryoner.

Principielle argumenter mod genmanipulation er stadig aktuelle

Flertallet mener, at der stadig er principielle argumenter, som har været fremført mod at ændre på arveanlæggene. Mindretallet mener derimod, at disse argumenter ikke er tungtvejende imod anvendelse af ændringer af de humane gener. Det drejer sig om fire argumenter:

NATURENS ORDEN: Det fremføres ofte, at den genetiske variation i befolkningen er der af en grund; for nogle er grunden, at denne orden er skabt af Gud, andre vil lægge vægt på, at natu-



Figur 1. Designer baby. Kilde: George Dvorsky: 'Here's Why We'll Eventually Have to Accept Designer Babies', gizmodo.com. <http://gizmodo.com/heres-why-we-ll-eventually-have-to-accept-designer-babi-1746216328>

ren i sig selv er styret af en orden eller nogle mekanismer, hvis kompleksitet overgår menneskers forståelsesevne. I begge tilfælde mener fortalerne, at mennesker ikke bør udfordre denne naturens orden, de bør indstille sig på, at der er grænser for, hvor indgribende de bør agere i forhold til naturen, og at der er visse ting, som er for komplekse og uoverskuelige for mennesker at blande sig i. Genetisk manipulation udgør efter flertallet i Rådets mening sådan en grænse.

Mindretallet mener derimod ikke, der er en bestemt grænse for, hvor langt mennesker må gå i deres manipulation af naturen. Vi manipulerer hele tiden med naturen, og grænser må derfor hele tiden sættes afhængigt af vores vidensniveau. Vi bør først gå videre med genetiske sygdomsinterventioner, når vi ved mere om risici og konsekvenser af de enkelte indgreb. Men når vi har denne viden, bør vi tage skridtet, fordi det vil gavne mange mennesker at kunne fjerne sygdomme.

BIOLOGISK MANGFOLDIGHED FREMMER TOLERANCEN: Et andet

argument, som flertallet i Rådet tilslutter sig, tillægger også den eksisterende orden i naturen værdi. Her fokuseres dog især på, at hvis antallet af afvigelser mindskes, hvis næsten alle var raske både fysisk og mentalt, vil vi miste forståelsen for det, der var anderledes, og vores tolerance overfor sygdom og svaghed vil mindskes.

Mindretallet er enig i, at det er vigtigt at fremme tolerance, men sygdom er ikke nødvendigt for, at et samfund kan være tolerant. Selvom man kunne fjerne alle sygdomme, ville der stadig være masser af afvigelser mellem mennesker, som kunne befordre tolerance. Derfor bør man ikke ofre individers mulighed for at leve det bedst mulige liv, dvs. liv uden sygdom, for at opnå et overordnet gode som tolerance for det anderledes.

AFVEJNING AF HENSYN TIL KOMMENDE BØRN OG TIL FORÆLDRE:

For flertallet i Rådet har det betydning, at det ikke er nødvendigt at ændre i arveanlæggene hos kommende børn, fordi der findes alternativer for par, hvor én eller begge er disponeret for alvorlige, arvelige sygdomme. Alternativer

som ægsortering (prænatal genetisk diagnostik), donation af kønsceller fra raske, adoption, eller slet ikke at få børn. Hvis man skal afveje hensynet til parret mod hensynet til det barn, der vil komme ud af de genetisk modificerede kønsceller eller embryoner, bør man lægge stor vægt på de store usikkerheder, der er i forhold til uforudsete bivirkninger for barnet.

Mindretallet finder derimod, at der bør lægges stor vægt på, at det er vigtigt for mange mennesker at kunne få deres egne børn, derfor bør denne mulighed også gives til dem, der har været så uheldige at blive født med alvorlige sygdoms anlæg. Syge mennesker er i forvejen stillet i en vanskeligere situation, end raske. Derfor bør samfundet forsøge at hjælpe dem til ligesom raske mennesker at kunne få deres egne børn. I stedet bør man tilgodese det kommende barn ved at forsøge at fjerne sygdoms anlæggene, hvis det er muligt.

GLIDEBANE MOD BEHANDLING AF NORMALE EGENSKABER: Vanskelighederne ved at trække grænsen mellem sygdom og normalitet er endnu

en grund for flertallet til at afvise brugen af genmodifikation på kommende mennesker. Medlemmerne frygter en glidebaneeffekt, hvor grænserne for, hvilke ændringer, man vil acceptere, hele tiden vil rykke sig i retning af stadig mere betænkelige ændringer. Derfor bør man i udgangspunktet slet ikke bevæge sig ned ad denne vej ved at forsøge at fjerne sygdomsanlæg hos kommende mennesker.

Mindretallet er enigt i, at det kan blive en udfordring at drage den præcise grænse mellem sygdomsfjernelse og forbedring af normalegenskaber, og at man ikke bør forsøge at ændre kommende børns normale egenskaber. De er dog samtidig uenige i, at åbning for genmodifikation til sygdomsbehandling vil føre til den nævnte glidebaneeffekt. Det er et vilkår for sundhedsvæsenet, at der hele tiden skal trækkes sådanne grænser; det skal der også i dag, men med de rette kontrolinstanser vil det være muligt at håndtere disse grænse-dragninger, også hvor det drejer sig om genetiske ændringer.

Risiko for uforudsete virkninger holder udviklingen tilbage

For mange er risikoen for uforudsete virkninger på ikke alene forsøgspersoner, men også for deres efterkommere, altså *det* afgørende argument mod at anvende genteknologi til at fjerne sygdomsanlæg fra kommende mennesker. Derfor bliver det formentlig afgørende for manges etiske stillingtagen, om der kan udvikles sikre og effektive behandlinger på kønscelle- eller embryostadiet. Det kan også få betydning, om der kan udvikles sikre og effektive behandlinger på somatiske celler, hvilket vil omfatte de syge celler/organer på det fødte individ. Hvis dette bliver tilfældet, vil behovet for at ændre på arveanlæggene på kønscelle- eller embryonstadiet blive mindre.

Ved den nyligt afholdte kongres i European Society of Human Genetics (ESHG) var der en session om 'CRISPR germline gene-editing' og etik. Kelly E. Ormond, Genetics Department, Stanford University, USA fremlagde et

udkast til en erklæring fra American Society of Human Genetics (ASHG), at det er for tidligt at udføre *germline* gen-editering med graviditet som formål, hvilket er i overensstemmelse med udtalelsen fra flertallet i Etisk Råd (1).

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from *Streptococcus pyogenes*. Thus, if a highly active catalytic domain was fused to dCas9, the transient interaction between dCas9 and DNA might create substantial un-intended modifications of the epigenome. Furthermore, the catalytic domains of epigenetic modifying enzymes *per se* could also unspecifically modify the epigenome, simply due to random protein-DNA interaction.

Studies based on chromatin immunoprecipitation (ChIP) with massive parallel DNA sequencing (ChIP-seq) have revealed that dCas9 can still

strongly bind to DNA even with over 10 mismatches between gRNA guide sequences and the off-target locus. Consistent with this, we have observed all aforementioned off-target effects in an on-going project in which we harness the dCas9-DNMT3A fusion protein for targeted inhibition of oncogenes in cancer cells.

The ultimate aim of these epigenetic tools is not merely for perturbation of gene functions but for precision epigenetic therapy of human diseases such as cancers. A better understanding of the

cause of these off-target effects might enable us to further improve epigenome editing tools with higher specificity and efficacy.

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