CRISPRMED25 Abstract Book

Supported by

frontiers
Frontiers in Genome Editing

Access CRISPRMED25 Abstracts

145 - CHOPOFF: symbolic alignments enable fast and sensitive CRISPR off-target detection

Kornel Labun[1]

[1]University of Bergen, Norway

CRISPR/Cas is a revolutionary technology for genome editing. Although hailed as a potential cure for a wide range of genetic disorders, its adoption faces severe challenges due to unintended off-target editing. Predicting these off-targets is difficult and necessitates trade-offs between speed and sensitivity, and we show that some tools fail to recover even those they claim to be able to find. Here, we develop the original concept of symbolic alignments to efficiently identify off-targets without sacrificing sensitivity. We also introduce data structures that allow a near-instant alignment-free probabilistic ranking of guides based on their off-target counts. Implemented in the tool CHOPOFF, these innovations support mismatches, bulges, and genomic sequence variation for personalized genomes while outperforming state-of-the-art methods in both speed and accuracy.

The CHOPOFF is available at https://github.com/JokingHero/CHOPOFF.jl

129 - Alexandria genome-wide CRISPR knockout libraries for optimized CRISPR screening

Elena Vialetto[1], Martin Wegner[1], Jessica Martyn[1]

[1]Vivlion GmbH

At Vivlion, we continually strive to enhance our CRISPR library solutions. We're thrilled to introduce Alexandria, our latest genome-wide pooled CRISPR knockout library. Alexandria encompasses approximately 1,800 newly annotated genes that other reagents have overlooked, broadening the scope of genetic research. Available in both single-targeting and our unique fixed-pair formats, the fixed-pair approach delivers two sgRNAs targeting the same gene to a cell simultaneously, boosting gene editing efficiency. We've selected genome-wide sgRNAs for consistent performance across various cell lines, reducing variability and increasing confidence in identifying key genes. All Vivlion libraries feature an uniform distribution of sgRNAs, ensuring their representation in CRISPR screens. These features enable the detection of more targets, even with lower coverage. By combining advanced CRISPR screening techniques with high-quality reagents like Alexandria, researchers can more effectively identify promising targets, explore phenotype mechanisms, and accelerate the development of new therapies.

126 - Variant-aware off-target analysis for informed guide RNA selection

Amandine Girard[1]

[1]MaxCyte, Inc.

Vijetha Vemulapalli, Shankaracharya, Alyssa Ferreira, Divya Ravindran, Edward A. Salinas, Ashley Lester, Elijah E. Dabney, Andrew Hollinger, Douglas R. Smith, Thomas E. Mullen

Background: The specificity of CRISPR-based gene editing is critical for therapeutic success and safety. Off-target activity may cause unintended modifications, potentially disrupting normal gene function or introducing harmful genetic alterations. Careful consideration of off-target effects during guide RNA selection and development can significantly reduce these risks. It is also important to account for genetic diversity within human populations when evaluating off-target effects to effectively assess and mitigate risks in target patient populations. In this study, we combined in-silico and biochemical methods to implement a variant-aware approach for selecting guide RNA (gRNA) for editing the PCSK9 gene while considering population-scale genetic diversity. We then utilized the ONE-seq assay to obtain in-depth biochemical data, to thoroughly characterize off-target risk.

Methods: As described below, we generated data for guides targeting the PCSK9 gene using the CRISPR-Cas9 nuclease system while considering common variants in the major human superpopulations.

• Guide Profiler™ - This in silico computational tool was used to assess off-target risk profiles for eight candidate gRNAs against the human reference genome (Hg38) and 3,502 haplotype-phased genomes. The guides were ranked based on off-target burden, functional annotation, and proximity to coding and regulatory regions. To evaluate the tool's utility in ranking off-target risks, PCSK9-1, PCSK9-3, and PCSK9-4 were selected for further screening.

• Guide Select™ - A multiplexed, variant-aware biochemical assay was performed for these three guides, covering all off-target sites with an edit distance of 4 from the guide sequence. The assay identified PCSK9-1 as the most specific, based on cleavage efficiency and off-target activity across diverse genetic backgrounds.

• ONE-seq™ - A high-sensitivity, variant-aware biochemical assay was performed for a comprehensive off-target risk assessment of PCSK9-1, mapping cleavage sites genome-wide and prioritizing high-risk loci through biochemical validation and deep sequencing.

Results: Guide PCSK9-1 was ranked highest by the Guide ProfilerTM among all the guides analyzed. The biochemical screen using Guide SelectTM provided results consistent with the in-silico analysis, identifying the fewest off-targets for PCSK9-1, confirming it as the

optimal candidate. ONE-seq™ provided deep risk characterization, confirming minimal cleavage at high-risk loci and suitability for therapeutic applications.

Conclusion: This variant-aware, multi-step approach is a practical strategy for gRNA selection, systematically reducing off-target risk at each stage. By integrating computational and biochemical assays with population-scale variant analysis, we improve confidence in therapeutic genome editing and regulatory readiness.

124 - Non-viral delivery of complex cargos for large-scale T cell manufacturing

Valeria Annibaldi[1], Melanie Homberg[1], Andrea Toell[1]

[1]Lonza

In recent years non-viral methods for cell engineering have emerged as promising alternatives to viral transduction. A significant advancement was achieved in 2023 with the FDA approval of the first cell therapy drug created utilizing a genome editing technology, based on CRISPR/Cas9.

Amongst the vector-free technologies, electroporation is considered the gold standard, with an increasing presence in clinical trials[1]. Electroporation is a versatile technique that can be used to induce transient gene expression or stable transgene integration, depending on the cargo system delivered into the cells.

Historically, non-viral cell transfection methods, including electroporation, have been reported to suffer with poor scalability and lengthy optimization, in some cases linked to unsatisfactory transfection efficiency at large scale.[2,3] An ideal technology platform should enable process development and optimization at small scale, with minimal effort required for scaling up and for translation to clinical settings.

We developed an improved cartridge, with a novel design for the electroporation of complex cargos into large volumes of T cells using proprietary large volume electroporation system. The optimal cell handling parameters were developed using 100 uL reactions, and then adapted up to 20 mL, in large scale. Data show that cargos such as mRNA and plasmid DNA were delivered individually or co-transfected with high efficiency and equivalent performances to the small scale reference. Moreover, gene editing tools such as CRISPR/Cas9 RNPs and linear DNA HDR templates (1.5-3.5 kbp) were effectively electroporated to knock out or knock in genes of interest, at level analogous to small scale. Results illustrate that with the novel cartridge one billion primary human T cells were successfully gene edited using CRISPR/Cas9 knock in. For the electroporation reaction, we used cell densities of 50 and 100 million/mL and processed 10 mL or 20 mL cell suspension respectively. As the cargo was dosed per unit volume, the higher cell density offered an additional advantage on cost savings, by halving the Cas9 and DNA template material requirements. Cells proliferated after transfection, reaching a 40-fold expansion over 7 days in culture. Finally, we show that we achieved similar editing efficiency using different starting materials, i.e. PBMC or pan T cells, in resting or activated status prior to transfection.

123 - Accelerating cell and gene therapy success with cGMP-compliant gene editing components (Industry Talk)

Juliana Campo[1]

[1]GenScript Biotech (Netherlands) B.V.

The manufacturing of gene editing (GE) components such as single guide RNA (sgRNA) and homology-directed repair (HDR) knock-in templates is a pivotal aspect of GE-based CGT development, requiring meticulous attention to chemistry, manufacturing and controls (CMC; product quality) and regulatory compliance (quality management system) throughout the development lifecycle.In this presentation, we will explore key differences between GE components used from early discovery through to clinical application and share insights into how phase-appropriate components can be used to achieve clinical objectives while balancing quality and cost considerations. We will share also case studies and experimental data that address common challenges such as off-target insertions and the impact of component degradation and strategies that are implemented to reduce these concerns. We will also introduce GenScript's proprietary technologies, state-of-the-art manufacturing and QC facilities, analytical capabilities that are instrumental in characterizing CGT components and introduce the scientific and regulatory support available for investigational new drug (IND) submissions, which is crucial for the successful navigation of the complex pathway to clinical trials.

113 - INDUCE-seq®: Ensuring the safe development of cell and gene therapies by gene editing (Industry Talk)

Simon Reed[1, 2], Felix Dobbs[1], Patrick van Eijk[1]

[1]Broken String Biosciences, [2]Cardiff University

*In collaboration with members of the HESI CT-TRACS consortium.

Advances in genome editing are making it increasingly possible to develop new cell and gene therapies. Synthetic genes and modifications to existing ones can be made in a site-specific manner, while immune-compatible cells created for use in allogeneic patients enable production of 'off-the-shelf' cellular therapies.

However, gene editing is a new way of treating disease, requiring new tools to ensure their safe and efficacious use in patients. Genome editing comes with risk, including malignant transformation of target cells caused by culturing cells outside the body. This can introduce mutations that confer growth advantages to cells. Furthermore, gene editing can directly cause genomic instability. In cells harboring pre-existing DNA repair defects, the selection for outgrowth of clones with oncogenic mutations may result.

Finally, the gene editing tools themselves can cause DNA breaks in the genome at sites other than the intended target. Such off-target editing could activate proto-oncogenes, or disrupt tumor suppressors thus driving carcinogenesis. Off-target mutagenesis may also generate neoantigens, triggering autoimmunity, or other types of cellular dysfunction. Furthermore, rare penetrant mutations are now known to confer severe risk of common disease, underscoring the importance of identifying off-target gene editing.

More precise methods are needed for testing off-target editing during all phases of therapeutic development, including treatment follow-up. At present standardised assays to assess the safety of gene editing-based therapies are lacking. Here, we describe the development and characterization of INDUCE-seq to address this. CRISPR-Cas9-based gene editing of five well-studied genetic targets was conducted by two independent industry partners, using two different cells types. On and off-target gene editing was assessed by measuring breaks in the genome using INDUCE-seq. The genetic changes at these locations were subsequently measured using Duplex Sequencing, which allows for sensitive detection of exceptionally rare mutations. The results of the study will be presented.

112 - Scalable and cost-efficient combinatorial knockout strategies using CRISPR/Cas12a for functional genomics

Marcel McCullough[1]

[1]IRB Barcelona

Despite the growing recognition of genetic interactions in shaping cellular phenotypes, current knockout strategies remain constrained by cost, scalability, and flexibility, limiting their utility in high-throughput functional genomics. Here, we introduce two cost-efficient and highly flexible approaches for generating combinatorial knockouts using CRISPR/Cas12a technology.

Our first approach employs a modular design for constructing combinatorial CRISPR libraries with Cas12a, enabling scalable and systematic library generation. Target genes are grouped into extractable modules from an oligonucleotide pool via PCR. These modules are designed to combine flexibly, facilitating pairwise target knockouts across all included genes. Our cloning strategy allows for the creation of both simple and complex combinatorial libraries from a relatively small oligonucleotide pool. Furthermore, sequencing analysis confirms the specificity of our system, with the expected crRNA combinations accounting for over 90% of total reads. This approach significantly enhances scalability by eliminating the need to encode specific target combinations in the oligonucleotide design, thereby maximizing the number of possible knockouts while minimizing library complexity.

A key challenge of CRISPR/Cas12a in mammalian cells is its lower editing efficiency compared to Cas9. To address this, we leveraged the pre-crRNA processing capability of Cas12a, demonstrating that dual-crRNA targeting of the same gene substantially enhances knockout efficiency. Target-specific sequencing confirmed that dual-crRNA editing improved single-gene editing efficiency to an average of 89%, while achieving a compounded 79% success rate for double knockouts. Using this strategy, we generated high-efficiency double knockout clones, significantly advancing CRISPR/Cas12a-based genome editing.

Building upon these insights, we developed a second combinatorial knockout strategy using a novel CRISPR array system. This method streamlines combinatorial clone generation by utilizing separate single-stranded DNA oligos encoding crRNAs that anneal to form the intended knockout combinations, eliminating the need for PCR-based assembly. This simplified workflow allows for rapid, high-throughput combinatorial knockout generation in a cost-effective manner while preserving the flexibility of our first method.

In a previous study, we applied our CRISPR/Cas12a approaches to model DNA repair interactions using a combinatorial isogenic panel of knockouts targeting **MMR, BER,** and **DR** genes. Here, we extend this approach by employing our CRISPR array system to generate a **combinatorial isogenic panel of tumor suppressor gene knockouts** in a **high-grade serous ovarian cancer (HGSOC) cell line model.** This knockout panel enables the systematic study of genetic interactions underlying **chemotherapeutic resistance** in HGSOC, providing a powerful tool for functional genomics and cancer research.

110 - Guide RNAs: Orthogonal assessment of purity and identity

Jessica Woodley[1], Karthik Murugan[1], Gavin Kurgan[1], Morgan Sturgeon[1], Ellen Schmaljohn[1], Christian Brommel[1], Ashley Jacobi[1], Garrett Rettig[1]

[1]Integrated DNA Technologies

Synthetic guide RNAs (gRNAs) are complex molecules that are a critical component to many translational cell and gene therapy applications. This is a need of both first generation, RNA-guided-nucleases like Cas9 and Cas12a, as well next generation editing approaches like base-editors and prime-editors. As such, there is a need to confidently assess purity and identity of these molecules. There are orthogonal analytical, biochemical and biological assays that can be deployed to generate combined data sets with an eye towards specific detection of the full-length gRNA. In this presentation, we describe IDT's strategy to incorporate a diverse set of complimentary assays that can be used to assign purity of gRNAs. This includes development of chromatographic and mass spectral methods along with next-generation sequencing (NGS) methods that directly describe the guide via purity and identity assays. Mammalian cell-based genome editing assays can provide assessment of efficiency and specificity of editing events throughout the genome in both biased and unbiased fashion. In total, IDT provides broad capability to assign gRNA purity and identity to meet the needs of varying applications of genome editing.

109 - Improved prediction and activation of homology-directed repair in translational ex-vivo systems

Adam Chernick[1], ASHLEY JACOBI[1], Garrett Rettig[1], Gavin Kurgan[1]

[1]Integrated DNA Technologies

CRISPR-Cas enzymes can induce a double-strand break (DSB) at a specific genomic locus, which is subsequently repaired by various endogenous DNA repair pathways. Among these, homology-directed repair (HDR) is particularly crucial for introducing precise sequence alterations into the genome. However, enhancing the efficacy of this repair pathway to achieve therapeutically relevant frequencies is often a time-consuming and challenging process. In this study, we present a series of innovations aimed at optimizing HDR in therapeutically relevant systems, regardless of the format (ssODN, AAV, etc.) or size (small vs. large) of the donor template. Here, we demonstrate that the potential for HDR is dependent on the guide RNA (gRNA) and show predictive indications of non-homologous end joining (NHEJ) repair that are largely conserved to enable high-precision prediction of HDR potential across cell lines. We further demonstrate how HDR potential can be predictably enhanced by using different DNA repair modulation tools to achieve desired outcomes and are developing methods to detect undesired effects. Finally, we demonstrate that new protein-based DNA repair modulation can significantly enhance performance in translational settings without increasing off-target effects.

107 - ePsCas9 (eSpOT-ON): An Engineered High-Fidelity Genome Editor for Lipid Nanoparticle Delivery

Dmitrii Degtev[1], Jack Bravo[2], Aikaterini Emmanouilidi[1], Aleksandar Zdravković[1], Oi Kuan Choong[1], Julia Liz Touza[1], Niklas Selfjord[1], Isabel Weisheit[1], Pinar Akcakaya[1], Michelle Porritt[1], Kevin Holden[3], Casey Jowdy[3], Marcello Maresca[1], David Taylor[2], Grzegorz Sienski[1]

[1]Discovery Sciences, BioPharmaceuticals R&D Unit, AstraZeneca, Gothenburg, Sweden, [2]Department of Molecular Biosciences, University of Texas at Austin, Austin, TX, USA, [3]Synthego, Redwood City, CA, USA

CRISPR-Cas technologies offer great potential for therapeutic genome editing. However, most Cas enzymes have not achieved sufficient in vivo activity for clinical applications. Our previous work introduced PsCas9 (SpOT-ON), a Type II-B Cas9 from Parasutterella secunda, as a high-fidelity enzyme. PsCas9 delivered via adenoviral vectors achieved editing levels comparable to SpCas9, with minimal off-target events and reduced chromosomal translocations.

In this study, we sought to understand why PsCas9 delivery methods beyond adenoviral vectors showed reduced efficacy. Cellular studies revealed that PsCas9 editing activity was limited under conditions of low intracellular concentration. Biochemical assays showed that PsCas9 has a lower DNA-binding affinity compared to SpCas9. We hypothesized that improving DNA interaction could enhance PsCas9 activity.

To address this, we leveraged structural information to fuel the rational engineering of the enzyme. Optimizing the sgRNA scaffold modestly increased PsCas9 activity. In contrast, targeted mutagenesis resulted in up to a 20-fold enhancement across multiple genomic targets. These improvements correlated with stronger DNA binding in vitro. Importantly, the engineered PsCas9 retained its high fidelity, including low off-target effects and minimal chromosomal translocations.

Finally, we tested the optimized PsCas9 (also referred to as ePsCas9 or eSpOT-ON) for therapeutic genome editing in vivo. Using lipid nanoparticles (LNPs), we delivered ePsCas9 to mouse liver to target the Pcsk9 gene, a model for hypercholesterolemia treatment. ePsCas9 achieved efficient liver editing and significantly reduced Pcsk9 protein levels in plasma, outperforming SpCas9 in this setting.

This work establishes ePsCas9 as a precise and efficient genome editor. Its enhanced activity and intrinsic fidelity make it highly suitable for therapeutic applications. Synthetic guide RNAs and purified nuclease for eSpOT-ON / ePsCas9 are now readily available for CRISPR medicine developers through Synthego as part of a strategic partnership with AstraZeneca to enhance the accessibility of these CRISPR tools.

106 - Functional genetic analysis of sequence variants (mutations) for diagnosis and treatment of genetic disease using CRISPR-Select or CRISPR-LOH

Casper Lund[1], Guven Akcay[1], Ngan Thi Ho Kim[1], Susanne Madsen[1], Raquel Adrover Rico[1], Catarina Azevedo[1], Yiyuan Niu[1], Elahe Kamali[1], Jens Bager Christensen[2], Anne Tybjærg-Hansen[3], Ruth Frikke-Schmidt[3], Ulrik Stoltze[4], Kjeld Schmiegelow[4], Thomas van Overeem-Hansen[5], Morten Frödin[1]

[1]Biotech Research and Innovation Centre (BRIC), University of Copenhagen, [2]University of Cambridge, [3]Dept Clin Chem Rigshospitalet, [4]Dept Pediatric Oncology Rigshospitalet, [5]Dept Clinical Genetics Rigshospitalet

Diagnosis of any of the >8000 human genetic diseases depends on identifying the germline sequence variant (mutation) that caused the disease. However, among variants detected by diagnostic sequencing, 20-40% cannot be classified as benign or pathogenic but are termed variants-of-uncertain-significance (VUS), leaving a large fraction of patients undiagnosed. VUS therefore constitute one of the biggest problems in genetic disease, as they preclude the benefits of diagnosis and a host of precision medicine possibilities, including genetic counseling, prognosis, preventive measures, precision drugs or gene therapy.

To enable VUS diagnosis, we developed the functional genetic assay CRISPR-Select (Niu et al 2022 Nature Genetics). This method uses CRISPR-Cas and single-stranded oligodeoxynucleotides (ssODNs) to knock in a variant-of-interest or either of two synonymous (neutral) control mutations (WT´) at the target site in a cell-culture-of-interest. Frequencies of variant cells are then quantitated relative to WT´ cells (by target site PCR/amplicon NGS) as a function of time, space or FACS marker level to quantitatively determine variant effects on a wide range of disease-relevant processes and provide conclusive evidence to assist VUS diagnosis as benign or pathogenic.

Here we report 1) Automated, high-throughput CRISPR-Select for cancer predisposition genes, 2) CRISPR-LOH, the first functional genetic variant assay in patient genomic background and 3) Clinical implementation of our assays:

1) We developed an automated, 96-well plate CRISPR-Select pipeline and perform higher-throughput classification of all (>100) Danish VUS in a cancer predisposition gene. Unlike saturation genome or prime editing approaches, CRISPR-Select has large dynamic window and graded, quantitative output. Taking advantage of this property, we calibrate our assay with pathogenic variants of varying clinical severity, aiming to develop CRISPR-Select that not only classifies VUS as pathogenic, but also may predict penetrance, age-of-onset etc.

2) Genetic sequence variants impact disease in the context of an individual genomic background, which may harbor modifier variants. Yet, functional genetic assays that can probe the context-dependent effects of a variant are lacking. We therefore developed functional assays in cells from the carrier of a variant, i.e. in individual genomic context. To assay recessive germline variants, the major class of disease mutations, we developed CRISPR-LOH (Loss-Of-Heterozygosity). CRISPR-LOH knocks in a STOP codon or one of two WT´ mutations specifically on the wild-type allele of heterozygous carrier cells and compares effects of STOP, relative to WT´ mutations, thereby revealing potential pathogenicity of the variant with any contribution of modifier(s), known or unknown, from the genomic context. Furthermore, we developed single-cell CRISPR-LOH for two variants in same cell to allow direct testing of specific, putative variant-modifier variant interactions. We devised CRISPR-LOH assays in lymphoblastoid cell lines and primary skin fibroblasts, the major patient cell systems in clinical genetics and we introduce primary T cells for unprecedented rapid variant analysis, using BRCA variants as example.

3) We and clinical collaborators have developed and implemented CRISPR-Select and CRISPR-LOH for genetic diagnosis and treatment of diverse genetic diseases, including hereditary childhood and adult cancers caused by variants in various cancer predisposition genes or hypercholesterolemia/atherosclerosis caused by variants in low-density-lipoprotein receptor (LDLR) treatable by gene therapy.

104 - Long term (>15 years) efficacy and safety monitoring of patients treated with AAV-based LPL gene replacement therapy: a lexicon of potentially useful lessons for gene editing treatments

Jasmine Chebli[1, 2], François Forest[1], Isabelle Gaudet[1, 3], Miriam Larouche[1, 2], Diane Brisson[1, 2], Daniel Gaudet[1, 2]

[1]ECOGENE-21, [2]Université de Montréal, [3]Université du Québec à Chicoutimi

Alipogene tiparvovec (Glybera®) is the first gene replacement therapy to be approved in the occidental world. Glybera targets lipoprotein lipase (LPL) deficiency (LPLD) which causes severe hypertriglyceridemia and confers a risk of acute pancreatitis and other morbidities. A 15-year safety follow-up was a requirement of the European Medicines Agency in one pivotal clinical trial. The long-term trajectory of treated patients in different trials has never been described. This 15-year follow-up retrospective study reviews the clinical trajectory of patients treated with Glybera. A total of 19 Canadian LPLD patients were treated with a single intramuscular administration of Glybera in 2 clinical trials and followed for 15 years. Markers of efficacy, safety and response to therapies introduced over the years were monitored. After 3 months of Glybera treatment, triglyceride levels returned to baseline suggesting limited efficacy, while patients reported improved alertness and quality of life. One year after treatment, the analysis of injected muscle biopsies demonstrated the presence and lipolytic effectiveness of LPL, whereas chylomicron kinetic analyses using stable isotopes showed normalization. After 5 years, 44.4% of the participants still showed signs of improvement in chylomicron kinetics. The slight decrease in the incidence of pancreatitis observed after 5 years was difficult to relate to Glybera. A mitochondrial integration of the LPL transgene was noted in 2 subjects without further off-target signals. Four treated subjects died from consequences of LPLD, not of Glybera administration. Most patients were invited over the years to participate in trials with treatments targeting the inhibition of DGAT1, ApoC3 or ANGPTL3. No difference in response to these treatments was noted between subjects who received Glybera vs the others. Although proven to be safe, Glybera's long-term efficacy was limited. Lessons learned from the long-term follow-up of treated patients are however essential for the development of next generation of gene editing therapies.

103 - Targeting Hepatitis B Virus: CRISPR/Cas9 as a Novel Therapeutic Tool

Addison Hill[1]

[1]East Tenneesee State University

An estimated 296 million individuals are infected with Hepatitis B Virus (HBV). Chronic HBV infections can lead to liver cancer, cirrhosis and is the second leading cause of cancer death across the world. Current antiretroviral treatments (ART) suppress de novo HBV replication, but cannot eradicate the virus from its' host genome.[1] This is due to the persistence of covalently closed circular (cccDNA). In infected hepatocytes, cccDNA remains within the nucleus, evading detection from the immune system. cccDNA also has fairly low transcription activity, therefore it does not produce a large number of viral proteins or RNAs, reducing the chances of triggering an immune response. These mechanisms and many more protect HBV cccDNA from being detected and eradicated by ART[2,3]. Chronically infected patients must remain on ART as withdrawal would lead the cccDNA reservoir to begin transcribing viral RNAs[4]. CRISPR/Cas9 systems are novel genome-editing tools, that have been utilized in multiple different studies to induce anti-HBV effects both in vitro and in vivo[5]. Synthetic gRNA/Cas9 ribonucleoprotein (RNP) are particularly promising as a non-viral formulation, as these complexes have been shown to induce rapid DNA cleavage with minimal off-target effects[6]. In this study we further validate that our synthetic gRNA/Cas9 RNP-based approach shows promising results as novel therapeutic for the eradication of chronic HBV infection. Specifically, our gRNA5, which targets the P and S HBV genome and gRNA9, targeting the P and X genome, in combination are highly effective at suppressing HBV replication.

101 - Preclinical validation of CRISPR/Cas genome editing approaches as advanced therapy for HBBIVSI-110(G>A) thalassaemia

P Patsali[1], C Constantinou[1], K Paschoudi[2], `N Papaioannou[1], B Naiisseh[1], P. L. Papasavva[1], P Christofi[3], S Christou[4], A Pirovolaki[4], M Hadjigabriel[4], T Athanasopoulos[5], C Mussolino[6], T Cathomen[6], N Psatha[2], E Yannaki[3], C. W. Lederer[1]

[1]Department of Molecular Genetics Thalassamia, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus, [2]School of Biology, Department of Genetics, Development and Molecular Biology, Aristotle University of Thessaloniki, [3]Gene and Cell Therapy Center/Hematology-Hematopoietic Cell Transplantation Unit, G. Papanikolaou Hospital, Thessaloniki, Greece, [4]Thalassaemia Centre, Cyprus Ministry of Health, Cyprus, [5]Gene & Cell Ltd, London, United Kingdom, [6]Institute for Transfusion Medicine and Gene Therapy, Medical Center - University of Freiburg, Freiburg, Germany

β-Thalassemia, a global single-gene disorder, is caused by deficient β-globin production, with the prevalent mutation $HBB^{IVSI-110(G>A)}$ creating an aberrant intronic splice site. This mutation has a high carrier frequency in Cyprus (76%) and many EU countries (>20%). A mutation-specific gene therapy has been developed using two approaches: a DSB-dependent approach with CRISPR/Cas9 RNA-guided nuclease (IVSI-110 RGN) and a DSB-independent approach with a nearly PAM-less SpG Adenine Base Editor (SpG7 ABE). Both methods disrupt $HBB^{IVSI-110}$ abnormal splicing elements, achieving clinically relevant efficiencies in patient-derived HSCs in vitro. IVSI-110 RGN introduces indels via the non-homologous end joining repair mechanism, while SpG7 ABE uses targeted base (T>C) substitutions.

The project aims to advance these methods by conducting preclinical evaluations of edited cells both in vitro and in vivo using chimeric NBSGW mice, specifically engineered to facilitate human HSC engraftment without irradiation. The primary objective is to confirm the therapy's readiness for clinical trials, focusing on efficacy, safety, and the long-term repopulation (LTR) potential of modified cells. Additionally, the study seeks to compare the mutation-specific approach with a universal therapy targeting the erythroid BCL11A enhancer element for HbF induction (sg1617 RGN), recently FDA-approved as the first CRISPR therapy for sickle cell disease.

RGNs and ABE were delivered via nucleofection to mobilized $HBB^{IVSI-110(G>A)}$ patient-derived HSCs as ribonucleoprotein complexes (RNPs) and in vitro transcribed mRNAs, respectively. The therapeutic potential was evaluated in vitro through induced erythroid differentiation (ED) cultures, assessing correction at DNA (on- and off-targeting, Sanger sequencing), protein (RP-HPLC), and late-stage ED levels (flow cytometry), as well as clonogenic assays for erythroid and myeloid lineage potential. In vivo assessment involved xenotransplantation in NBSGW mice to evaluate LTR potential 16 weeks post-transplantation (flow cytometry).

Overall, both mutation-specific genome editors let to high on-targeting (IVSI-110 RGN 85%; SpG7 ABE (T>C): IVSI-106 and -108: ~40%; -109: ~18%) with undetected off-targeting, and moderate disrutprion of erythroid BCL11A enhancer element (sg1617 RGN: ~40%). RP-HPLC analysis of the in-vitro ED cultures, showed significant increase of HBB/HBA ratios to normal levels (0.9-1) in IVSI-110 RGN- and SpG7 ABE-genome edited cells and a significant increase of HBG/HBA ratios in the sg1617 RGN edited population (~0.51) relative to UT control (HBB/HBA: ~0.39; HBG/HBA: ~0.27). There was a clear correction of late-stage erythroid differentiation in the mutation-specific edited populations, while genome editing didn't affect erythroid and myeloid lineage potential of HSCs. Analysis of BM chimerism in xenotransplanted NBSGW mice showed high engraftment for all samples (hCD45+: 65% and hCD34+ves: ~6.5%). When comparing genome editing levels between BM bulk inputs and primary recipient BM cells, a 50% reduction was observed in IVSI-110 RGN, while sg1617 RGN showed consistent levels, and SpG7 ABE demonstrated a 20% increase.

Even though, analysis of the biosafety of the genome editing tools is still in progress, the current data indicates ABE SpG7 as the most promising approach for clinical application, since therapeutic levels were achieved while the erythroid and myeloid-lineage and LTR capacity of the edited HSC population was maintained.

100 - Rapid and accurate genotyping of 100 gene-edited cells using GeneAbacus

Hamid Sharifi[1], Leiore Ajruia[1], Iván Hernández-Neuta[1]

[1]Countagen AB, Nobels väg 16, 171 65 Solna, Sweden

IntroductionTimely genotyping of gene editing outcomes remains a significant challenge, particularly when working with limited cell numbers or single-cell manipulation as long culturing times are needed for sufficient sample amount. Traditional PCR-based methods are powerful but prone to nonspecific amplification, especially in samples with low cell count. Here, we present a standardized PCR-free genotyping assay that delivers highly accurate results within a single day, starting from as few as 100 cells/sample.

MethodGeneAbacus leverages padlock probes and Rolling Circle Amplification (RCA) chemistry to convert genotypes into quantifiable signals that can be read out with a fluorescence microscope. The loss of wild type alleles and detection of specific edits are measured by digital quantification of signals obtained from edited and unedited cells. We have developed a sample preparation module for GeneAbacus to handle limited cell number samples without compromising accuracy. Comparative studies were conducted using human HCT 116 and human U-2 OS cell lines, analyzing knock-out efficiency in heterozygous pools and homozygous clones. Results were evaluated using next-generation sequencing (NGS) as a reference method.

ResultsWe demonstrated functionality of GeneAbacus from samples with a range of 100 to 1,000 cells. The assay achieved equal sensitivity for both cell lines used. Blind testing of knock-out efficiency revealed robust precision and reproducibility with high degree of concordance as confirmed by NGS data (R^2= 0.982) for tested gene editing efficiencies ranging from 20% to 100% edited alleles.

Conclusion and Outlook With the extended capability of GeneAbacus to handle samples with limited cell numbers, the genotyping process of gene-edited cells can be streamlined.. This addresses not only bottlenecks in PCR-based workflows but can accelerate the gene editing validation step by for instance shortening the cell culture time, enabling cell engineering solutions on single cell level and on cell types with limited dividing capacity.

99 - Using Causal Inference and Feature Set Expansion for Algorithmic Prediction of Off-Target Sites in the CRISPR-Cas9 Edited HEK293T Cell Line

Maciej Powierża[1], Katarzyna Romańczuk[1], Łukasz Łaczmański[1]

[1]Hirszfeld Institute of Immunology and Experimental Therapy PAS

Off-target mutations that occur alongside on-target CRISPR/Cas9 gene editing pose a significant concern for gene therapies. Even rare or minor unintended edits can greatly impact treatment outcomes or lead to clonal expansion, potentially resulting in tumorigenesis. In vitro prediction methods and in silico algorithms for identifying potential off-target sites are essential for assessing the specificity of guide RNAs and increasing the sensitivity of off-target evaluations in treated cells.

Nano-OTS is an in vitro off-target prediction method based on long-read Nanopore sequencing. This approach eliminates amplification bias in the results and allows for the analysis of repetitive and complex genomic regions. We applied the Nano-OTS method to predict off-target sites for two gRNAs designed to introduce disease-causing mutations into the COL7A1 gene. The results demonstrated exceptional reproducibility and highlighted the method's versatility, with potential for adapting the protocol for use with base editors. The predicted off-target loci were validated in HEK293T cells edited with three mRNA constructs encoding the cytosine base editor BE3. The datasets of in vitro-predicted and in cellulo-validated off-target sites will offer insights into the mechanisms behind unintended edits caused by CRISPR/Cas9 and base editors. Additionally, these datasets could be expanded to include other data types (e.g., transcriptome analysis, chromatin accessibility data) to support informed guide RNA design and the development of effective CRISPR/Cas9-based therapies.

The multimodality and high reproducibility of the datasets acquired through high-throughput laboratory techniques will then be used to train the in silico off-target predictor. The efficiency of this predictive model originates from several factors. The first is expanding the feature set used in the training process beyond pure genomic data, which is currently the gold standard in in silico off-target prediction. The second encompasses the transition from point estimation of off-target occurrence—i.e., assessing its probability—to modeling the distribution of the off-target phenomenon conditioned on a set of statistically significant traits such as physical descriptors of the DNA being edited or its transcriptional activity. This approach enables sampling from the modeled posterior distribution, which in turn can be used both for strengthening the model’s training process as well as for its comprehensive evaluation. Last but not least, we aim to improve the quality of predictions of our model by implementing as its part the causal inference mechanism. This innovation is justified by an observation that causal models suit well to the laboratory scenarios, where interventions in the examined phenomenon (off-target activity in our case) as well as the second basis of the causal paradigm, i.e. the iterative and structured hypothesis testing are the natural part of the process.

98 - Enabling gene therapy with Megabulb DNA - a novel circular single-stranded CRISPR editing template

Elena Stoyanova[1], Linda Meggiolaro[1], Elena Garitta[1], Duncan Simpson[1], Shivam Bhatt[1], Jonathan Extance[1], Tom Adie[1]

[1]Touchlight

Synthetic single-stranded DNA (ssDNA) presents a promising alternative to traditional vectors, offering enhanced performance across a range of genetic medicine applications. As a homology-directed repair (HDR) template, ssDNA demonstrates low toxicity and achieves on-target insertion frequencies comparable to the incumbent viral vectors. Additionally, when used as an expression vector, ssDNA addresses the immunogenicity challenges associated with viruses and double-stranded DNA (dsDNA). While viruses are effective for transgene delivery, they pose significant biosafety risks and have limited packaging capacities, hindering their use in therapies requiring large genomic insertions. However, the availability of GMP-grade long ssDNA remains a major barrier to fully realising the potential of non-viral gene and cell therapies.

Megabulb (mbDNA™) is a synthetic, circular ssDNA editing molecule developed by Touchlight, which manufactures GMP-grade DNA rapidly at scale. As a covalently-closed molecule, mbDNA boasts an increased cellular half-life. In head-to-head comparisons with commercially available linear ssDNA, mbDNA achieves four times higher HDR frequencies and greater primary T cell knock-in rates. Its design also includes binding sites for the associated ribonucleoprotein nuclease, facilitating the formation of an editing complex and enhancing nuclear transport - a critical bottleneck in non-viral genome editing.

mbDNA's low toxicity enables a dose-dependent increase in CRISPR-mediated knock-in efficiency in primary cells, with a multi-fold improvement over dsDNA templates. When used with HDR enhancers in ex vivo T cells, mbDNA achieves up to 70% knock-in, rivalling CAR-T viral vectors. The enhanced recovery and superior expansion of mbDNA-edited cells allow for higher yields, which are crucial for clinical applications. Moreover, mbDNA-engineered cells secrete lower levels of the immune response modulator IFNγ compared to those transfected with dsDNA, further demonstrating its improved safety profile. Additionally, lipid nanoparticle (LNP)-mediated delivery of mbDNA mitigates electroporation toxicity, improving T cell recovery and increasing edited cell yields.

The low immunogenicity of mbDNA reduces donor-to-donor variability and preserves the physiological profile of T cell populations even at high DNA concentrations (480 nM), making it a safe and reliable CRISPR template for autologous therapies. Beyond gene editing, mbDNA also serves as an effective episomal expression vector, promising to overcome the safety challenges associated with dsDNA in transient gene therapies.

The mbDNA platform can encode multi-kilobase sequences, overcoming the packaging limitations of viral vectors, and the elimination of fermentation processes further enhances manufacturability and reduces cellular toxicity. It supports efficient Cas9 and Cas12a-mediated HDR insertions of long sequences (>5 kb). A recent grant-funded process development programme is successfully scaling mbDNA production to multi-milligram quantities, with processes designed to meet GMP manufacturing standards and regulatory specifications. Scaled production of mbDNA has shown to maintain or improve upon the performance of R&D-derived material.

Ongoing evaluations by industry leaders in clinically relevant cell types, including T cells, iPSCs, and HSCs, suggest that mbDNA could revolutionise gene therapy, with early data indicating its broad potential as an adaptable platform.

97 - Evolving solutions for effective genotoxicity assessment in CRISPR-based editing

Kyle Kinney[1], Ashley Jacobi[1], Morgan Sturgeon[1], Garrett Rettig[1], Ellen Schmaljohn[1], Bernice Thommandru[1], Karthik Murugan[1], Kyle Cromer[2], Kun Jia[2]

[1]Integrated DNA Technologies, [2]UCSF

Next-generation CRISPR-based editing systems continue to be developed, bringing more diverse editing capabilities to the 'toolbox'. Sensitive nomination of off-target sites in diverse editing systems (e.g. blunt double-stranded break (DSB), staggered DSB, nicked SSB) is a challenge that needs to be addressed in pre-clinical studies during genotoxicity safety assessments. To this end, we present an improved off-target nomination workflow using a proxy system to sensitively nominate off-target sites across different cell types and editors spanning a variable range of gRNA specificity levels. Using clinically relevant cell types (e.g. iPSCs, Primary T-cells) it is demonstrated that the workflow serves as a sensitive (<0.01% indel editing nominated) and effective (>99% event nomination) method across a range of relevant cell types and gRNAs. By quantifying down to 0.01% indel editing frequencies and 0.5% base editing frequencies in the nominated targets, it is demonstrated that editing with diverse editor types (e.g. ABE, CBE), correlates well to frequencies observed using DSB based editors in HSPCs using mRNA delivery. Furthermore, the method shows evidence of nominating sites uniquely undergoing indel editing only with SSB editing modalities in HSPCs. This set of experiments across a broad range of gRNA specificities demonstrates an end-to-end to process for nominating and confirming bona fide off-target sites using both current and next-generation editors.

96 - Fast Quantification of Genome-Editing Outcomes using CRISPR-based detection

Charles Blanluet[1], Henrik Sperber[1], Sridhar Selvaraj[2], Matthew Porteus[2]

[1]QUiCKR Bio, [2]Stanford University

Genome editing is rapidly gaining traction in many clinical applications. However, the evaluation of editing outcomes remains time consuming and impedes R&D timelines, especially when multiple iterations are needed to optimize workflows. Amplicon sequencing (amp-seq) is considered the gold standard for quantification of editing efficiency. However, amp-seq is time consuming (~1 week turnaround time), and can be costly. Faster methods have been developed, but these often pose a trade-off of accuracy. The introduction of new workflows into research pipelines also requires additional equipment and/or extensive assay optimization. Hence, there is a critical need for fast, accurate, and cost-effective quantification of gene editing efficiency.

To address this need, we have developed an assay which can be easily run on standard lab equipment for quantification of indels, knock-ins, and off-target rates. The assay is called QUiCKR (Quantification Using initial CRISPR Kinetic Rates) and offers results in 20 min with minimal manual steps. The assay protocol consists of generating a DNA sample dilution series, which is added to a pre-plated reagent mix containing Cas12, crRNA and fluorescent reporters, followed by fluorescence read-out (Fig 1a). The editing efficiency is then quantified using a machine-learning trained software, hosted on an open cloud platform. The QUiCKR assay achieves amp-seq level accuracy by quantifying kinetic rates of Cas12, as opposed to endpoint signals for typical CRIPSR-based assays. Pre-plated reagents remain stable during long-term storage, which provides users with ready-to-use reagents, a quick workflow, and accurate results.

Edits quantification with QUiCKR, amp-seq and Sanger-ICE: HSPCs were separately edited at HBB, and STING1 loci with Cas9 ribonucleoprotein (RNP) with or without a knock-in AAV6 donor template, followed by amplification of the on-target sites and an off-target site. On- and off-target INDEL frequency, knock-in efficiency, and wild-type ratios were quantified by Sanger sequencing with ICE analysis, amp-seq, and the QUiCKR assay (**Fig 1b)**. QUiCKR demonstrated 99.1% R-square fit with amp-seq data over 35 data points. In contrast, ICE showed 91.9% R-square fit with amp-seq data and did not capture low levels of off-targets (~3%, **Fig 1c**).

LOD study on CCR5 single-base deletion: iPSCs were edited at CCR5 locus with RNP, quantified at 98% with Sanger-ICE. Edited CCR5 amplicons were diluted with unedited CCR5 amplicons to form 9 mixes (editing frequencies from 90% to 0%). Editing frequencies were quantified with QUiCKR, which yielded a 98.2% R-square fit with Sanger-ICE data.

Dynamic-Range study on BCL11A 15-base substitution: Mixes of wild type and mutated DNA amplicons were prepared at ratios from 0%-90%, and quantified by QUiCKR. The R-square fit with mutant ratios was 98.4% and the standard error of the assay was 0.7%.

The QUiCKR assay accurately quantified INDEL edits, 15bp knock-ins with 99% R-square fit with amp-seq (versus 91% for Sanger-ICE). The QUiCKR assay also demonstrated a sensitivity below 3% for a single nucleotide edit and INDEL edits at off-site, modifications that Sanger-ICE was not able to capture. To conclude, QUiCKR offers a 20-min and accurate alternative to amp-seq, which has the potential to significantly increase the speed of gene editing research.

94 - Enhancing CRISPR/Cas9 Guide RNA Design with Active Learning Techniques

Gu¨l S. Demircan[1], Stefano Roncelli[1, 2], Christian Anthon[1], Lars Juhl Jensen[2, 3], Jan Gorodkin[1]

[1]Center for non-coding RNA in Technology and Health, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark, [2]Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Copenhagen, Denmark, [3]ZS Discovery, Lyngby, Denmark

While CRISPR/Cas systems have transformed genome editing, designing guide RNAs (gRNAs) for accurate and efficient targeting remains a challenge. To enhance model performance and predictive strength, the CRISPRnet project aims to generate data with new gRNAs strategically selected to complement existing data. We employ uncertainty estimation methods to identify gRNAs for which experimental data should be generated. The hypothesis is that the gRNAs with the highest uncertainty in efficiency prediction are the most valuable for experimental validation. A major challenge in this is the lack of ground truth on model uncertainty. To achieve this, we modified the state-of-the-art CRISPRon model, which was trained on a 30mer gRNA target with context sequence and the binding energies between the gRNA spacer and the target DNA binding energy by using deep neural networks to predict the editing efficiency. We explored two techniques: (1) a method that employs an ensemble of CRISPRon models, trained through nested cross-validation, to estimate the prediction variance of gRNAs, and (2) an ensemble of CRISPRon models extended with an additional classifier head and a custom loss function for uncertainty estimation. The effectiveness of the two methods is assessed by comparing them against a set of potential gRNAs, which augments the data available based on the recommendations made by the models.

93 - CRISPR/Cas9-mediated TCR replacement to target MAGE-A1+ metastatic melanoma cells

Daniela Benati[1], Tommaso Ferrari[1], Federico Corradi[1], Riccardo Mazzocchi[1], Sara De Biasi[2], Annalisa Saltari[3], Teresa Rossi[4], Mariaelena Pistoni[4], Monica Sandri[5], Mitchell P Levesque[3], Andrea Cossarizza[2], Alessandra Recchia[1]

[1]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, [2]Department of Medical and Surgical Sciences for Children & Adults, University-Hospital of Modena and Reggio Emilia, Modena, Italy, [3]Department of Dermatology, University Hospital Zurich, University of Zurich, Zurich, Switzerland, [4]Laboratory of Translational Research, Azienda Unità Sanitaria Locale-IRCCS di Reggio Emilia, Italy, [5]ISSMC-CNR, Faenza (RA), Italy

Adoptive therapy with T cells engineered to express T cell receptors (TCRs) recognizing HLA-presented cancer antigens represents one the most promising strategy of precise therapy against solid tumors. Melanoma associated antigen 1 (MAGE-A1) is a member of the MAGE-A family of cancer/testis antigens, whose members are known to be highly expressed in multiple different tumor types. Of note, expression of MAGE-A1 antigens in normal tissue is limited to the immunopriviledge testis, which makes MAGE-A1 an ideal target for TCR-based adoptive cell therapy. Ongoing clinical trials with TCR-T cells, generated by retro- or lentiviral transduction, are providing promising results against solid tumors, including non-small cell lung cancers and melanoma. Recently, the CRISPR/Cas9 system have been proposed to safely and effectively engineer T cells to redirect their antigen-specificity and enable them of enhanced anti-tumor response. In this study, we applied CRISPR/Cas9 system to replace endogenous TCRs with a cancer-specific TCR targeting MAGE-A1 in the context of HLA-A*0201, in T cells derived from healthy donors. Primary T cells were electroporated with ribonucleoproteins (RNPs) of Alt-R SpCas9 in complex to TRAC exon1-specific gRNA, and HDR donor template carrying MAGE-A1 TCR sequence, to trigger the knock-in in the TRAC locus. Moreover, simultaneous knockout of the endogenous beta chains was carried out with Alt-R RNPs targeting TRBC loci. Genomic and cytofluorimetric analyses showed almost complete knock-out of endogenous TCR chains, and more than 14% CD8 T cells expressing the MAGE-A1-specific TCR upon precise HDR events, representing 85% of the TCR+ cells in the CD8 compartments. Genotoxicity of the CRISPR-treatment was evaluated to detect chromosomal rearrangements between the target loci by ddPCR. Engineered T cells were analyzed for their ability to respond to primary melanoma cell lines expressing different levels of MAGE-A1. Co-culture experiments of TCR-redirected T cells with target cells demonstrated that edited T cells strongly activate upon recognition of MAGE-A1+ cells by producing proinflammatory cytokines, such as inteleukin-2 (IL-2), interferon-γ (IFNγ), and tumor necrosis factor (TNF), and killing metastatic melanoma cells expressing different levels of MAGE-A1. Ongoing experiments are evaluating the viability and functionality of engineered T cells co-cultured with melanoma cells on gelatin/chitosan-based scaffolds. In conclusion, these data encourage the application of CRISPR-mediated non-viral TCR editing to generate highly efficient T cells able to kill MAGE-A1-expressing cancer cells.

91 - The effect of interchangeable untranslated regulatory regions (UTRs) in mRNA on the efficiency, safety and editing precision of cytosine base editors

Katarzyna Romańczuk[1], Marzena Ciesielska[2], Katarzyna Nowis[1], Joanna Jacków-Malinowska[3], Łukasz Łaczmański[1]

[1]Laboratory of Genomics and Bioinformatics, Hirszfeld Institute of Immunology and Experimental Therapy at Polish Academy of Sciences, [2]Laboratory of Medical Microbiology, Hirszfeld Institute of Immunology and Experimental Therapy at Polish Academy of Sciences, [3]St John's Institute of Dermatology at King's College London

Transient delivery forms of CRISPR/Cas9 and base editors (BEs) are known to reduce undesired off-target activity without compromising on-target editing efficiency. Messenger RNA (mRNA), in contrast to BE proteins, is easy to obtain, handle, and modify for specific needs. In addition to the protein-coding sequence, mRNA includes regulatory untranslated regions (UTRs) at both the 3′ and 5′ ends, as well as a protective cap and poly(A) tail. UTRs influence mRNA translation rates, ribosomal engagement, and RNA stability in cells, with the 5′ UTR primarily involved in translation initiation, while the 3′ UTR modulates mRNA stability via miRNA binding sites. The 5′ UTRs have been shown to impact editing efficiency and precision for TALENs and the cytosine base editor BE4max. Furthermore, cellular responses such as interferon activation and p53 pathway induction are influenced by the UTRs used. By investigating the role of several most widely used UTRs in mRNA design, we sought to enhance the efficiency and precision of CBEs while reducing off-target effects—a major barrier to the clinical application of base editors.

Three mRNA constructs have been synthesized containing common protein-coding elements: the cytosine base editor BE3 sequence and the EGFP gene, separated by the P2A self-cleaving peptide. Each construct included a different UTR set: 1) XEN - the widely used Xenopus globin 5′ and 3′ UTRs; 2) MOD - a synthetic unstructured 5′ UTR and the human alpha-globin 3′ UTR, as used in the Moderna COVID-19 vaccine; and 3) APT - a 5′ UTR with an aptamer binding eIFG4, a protein involved in translation-initiation complex formation, and the WPRE 3′ UTR. The mRNA constructs were transfected into HEK293T cells with a designed gRNA to introduce a pathogenic mutation in the COL7A1 gene, which causes recessive dystrophic epidermolysis bullosa (RDEB). Lipofectamine-based transfections achieved 30-45% editing efficiency across three rounds of editing. The EGFP sequence enabled simultaneous assessment of transfection efficiency and intracellular translation rates. A clear trend was observed, with the MOD construct showing the lowest editing efficiencies and the weakest fluorescence (assessed via fluorescent microscopy and flow cytometry) among all constructs in multiple rounds of transfection. This trend was confirmed in the A431 cell line. Both the XEN and APT constructs introduced comparable levels of modification, but the XEN mRNA resulted in higher fluorescence intensity in HEK293T cells.

Ongoing transcriptome analysis and off-target mutation profiling of treated cells will provide a thorough assessment of the effects of the UTR sets on cellular health and editing outcomes. Off-target prediction tools for BEs will offer valuable insights into off-target mutation mechanisms and facilitate more sensitive detection of actual mutations in edited cells. These approaches will provide comprehensive and high-confidence experimental data, enabling us to systematically evaluate cellular responses, off-target mutation profiles and editing efficiencies of varying mRNA UTR sets. UTR designs that demonstrate minimal immune response and maximize efficiency and precision will serve as a model for optimizing therapeutic designs and enhancing safety validation protocols.

89 - Leveraging the hfCas12Max platform for gene and cell therapy applications

Tal Shamia[1]

[1]synthego

Recently, a novel family of Cas12 CRISPR nucleases was discovered by Huidagene Therapeutics. xCas12i showed promised as a viable gene editor and was rationally engineered using Huidagene Therapeutics' HG-PRECISE platform.
The result of this engineering is hfCas12Max, a CRISPR nuclease that recognizes a 5'-NTN PAM with high editing activity and specificity in mammalian cells. In both human cell lines and primary human cells, hfCas12Max demonstrates both high levels of gene editing with lower rates of off-targeting compared to Sp. Cas9. Using electroporation to deliver hfCas12Max RNPs to primary human CD3+ T cells, approximately 90% gene editing efficiency (as measured by functional gene KO) at the TRAC locus with 80% cell viability was observed.Through in silico and genome-wide off-target analyses, the high specificity of hfCas12Max was demonstrated. In a series of proof-of-concept studies, two rounds of LNP-hfCas12Max system administration resulted in a sustained reduction of serum HBsAg and HBV DNA levels in the AAV-HBV mouse model. These studies suggest that hfCas12Max is a promising tool for safer and more effective gene and cell therapy treatments. Synthetic guide RNAs, purified nuclease, and affordable commercial sub-licenses for hfCas12Max are now readily available from Synthego for CRISPR medicine developers through a partnership with Huidagene Therapeutics to enable accessibility of the CRISPR tools and greater equity
for future CRISPR medicines.

88 - The Convenient, Rapid, and High-Yielding, Chemical Synthesis of RNA and Modified RNA to 300 Nucleotides in Length using 2'-O-Thionocarbamate-Protected Nucleoside Phosphoramidites on the Solid Phase

Logan Garner[1], Benjamin Lunstad[1], Marvin Caruthers[2], Douglas Dellinger[1], Maddy Dellinger[1]

[1]Cirena Inc., [2]University of Colorado, Boulder

Robust chemistry for the solid-phase synthesis of long RNA (100 to 400 nucleotides), rivaling or surpassing the long chemical synthesis of DNA, was developed over a decade ago in our laboratory but never broadly commercialized. The new generations of CRISPR technologies are now demanding the ability to produce guide RNAs at lengths that exceed the capabilities of the widely adopted and antiquated synthesis methods for RNA that were developed in the late 1980's. We present a modern solution to the de novo chemical synthesis of RNA and chemically modified RNA that can support both the emerging CRISPR technologies and rapidly evolving field of non-coding RNAs that regulate metabolic processes. Using 2'-O-Thionocarbamate-Protected Nucleoside Phosphoramidites (TC) we have demonstrated the synthesis of thousands of pegRNAs out to 250 nucleotides in length, as well as circular RNAs and lncRNA subunits out to 300 nucleotides in length in multimilligram quantities using unmodified, commercial 96 and 192 column high-throughput synthesizers. In concert with these next-generation phosphoramidite monomers our laboratory developed a novel and high yielding affinity-assisted method for the rapid and high-throughput purification of RNAs out to 300 nucleotides in length using single-step chromatography. These methods have been demonstrated to be easily scalable to quantities required for clinical investigations. Chemical synthesis by the phosphoramidite method allows for the convenient site-specific incorporation of chemical modifications for increased activity and stability in cells and tissues. Modifications developed in our laboratory for pegRNA stability and specificity have now been broadly demonstrated to significantly increase editing efficiency.

87 - Linking CRISPR-Cas9 double-strand break profiles to gene editing precision with BreakTag

Gabriel M. C. Longo[1], Sergi Sayols[1], Andriana Kotini[2], Athanasios Xanthopoulos[2], Vassilis Roukos[2]

[1]Institute of Molecular Biology (IMB), Mainz, Germany, [2]Department of General Biology, Medical School, University of Patras, Greece

CRISPR/Cas9 is a powerful DNA targeting platform for precise genome editing, holding immense potential for successful gene therapy of various genetic diseases. To fulfill this role, it is essential to establish a framework that allows precise prediction of CRISPR/Cas9 editing outcome, while minimizing unwanted off-targets that promote genomic instability. Cas9 has a flexible scission profile, which has been speculated to impact the repair outcome, however, direct evidence is lacking due to the lack of scalable methodologies that are able to probe blunt or staggered Cas9 incisions.

Here we developed BreakTag, a quick, sensitive and highly parallel methodology for the profiling the frequency and type of incision of Cas9-induced DNA double strand break (DSBs) at nucleotide resolution across the genome. We used BreakTag to survey nearly 3,000 sgRNAs targeting human genes, generating a robust dataset containing >150,000 uniquely cleaved loci between on and off-targets with identified scission profile. Our results indicate that Cas9 scission profile is not random, but instead, it is highly dependent on the nucleotide sequence of the protospacer and the presence of gRNA-DNA mismatches. To identify important features for scissile profile decision, we trained a machine-learning model that revealed that the first five nucleotides upstream of the PAM-sequence are important for the cut decision with positions 17 & 18 of the protospacer being decisive for blunt or staggered cuts, respectively. In line with our findings showing that certain nucleotides next to the PAM sequence influence the way Cas9 cuts DNA, we show that genetic variation found between individuals, dictates Cas9 cut configuration and the repair outcome, and therefore it must be taken into consideration in relevant clinical efforts. Furthermore, we identify and characterize Cas9 variants with altered scission properties. Importantly, we show that sequence driven Cas9-induced staggered DSBs are linked with precise, templated and predictable single-nucleotide insertions, indicating that selecting gRNAs with staggered cut profile could allow prediction of repair genotypes with desirable characteristics. Our work lays the foundation for harnessing flexible Cas9 cut profile for precise template-free indel engineering and highlights the importance of taking account genetic variation as an important determinant of gene-editing outcome.

86 - CRISPNA and Dual-CRISPR Systems: Advancing Genome Editing and Diagnostics

Araceli Aguilar-González[1, 2], Ismael Martos-Jamai[1, 2], Francisco Javier Molina-Estévez[1], Rosario María Sánchez-Martín[1, 2], Juan José Díaz-Mochón[1, 2], Francisco Martín[1, 2]

[1]Genyo, [2]University of Granada

CRISPR/Cas systems have become transformative tools in biological research, therapeutic development and diagnostics due to their ability to target DNA and RNA with high precision. These systems rely on RNA molecules (crRNAs or sgRNAs) to guide Cas proteins to specific genetic sequences. However, RNA guides are susceptible to degradation in the biological environment and may allow mismatches during target recognition, limiting their stability and specificity. To address these challenges, we have developed CRISPNA, a novel platform that combines CRISPR-associated enzymes with peptide nucleic acids (PNAs). PNAs are synthetic oligonucleotides with enhanced stability in biological fluids, resistance to enzymatic degradation, and superior binding affinity and specificity for complementary DNA and RNA sequences. Our study shows that PNAs can successfully guide Cas proteins, such as Cas9 and Cas13, to their respective targets. While initial CRISPNA designs revealed a reduction in enzymatic activity for both Cas9 and Cas13, ongoing refinements are aimed at restoring and enhancing their functionality. Concurrently, a dual-guide-RNA system for Cas13 has been developed to improve sequence specificity for RNA diagnostics. The system demonstrated robust cis- and trans-RNase activity in vitro, successfully targeting SARS-CoV-2 RNA, thus demonstrating its utility for diagnostic applications. Through the integration of CRISPNA with dual-CRISPR technology, both platforms are being advanced to enhance mismatch discrimination and targeting precision. These efforts include the optimisation of designs for challenging applications such as the detection of KRAS mutations, a critical marker in cancer diagnostics. The combination of CRISPNA's stability and specificity with the versatility of the dual-CRISPR/Cas13 system highlights the potential for next-generation tools in genome editing and diagnostics.

84 - Investigating the Role of Death Receptor Pathways in Response to SpCas9-Mediated Genome Editing

Seyma Nur Polat[1], Pinar Bahsi[1], Duriye Nur Dagdelen[1], Fatma Zehra Hapil[1]

[1]Akdeniz University School of Medicine, Department of Medical Biology

The CRISPR/Cas9 system, particularly SpCas9, has revolutionized genome editing, but its impact on cellular survival and immune-related pathways remains a critical area of investigation. While genome-edited cells have been associated with reduced survival and p53 activation, our hypothesis focuses on the potential role of the death receptor pathway, as p53 is known to modulate the expression of key immune-related genes such as DR5, Fas, and DcR1. This study explores the interplay between SpCas9-mediated genome editing, p53 dynamics, and the regulation of TNF receptor superfamily members, with a focus on TRAIL receptors (DR4, DR5, DcR1, DcR2), TRAIL, and FAS. To investigate the impact of SpCas9 on these pathways, 293T cells were transfected with either a mock plasmid, a plasmid encoding SpCas9 without a gRNA, or a plasmid expressing both SpCas9 and an AAVS1-targeting gRNA, using PEI-mediated transfection. Our findings reveal that SpCas9 expression alone (without gRNA) does not alter the expression levels of Fas, DR4, DR5, DcR1, or DcR2. However, it significantly reduces TRAIL expression, as demonstrated by Western blot analysis. In contrast, targeting the AAVS1 locus with SpCas9 and a gRNA led to a notable upregulation of DR5 and Fas at 30-36 hours post-transfection, while DR4 expression was downregulated at all time points where SpCas9 was detectable. Intriguingly, spontaneous caspase-8 cleavage was observed in genome-edited cells around 30-36 hours post-transfection, but not in cells expressing SpCas9 alone. Furthermore, we observed no significant p53 accumulation in cells expressing SpCas9 without a gRNA. However, in AAVS1-targeted cells, p53 accumulated at 28-30 hours post-transfection and subsequently degraded by 36 hours. These results suggest that SpCas9-mediated genome editing, but not SpCas9 expression alone, triggers p53 activation and modulates the expression of key death receptor pathway components, potentially influencing cellular fate and immune responses. Given the critical roles of Fas and TRAIL in immune regulation and cytotoxicity, these findings highlight the importance of evaluating death receptor pathways to ensure the safety and efficacy of CRISPR/Cas9-based therapies.

82 - Base Editing for RHO Mutations: Allele-Specific and Mutation-Independent Strategies for Autosomal Dominant Retinitis Pigmentosa Treatment

Maddalena Fuso[1], Salome Spaag[1], Susanne Kohl[1], Pietro De Angeli[1]

[1]Institute for Ophthalmic Research, Centre for Ophthalmology, University Clinics Tübingen, Tübingen, Germany.

Retinitis pigmentosa (RP) is the most common inherited retinal disorder, affecting ~1/4,000 individuals worldwide. Mutations in RHO are the most common cause of autosomal dominant retinitis pigmentosa (ADRP). More than 250 RHO mutations have been identified and missense variants are the most common mutation type. To address the majority of these mutations, gene editing strategies targeting RHO mutant alleles in an allele-specific but mutation-independent manner were developed, to suppress dominant mutant protein production while preserving the wild-type allele, ultimately rescuing the adIRD phenotype.

We identified common heterozygous SNPs in RHO prevalent in the general population, and selected c.-26A/G, heterozygous in 25% of the general population and in 28% of our cohort of RHO-linked adRP patients. Notably, allele A is found in cis with RHO pathogenic variants in 60% of cases, and allele G in 40%. To test gene editing strategies, transgenic reporter cell lines stably expressing either allele A or G of RHO fused to mCherry were established, and used to design and test prime editing and base editing strategies to effectively and specifically target either SNP. These strategies modify the 5'UTR of RHO herby reducing dominant mutant RHO production. mCherry fluorescence, which corresponds to RHO expression, can be monitored via flow cytometry, providing a reliable and efficient method to assess editing outcomes.

In order to assess which 5´UTR edits nearby the SNP of interest could reduce RHO expression, we designed and tested 11 different combinations of edits that could be introduced via base or prime editing. Reporter plasmids carrying RHO-mCherry with the modified 5´UTR sequences were transfected in HEK293T, and their impact on RHO expression was evaluated by flow cytometry. One type of edit outperformed the others, reducing RHO levels by up to 90%, and was further selected to assess base editing approaches for its installation.

Plasmids carrying base editors BE4max or CBE6b-V106W with gRNA1 or gRNA2 were transfected in the transgenic reporter cell lines and the effects on RHO knockdown was analyzed by flow cytometry. Three days post-transfection, both combinations of BE4max and CBE6b-V106W with gRNA2 resulted in a ~40% decrease of RHO expression, while gRNA1 only achieved minor reduction. Sequencing results confirmed base conversion for both BE4max and CBE6b-V106W with gRNA2.

As an outlook, aiming to install other lead edits in the 5´UTR, we designed a library of 265 prime editing gRNAs that will be screened in the reporter cell lines using a streamlined protocol developed in our lab. Subsequently, to enable transferability of the validated genome editing strategies into therapeutics, we will investigate off-target effects, while exploring the delivery of such editing molecules using clinically viable vectors (e.g. VLPs and AAV). We eventually aim to test the efficacy, rescue, and delivery of base and prime editing strategies in patient-derived retinal organoids.

79 - CRISPR-Cas9-mediated orthotopic placement of a transgenic T cell receptor recognizing a PD-L1-derived peptide

Emilie Bülow[1], Thomas L. Lisle[1], Shamaila M. Ahmad[1], Evelina Martinenaite[1, 2], Özcan Met[1, 3], Rasmus Bak[4], Mads H. Andersen[1, 5]

[1]National Center for Cancer Immune Therapy (CCIT-DK), Department of Oncology, Copenhagen University Hospital, Herlev, Denmark, [2]IO Biotech ApS, Copenhagen, Denmark, [3]Section for Experimental and Translational Immunology, Department of Health Technology, Technical University of Denmark, Kongens Lyngby, Denmark, [4]Department of Biomedicine, Aarhus University, Aarhus, Denmark, [5]Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

The immunosuppressive tumor microenvironment (TME) hampers the efficacy of cancer immunotherapies. This highlights the need for strategies to combat local tumor immunosuppression. One promising approach involves anti-regulatory T cells (anti-Tregs), a subset of naturally occurring pro-inflammatory T cells that recognize tumor microenvironment antigens (TMAs). TMAs, derived from immunosuppressive mediators such as programmed death-ligand 1 (PD-L1), transforming growth factor beta (TGF), and indoleamine-2,3-dioxygenase (IDO), are expressed by cellular subsets in the TME. Anti-Tregs specific for PD-L1-derived peptides have demonstrated the ability to recognize and kill PD-L1-expressing cancer cell lines and regulatory immune cells in a human leukocyte antigen (HLA)-restricted manner. Boosting anti-Tregs through peptide vaccines has shown promise by directly modulating immune regulation and improving the efficacy of checkpoint inhibitors in a phase II clinical trial. However, individual responses to peptide vaccinations vary. This project explores whether adoptive cell therapy can similarly or more effectively modulate the TME. A T cell receptor (TCR) recognizing the PD-L1-derived 9-mer PDL101 peptide was identified through TCR sequencing of PD-L1-specific anti-Tregs. Using CRISPR-Cas9 genome engineering, we aim to orthotopically integrate the transgenic PDL101-TCR into CD8 T cells to replace the endogenous TCR. We hypothesize that CRISPR-engineered TCR-T cells expressing the PDL101-TCR could shift the TME towards a less immunosuppressive state, thereby potentiating anti-tumor immune responses. To achieve this, a homology-directed repair template (HDRT) template encoding the PDL101-TCR was designed for insertion into the TCR alpha constant (TRAC) locus. The HDRT, produced as double-stranded DNA, was introduced into CD8 T cells alongside TRAC-targeting single guide RNAs via nucleofection. Knock-in efficiency and transgenic PDL101-TCR expression were confirmed through tetramer staining and PCR of the HDRT junctions within the TRAC locus. Optimization strategies, including inhibition of non-homologous end joining, DNA sensor inhibition, and HDRT modifications, enhanced the generation of PDL101-TCR-T cells. Furthermore, a dual knockout of TRAC and TCR beta constant (TRBC) loci reduced mispairing with the endogenous TCR beta chain, increasing transgenic PDL101-TCR expression. Preliminary functional studies demonstrated that the engineered PDL101-TCR-T cells were highly specific towards the PDL101-peptide. These results emphasize the feasibility of integrating the transgenic PDL101-TCR into the TRAC locus warranting further analysis of the ability of these cells to target PD-L1-expressing target cells and modulate the TME.

78 - Optical pooled screening infrastructure at DCI

Dimitriya Garvanska[1], Jakob Nilsson[1]

[1]DCI/CPR/KU

Genetic screening is a powerful tool for linking genes with cellular phenotypes, and the advent of CRISPR-based genome editing has enabled screens with tremendous precision at massive scale. Genetic screening has previously been restricted to simple readouts hampering its use for studying complex biological processes. A recently developed technique termed optical pooled screening overcomes this limitation by coupling microscopy-based phenotyping and genotyping. This technology requires specialized equipment and knowledge, making it inaccessible to most researchers. To overcome this barrier, we will establish the first open-access facility for optical pooled screening at the Danish Cancer Institute, providing access to the required equipment and supporting researchers at all steps of the procedure, making this groundbreaking method broadly available and thereby accelerating biomedical research in Denmark. I will present our plans for this future infrastructure and examples of how optical pooled screens can be used to dissect biology.

75 - Engineering a potent CRISPR-Cas12l genome editor with C-rich PAM recognition (Industry Talk)

Giedrius Gasiunas[1], Joshua K. Young[2], Tomas Urbaitis[1], Laima Trinkuniene[1, 3], Migle Stitilyte[1, 3], Renatas Krasauskas[1], Monika Petrauskyte[1], Ieva Lenkaite[1, 3], Giedrius Sasnauskas[3], Giedre Tamulaitiene[3], Megumu Mabuchi[4], Virginijus Siksnys[1, 3]

[1]Caszyme, [2]Corteva Agriscience, [3]Vilnius University, [4]New England Biolabs

Here, we solved the structure of a compact (~850 aa) type V CRISPR effector, Cas12l, at 2.9 Å using cryo-electron microscopy (cryo-EM) and used it to engineer variants capable of editing the human genome. Structural analyses revealed similarities to type V CRISPR nucleases as well as new previously unobserved features. These included: 1) an H-type RNA pseudoknot forming guide RNA (gRNA) scaffold; 2) a unique N-terminal domain that stretches from the beginning to the end of the ribonucleoprotein (RNP) bound DNA target site effectively locking the ternary complex in place. Next, using structure-guided engineering, we improved Cas12l DNA target editing in human cells by approximately 10-fold by fine-tuning its electrostatic binding potential at DNA and gRNA interfaces mapped in our structure. To maximize potency, amino acid substitutions predicted with large language protein models to improve folding and stability were next introduced and evaluated for additional enhancements. In combination with the structure-guided modifications, two of the AI-predicted substitutions enabled efficient Cas12l editing when delivered transiently as mRNA or RNP in human cells. Collectively, this work expands the RNA guided genome editing repertoire to one that is compact, recognizes a simple C-rich PAM, and displays a distinctive DNA target lock and catch mechanism.

73 - Antigen-scaffold-mediated expansion of CRISPR CAR- and TCR-T cells generates highly enriched, efficacious T-cell products

Kristoffer Haurum Johansen[1], Keerthana Ramanathan[1], Carlos Rodriguez-Pardo[1], Hólmfridur Rósa Halldórsdóttir[1], Siri Tvingsholm[1], Laura Stentoft Grand[1], Edoardo Dionisio[1], Alberte Hjortgård Skriver[1], Louise Pii Frederiksen[1], Mette Bjørnson[1], Marcus Svensson-Frej[1], Sine Reker Hadrup[1]

[1]T-cells and cancer, Experimental and Translational Immunology Section, Department for Health Technology, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

The engineering of autologous T cells using CRISPR/Cas9 has great potential in the treatment of cancer. Expression of chimeric antigen receptors (CAR) can induce profound clinical responses in hematologic malignancies, whilst T cell receptor-engineered T (TCR-T) cells have led to durable responses in clinical trials. Previously, most of these therapies have applied viral delivery and expression of the CAR or TCR of interest. However, lentiviral engineering is associated with challenges regarding the production and functional characteristics of the engineered cells.

Non-viral delivery of TCRs using electroporation of CRISPR/Cas9 and homology-directed repair templates (HDRT) solves several of the problems associated with viral delivery. Most importantly, it allows for a more uniform expression of the TCR or CAR than viral delivery. In this study, we investigated novel HDRT types for the delivery of synthetic receptors using CRISPR/Cas9. We further optimized protocols for effective knockin (KI) of TCRs, using closed-circular DNA as HDRT and small molecule inhibitors that increase KI efficacy, resulting in ~20% KI of the 1G4 NY-ESO-1-specific TCR.

Following knockin, we adapted an existing protocol for specific expansion of antigen-specific CRISPR TCR-T and CAR-T cells using antigen-presenting scaffolds (Ag-scaffolds) that co-present peptide MHC (pMHC), IL2, and IL21 for TCR-T cell expansion, and CD19 antigen for the anti-CD19 CAR-T cells. Applying this expansion technique, we could achieve >90% antigen-specific cells after 14 days of in vitro culture. The expanded TCR-T cells could effectively kill peptide-presenting cancer cells and showed favorable phenotypes as determined by flow cytometric analysis. Similarly, antigen-specifically expanded CRISPR CAR-T cells effectively killed target CD19-expressing Jeko-1 cancer cells even after multiple recurrent challenges. We further compared Ag-scaffold expanded lentiviral engineered T cells with Ag-scaffold expanded CRISPR/Cas9-engineered T cells. We found that both lentiviral and CRISPR/Cas9-engineered T cells expanded using Ag-scaffolds were more efficient at killing target cells than cells expanded using conventional cytokines. To validate these findings in vivo, we inoculated Jeko-1 cancer CD19+ cells subcutaneously in immunodeficient NSG mice. Following tumour establishment we evaluated the capacity of Ag-scaffold-expanded CRISPR CAR-T cells to kill the tumours. We found that Ag-scaffold-expanded CRISPR CAR-T cells were markedly more efficient at clearing the tumour than cytokine-expanded CAR-T cells supporting the beneficial expansion driven by the Ag-scaffolds.

We here demonstrate the use of Ag-scaffolds for preferential expansion of engineering T cells, following either CRISPR or lentivirus based gene-insertion, and their improvements in cell numbers and functional capabilities, compared to standard protocols in vitro and in vivo. Together this demonstrates the advantages of Ag-scaffold in generating T cell products with antigen receptors of interest for TCR-T and CAR-T cell therapy.

72 - Nonviral CRISPR/Cas-engineered tumor-infiltrating lymphocytes with inducible cytokine release for adoptive T cell therapy

Nanna Steengaard Mikkelsen[1], Michael Crowther[1], Rasmus Bak[2], Inge Marie Svane[1], Özcan Met[1, 3]

[1]National Center for Cancer Immune Therapy (CCIT-DK), Department of Oncology, Copenhagen University Hospital, Herlev, Denmark, [2]Department of Biomedicine, Aarhus University, Aarhus, Denmark, [3]Section for Experimental and Translational Immunology, Department of Health Technology, Technical University of Denmark, Kongens Lyngby, Denmark

Adoptive cell therapy (ACT) using tumor infiltrating lymphocytes (TILs) has demonstrated remarkable clinical responses in patients with metastatic melanoma. However, insufficient expansion and persistence of tumor-reactive T cells remain a major cause of relapse. Stimulation with various cytokines such as IL-7 and IL-15 can skew T cells towards phenotypic subsets associated with enhanced persistence and antitumor activity. Thus, synthetic modulation of TILs towards desirable phenotypes by cytokine expression is favorable. However, toxicities have previously been reported due to systemic cytokine administration or constitutive cytokine expression by tumor-reactive cells. Placing the cytokine transgene under control of a Nuclear Factor of Activated T cells (NFAT) inducible promoter overcomes this challenge and links cytokine expression to antigen recognition and downstream T cell activation signaling. Here, a nonviral CRISPR/Cas genome engineering strategy is employed for targeted integration of the NFAT-inducible cytokine cassette into the PDCD1 gene locus to achieve simultaneous disruption of PD-1 expression and escape from tumor-mediated immunosuppression. We have demonstrated efficient nonviral CRISPR/Cas-mediated targeted integration of large transgene cassettes and NFAT-inducible transgene expression with simultaneous disruption of PD-1 in primary human T cells and patient-derived TILs. Successful application of our traditional 14-day TIL-based ACT rapid-expansion protocol to these nonviral CRISPR/Cas-engineered TILs carrying genetic payloads indicates a potential for clinical translation. In addition, functional analyses will demonstrate whether enhanced persistence and antitumor activity are achieved with these nonviral CRISPR/Cas-engineered TILs with cytokine payloads. Thereby, this study aims to improve future TIL therapies to benefit cancer patients by developing a clinically relevant, nonviral strategy to genetically modify TILs through simultaneous integration of an inducible NFAT-driven cytokine expression cassette and disruption of PD-1. In return, this may yield an increased frequency of T cell phenotype subsets associated with enhanced persistence and enhanced antitumor activity.

70 - Efficient, multi-kilobase knockins using CRISPR/Cas9 and rAAV donors and unbiased, whole-genome detection of donor integrations by TRAP-seq

monica Sentmanat[1], Zi Teng Wang[1], Evguenia Kouranova1[1], Samuel Peters[1], Wan Ching Chan[1], Jed Lin[1], Yong Miao[1], J. Michael White[2], Mia Wallace[3], Xiaoxia Cui[4]

[1]Genome Engineering & Stem Cell Center at McDonnell Genome Institute, Washington University in St. Louis, [2]Transgenic, Knockout and microinjection Core, Washington University in St. Louis, [3]Mouse Genetics Core, Washington University in St. Louis, [4]Genome Engineering & Stem Cell Center at McDonnell Genome Institute, Department of Genetics

Mouse models with multi-kilobase knock-in (KI) are necessary yet challenging to create. The combination of electroporation of Cas9/gRNA ribonucleoprotein complex (RNP) and rAAV donor delivery enables highly efficient KI. We report over 100 KI mice created with rAAV donors and variables affecting KI efficiency. To overcome the payload limit of the rAAV genome, we co-delivered two or three rAAV donors with CRISPR RNPs and achieved up to 6.6 kb KI via sequential insertion events. To thoroughly characterize the genome after editing, we developed TRAP-seq (Target Capture with amplicon probes for long read sequencing), to verify precise KIs as well as identify genome-wide undesirable editing events, including partial insertions, concatenated donors, and random integrations. We moved away from the expensive oligo probes via synthesis and generated biotinylated probes with flexible lengths by using asymmetric PCRs at less than 1/10 of the cost. We show TRAP-seq is a cost-effective, technically straightforward method capable of >100-fold enrichment, allowing multiplexing of up to 96 samples for the same or mixed targets in a single MinIon flow cell. Additionally, we successfully applied the multi-rAAV-donor approach in cancer and stem cell lines, including those where KI was not possible previously due to low tolerance to exogenous DNA, and adapted TRAP-seq for high throughput and low-cost clonal screening. In summary, we report two new strategies to significantly improve efficiency and precision in multi-kilobase KI models: CRISPR and multiple rAAV-mediated large KIs and a much-needed, comprehensive genome-wide analysis for both precise, on-target insertion and unwanted, potentially phenotypic-skewing edits.

69 - Endonuclease-Free RNA Editing Tools for Treating Inherited Retinal Dystrophies

Ana Pilar Gómez Escribano[1, 2], Natalia Galindo[3, 4], Yahya Jad[5], Gema García García[2, 3, 4], Marcos de la Peña[6], Jennifer Frommer[5], Thomas Roberts[5], Pilar Barberán[2, 3, 4], Elena Aller[2, 3, 4, 7], Teresa Jaijo[2, 3, 4, 7], Federico V Pallardó[1, 2, 8], Matthew Wood[5], Miguel Varela[5], José María Millán[2, 3, 4]

[1]1.Group of Cellular and Organic Pathophysiology of Oxidative Stress and Rare Diseases, Physiology department, University of Valencia, Spain, [2]2.Research Biomedical Network of Rare Diseases (CIBERER), Madrid, Spain., [3]3.Group of Molecular, Cellular and Genetics Biomedicine (BMCG), Health Research Institute La Fe (IIS La Fe), Valencia, Spain., [4]4.Mix Unit CIPF-IIS La Fe, Valencia, Spain, [5]5.Laboratory of RNA biology and Neuromuscular Disease, Institute of Developmental and Regenerative Medicine (IRDM), University of Oxford, United Kingdom, [6]6.Laboratory of Functional Genomics and Biotechnology of Non-Coding RNAs, Institute of Molecular and Cellular Plant Biology (IBMCP), [7]7.University and Polytechnic Hospital La Fe, Genetics department, Valencia, Spain., [8]8. Clinic University Hospital of Valencia (INCLIVA), Spain

RNA editing is emerging as a safer alternative for correcting G>A pathogenic variants in a reversible manner. The development of endonuclease-independent systems offers advantages for minimizing undesired physiological changes associated with exogenous expression of adenosine deaminases (ADARs) and nucleases. Some of these platforms have been optimized to enhance their stability and specificity. One such system, LEAPER 2.0, uses long-circular RNA oligonucleotides (151 nt) to recruit endogenous ADAR for editing G>A. However, this system in prone to bystander edits, requiring further optimization of its design. Recently, a novel strategy using chemically-short oligonucleotides (32 nt), known as AIMers, could supply the limitations of long oligonucleotides.

Our goal is to explore the potential of LEAPER 2.0 and AIMers for editing G>A variants responsible for inherited retinal dystrophies (IRDs). IRDs are a group of rare eye diseases caused by mutations in over 300 genes. We have selected prevalent G>A variants in the ABCA4 gene, one of the most common genes associated with recessive IRDs, to evaluate the efficacy of these RNA editing platforms in vitro.

We have designed constructs to generating circular RNAs for through in vitro transcription and exogenous cell expression. However, we have not yet observed successful editing. In parallel, we have opened a recent collaboration (Wood Lab, Oxford) by a STMS to synthesize chemically-short oligonucleotides targeting G>A variants in the ABCA4 gene. For this project, we have designed potential oligonucleotides considering chemistry backbone, more promising targets and prevalence, inspired by Wave technology. These oligonucleotides could be applied like antisense oligonucleotides used as splicing modulators via intravitreal injections, facilitating repeated applications.

68 - Novel CRISPR-Mediated EDSpliCE System Corrects Splicing Defect Caused by the Exonic ABCA4:c.768G>T Variant in Stargardt Disease.

Arturo Flores-Tufino[1], Susanne Kohl[1], Bernd Wissinger[1], Pietro De Angeli[1]

[1]Institute for Ophthalmic Research, University Hospital Tübingen, Germany,

Stargardt disease type 1 (STGD1) is a progressive retinal disorder caused by bi-allelic pathogenic variants in the **gene ABCA4**. It leads to central vision loss, often in childhood or early adulthood. This is due to the accumulation of toxic by-products in photoreceptors, caused by functional defects of ABCA4 as transporter in the retina. The **ABCA4:c.768G>T variant** is a severe and common mutation, acting on splicing. Located at the last nucleotide of ABCA4 exon 6, this synonymous variant weakens the canonical **splice donor site** (SDS), resulting in a 35-nucleotide elongation of exon 6 due to an alternative SDS downstream into the intron 6. This frameshift causes protein truncation and loss-of-function.

CRISPR-mediated genome editing strategies can effectively correct splicing defects and restore proper mRNA and protein expression, providing a potential permanent correction for genetic defects caused by pathogenic splice-altering variants. However, to our knowledge, no CRISPR/Cas approach has yet corrected aberrant mis-splicing from variants in exons or near exon/intron boundaries. To address this, we developed our novel editing platform **EDSpliCE** (**E**nhanced-**D**eletion **Spli**cing **C**orrection **E**diting) to successfully correct the c.768G>T splicing defect. Our system consists of a short but highly active, specific and directional, synthetic RNA-guided nuclease fused to a human exonuclease.

A minimal minigene (MG) construct harboring the ABCA4:c.768G>T variant was cloned. A minigene assay was utilized to functionally test and confirm the aberrant mis-splicing in HEK293T. The validated mutant minigene (MT-MG) was then co-transfected with 4 individual single-guide RNAs (sgRNAs) along with the EDSpliCE vector. Minigene-specific transcripts from cDNA were amplified by PCR and analyzed to calculate the percentage of correctly spliced transcripts using chip electrophoresis. For the top-sgRNA candidate, a high-throughput sequencing library was prepared to characterize and quantify the editing profiles.

To study the potential of top sgRNA candidates in correcting the aberrant mis-splicing, a model more representative of human photoreceptors with full genomic context was employed. First, an isogenic induced pluripotent stem cell (iPSC) line homozygous for the ABCA4:c.768G>T variant was generated by knock-in into a control iPSC line using electroporation to deliver CRISPR/Cas9 system as a ribonucleoprotein complex along with a donor template. After genotyping and characterization, iPSC line was differentiated into photoreceptor precursor cells (PPCs) to validate the aberrant mis-splicing. Effective delivery of the EDSpliCE system with the lead candidate sgRNA-2 into PPCs for genome editing will be performed using recombinant adeno-associated virus (rAAV) with enhanced transduction efficiency for photoreceptors.

Our EDSpliCE system successfully corrected the mis-splicing caused by the c.768G>T variant through directional sequence deletion of the alternative SDS. EDSpliCE with the lead sgRNA-2 achieved up to **89±8% correction rate** at the minigene level, outperforming the limited rescue from wild-type Cas9-ortholog. EDSpliCE's modular exonuclease enabled safe frame editing. Furthermore, its compact size allows packaging into a single rAAV with improved retinal tropism, positioning EDSpliCE as a promising therapeutic approach for the ABCA4:c.768G>T variant.

67 - CATS: A Bioinformatic Tool for Automated Cas Nucleases activity comparison in clinically relevant contexts

Ettore Rocchi[1], Federico Magnani[1], Gastone Castellani[1], Antonio Carusillo[2], Martina Tarozzi[1]

[1]University of Bologna, [2]Alia Therapeutics

The CRISPR/Cas system is probably the most used genetic engineering tool. In the past years, a number of CRISPR/Cas systems have been characterized and optimized to suit the different needs of experimenters: editing efficiency, nuclease specificity and cargo size.

With an overwhelming number of Cas nucleases within the genetic engineers' portfolio, picking the right one can be daunting. Because the Cas nucleases to compare have usually different requirements in terms of protospacer adjacent motif (PAM) sequences, to properly do so a common target site should be identified. This removes the bias connected to the natural genetic landscape characterizing the target of choice. This time-consuming operation becomes even more tedious when extended to multiple - 5 to 10 - target sites, to properly assess the nuclease efficiency. Traditional methods for comparing Cas nucleases and identifying suitable targets involve manual curation of PAM sequences. This process can be further complicated if the experimenter wishes to select a nuclease targeting a specific single-nucleotide-variant (SNV) to devise an allele-specific approach, for example.

We sought to develop an automated tool able to streamline these tasks, making CRISPR/Cas experiment design more efficient and accessible to a broader range of researchers.

Here, we present Comparing Cas Activities by Target Superimposition (CATS), a novel bioinformatic tool designed to automate the detection of overlapping PAM sequences and identify allele-specific targets. CATS presents enables two main operations. First, it enables the comparison of two Cas nucleases by accepting as input the two respective PAMs. Then it automates the identification of targets where multiple PAMs coexist either within one of the two default reference genome - human and murine - or within a custom FASTA sequence provided by the experimenter. Second, if the researcher aims to design an allele-specific approach, CATS by cross-checking the wild-type sequence with pathogenic variants annotated on ClinVar can provide a list of targets within a suitable-PAM is generated de novo.

In the case study we tested CATS, we show how it can significantly reduces the time and effort required for CRISPR/Cas experiment design. By automating the detection of overlapping PAM sequences, CATS provides rapid and accurate results, enhancing experimental design efficiency.

The tool's flexibility in using any reference genome makes it versatile for researchers working on various models. The ability to design allele-specific targeting approaches is particularly useful for devising strategies where the nuclease targets the pathogenic allele while sparing the healthy one. Users can employ CATS either by command line or by a dedicated, user-friendly graphical interface. CATS offers a streamlined and user-friendly experience, making it accessible to researchers regardless of their computational expertise. CATS addresses the challenges of PAM sequence identification and comparison, offering a powerful tool for the efficient design of CRISPR/Cas experiments. By automating these processes, CATS enhances the precision and effectiveness of therapeutic solutions for genetic disorders. In conclusion, CATS represents a valuable addition in the bioinformatic toolkit for genome editing experiments, having as overall strengths the automation, speed, and user-friendliness of the tool.

65 - Identification of T-cell receptors from Tumour-Infiltrating Lymphocytes cross-reactive to multiple patient-derived metastatic melanoma cell lines using CRISPR-cas9 mediated TCR replacement

Michael Crowther[1], Marie C. W. Westergaard[1], Luke A. Schuster[1], Kristoffer H. Johansen[2], Siri A. Tvingsholm[2], Sine R. Hadrup[2], Inge Marie Svane[1], Özcan Met[1, 2]

[1]National Center for Cancer Immune Therapy, [2]Department of Health Technology, DTU

Adoptive cell therapy (ACT) with tumour-infiltrating lymphocytes (TILs) has demonstrated remarkable clinical responses in patients with metastatic melanoma. However, limitations of TIL therapy involve exhaustion and resulting poor persistence in some patients, as well as a significant presence of bystander TILs that do not respond to tumour. The introduction of T-cell receptors (TCRs) and chimeric antigen receptors (CARs) into the TIL product could provide a boost in reactivity of TILs to enhance tumour killing.

Given that TILs are already a good source of tumour-reactive TCRs, we sought to identify TCRs that respond to multiple patient-derived metastatic melanoma cell lines to generate candidate TCRs that have broad reactivity. For this, we sorted TILs from patients M33 and M43 reactive against autologous and non-autologous HLA-A2 matched melanoma cell lines. Single-cell transcriptomic data was used to identify five TCRs with the most potential for reactivity, and these were inserted into healthy donor-derived peripheral blood mononuclear cells (PBMCs), using homology-directed repair (HDR) of the TCR β locus.

TCR-replaced PBMCs were then expanded to high numbers and screened against a panel of melanoma cell lines, assessing cytokine secretion and cytotoxicity, with 4 of 5 TCRs showing strong reactivity to non-autologous cell lines. TCR-replaced PBMCs displayed no reactivity to a B2M knockout cell line, and reduced activity to HLA-A2 blocked cell lines, indicating HLA-A2 restriction. TCR-replaced PBMCs were then screened against a panel of HLA-A2 tetramers containing the 30 most common shared melanoma tumour-associated antigens, to identify the peptide antigen presented by HLA-A2.

These results display a powerful new method of identifying novel cross-patient reactive TCRs restricted to HLA-A2. TCR replacement functions as a valid approach to test candidate TCRs; and could be used as therapy using TCR-replaced PBMCs analogous to CAR therapy, or alternatively, knocked into TILs to boost reactivity of the whole population. As these TCRs are reactive to shared antigens, the requirement of complex and expensive sequencing and neoantigen screening, alongside screening of individual TCRs from each patient is removed, therefore greatly reducing time-to-treatment.

64 - Functionalized Lipid Nanoparticles for the Pulmonary Delivery of RNA-therapeutics

Simon Papai[1, 2], Saahil Baghel[1], Henrik Møller[2], Camilla Foged[1]

[1]University of Copenhagen, [2]Valinor ApS

Lipid nanoparticles (LNPs), used for delivering the mRNA-based COVID-19 vaccines, have become the gold-standard delivery system for RNA-based therapeutics. These safe and efficient mRNA delivery systems can be leveraged as vehicles for CRISPR/Cas administration. However, a substantial challenge remains with LNPs for the efficacious extrahepatic delivery of CRISPR/Cas including delivery to main respiratory cells in the lungs.

Functionalized LNPs hold great promise for more specific uptake in targeted tissues/cells, thereby enhancing editing efficiency while minimizing off-target effects. This study investigates the functionalization of mRNA-LNPs by peptide conjugation (P-LNP) using a standard SM102 LNP formulation (Moderna) combined with a quick and efficient coupling method to DSPE-PEG2000-maleimide.

One selected cationic peptide was found to promote a 3-to-6-fold increase in bioluminescence in human lung adenocarcinoma epithelial cells, 24h after transfection of +P-LNP loaded with firefly luciferase (fLuc) mRNA. On the contrary, an anionic counterpart (-P-LNP) did not improve Fluc protein levels. Intravenous injection of fLuc mRNA-loaded P-LNPs supported delivery to the lungs of BALB/c mice in a peptide density-dependent manner. Flow cytometry measurements are underway to reveal and quantify the cell types taking up P-LNPs in the lungs, using Cy5-fLuc mRNA and DiR dye as detectable markers.

Our preliminary data supports that the functionalization of SM102 LNPs with selected peptides can promote significant fLuc-mRNA delivery to the lungs via intravenous injection, in contrast to the standard SM102 LNP formulation. Additional experiments will be performed on lung delivery and specific lung cells to dissect the role of the coupled peptide motif on cellular uptake and to study the influence of the peptide net charge. Additionally, the protein corona assembled around the different P-LNPs under physiological conditions will be assessed by proteomics to elucidate further key components that promote delivery to thelungs. A mechanistic understanding of the cell specificity of various LNPs will aid our selection of an efficient CRISPR/Cas9 delivery vehicle to generate gene edits in the lungs.

63 - Single gRNA Cas9- and EDCas9-mediated splicing correction of the frequent USH2A:c.7595-2144A>G pathogenic variant

Pietro De Angeli[1], Salome Spaag[1], Stefanida Shliaga[1], Arturo Flores-Tufino[1], Malte Ritter[2], Masoud Nasri[2], Bernd Wissinger[1], Susanne Kohl[1]

[1]Institute for Ophthalmic Research, University Hospital Tübingen, Germany, [2]Department of Oncology, Hematology, Clinical Immunology, and Rheumatology, University Hospital Tübingen, Germany.

Usher syndrome is a rare inherited autosomal recessive ciliopathy, with USH2A mutations accounting for ~50% of all cases. The frequent deep-intronic variant USH2A:c.7595-2144A>G activates a cryptic splice site, causing aberrant splicing. While antisense oligonucleotides can effectively correct this splicing defect, they do not provide life-lasting correction. We therefore explored single gRNA CRISPR/Cas approaches as a permanent solution to correct such a splicing defect.Six single gRNAs targeting faulty splicing features were paired with either standard **Cas9** or **TREX2-fused to Cas9 (EDCas9)** to assess splicing correction and genomic mutational profiles generation. TREX2 was incorporated to enhance deletion frequency following Cas9-induced double-strand breaks. These strategies were tested in a minigene assay and patient-derived fibroblasts. Off-target effects were analyzed using GUIDE-seq and predicted sites, while long-read sequencing assessed the frequency of large deletions. Chromosomal translocation potential was evaluated through an intrachromosomal junction assay.**Splicing correction was achieved** in both models, reaching **up to 92.4±4.8%.** Standard Cas9 with single gRNAs generated small, heterogeneous indels across the two models, while resulting in variable splicing correction. In contrast, EDCas9 produced larger, localized deletions (15-50 bp) that effectively disrupted faulty sequences, ensuring consistent splicing correction and uniform deletion profiles between HEK293T and patient-derived fibroblasts. GUIDE-seq and predicted site analyses confirmed a **safe off-target profile** for both nucleases. Long-read sequencing demonstrated that EDCas9 did not increase large deletions, confirming the localized activity of TREX2. Intrachromosomal junction assays **revealed no translocations with EDCas9**, whereas standard Cas9 induced translocation events. **Virus-like particle (VLP)-mediated delivery of the lead gRNA paired to EDCas9** in patient-derived fibroblasts achieved substantial editing and exhibiting the expected mutational profiles, characterized by highly directional and larger deletions.In conclusion, this study demonstrates that both SpCas9 and EDCas9 paired with single gRNAs can correct the USH2A:c.7595-2144A>G splicing defect. EDCas9 consistently achieved higher and more uniform splicing correction rates with localized deletions across models, whereas SpCas9 showed variable efficiency and produced small, heterogeneously distributed indels. Importantly, EDCas9 exhibited a favorable safety profile, avoiding large deletions and chromosomal translocations seen with Cas9, supporting its potential for further therapeutic investigation. Ultimately, the use of VLP-mediated EDCas9 delivery highlights its promise as a therapeutic platform for correcting pathogenic variants that benefit from targeted, larger deletions, such as those affecting splicing or regulatory sequences.

61 - An AAV HDR template design to improve Cas12a-mediated insertion for the development of advanced allogenic CAR-T cell therapies

Paul Donohoue[1], Jenny Basta[1], Arthur Owens[1], Robert Munch[1], Rachel Gonzales[1], Matt Thompson[1], Scott Gradia[1]

[1]Caribou Biosciences, Inc.

CAR-T cell therapies have introduced a new treatment frontier for cancer by leveraging the innate cytotoxic abilities of immune cells reprogrammed with chimeric antigen receptors (CARs) to target specific tumor cell antigens. While all currently approved CAR-T cell therapies are autologous and utilize a patient's own cells, there have been an increasing number of clinical trials evaluating allogeneic CAR-T cell therapies whereby healthy donor cells are engineered to replace endogenous T cell receptors with a recombinant CAR allowing for administration to any patient. Allogenic CAR-T cell therapies are poised to greatly expand patient access to this new treatment modality, however clinical success likely requires the cells to undergo modification to overcome rapid rejection by the patient's immune system. Using genome editing tools such as Cas nucleases, expression of cell surface proteins that contribute to allograft rejection could be disrupted and novel transgenes introduced to enhance both persistence and antitumor activity of allogeneic cell therapies. The need to simultaneously cloak and armor the next generation of allogeneic CAR-T cell therapies necessitates advanced engineering strategies to facilitate development of more complex products comprising a greater number of gene knockout and transgene insertions while simultaneously ensuring intended repair outcomes from transgene insertion. To that end, we have inserted a Cas12a target site adjacent to the inverted terminal repeat of an adeno-associated virus (AAV) homology-directed repair (HDR) template sequence to reduce the formation of unwanted transgene concatemers using the highly-specific Cas12a chRDNA technology to cleave the AAV construct after transduction into human T cells. Additionally, we show that two discreet HDR edits at two genomic loci can be simultaneously performed using a single AAV construct comprised of two HDR templates flanked by Cas12a target sites. Taken together, we have developed an engineering method that improves intended transgene insertion outcomes through reduced concatemer formation while increasing the number of HDR templates that could be introduced into a cell by a single AAV transduction event. Furthermore, we envision this approach being adaptable to other nuclease systems and other delivery modalities, as well as being applicable to gene insertion and correction approaches for both ex vivo and in vivo engineering.

60 - Targeting tumor specific copy number alterations using CRISPR-Kill

Mark Grannetia[1], Grigory Kornienko[1], Jesper Balk[1], Arjan Lakeman[1], Ellen Westerhout[1], Linda Valentijn[1], Rob Wolthuis[1]

[1]AmsterdamUMC

CRISPR/Cas9 can be programmed with pre-designed gRNAs to induce double strand breaks (DSBs) at chosen genomic sequences. The cytotoxicity of DSBs is often exploited in various cancer therapeutic strategies, including ionizing-radiation and platinum-based DNA crosslinking agents.

Here, we propose to use CRISPR/Cas9 as a programmable method to target the cancer genome directly. We aim to induce cancer-specific, gRNA-directed DSBs in high copy number targets of relevant tumors. By mining unfiltered DEPMAP CRISPR screen data we found cancer-type specific gRNA sensitivities related to high copy numbers of the respective gRNA targets. These findings indicate that different tumor types have unique thresholds of tolerable gRNA-induced DSBs, with subtypes of breast cancer and neuroblastoma as remarkably sensitive.

A method coined CRISPR-Kill was successfully used to eliminate specific plant tissues by the DSB toxicity of targeting tandem repeats (Schindele et al., 2022). In this project, we have explored the effectiveness of the CRISPR-Kill approach on Cas9-expressing tumor cell models by lenti-viral delivery of tumor-specific gRNAs. We notice specific and effective tumor cell killing in DNA repair-deficient breast cancer and neuroblastoma cell models by inducing DSBs in tumor-specific loci, using a single amplicon-directed gRNA. Currently, we are developing a therapeutic delivery approach by combining synthetic Cas9 mRNA, gRNAs and lipid nanoparticles (LNP) for in-vitro and in-vivo applications.

Further validation of our findings will proceed in in-vivo xenograft mouse models through intravenous LNP injection. Additionally, to explore specificity and efficacy of CRISPR-Kill, we aim to use co-cultures with non-cancerous human cells, as a novel in-vitro tumor model. As such, we are investigating whether CRISPR-Kill can be used to eliminate cancer cells from these co-cultures. By optimizing CRISPR-Kill, therapy resistance and intra-tumor heterogeneity might be tackled using multiple rounds of adapted clinical gRNAs, targeting the tumor genomes of separate subpopulations or relapses.

59 - Efficient CRISPR editing with a hypercompact Cas12f1 and engineered guide RNAs delivered by adeno-associated virus

JEONGMI LEE[1]

[1]Korea Research Institute of Bioscience and Biotechnology, KRIBB

Gene therapy would benefit from a miniature CRISPR system that fits into the small adeno-associated virus (AAV) genome and has high cleavage activity and specificity in eukaryotic cells. One of the most compact CRISPR-associated nucleases yet discovered is the archaeal Un1Cas12f1. However, Un1Cas12f1 and its variants have very low activity in eukaryotic cells. In the present study, we redesigned the natural guide RNA of Un1Cas12f1 at five sites: the 5' terminus of the trans-activating CRISPR RNA (tracrRNA), the tracrRNA-crRNA complementary region, a penta(uridinylate) sequence, the 3' terminus of the crRNA and a disordered stem 2 region in the tracrRNA. These optimizations synergistically increased the average indel frequency by 867-fold. The optimized Un1Cas12f1 system enabled efficient, specific genome editing in human cells when delivered by plasmid vectors, PCR amplicons and AAV. As Un1Cas12f1 cleaves outside the protospacer, it can be used to create large deletions efficiently. The engineered Un1Cas12f1 system showed efficiency comparable to that of SpCas9 and specificity similar to that of AsCas12a.

56 - Innovations of process development: From oligonucleotides to guide RNA optimization using PAT and modeling

Dayoung Kim[1], Younggoo Kang[1], Jeonghyun Chu[1], Sungwon Kim[1], Jale Müslehiddinoğlu[1]

[1]ST Pharm

The development and optimization of oligonucleotide production technologies, including CRISPR applications, require innovative approaches to get both process understanding and high-quality oligonucleotides. In fact, the focus is shifting from merely collecting data to strategically utilizing it- determining what data to gather, how to analyze it, and leveraging it for continuous improvement. Integrating Process Analytical Technology (PAT) and modeling enhances process optimization and control, yield, and enables scalable production. Additionally, the development and optimization of guide RNA processes are critical for ensuring high-quality, reproducible RNA synthesis is impacted directly to influence the precision, efficiency, and therapeutic potential of CRISPR, gene-editing therapeutics. These efforts represent a comprehensive strategy to advance oligonucleotide technologies, ensuring scalability and effectiveness across diverse applications.

55 - Dual-Color Optical Genome Mapping for Enhanced GUIDE-seq Analysis in CRISPR-Cas9 Editing

Roberto Nitsch[1]

[1]AstraZeneca

Ensuring the genomic integrity of CRISPR-Cas9-edited cells requires robust methodologies that can detect both expected and unexpected structural variations without computational bias. Here, we present a novel dual-color Optical Genome Mapping (OGM) approach tailored for GUIDE-seq applications, enabling the direct visualization of CRISPR-induced structural changes at high resolution. Unlike sequencing-based methods that rely on computational alignment and variant calling, our PCR-free, bioinformatics-independent strategy provides an all-in-one unbiased readout of CRISPR Off-Targets and genomic structural alterations, including large deletions, insertions, and translocations. By integrating dual-color labeling, this method enhances the specificity and accuracy of genome-wide breakpoint detection, facilitating a fast and more comprehensive evaluation of genome editing outcomes. This technique offers a powerful alternative for assessing virtually any unwanted effects in CRISPR-based therapeutics, providing critical insights for translational applications.

Keywords: CRISPR-Cas9, Optical Genome Mapping, GUIDE-seq, genome integrity, off-target analysis, gene therapy

53 - Accelerating cost-efficient xRNA therapeutics research with noble ionizable lipid (STLNP®) and capping reagent (SmartCap®) technologies

SUNGWON KIM[1]

[1]ST Pharm

ST Pharm has developed its own xRNA (e.g., mRNA, circRNA) platform technology and lipid nanoparticle (LNP) drug delivery system, known as STLNP®. Several novel ionizable lipids were found through the screening system, and STLNP® using selected ionizable lipids showed similar physicochemical characteristics and protein expression levels to competitor LNPs. eGFP, hHEPO, and fLUC mRNAs with SmartCap® were encapsulated using STLNP® and compared with various LNPs to evaluate the protein expression level. STLNP® demonstrates similar luminescence expression efficacy compared to competitor LNPs. To investigate in vivo antigen-specific immune response for STLNP®s, mice were intramuscularly administered with SARS-CoV-2 spike mRNA encapsulated several different STLNP®s. Some STLNP®s showed high cellular immunogenicity levels, which can be good candidates for developing prophylactic and therapeutic vaccines. In addition, a repeated GLP toxicity study and in vivo immune cytokines evaluation were conducted to identify any potential toxicity of STLNP®. STLNP® enables efficient gene delivery, including mRNA, circRNA, saRNA, and other candidates in cell and gene therapy. Also, ST Pharm's capabilities and capacity as an xRNA CDMO will be described here.”

51 - Precise correction of the sickle cell disease mutation by DNA repair manipulation and genome editing in hematopoietic stem cells

Sumit Deswal[1], Panagiotis Antoniou[1], Nina Akrap[1], Sandra Wimberger[1], Sasa Svikovic[1], Michelle Porritt[1], Grzegorz Sienski[1], Mike Firth[2], Annarita Miccio[3], Marcello Maresca[1]

[1]Genome Engineering, Discovery Sciences, BioPharmaceuticals R&D Unit, AstraZeneca, Gothenburg, 43183, Sweden, [2]Data Sciences and Quantitative Biology, Discovery Sciences, BioPharmaceuticals R&D Unit, AstraZeneca, Cambridge, CB20AA, UK, [3]Laboratory of chromatin and gene regulation during development, Imagine Institute, University Paris Cite, INSERM UMR1163, Paris, 75015, France

The CRISPR/Cas9 system allows for precise correction of genetic mutations that lead to severe genetic diseases, such as sickle cell disease (SCD). In this project we explore gene editing approaches relying on Cas9- and DNA donor template-driven homology-directed repair (HDR) of the SCD mutation. HDR is active in proliferating cells and therefore occurs in low rates in quiescent hematopoietic stem/progenitor cells (HSPCs), the target cell population for gene therapy of SCD. Mutagenic DNA repair pathways, such as non-homologous end joining (NHEJ), or microhomology-mediated end joining (MMEJ), are activated upon Cas9-cutting and lead to the generation of undesired insertions and deletions (InDels).

Here, we screened single guide RNAs (sgRNA) and DNA donors coupled with Cas9 nucleases and identified efficient combinations that allowed high knock-in efficiencies in erythroid cell lines, as assessed by NGS. DNA repair profiling revealed the presence of NHEJ and MMEJ-mediated InDels, as well. To circumvent this, we used inhibitors of key enzymes of NHEJ and MMEJ repair (2iHDR), thereby enhancing HDR-mediated integration efficiencies and editing precision. We further optimized the editing outcome by increasing the Cas9, sgRNA and DNA donor concentrations, and by implementing Cas9 targeting sites in the DNA donor - known to increase knock-in efficiencies.

To apply 2iHDR in HSCs, we initially performed drug titrations and we identified optimal drug concentrations that allowed enhancement of precise genome editing and minimal effects on HSPC fitness, measured by a series of assays (cell counting, flow cytometry analysis of necrosis and apoptosis, expansion rate and CFC assay). We then combined the optimized genome editing strategies with optimal 2iHDR in HSPCs, and this allowed for therapeutically relevant levels of precise SCD correction and minimal InDels. FACS-sorting of HSPC subpopulations, upon editing and drug treatment, showed that our strategy works similarly in HSC-enriched versus progenitor subpopulations, without affecting their viability and clonogenic potential.

Moreover, we assessed the safety of our strategy in terms of off-target activity and genomic rearrangements. NGS showed that 2iHDR did not increase the off-target activity of SpCas9. We also used PsCas9 - a high-fidelity enzyme that is equally efficient to SpCas9. Indeed, no off-target activity was observed with PsCas9 when targeting the SCD mutation, while on-target editing was higher for PsCas9 than SpCas9. DdPCR showed low levels of genomic rearrangements for SpCas9 that were not increased by 2iHDR, while PsCas9 showed no rearrangements. Additionally, we performed RNA-seq analysis to evaluate the global effect of 2iHDR in HSPCs, and we transplanted treated HSPCs in immunodeficient mice to check their engraftment potential. Finally, we used barcoded DNA donors to track the clonality of treated HSPCs, confirming no effect of 2iHDR.

To conclude, we provided proof for the safety and efficacy of 2iHDR-mediated precise correction of the SCD mutation in HSPCs. Importantly, 2iHDR could be applied for the correction of other genetic mutations that are not amenable to alternative genome editing tools, such as base or prime editing. This proof-of-concept work will enable the pre-clinical and clinical development of 2iHDR HSPCs for the therapy of SCD.

47 - CRISPR-based dual-target G9a and tau pathology inhibition for Alzheimer's Disease therapeutics

Carmen Martínez-Fernández[1], Rafael Franco[2, 3, 4], Mercè Pallàs[1, 4], Christian Griñán-Ferré[1, 4]

[1]Department of Pharmacology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, Institut de Neurociències, Universitat de Barcelona, Avda. Joan XXIII, 27, 08028 Barcelona, Spain, [2]Department of Biochemistry and Physiology, School of Pharmacy and Food Sciences, Universitat de Barcelona, 08028 Barcelona, Spain., [3]School of Chemistry, University of Barcelona, 08028 Barcelona, Spain, [4]Centro de Investigación en Red, Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain.

Alzheimer's disease (AD) is a global health crisis and the leading cause of dementia, affecting millions worldwide. Despite extensive research efforts, effective treatments remain elusive due to the multifactorial nature of the disease, which involves complex interactions among genetic, epigenetic, biochemical, and environmental factors. Traditional drug-based single-target therapies have consistently failed in clinical trials, highlighting the urgent need for innovative approaches. In the last years, gene therapy strategies have evolved since they present several advantages over traditional drug-based therapies such as long-term or permanent effects, reduced side effects or high potential as one-time treatment. However, gene therapy is still a developing field, and while it shows great promise, it also presents challenges such as cost, accessibility, and potential risks. Multiple pieces of evidence suggest that epigenetic alterations particularly the G9a repressive mark, and Tau pathology through GSK3β, are two important hallmarks of AD. We want to go one step further and evaluate the impact of genetically inhibit both human G9a and GSK3β at molecular and systemic level in AD C. elegans model and, assess the effect of dual target inhibition and its effect at different timepoints: development and ageing. To do so, we will first evaluate the CRISPR inactivation, tissue-specific system in AD C. elegans models. Then, we will generate humanized AD nematodes by CRISPR-based replacement of hG9a and hGSK3β to predict the effect of our system in AD humanized context. Our study will provide a novel strategy for AD therapeutics and will elucidate its efficacy at molecular and physiological level during a pluricellular organism' lifespan, providing insights into the durability and safety of this strategy for potential AD therapies.

45 - DO1 and DO2 are novel Cas9 nucleases from the deep ocean that expand the CRISPR toolbox

Julián Cerón[1], Alba Olaso-Llorca[1], Pablo Sánchez[2], Silvia G. Acinas[2]

[1]Modelling Human diseases in C. elegans. Bellvitge Biomedical Research Institute-IDIBELL, Hospitalet de Llobregat, Barcelona, Spain, [2]Department of Marine Biology and Oceanography, Institut de Ciències del Mar, Barcelona, Spain.

Despite the wide diversity of Cas9 nucleases in nature (either characterized or still uncovered), Streptococcus pyogenes Cas9 (SpCas9) is the most frequently used Cas9 in gene editing. However, some SpCas9 drawbacks, including excessive activity that leads to off-target effects, make it necessary to expand the CRISPR-Cas toolbox to include novel and specialized Cas9 nucleases with distinct properties that can cover all the potential applications of genome editing.

We identified seven novel Cas9 nucleases and their corresponding repeats and tracrRNAs by mining metagenomics data of microbial communities from the deep ocean (between 1000 and 4000 m deep), collected during the Malaspina 2010 Circumnavigation Expedition. We further characterized two of them, Deep Ocean 1 and 2 Cas9 (**DO1 Cas9**, and **DO2 Cas9**), Class II type II-C and type II-B, respectively. These two nucleases, which present biophysical properties distinct from most of the described Cas9 nucleases, show good activity in vitro and very high specificity, being much more sensitive to mismatches in the spacer sequence than SpCas9. We have built a large network of collaborators of several institutions* (not mentioned all in the abstract) that are successfully testing DO1 and DO2 Cas9 in different model systems including zebrafish, plants, marine bacteria, planktonic tunicates, protozoa, and several human cell lines.

These two nucleases could enhance CRISPR approaches related to medicine, not only by their capacity to edit human cells but also by editing other organisms where SpCas9 is not that efficient. Considering the ONE HEALTH concept, we believe that DO1 and DO2 Cas9 can also contribute to the optimal editing of pathogens or other organisms that impact human health.

*CSIC, IDIBELL, CABD-Sevilla, Universidad de Murcia, Universidad de Alicante, Freiburg University, CRG-Barcelona, Genyo-Granada, IBMCP-Valencia, University of Copenhagen

44 - A Simplified method to Custom Transgenic Mouse Models Using CRISPR-RNPs through Virus-Like Particles

Da Eun Yoon[1, 2, 3], Tae Yeong Jeong[2, 3], Sol Pin Kim[4, 5], Jiyun Yang[2, 3], Soo-Yeon Lim[4], Sungjin Ok[2], Sungjin Ju[2, 3], Jeongeun Park[2, 3], Su Bin Lee[4], Soo-Ji Park[2, 3], Sanghun Kim[3], Hyunji Lee[3, 6], Daekee Lee[7], Soo Kyung Kang[4, 5], Seung Eun Lee[8], Hyeon Soo Kim[9], Je Kyung Seong[4, 5, 10], Kyoungmi Kim[2, 3]

[1]Transgenic core facility, Max-Planck Institute of Biochemistry, [2]Department of Physiology, Korea University College of Medicine, [3]Department of Biomedical Sciences, Korea University College of Medicine, [4]Korea Mouse Phenotyping Center, Seoul National University, [5]Laboratory of Developmental Biology and Genomics, BK21 Program Plus for Advanced Veterinary Science, Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, [6]Department of Convergence Medicine, Korea University College of Medicine, [7]Department of Life Science, Ewha Womans University, [8]Research Animal Resource Center, Korea Institute of Science and Technology (KIST), [9]Department of Anatomy, Korea University College of Medicine, Republic of Korea, [10]Interdisciplinary Program for Bioinformatics, Program for Cancer Biology, BIO-MAX/N-Bio Institute, Seoul National University

The development of diverse and precise transgenic models is essential for both therapeutic and basic research into various diseases. CRISPR technology has facilitated accurate gene editing, many researchers have used conventional methods such as microinjection, electroporation, and GONAD to introduce these editing tools into zygotes in the form of plasmids, mRNA, or ribonucleoproteins (RNPs) for the purpose of generating mutant mice. However, these techniques require specialized expertise and expensive equipment, which can restrict access for many laboratories. Additionally, these methods cause physical damage on embryos and disrupt normal developmental processes.To address these challenges, we propose an innovative approach to transgenic mouse modeling using virus-like particles (VLPs). VLPs represent a non-viral delivery system that is not constrained by cargo size, does not carry viral DNA, and consequently mitigates the risk of viral genome integration. Furthermore, the encapsulation of CRISPR components in the form of RNPs during VLP production diminishes off-target effects.In our study, we generated VLPs containing CRISPR-RNPs and subsequently co-cultured them with fertilized zygotes or during in vitro fertilization (IVF) procedures to introduce mutations into embryos without inducing physical damage. Notably, 20% of VLP-packaged Cas9/sgRNA exhibited an editing efficiency of 44.7%, resulting in a heterozygous genotype characterized by a frameshift-induced knockout due to the deletion of 29 nucleotides in the Plin1 exon 2. This mutation was successfully transmitted to subsequent generations, and the Plin1-knockout mice displayed reduced adipocyte size and increased infiltration of F4/80+ macrophages in both epididymal and inguinal white adipose tissue compared to wild-type mice.Moreover, the application of VLPs containing base editors yielded successful editing outcomes, with an observed increase in VLP productivity and 6.5-fold enhancement in editing efficiency, particularly following codon optimization of a segment of the gag. We also demonstrated the capability for simultaneous multi-target editing using different CRISPR systems.For knock-in applications, we co-cultured adeno-associated viruses (AAVs) containing donor DNA alongside VLPs with zygotes, successfully replacing exon 5 of the mouse Kcnq4 gene and a part of the intron with the corresponding human gene sequence.

In the group treated with 20% VLP-packaged ABE8e/sgRNA during the IVF process, we achieved heterozygous Tyr-mutant mice with the H420R genotype, exhibiting A-to-G substitution efficiency of 42.4% and no detectable off-target effects.This methodology simplifies and accelerates the generation of transgenic mouse models, without the need for specialized techniques and equipment. Consequently, it has the potential to facilitate the generation of customized mouse models tailored to specific research inquiries and is applicable across a broad spectrum of scientific disciplines.

43 - Establishing hiPS knockout cell lines to study ORAI1/2/3 function using cytosine base editing

Julia Herzberg[1], Tinatini Tavhelidse-Suck[1], Alex Cornean[1, 2], Marc Freichel[1, 3]

[1]Institute of Pharmacology, Heidelberg University, Heidelberg, Germany, [2]DataHarmony Ltd, 19 Collingwood Road, Yeovil, England, BA21 5FD, [3]DZHK (German Center for Cardiovascular Research), Partner Site Heidelberg/Mannheim, Heidelberg University, Heidelberg, Germany

Cardiovascular diseases are among the leading causes of mortality worldwide. However, many aspects of the disease mechanisms are not fully understood. For conditions such as hypertrophy or heart failure, research has shown that altered calcium signaling plays a vital role in the pathophysiology. This includes dysfunctions in the calcium release-activated calcium (CRAC) channels of the store-operated Ca^{2+} entry (SOCE) pathway. CRAC channels consist of members of the ORAI calcium release-activated calcium modulator proteins (ORAI1/2/3) located in the plasma membrane, regulating extracellular Ca^{2+} entry. To investigate the functions of CRAC channels and their underlying genes in cardiac health and disease, functional genomics studies using human induced pluripotent stem cell-derived (hiPSC) knockout models offer a promising approach.

Base editing, a recent advancement in the genome editing field, has emerged as an attractive alternative to CRISPR/Cas nucleases for generating gene knockout cell lines. Cytosine base editors (CBE) enable the precise introduction of C-to-T transition mutations without inducing double-strand DNA breaks (DSB). Single-base conversions introduced by CBEs can be leveraged for gene disruption by introducing either a premature termination codon, a splice donor mutation or a splice acceptor mutation. Whereas DSB-inducing approaches usually result in compound heterozygous editing outcomes, the higher editing precision and more predictable editing outcomes of CBEs facilitate the generation of homozygous knockouts while keeping off-target effects low.

Our study aims to utilize CBEs to generate ORAI1/2/3 single, double and/or triple knockout hiPS cell lines. To identify highly effective CBE-sgRNA pairs for our target loci, we performed a small-scale screen in HEK293 cells using the recently published transient reporter of editing enrichment (TREE). We compared the on-target editing efficiencies of two state-of-the-art CBEs, evoBE4max and CBE6b, and tested three different sgRNAs per target locus. The screen revealed highly efficient CBE-sgRNA pairs for each target locus. Similarly, also in hiPSCs these pairs showed high on-target editing efficiencies for ORAI1/2/3 when combined with the TREE system. Notably, we also observed high editing efficiencies when targeting two or three ORAI genes simultaneously. Next, we will generate monoclonal hiPS knockout cell lines to study ORAI1/2/3 function in models derived thereof.

42 - CRISPR/Cas9-based pipeline to introduce custom edits in autologous T cells

Katariina Mamia[1], Frida Høsøien Haugen[1, 2], Oline Eriksen Rio[1], Solrun Kolbeinsdottir[3], Zhuokun Li[1], Kornel Labun[4], Anna Komisarczuk[1], Salla Keskitalo[5], Ganna Reint[1], Britt Olaug Lindestad[1], Thea Johanne Gjerdingen[2], Antti Tuhkala[5], Carolina Wieczorek Ervik[1], Pavel Kopcil[1], Nail Fatkhutdinov[1], Siv Skundberg Jensen[6], Monika Szymanska[1], Eero Tölö[7], Virpi Glumoff[8], Janna Saarela[1, 2, 5], Trond Melbye Michelsen[8], Camilla Schalin-Jäntti[9], Johanna Olweus[1, 2], Eira Leinonen[5], Karen Helene Martinsen[2], Hans Christian Erichsen[1, 2], Børre Fevang[1, 2], Timo Hautala[8], Martin Enge[3], Timi Martelius[9], Shiva Dahal-Koirala[1], Emma Haapaniemi[1, 2]

[1]University of Oslo, [2]Oslo University Hospital, [3]Karolinska Institutet, [4]University of Bergen, [5]University of Helsinki, [6]OsloMet, [7]Ministry of Finance, Oslo, [8]Oulu University Hospital, [9]Helsinki University Hospital

CRISPR/Cas9 gene editing technology is a promising tool for correcting pathogenic variants in Inborn Errors of Immunity (IEIs). Current IEI correction strategies rely on cDNA knock-in or gene knockout, which can disrupt gene expression and need extensive optimization. To address this, we developed a safe, efficient CRISPR/Cas9-based T cell editing pipeline using HDR for SNV correction.

We observe a rapid and selective expansion of CD3+ T cells from patient peripheral blood following an 8-day cytokine stimulation. To enhance HDR outcomes, we explored multiple strategies and developed an optimized T cell pipeline capable of achieving up to 80% HDR. We found no pre-malignant off-target effects or karyotypic, transcriptomic, or proteomic abnormalities in patient T cells analysed with GUIDE-seq, scRNA-seq, long-read WGS, and proteomics. We observed functional correction of the disease phenotype in patient T cells demonstrated on transcriptomic level by single cell RNA sequencing, on proteomic level by Mass Spectrometry and in cell culture-based assays.

So far, we have implemented the pipeline and corrected 12 unique SNV mutations causing 8 diverse IEIs. This platform is designed to streamline CRISPR tool discovery/optimization and can be further developed into a GMP-compatible, scaled-up process for salvage therapy in T cell-related IEIs.

41 - Identifying epigenetic features that influence Cas9-mediated translocations.

Eve Stalker[1, 2], Anne Willis[2], Giando Turchiano[3], Luisa Loiacono[3], Roberto Nitsch[3], Lajos Kalmar[2]

[1]University of Cambridge, [2]MRC Toxicology Unit, [3]AstraZeneca

Gene editing therapies can provide promising treatment options for genetic disorders. However, they present their own unique safety assessment challenges. In the case of Cas-9 editing rare chromosomal translocation events have been identified and it is essential to both characterise these events and understand how they arise. Cas9 directly interacts with the genome, therefore it is critical to understand how the epigenome influences Cas9-mediated safety concerns. The epigenome is a highly important form of protection for the genome. Not only in shaping the chromatin structure and therefore accesibility but also highly invovled in faciltating DNA repair. Currently very little is understood about how understood about how Cas9 interacts with the epigenome. As the risk of chromosomal translocations forms a signficant barrier to the progression of cell and gene therapies understanding all fatcors that might influence them is key.

We aimed to identify regions that are prone to translocation and in parallel with associated epigenetic markers to understand the interplay between the epigenome and translocation events. First, quantified chromosomal aberrations derived from on-target and off-target activities of Cas9 were identified by CAST-seq. By using a range of ChIP-seq data collated by the ENCODE consortium the overlap between translocations and histone peaks were characterised in K562 and HEPG2 cell lines. Several histone marks associated with open chromatin and moreover transcriptional start sites emerged as potential predictors of Cas9-mediated translocations. The histone modification H3K4me1 was significantly enriched in identified translocated regions over predicted off-target sites based on sgRNA sequence similarity. H3K4me1 has also previously been linked to chromosomal translocations in hematological malignancies. This information will be used improve sgRNA design to ensure the on-target and off-target binding avoids these regions. The human epigenome is highly variable within the population therefore it is important to understand the implications of this interaction to enable accurate prediction and mitigate toxicity on a patient-by-patient basis

40 - CRISPA: A Novel Anthrax Toxin-Based Platform for Transient Cas9 Delivery

Maximilian Hirschenberger[1, 2], Nicole Stadler[1], Maximilian Fellermann[1], Konstantin M J Sparrer[2, 3], Frank Kirchhoff[2], Holger Barth[1], Panagiotis Papatheodorou[1]

[1]Institute of Experimental and Clinical Pharmacology, Toxicology and Pharmacology of Natural Products, Ulm University Medical Center, Ulm, Germany, [2]Institute of Molecular Virology, Ulm University Medical Center, Ulm, Germany, [3]German Center for Neurodegenerative Diseases (DZNE), Ulm, Germany

Current CRISPR/Cas9 gene editing applications are limited by inefficient and often unsafe delivery methods. Here, we describe CRISPA, a novel non-viral strategy for transient Cas9 delivery into human cells utilizing the anthrax toxin translocation machinery. We demonstrate that Cas9 variants fused either to the N-terminus of the anthrax toxin lethal factor or a hexahistidine tag can be transported into human cells via channels formed by PA (protective antigen), the anthrax toxin binding and translocation component. Successful gene editing was achieved as proof-of-concept in human cell lines, including the knockout of LSR in HCT116 and GFP in GFP-expressing 293T cells. Importantly, PA can be engineered for cell-type specific delivery of Cas9 and other cargo proteins. Thus, our CRISPA approach might represent a significant advancement towards the safe and effective translation of the CRISPR/Cas9 technology into clinics.

38 - Type IV-A3 CRISPR-Cas System from Klebsiella pneumoniae: Nuclease-Independent Approach for Modulating Gene Expression in Mammalian Cells

Neringa Daugelaviciene[1, 2], Rimvyde Cepaite[1], Patrick Pausch[1], Urte Neniskyte[1, 2]

[1]VU LSC-EMBL Partnership for Genome Editing Technologies, Life Sciences Center, Vilnius University, Vilnius, Lithuania, [2]Institute of Bioscience, Life Sciences Center, Vilnius University, Vilnius, Lithuania

Identifying CRISPR-Cas tools as precise genome editors has opened new possibilities for treating various human diseases. However, traditional CRISPR methods rely on generating site-specific DNA double-strand breaks (DSBs), which carry inherent risks, including unspecific mutations at the break site and extensive genomic alterations. As a result, alternative approaches to DNA cutting and editing are needed. The recently described type IV-A3 CRISPR-Cas system from Klebsiella pneumoniae offers a promising solution, enabling targeted gene expression modulation without DSBs. This system, composed of a multi-subunit effector complex and a CasDinG helicase, employs a nuclease-independent transcriptional interference pathway to suppress gene expression. While the type IV-A3 CRISPR-Cas system has demonstrated gene repression activity in bacterial cells, its application in mammalian cells remains unexplored.

In this study, we expressed the type IV-A3 CRISPR-Cas system and non-targeting RNA in human embryonic kidney HEK293T cells. Western blot analysis was performed to confirm the expression of individual type IV-A3 system subunits in HEK293T cells and the proportion of cells expressing type IV-A3 complex proteins quantified using immunofluorescent labeling. To apply the type IV-A3 CRISPR system for transcriptional interference in HEK293T cells, we targeted the highly conserved PPIB gene, commonly used as a benchmark for assessing gene expression modulation. For PPIB expression interference, we employed ten RNA guides targeting the gene's coding and non-coding regions. Transcriptional interference was assessed using quantitative real-time PCR, while transfection efficiency was evaluated via immunofluorescence microscopy.

Our findings demonstrate that the type IV-A3 CRISPR system can be used for targeted genome modulation in HEK293T cells. This application in mammalian cells highlights the potential to expand the CRISPR toolbox with safer, targeted techniques that modify the genome without introducing DSBs.

37 - Zebrafish Avatars: Advancing Diagnosis and Personalized Therapies for Patients with Rare Diseases

Diana García Moreno[1, 2], Isabel Cabas Sánchez[2], Alicia Martínez López[1, 2], Francisca Alcaraz Pérez[1, 2], Jesús García Castillo[1, 2], Beatriz Bernal Bermúdez[1, 2], Haleh Nikzamir[1, 2, 3], Francisco Juan Martínez Navarro[1, 2], Naomi Bellido Alcolea[1], María Luisa Cayuela Fuentes[1, 2], Victoriano Mulero Méndez[1, 2, 3]

[1]Instituto Murciano de Investigación Biosanitaria Pascual Parrilla (IMIB-PP), Murcia, Spain., [2]Centro de Investigación Biomédica en Red de Enfermedades Raras, ISCIII, Spain, [3]Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Spain.

Due to the need for new therapeutic options in rare diseases, a research platform was born for the repositioning of drug and personalized treatment of rare diseases, within Plan Integral de Enfermedades Raras de la Región de Murcia, using the zebrafish animal model. This model offers many advantages such as simple genetic manipulation and visualization of biological processes in vivo. The specific objectives are: (1) Generation of zebrafish models using genetic editing techniques (CRISPR-Cas9) that reproduce the possible genetic variants causing the disease (Avatar). (2) Identification of the genetic variant causing the disease: functional characterization of the developed models. (3) Drug repositioning for personalized therapies: screening of drug libraries approved for clinical use in Avatar models. From the beginning, zebrafish models have been generated for several rare diseases, including certain intellectual disabilities, type I interferonopathy, and congenic dyskeratosis, among others. A total of 30 disease models, involving some 34 genes, are being carried out, some of them in collaboration with other centers. In addition, the specific genetic cause for several pediatric patients has been modeled in zebrafish. Many of the models previously mentioned have been validated and are being prepared to perform a drug screening using compounds approved by the FDA and EMA. These advances could provide an alternative for precision therapy and medicine in these diseases, in addition to its modeling and characterization.

In Spain there are more than 3 million people whosuffer from rare diseases. From the ZEBER platform we develop unique preclinical modelsusing CRISPR-Cas9 technology to edit thezebrafish genome and find new drugs for thetreatment of these pathologies. The preclinicalmodels developed will serve to characterize themechanisms responsible for these syndromes, allowing the identification of new diagnostic and prognostic markers as well as the identification, validation and improvement of new drugs.

35 - A field-deployable CRISPR diagnostic platform for early and noninvasive canine cancer detection using droplet microfluidics

Aditi Dey Poonam[1], Peyton Blosser[1], Tianyi Gao[2], Zach Hetzler[1], John Vargas[1], Matthew Breen[1], Qingshan Wei[1]

[1]North Carolina State University, [2]Tsinghua University

Canine urothelial carcinoma (UC), one of the most common bladder malignancies in canines, affects tens of thousands of dogs a year in the US. The overlapping clinical symptoms of UC with other conditions causes delayed diagnostics in over 2 million dogs, which lead to overt metastasis in nearly 20% of cases, significantly reducing dogs' survival rates. Current molecular diagnostics require invasive biopsies and specialized medical equipment, resulting in further diagnostic delays and limited accessibility. Therefore, a non-invasive, rapid, sensitive, and field-deployable diagnostic tool is urgently needed to improve detection and mortality rates. Here, we developed a point-of-care (POC) diagnostic platform leveraging the CRISPR technology for detecting UC-associated BRAF mutations (V595E) directly from canine urine. The V595E mutation, present in 85% of canine UCs, hyperactivates the MAPK pathway, driving cancer progression. Different from conventional CRISPR diagnostics, we developed a novel CRISPR assay that significantly improves assay sensitivity to point mutations (variant allele frequency (VAF) detection limit of 0.1%) without pre-amplification, and a droplet-based microfluidic system that compartmentalizes CRISPR reaction into pico-liter sized droplets to further improve the limit of detection (LOD) down to < 1 fM. The microfluidic assay platform is capable of generating 800,000 monodisperse droplets (~50 μm) in under 30 minutes, providing a portable, high-resolution, and noninvasive solution for early UC diagnosis. The technology also shows promising potential for translation to the detection of human prostatic carcinoma, given its significant molecular similarities to canine UC, therefore, paving the way for broader applications in precision oncology and advancing the future of noninvasive cancer diagnostics.

33 - AlPaCas: allele-specific CRISPR gene editing through a protospacer-adjacent-motif (PAM) approach

Serena Rosignoli[1], Elisa Lustrino[2], Alessandra Fabrizi[1], Katia Russo[1], Alessandro Paiardini[2], Laura De Rosa[1], Michele De Luca[1]

[1]Centre for Regenerative Medicine “Stefano Ferrari”, Department of Life Sciences, University of Modena and Reggio Emilia, 41125 Modena, Italy, [2]Department of Biochemical Sciences “A. Rossi Fanelli”, Sapienza University of Rome, Rome 00185, Italy

Gene therapy for dominantly inherited genetic disorders requires either the selective disruption of the mutant allele or precise editing of the mutation [1]. The CRISPR-Cas system offers significant potential for correcting single nucleotide variants (SNVs), including those driving dominant mutations. The current strategy to achieve selectivity involves designing gRNAs complementary to the target genomic sequence. However, accurately distinguishing single-nucleotide variations in a pathogenic genomic context remains a significant challenge, as the inherent tolerance of mismatches can lead to unintended editing of the wild-type allele, posing a major obstacle to the clinical application of CRISPR/Cas gene editing [2].

The presence of a protospacer adjacent motif (PAM) specific to the mutant allele can enable its precise targeting while preserving the functionality of the wild-type allele [3, 4]. To address this, we developed **AlPaCas** ("Aligning Patients to Cas") [5], a computational pipeline designed to identify and analyze SNV-derived PAMs through sequence-based and structural approaches. An SNV-derived PAM arises when an SNV coincides with a PAM sequence, facilitating the discrimination of the mutant allele from the wild-type counterpart .

Given a gene or SNV input, AlPaCas can: (i) detect SNV-derived PAMs; (ii) generate a list of compatible Cas enzymes that recognize the identified SNVs; and (iii) suggest mutational Cas-engineering strategies to improve selectivity for the SNV-derived PAM. Notably, AlPaCas is the only available tool offering this functionality, as existing platforms and web servers for allele-specific CRISPR-Cas system engineering are limited to gRNA design.

AlPaCas features a user-friendly interface, catering to users of varying expertise and removing the need for manual data processing. It is freely-available at: https://schubert.bio.uniroma1.it/alpacas

[1] Humbert, O. et al. (2012) Targeted gene therapies: tools, applications, optimization. Crit. Rev. Biochem. Mol. Biol. DOI: 10.3109/10409238.2012.658112

[2] Zheng,T. et al. (2017) Profiling single-guide RNA specificity reveals a mismatch sensitive core sequence. Sci. Rep. DOI: 10.1038/srep40638

[3] Christie, K.A. et al. (2017) Towards personalised allele-specific CRISPR gene editing to treat autosomal dominant disorders. Sci Rep. DOI: 10.1038/s41598-017-16279-4

[4] Courtney, D.G., et al. (2016) CRISPR/Cas9 DNA cleavage at SNP-derived PAM enables both in vitro and in vivo KRT12 mutation-specific targeting. Gene Ther. DOI: 10.1038/gt.2015.82

[5] Rosignoli, S. et al., (2024) AlPaCas: allele-specific CRISPR gene editing through a protospacer-adjacent-motif (PAM) approach. Nucleic Acids Res. DOI: 10.1093/nar/gkae419

31 - High resolution evaluation of the N6-methyladenosine RNA modification pathway as a driver of tumor proliferation and drug resistance

Martina Scichilone[1, 2], Cinzia Cocola[2], Paride Pelucchi[2], Alessandro Orro[2], Arianna Consiglio[2], Tommaso Selmi[2]

[1]University of Milan, [2]ITB-CNR

The methylation of RNA adenosines into N6-methyladenosine (m^6A) is the most abundant internal mRNA modification in mammalian cells. It is regulated by writers (e.g., methyltransferase-like 3 (METTL3)) and erasers, while readers mediate its molecular effects at the level of different biological processes (e.g., RNA translation and decay). Consequently, m^6A affects multiple cellular functions like cell proliferation. Indeed, downregulation of METTL3 significantly reduces CRC cell proliferation and targeting METTL3 with a small molecule inhibitor (METTL3i) affects acute myeloid leukemia (AML) cell proliferation. In addition, CRISPR-KO screening in AML cell lines identified different m6A effector as essential.

This opens new questions regarding the mechanisms by which these effectors contribute to the function of m^6A in cancer and in drug resistance, which may be investigated with CRISPR gene editing approach. In particular, CRISPR-cytosine base editing (CBE) and -KO can create respectively single nucleotide changes (C>T) and insertion/deletions within the genomic sequence of the target genes.

The aim of this project is to study m^6A in cancer cell proliferation and drug resistance by means of a high-throughput pooled CRISPR screening. We will develop a CRISPR-base editing and KO platform for the high-density mutagenesis of the genes that are active in the m^6A-pathway under METTL3i treatment and in control conditions. The following enrichment analysis of the sgRNAs will determine the association between specific single-nucleotide mutations, or gene deletions, and the proliferation phenotype.

We prepared an in-silico sgRNA library targeting m^6A and control genes (essential and non-essential genes, non-targeting and intergenic sequences). We applied a tiling strategy (i.e., multiple sgRNAs targeting different gene positions) to study the targeted sequences in HT-29 (CRC) and AML (MOLM-13, NOMO-1) cell lines.

This will define functional domains associated with the proliferation phenotype, as well as variants that confer resistance, or sensitiveness, to METTL3i. By combining the KO and CBE data, we aim at improving our understanding of how m^6A effectors cooperate in supporting cancer cell proliferation and drug resistance.

30 - Functional correction of the CFTR 1717-1G>A mutation using a precise adenine base editor

Alessandro Umbach[1], Annalisa Santini[1], Mattijs Bulcaen[2, 3], Daniela Guidone[4], Sam Thierie[2], Giulia Maule[1], Irene Carrozzo[1], Matteo Ciciani[1], Luis Galietta[1], Marianne Carlon[2], Anna Cereseto[1]

[1]Department CIBIO, Laboratory of Molecular Virology, University of Trento; Trento, Italy., [2]Molecular Virology and Gene Therapy, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven (Catholic University of Leuven); B-3000 Leuven, Belgium., [3]BREATHE, Department of Chronic Diseases and Metabolism, KU Leuven (Catholic University of Leuven); B-3000 Leuven, Belgium., [4]Telethon Institute of Genetics and Medicine (TIGEM); Naples, Italy.

The 1717-1G>A is a prevalent splicing mutation causing cystic fibrosis (CF) for which no pharmacological treatments have been approved. This specific mutation disrupts a conserved AG dinucleotide at the 3' end acceptor splice site in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leading to exon 12 skipping or activation of a newly formed cryptic splice site. Both aberrant splicing events result in a premature stop codon, preventing the correct CFTR protein synthesis.This study aims to develop an innovative CRISPR/Cas-based genome engineering to correct the 1717-1G>A mutation, leveraging adenine base editing (ABE) technologies.A cellular model of the mutation was established in HEK293 cells. After evaluating several editing tools, we selected ABE9 with the PAM-relaxed SpRY variant (SpRY-ABE9), as the optimal tool for this mutation. Plasmid-based transfection of SpRY-ABE9 achieved over 30% A>G correction, with minimal bystander editing, restoring CFTR protein localization at the cell membrane.To maximize the efficacy of the editing strategy through RNA delivery, we extensively optimized the sgRNA scaffold and mRNA capping encoding SpRY-ABE9. RNA delivery of the SpRY-ABE9 in 1717-1G>A patient-derived bronchial epithelial cells and intestinal organoids showed genetic and functional correction, with minimal off-target editing.In summary, our data indicates SpRY-ABE9 as a promising strategy, balancing editing efficiency and minimizing bystander/off-target editing. This approach has potential for restoring CFTR function, addressing the challenges of the 1717-1G>A mutation in CF.

29 - Exploring Quantum-Enhanced CRISPR-Based Epigenome Editing: A Case Study on TP53 Demethylation Using NV-Centered Diamond Nanoparticles

Asra Hamidi[1], Sunil Joshi[1], Amir A. Ahmad Chali[2]

[1]QuantumImmunogenix-A Third Eye Network Inc., USA, [2]The Ministry of Higher Education and Scientific Research of Kurdistan Region of Iraq

Off-target effects pose a major challenge in CRISPR-based epigenome editing, especially in therapeutic applications requiring precision. This study presents a quantum-assisted strategy for DNA-demethylation at position 248 of TP53, a gene mutated in over 50% of human-cancers and crucial for genomic stability. Position 248, a mutational-hotspot implicated in various cancers, was chosen as a model to demonstrate the precision and efficacy of the proposed quantum-enhanced demethylation-technique. To achieve precise targeting, spherical nano-diamonds (5nm) with nitrogen-vacancy (NV) centers were employed. NV-centers are atomic-scale defects in the diamond lattice where a nitrogen atom replaces a carbon atom, leaving an adjacent vacancy. These centers exhibit unique quantum-properties, including stable spin-states (a particle's intrinsic angular momentum, which can encode information) and the ability to exist in multiple states simultaneously (superposition). By leveraging quantum-entanglement—a phenomenon where particles become interlinked such that the state of one directly influences the other, regardless of the distance between them—this system establishes a synchronized relationship between engineered guide-RNA (egRNA) and the target-methylation site on DNA. This synchronization enhances targeting specificity, while the CpG-site further modulates the quantum-state to achieve greater precision. Moreover, a smart-linker system was incorporated to anchor the diamond-nanoparticle on the egRNA scaffold during the targeting phase. Responsive to external electric-fields, the linker enables controlled repositioning of the nanoparticle post DNA binding, minimizing steric hindrance, preserving enzymatic accessibility, and further improving precision. Preliminary results show substantial improvements over conventional CRISPR-methods. The quantum-enhanced system achieved a fidelity of 95%-99.9%, surpassing the 80%-95% of current systems. Quantum-fidelity reflects the system's ability to maintain accuracy, minimize noise, and optimize interactions. Off-target effects were reduced by 90%-99%, compared to 50%-80% in conventional-systems. Additionally, targeting accuracy improved to 97%-99.9%, up from 85%-95% in existing methods. These results showcase the transformative role of quantum-optimization in improving genetic editing precision. These findings underscore the quantum-assisted approach's potential to enhance targeting precision in epigenome-editing. Simulations in cryogenic-environments were conducted to stabilize NV-center quantum-states and maintain entanglement. While effective in preserving quantum-properties, cryogenic-conditions present challenges for intracellular molecular-activity. This study lays the groundwork for specialized cryogenic-systems tailored to ex-vivo applications. Future strategies aim to develop environments near absolute-zero for high precision editing, where edited cells may be reinfused or transferred using cell therapy. Although, this in-silico study demonstrates promising outcomes, translating quantum-technologies into experimental and clinical applications remains challenging due to high costs, limited infrastructure, and the need for specialized cryogenic-systems. Maintaining quantum-coherence (preserving quantum-states for functionality) under physiological-conditions also demands multidisciplinary collaboration and innovative engineering. Overcoming these challenges is essential to realizing the transformative potential of precision-medicine. This quantum-enhanced system integrates quantum biology, molecular genetics, and nanotechnology, redefining precision in epigenome editing. Current research emphasizes cryogenic cell cultures and ex-vivo editing, while future studies may explore in-vivo applications and optimization under physiological-conditions. Such advancements must address ethical issues like safety, accessibility, and regulatory compliance, with applications in organoid-models and clinical settings. This research paves the way for precision medicine and provides a platform for addressing epigenetic disorders, including neurodegenerative and metabolic diseases, beyond cancer therapy.

28 - Quantum-Enhanced Precision and Real-Time Monitoring of Prime Editing in Bone Marrow Transplantation for AML

Sunil Joshi[1], Asra Hamidi[1]

[1]QuantumImmunogenix-A Third Eye Netwok Inc., USA

Bone marrow transplantation is a cornerstone treatment for acute myeloid leukemia (AML). However, donor availability is often limited by genetic conditions, such as sickle cell disease, which pose additional risks to transplantation. This study introduces a dual-framework therapeutic approach, combining Prime Editing for genetic correction and quantum-enhanced immunological profiling to ensure safe and effective transplantation outcomes. In this case, the only HLA-compatible donor for an AML patient was diagnosed with sickle cell disease. Bone marrow was harvested and subjected to ex vivo genome editing using Prime Editing to correct the β-globin mutation responsible for the sickle cell phenotype. Quantum biosensors were integrated into the editing process, providing real-time monitoring of guide RNA-DNA binding stability, enzymatic activity, and editing accuracy. Results demonstrated 92% editing efficiency, with minimal off-target effects confirmed by high-throughput sequencing. Following genetic correction, quantum immunological profiling was employed to evaluate the immune response and safety of the edited cells. This innovative approach enabled precise monitoring of key immunological markers, including cytokine dynamics, HLA compatibility, and immune signaling pathways, ensuring a comprehensive understanding of post-editing immunogenic risks. Edited cells exhibited restored hematopoietic function and a significant 75% reduction in immunogenic markers, highlighting their compatibility with the patient's immune system. This case study demonstrates the feasibility and transformative potential of integrating Prime Editing with quantum-enhanced monitoring in a real-world clinical scenario. While this study highlights the success of a single case, further research is underway to validate the scalability and applicability of this approach across broader patient cohorts, paving the way for safer and more effective genome editing applications in transplantation medicine.

26 - Streamlining CRISPR/Cas9-based gene editing in human iPSCs: a core facility pipeline for standardisation and quality control

Magdalena Madej[1], Claudia De Guidi[1], Qianren Jin[1], Pia Johansson[1]

[1]Cell & Gene Therapy Core, Lund Stem Cell Center, Faculty of Medicine, Lund University, Sweden

The discovery of human induced pluripotent stem cells (iPSCs) and the development of CRISPR/Cas9 gene editing technology have revolutionized biomedical sciences, unlocking new possibilities for both basic research and translational medicine. However, gene editing in iPSCs presents unique challenges due to their sensitive nature, necessitating careful evaluation, standardisation, and robust quality control. Recently, core facilities have emerged as critical players in addressing these challenges, serving as knowledge hubs that provide expertise and standardise protocols in these rapidly evolving fields.

Here we present our core facility's refined pipeline for CRISPR/Cas9-mediated genome editing in human iPSCs, specifically focusing on gene knockouts, single-nucleotide editing and generation of tagged endogenous loci, followed by a thorough quality control strategy. Gene knockouts and single-nucleotide editing are achieved by nucleofecting cells with Cas9/single guide RNA (gRNA) ribonucleoprotein complexes (RNPs) for gene knockouts or by additionally introducing a repair template such as single-stranded oligonucleotides (ssODN), for precise nucleotide changes. Our gene-tagging/large insertion approach involves an initial lipid-mediated transfection of iPSCs with a donor plasmid (containing the insert and target-specific homology arms) on day 1, followed by nucleofection with Cas9/sgRNA RNPs on day 2. After clonal expansion and identification of successfully edited clones, we perform a comprehensive set of quality control measures that includes copy number verification (CNV), mycoplasma testing, short tandem repeat (STR) analysis, assessment of iPSCs undifferentiated status, morphology and growth pattern analysis, evaluation of genome stability through molecular karyotyping and G-banding, and screening for potential off-target effects.

Through this streamlined pipeline, we aim to contribute to ongoing efforts to implement standardised and reproducible CRISPR/Cas9-based technologies for iPSC manipulation. Ultimately, we emphasize the pivotal role of core facilities in advancing the practical application of these cutting-edge techniques and ensuring their reliable use in both research and clinical settings.

25 - CRISPR-Cas9 Screening and Spatial Transcriptomics Reveal Driver Genes Mediating Chemoradiation Resistance in HPV-Negative HNSCC

Martina Franchini[1, 2, 3], Hugo Jean Victor Legenne[1, 2, 3], David Miguel Ferreira Francisco[4], Joana Carlevaro Fita[4], Simon Häfliger[1], Daniel Hendrik Schanne[5], Roland Giger[6], Olga Sara Stanowska[7], Adrian Ochsenbein[1, 2], Carsten Riether[1, 2]

[1]Department of Medical Oncology, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland, [2]Department for BioMedical Research (DBMR), University of Bern, Bern, Switzerland, [3]Graduate School of Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland, [4]Interfaculty Bioinformatics Unit and SIB Swiss Institute of Bioinformatics, University of Bern, Bern, Switzerland, [5]Department of Radiation Oncology, Inselspital, Bern University Hospital, and University of Bern, Bern, Switzerland, [6]Department of Otorhinolaryngology, Head and Neck Surgery, Inselspital, Bern University Hospital, University of Bern, Bern, Switzerland, [7]Institute of Tissue Medicine and Pathology (ITMP), University of Bern, Bern, Switzerland

Head and neck squamous cell carcinoma (HNSCC) remains a leading cause of cancer-related mortality, with human papilloma virus (HPV)-negative subtypes being associated with poor therapeutic outcomes. Standard-of-care chemoradiation therapy (CRT) often fails due to the emergence of treatment-resistant disease, underscoring the urgent need to elucidate the molecular underpinnings of resistance mechanisms. Our study employs a multifaceted approach that integrates genome-wide CRISPR-Cas9 screening with spatial transcriptomics to systematically investigate the cellular and molecular architecture of treatment-resistant HNSCC and identify potential therapeutic targets. To elucidate the genetic factors involved in CRT resistance, we performed a genome-wide CRISPR-Cas9 knockout screen in two HNSCC cell lines, UM-SCC11A and UM-SCC14A, derived from the larynx and the oral cavity, respectively. These lines, known to harbor hallmark genomic aberrations that recapitulate HNSCC carcinogenesis, were treated with single IC_{50} doses of radiation, cisplatin and their combination. Seven days post-exposure, DNA libraries were subjected to deep sequencing. While still ongoing, preliminary data from this unbiased functional genomic approach identified several candidate genes whose disruption influenced therapy response. Notably, 45 negatively selected genes were consistently identified across treatment samples, highlighting nodal modulators of CRT adaptation currently under investigation. Ongoing validation studies using a single-gene knockout strategy in HNSCC organoid models and cell lines will further evaluate the functional relevance of these candidate genes in the context of therapy resistance, employing biologically relevant systems for deeper insight. Complementary to our functional screening, spatial transcriptomics was applied to patient-derived, matched primary and recurrent specimens isolated from stage III-IV HPV-negative HNSCC tumors to contextualize our CRISPR findings within a clinically relevant, spatially resolved setting. While still ongoing, this approach has enabled us to assess whether the genes identified as negative hits in the CRISPR screen are spatially enriched or active in therapy-resistant regions of patient tumors, providing critical insight into their potential clinical relevance. By mapping these genes within distinct tumor compartments, we will evaluate their association with key resistance-associated transcriptional programs identified by the spatial analysis, including epithelial-to-mesenchymal transition (EMT) and cancer stem cell-associated pathways. This integration of CRISPR screening results with spatial transcriptomic data offers a cohesive framework for linking functional genomic hits to spatially defined resistance phenotypes. In summary, this study highlights the potential of a combined approach using both CRISPR-Cas9 genome-wide screening and spatial transcriptomics as a powerful tool for uncovering critical modulators of CRT resistance. While definitive conclusions are still pending, this integrated approach provides a clear roadmap for identifying vulnerabilities in treatment resistant HNSCC whose modulation may enhance the therapeutic outcome.

23 - High throughput single-cell assessment of genome integrity and toxicity events associated with edited cells

CY LI[1], Saurabh Gulati[1], Saurabh Parikh[1], Dong Jo Ban[1], Ozcan Sahin[1]

[1]mission bio

Advances in cell and gene therapy are revolutionizing the treatment and potential cure of diseases such as cancer and rare genetic disorders. Precision gene editing technologies like CRISPR-Cas9, TALENs, and ZFNs have surpassed traditional methods, enabling new strategies to correct genetic defects at their source. However, despite these advancements, the editing process yields heterogeneous populations where some cells may have undesired outcomes that bear the risk of genome toxicity leading to potential malignancies. These adverse effects can include the introduction of off-target edits, copy number variations, and chromosomal aberrations like translocations or large indels. Consequently, the success of effective gene therapies depends on the ability to accurately measure and understand these events. Furthermore, since “cells” are the functional units of gene editing products, it is prudent to measure the co-occurrences of editing results and potential genotoxicity events in a single-cell context.

In this study, we demonstrate the use of an advanced single-cell technology that combines microfluidics and multiplex PCR to simultaneously measure several critical aspects of gene editing. The assay analyzes the co-occurrence and zygosity of on-target edits, off-target edits, quantitatively detect translocations between predicted edit sites, and map the genomic copy number variation (CNV) landscape, all within over 10,000 cells in parallel. Due to the assay’s single-cell resolution, this method delivers a detailed and comprehensive view of the heterogeneous editing profiles present in gene-edited products. It allows for precise and rapid evaluation of both the editing outcomes and the overall genomic integrity, facilitating the identification of potential malignant events and improving the safety and efficacy of the gene therapy.

To evaluate the robustness of this workflow, we utilized a variety of samples with different levels of adverse effects from multiple gene editing experiments. These samples were validated orthogonally using bulk NGS (DNA-seq), rhAMPseq, and whole-genome sequencing for CNV. Employing a targeted sequencing panel (611-plex), in a single assay, we measured on- and off-target editing activity, detected structural variations, identified gene editing-induced translocation events, and assessed genome-wide CNVs at a single-cell level in these samples. The resulting data were analyzed using a novel bioinformatics pipeline specifically developed to simultaneously and accurately measure all these modalities. Additionally, for locus-level CNV detection, we demonstrated the performance of the single-cell CNV assay at a focal level. This was exemplified by confirming a Chr20q copy number alteration in an iPSC cell line, which conventional methods such as G-banding and CMA failed to detect.

This high-throughput single-cell NGS assay represents a significant advancement in gene editing analysis, measuring both intended and unintended consequences of genome editing across a variety of resolutions. This dual functionality not only aids in optimizing gene editing protocols but also represents a streamlined and thorough process for assessing the safety aspects of gene-modified therapies.

22 - TLR4 gene polymorphism and biochemical markers as a tool to identify risk of osteoporosis in women from Karachi

Rozeena Baig[1], Rehana Rehman[2], Rifat Zubair Ahmed[3]

[1]Aga khan university hospital Pakistan, [2]Aga khan university hospital y, [3]University of Karachi

Background: Osteoporosis, characterized by low bone mineral density, poses a global health concern. Diagnosis increases the likelihood of developing osteoporosis, a multifactorial disorder marked by low bone mass, elevating the risk of fractures in the lumbar spine, femoral neck, hip, vertebras, and distal forearm, particularly in postmenopausal women due to bone loss influenced by various pathophysiological factors.

Objectives: To investigate the association of serum cytokine, bone turnover marker, bone mineral density and TLR4 gene polymorphism in pre and post-menopausal women and to find if any of these can be the potential predictor of osteoporosis in postmenopausal women.

Material and methods: Study participants were consisting of Group A (n=91) healthy pre-menopausal women and Group B (n= 102) healthy postmenopausal women having ≥ 5 years' history of menopause. ELISA was performed for cytokine (TNFα) and bone turnover marker (carboxytelopeptides), respectively. Bone Mineral Density (BMD)was measured through dual X-ray absorptiometry (DEXA) scan. Toll-like Receptors 4 (TLR4) gene polymorphisms (A896G; Asp299Gly) and (C1196T; Thr399Ile) were investigated by PCR and Sanger sequencing.

Results: Statistical analysis reveals positive correlation of age and BMI with T scores in premenopausal group whereas in post-menopausal group found a significant negative correlation between age and T-score at hip (r = - 0.352**), spine (r = - .306**), and femoral neck (r = - 0.344**) and a significant negative correlation of BMI with TNF-α (-0.316**).No association and significant differences were observed for TLR4 genotype and allele frequencies among studied groups However, both SNPs exhibited significant association with each other.

Conclusions: This study concludes that BMI, BMD and TNF-α are the potential predictors of osteoporosis in post-menopausal women. However, CTX and TLR4 gene polymorphism did not appear as potential predictors of bone loss in this study and apparently cannot help in predicting bone loss in post-menopausal women.

19 - Leveraging of circular RNA synthesis in CRISPR-based therapeutics

Jeong-Ki Kim[1], Minju Park[1], Sunhee Lee[1], Yeon-Jin Yoo[1], Jun Young Choi[1]

[1]STPharm

Circular RNA (circRNA) is a type of single-stranded RNA with a covalently closed loop structure, which confers resistance to exonuclease-mediated degradation. This unique structure provides several advantages over linear mRNAs, including enhanced stability, longer half-life, and improved protein expression. These properties make circRNA an attractive candidate for gene editing applications. To harness circRNA for therapeutic purpose, we have developed a novel method for producing circRNAs with high protein expression, utilizing ribozymes and RNA ligase. Our technology significantly improves circularization efficiency compared to traditional Group I permuted intron-exon (PIE) systems, especially for large genes like Cas9 gene. Using firefly luciferase as a model, we demonstrated that the activity of the protein by the circRNA was 80-fold and 7.6-fold higher compare to conventional mRNA in vitro and in vivo, respectively. Most notably, our circRNA technology has been successfully applied into CRISPR/Cas9 gene editing system. Circular Cas9, when expressed via circRNA, showed significantly higher and prolonged protein expression in cells compare to Cas9 mRNA. Moreover, editing efficiency with circular Cas9 was significantly higher than with linear mRNA Cas9. Taken together, the circRNA-based systems could offer a promising alternative to traditional mRNA-based CRISPR.

16 - CRISPR/Cas-mediated disruption of aberrant PCCA pseudoexon inclusion rescues enzyme activity in the rare metabolic disease propionic acidemia

Mar Alvarez[1], Ainhoa Martínez-Pizarro[1], Eva Richard[2], Lourdes R Desviat[2]

[1]Centro de Biología Molecular Severo Ochoa CSIC-UAM, [2]Universidad Autonoma de Madrid, Centro de Biologia Molecular Severo Ochoa

Pseudoexons are nonfunctional intronic sequences that can be activated by deep intronic variants, resulting in an aberrant transcript coding for a non-functional protein. The PCCA c.1285-1416A>G variant activates an 84 bp pseudoexon and causes the severe metabolic disorder propionic acidemia, by deficiency of the propionyl-CoA carboxylase enzyme, composed of PCCA and PCCB subunits. The PCCA pseudoexon shows relatively high levels of inclusion in the wild-type context, resulting in a high proportion of unproductive splicing. Using splice switching antisense oligonucleotides to block the inclusion of the pseudoexon, normal splicing is restored, increasing both PCCA and PCCB protein levels and enzyme activity, not only in patient fibroblasts with the variant but also in cells harboring PCCA and PCCB hypomorphic missense variants. Now, we have investigated the potential of a one-time therapy using CRISPR/Cas12 to excise the PCCA pseudoexon or abrogate its inclusion, using a dual gRNA strategy or a single gRNA targeting a splice enhancer in the pseudoexon. We were able to rescue correct splicing by up to 35% in edited HepG2 cells with the c.1285-1416A>G variant, recovering protein and enzyme activity to therapeutically relevant levels. The single gRNA approach resulted in higher levels of corrected transcript. In wild-type HepG2 and fibroblast cells the extent of unproductive splicing (pseudoexon inclusion) was decreased. Overall, the results offer initial evidence of CRISPR/Cas genome editing potential as a therapeutic tool to permanently correct aberrant pseudoexon inclusion in propionic acidemia, reducing unproductive splicing to increase productive transcript and protein levels. This provides a unique approach to treat the disease caused by the deep-intronic variant or by hypomorphic alleles.

15 - Defining novel genes controlling CD4+ T cell differentiation in vivo during blood-stage malaria infection

Takahiro Asatsuma[1], Marcela Moreira[1], Cameron Williams[1], Hyun Lee[1], Oliver Skinner[1], Saba Asad[1], Shihan Li[1], Thomas Burn[1], Lynette Beattie[1], Ashraful Haque[1]

[1]Department of Microbiology and Immunology, Peter Doherty Institute, University of Melbourne, Parkville, Victoria, Australia

Malaria remains a global health burden, responsible for approximately 247 million cases and 619,000 global deaths in 2021. Due to the lack of effective vaccines and immunotherapy treatments against malaria infection, there is an urgent need for the development of new therapeutic agents and drugs. During blood-stage malaria infection, CD4+ T cell immunity plays a suboptimal but critical role in both humans and experimental mice. Two subsets, T-helper 1 (Th1) and follicular helper T cells (Tfh), partially control Plasmodium parasites via IFNγ or by supporting high-affinity antibody production, respectively. Discovering the mechanisms controlling Th1/Tfh fate may offer new opportunities for accelerating the onset of immunity to malaria; however, the genetic mechanisms governing CD4+ T cell differentiation during blood-stage malaria are not fully understood. Therefore, the aim of this study is to define the genetic factors controlling CD4+ T cell differentiation in vivo during blood-stage malaria infection. With single-cell RNA sequencing (scRNA-seq) and the utilization of TCR-transgenic CD4+ T cells (PbT-II) specific for a Plasmodium epitope, we previously defined the transcriptional dynamics of Plasmodium-specific CD4+ T cell differentiation over the course of blood-stage malaria infection in mouse models. Given that we observed early co-expression of chemokine receptors CXCR3 and CXCR5 by PbT-II cells, prior to their bifurcation towards either Th1 or Tfh fates, we hypothesized that competition between CXCR3 and CXCR5 influences Th1/Tfh fate in malaria. To test this, genes encoding CXCR3, CXCR5, or CXCR6 were disrupted alone or simultaneously in naïve PbT-II cells via cutting-edge CRISPR/Cas9 gene-editing techniques and examined for effects on differentiation in vivo. Strikingly, none of these chemokine receptors, either alone or in combination, substantially influenced either PbT-II expansion or Th1 differentiation, while interactions via LFA-1 or IL-2 signalling via CD25 were required for optimal clonal expansion and Th1 differentiation. In addition, consistent with our spatial transcriptomic analysis, which suggested a role for monocyte-Th1 interactions via CCR5, CRISPR/Cas9-mediated disruption of Ccr5 reduced clonal expansion and Th1 differentiation in our model. Hence, we propose that CCR5-dependent monocytic interactions of IL-2-primed CD4+ T cells promote Th1 immunity in malaria. Taken together, these data provide mechanistic insight into how Th1 responses are optimally generated in vivo during blood-stage malaria infection and hold hope for the development of therapeutic targets against malaria infection.

10 - CRISPR-CAS9 MEDIATED EDITING OF ESCHERICHIA COLI FOR OPTIMIZING LIPASE PRODUCTION USEFUL FOR DELIVERY OF POTENTIAL COVID-19 DRUGS USING POTATO PEEL AS A GLUCOSE SOURCE

JOSEPH MINARI[1], Samuel Dada[1], Gift NWOSU[1], Dhikrullah ABDULAZEEZ[1]

[1]University of Lagos

ABSTRACT

Lipases are one of the most used enzymes in pharmaceutical industry. The enzyme has been known to assist in the stimulation of controlled release of some drugs anti-SARS CoV 2 potential compounds such as DL-limonene. In spite of lipase enormous importance in the pharmaceutical industry, the cost of the production and purification of the enzyme is cost intensive. Potato peels that are disposed of improperly and cause damage to the environment can result into potential production of beneficial enzymes like lipase. This study aimed to genetically engineer Escherichia coli with the CRISPR Cas9 system to carryout submerged fermentation of potato peels for optimizing lipase production. Multiplex PCR and Gel electrophoresis were used to evaluate the editing efficiency. Submerged fermentation of potato peels was carried out in three different temperature condition (10°C, 30°C, 45°C) with CRISPR Cas9 edited E. coli and unedited E. coli while ammonium sulphate was used to purify the crude enzyme. Enzyme characterization was carried out at different pH and temperature. This study shows E. coli subjected to CRISPR-Cas9 system with Cas9 but without the single guide RNA (sgRNA) and arabinose, revealed a unique blue phenotype in 116 colonies. The E. coli with sgRNA, Cas9 but without arabinose shows no observable colonies. The E. coli colonies subjected to Cas9, arabinose but without sgRNA exhibit 239 colonies of distinct blue phenotype. The E. coli subjected to Cas9, sgRNA and arabinose showed 79 colonies with a characteristic white phenotype. The electrophoretic band pattern obtained from the CRISPR-Cas9 experiment shows that the E. coli colonies subjected to Cas9, arabinose has 2 distinct bands size of 650 bp. Two bands, originating from E. coli with a blue phenotype, were also detected in E. coli subjected to CRISPR-Cas9 system with Cas9 but without the single guide RNA (sgRNA) and arabinose with size of 1,100 bp. In the positive control, three distinct bands were observed with 1,100 bp, 650 bp and 350 bp whereas in the negative control, no bands were detected. The growth of CRISPR Cas9 edited and unedited E. coli in relation to temperature in the potato peel medium under 15 days of incubation shows an increased cell mass at 10°C. However, CRISPR Cas9 edited E. coli showed a clearer zone than the unedited. When temperature was raised from 25°C to 35°C, the activity of the enzyme produced by the CRISPR Cas9 unedited and edited Escherichia coli increased significantly ($p<0.05$). Significant ($p>0.05$) decreases in enzyme activity were observed in both purified lipase enzymes produced by CRISPR Cas9 edited and unedited from 45°C to 65°C with further temperature increase. A significant increase($p>0.05$) in enzyme activity was observed with increasing pH from pH 4 to pH 7. A significant reduction ($p<0.05$) was observed in activity from pH 7 to pH 8. This study revealed that the CRISPR Cas9-mediated lacZ gene editing in Escherichia coli enhances its ability to utilize waste substrate to increased lipase production.

8 - Democratizing CRISPR in its public-private ecosystem

Franziska Bächler[1]

[1]University of Basel

With the rapid and promising development of CRISPR, demands for adequate regulation are growing. In this article, we show why the existing legal standards are not sufficient to provide access to CRISPR-Cas9 as democratic technology and why the right to science could provide a remedy. We justify the need for a right to science as an alternative legal instrument to more traditional issues such as patent exceptions and licensing, to facilitate the widest possible access to and dissemination of CRISPR technology. Therefore, we examine the extent to which the right to science can serve as a normative framework for CRISPR to ensure that scientific advances are used responsibly and for the benefit of society.

7 - Precise Gene Writing System for CAR-T cell therapy generation

Avencia Sanchez-Mejias[1], Ivan Fernandez Muñoz[1], Alejandro Ragel Lopez[1], Paolo Petazzi[1]

[1]Integra Therapeutics

The development of effective, precise, and safe gene-editing technologies for ex vivo therapies, particularly in hematopoietic stem cells (HSCs) and chimeric antigen receptor T cells (CAR-Ts), poses a critical challenge. Current platforms face technical limitations and safety concerns, impeding the realization of their full therapeutic potential. To address these challenges, FiCAT emerges as a groundbreaking gene-writing platform with the aim of providing a flexible and universal solution for safe editing of small regions and efficient, programmable editing of large genes in clinically relevant primary cells.

FiCAT is composed of a CRISPR-Cas nuclease and propietary engineered transposase, with outstanding efficiency reached through rational protein engineering and an unsupervised variability generation approach. Over 400,000 variants are tested using a proprietary high-throughput screening system grounded in synthetic biology principles. In practical application, FiCAT RNPs had been produced and validated in T cells and HSCs using nucleofection for targeted integration of a minimal reporter. Our system compared favourably against existing systems like homology-directed repair (HDR) or homology-independent targeted insertion (HITI) using CRISPR-Cas9 alone. To enhance both efficiency and viability, electroporation is coupled with inhibitors targeting toxicity associated with double-stranded exogenous DNA. In addition, FiCAT therapeutic potential has been shown with the successful integration of CAR19 in a relevant locus (TRAC) for T cells . In vitro Functional validation of FiCAT generated CAR-T cells revealed comparable cytotoxicity with LV-generated CAR-T cells.

These results open up new possibilities with enhanced cargo size integration in HSCs and T cells, surpassing current gene editing techniques . This is of critical importance for both HSCs (i.e. correction of monogenic blood diseases) and T cells, since new generation of immunotherapies relies on targeting more than one epitope (Dual CAR) but also benefits from the inclusion of immunomodulators to enhance CAR-T cells efficiency and persistence.

FiCAT is designed to overcome precision and safety limitations in gene editing, streamlining the cell engineering process into a single step. Its potential to reduce costs and eliminate the need for viral vectors promises expanded accessibility to treatment. In summary, FiCAT represents a significant advance in gene editing, poised to enhance precision, safety, and cost-effectiveness in clinical applications.

5 - Innovative Delivery Strategies for Efficient Genome Editing in Mammalian Cells

Eimina Dirvelyte[1, 2], Modestas Mazerimas[3], Simonas Kutanovas[1, 2], Rima Budvytyte[3], Urte Neniskyte[1, 2]

[1]Institute of Bioscience, Life Sciences Center, Vilnius University, [2]Life Sciences Center - European Molecular Biology Laboratory (EMBL) Partnership Institute, Vilnius University, [3]Institute of Biochemistry, Life Sciences Center, Vilnius University, Vilnius, Lithuania

The discovery of CRISPR-Cas tools broadened the possibilities of repairing various mutations that cause genetic diseases. However, genome editing tools remain challenging to implement in personalized therapy, primarily due to the lack of efficient delivery methods. In particular, this limitation is critical for large genome editors and their in vivo applications. Current adeno-associated viral vectors are limited in size and struggle to accommodate both Cas and sgRNA or advanced tools such as prime editors (PE). Addressing this limitation, liposomal nanoparticles (LNP) and herpes viral (HSV) vectors emerge as promising alternatives, capable of delivering even very large genome editors across various platforms, including in vivo applications.

Our study focused on exploiting proprietary LNPs for Cas9 and sgRNA RNP delivery to mammalian cells. We assessed the delivery efficiency by identifying cell nuclei with GFP fluorescence after cell culture was incubated with Cas9-GFP RNP complexes encapsulated in LNPs. We also demonstrated that proprietary LNP-mediated delivery achieved indel formation levels comparable to those obtained with commercial transfection reagents. Additionally, we explored the potential of proprietary LNPs to traverse the blood-brain barrier using an in vitro brain endothelial cell transcytosis model.

The second approach involved HSV, delivering state-of-the-art genome editing tools like prime editors. We started by characterizing the cell tropism of HSV in various cell cultures and determining the timeline of expression in mouse organotypic hippocampal slice cultures. We then constructed a single HSV vector capable of delivering all components of the prime editing system by employing an extensible mammalian modular kit. This designed HSV vector was validated on an established reporter cell line within the laboratory, editing genome-encoded GFP to BFP. This technology is now being applied to repair mutations associated with neurodegenerative lysosomal storage diseases. We are optimizing pegRNA designs by evaluating different lengths of primer binding sites (PBS) and reverse transcription templates (RTT), followed by fine-tuning the editing process with nicking sgRNAs.

Our findings highlight LNPs and HSV vectors as versatile and efficient platforms for delivering genome editing tools, from nucleases to prime editors. The successful implementation of HSV-delivered prime editors for repairing the mutations causing neurodegenerative diseases could pave the way for further advancements in CRISPR/Cas-based therapies.

4 - Evaluation of Efficient Delivery Vectors for CRISPR Therapy Using Smaller Cas12f Protein

Karim E. Shalaby[1], Issam Hmila[2], Nasser H. Zawia[3], Omar M. A. El-Agnaf[2], Mustapha Aouida[4]

[1]Laboratory of Pluripotent Stem Cell Disease Modeling, Translational Medicine Department, Research Branch, Sidra Medicine, Doha, Qatar, [2]Neurological Disorder Research Center, Qatar Biomedical Research Institute (QBRI), Hamad Bin Khalifa University (HBKU), Qatar Foundation, Doha, Qatar, [3]Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI 02881, USA, [4]Biological and Biomedical Sciences Division, College of Health & Life Sciences, Hamad Bin Khalifa University, Doha, Qatar

The clinical translation of CRISPR genome-editing therapies is often hindered by inefficient delivery of the CRISPR-Cas RNA-protein complex into target cells. To address this challenge, we explored the potential of the smaller CRISPR-associated protein Cas12f, which, due to its reduced size compared to the widely used Cas9, may offer improved cellular uptake and delivery efficiency. In this study, we tested non-viral delivery vectors previously designed for Cas9 and evaluated their capacity to deliver Cas12f into cells. Our findings demonstrate that Cas12f, with its smaller size, facilitates more efficient cellular penetration and delivery than Cas9, making it a promising alternative for CRISPR-based therapies. By enhancing the efficiency of delivery, this work advances the development of CRISPR technologies for therapeutic applications, with implications for treating complex diseases such as neurodegenerative disorders, cancer, and diabetes.

2 - CRISPR/Cas-Based Diagnostic tool for Early-Stage Diagnosis of Diabetic Complications

Giriprasad Gangadari[1], Preety Rawal[1], Deepak Chitkara[1]

[1]BITS-PILANI, PILANI CAMPUS

Abstract: CRISPR technology is revolutionizing the field of gene editing and demonstrating various promising applications in diagnostics. Epigenetic alterations, particularly DNA methylation within promoter regions, have been implicated in diabetes complications like Diabetic Nephropathy (DN). Hence, herein we evaluated the methylation status of cell-free DNA (cfDNA) in blood, serum, and urine as a potential biomarker in early-stage detection of DN. This study investigates CRISPR-based diagnostic platform, such as the **R**eady to **U**se **C**RISPR-based **M**ultitarget **D**etection **A**ssay **(RuCMDA)**, for the rapid and cost-effective detection of epigenetic changes (DNA methylation) in Diabetic Mellitus patients. In this study, we utilized cfDNA as a pathological marker, offering insights into early-stage disease development and employed the cis-trans cleavage activity of CRISPR/Cas12b to assess the methylation status of cfDNA extracted from the blood of Diabetic Mellitus patients. We successfully expressed and purified the Cas12b protein from E. coli achieving a concentration of 1.5 mg/ml. The cfDNA from healthy and diabetic volunteers were subsequently analyzed and it was observed that the expression of genes ELMO1 and KCNJ11 were elevated in diabetic patients compared to healthy controls. Thus, our results revealed that ELMO1 and KCNJ11 were corroborated with DN prevalence. Thereafter, guide RNAs for the target genes were synthesized to generate ribonucleoprotein (RNP) complexes. The cis-trans cleavage activity of CRISPR/Cas12b RNPs was then evaluated using fluorescent-labeled reporters. Currently, we are optimizing this system for DNA methylation screening of cfDNA obtained from diabetic patients. Future work aims to develop a point-of-care device based on this technology.

Keywords: CRISPR diagnostics, diabetic complications, disease detection, trans-cleavage activity, early diagnosis

1 - Fluorescence-Based In Vitro Assays for Predicting Off-Target CRISPR/Cas9 Cleavages: A Cost-Effective Alternative to Sequence Approaches

Xinlai Cheng[1]

[1]Goethe university frankfurt

The precision of CRISPR/Cas9 genome editing is crucial for both research and therapeutic applications, yet it is often compromised by off-target effects that pose significant clinical challenges. In this study, we introduce a series of novel chemical modulators that substantially enhance the accuracy of CRISPR/Cas9-mediated genome and epigenome editing. These modulators were identified through targeted chemical screens and have proven effective in reducing unintended genetic modifications.

Exploiting these discoveries, we have developed a fluorescence-based in vitro assay that predicts off-target cleavages with high accuracy. This innovative assay mimics CRISPR/Cas9 interactions dynamically and offers a rapid, cost-effective alternative to traditional sequencing methods such as Circle-Seq. Comparative analyses have shown that our assay achieves similar predictive performance to these sequence-based approaches while reducing costs by approximately 90%.

Our advancements provide a valuable tool for improving the fidelity of gene editing techniques and present a scalable, economically viable solution for both basic and clinical research settings. The development of this assay marks a significant step toward enhancing the safety and reliability of CRISPR/Cas9 applications, potentially facilitating faster clinical translations.