DG-GT ANNUAL MEETING

NUCLEIC ACID THERAPEUTICS:
GENETIC INDICATIONS AND BEYOND

TranslaTUM, Munich
6-8 March 2024

DG-GT
Deutsche Gesellschaft für Gentherapie e.V.

ABSTRACT BOOK

INV03

Modulation of host immunity in the airways with interferon lambda encoding mRNA

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Type III interferons play an important role in the innate antiviral, antifungal and antiprotozoal defences of mucosal barriers and enhance adaptive immune responses in the respiratory mucosa. Based on its proprietary Stabilized Non-Immunogenic mRNA (SNIM®RNA) and lipidoid nanoparticle delivery platforms, Ethris has developed interferon lambda encoding mRNA as a drug candidate for prophylactic and therapeutic administration to the airways for prevention and treatment of viral infections. In the presentation, formulation of mRNA suitable for administration to the airways as an aerosol will be discussed. Furthermore, preclinical proof of concept data will be presented, demonstrating the potency of interferon lambda endcoding mRNA in mouse and ferret influenza A and SARS-CoV-2 virus challenge models in mice and ferrets when administered to the airways.

INV28

Affinity peptide mediated retargeting of AAV9 to cardiac interstitial cells

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Interstitial cells of the heart are remarkably resistant to AAV9-transduction. To overcome this limitation, we have employed a novel methodology for AAV9 vector-retargeting with a combination of polyamidoamine dendrimers (PAMAM) and cell type specific peptides and promoter specificity.
In a proof-of-concept study, we have demonstrated that endothelial cells of cardiac microvasculature can be transduced with modified vectors in different animal models. We identified an endothelial-affine peptide by bio-panning a phage display library, which then we linked to PEGylated PAMAM dendrimers, which were used to coat AAV9 vectors with Endoglin promoter-driven transgenes. After assessing transduction efficacy in reporter animals, we used the modified AAVs to deliver functionally relevant transgenes: an adhesion molecule (S1FG), where expression in cremaster endothelial cells increased leukocyte adhesion; an anti-inflammatory peptide (Anxa1), where its expression reduced long term leukocyte recruitment in carotid artery; and sgRNA targeting the vasodilatory enzyme eNOS, where Cas9 mediated knockout of eNOS caused significant increase of blood pressure. Moreover, using peptides targeting pericytes and fibroblasts and suitable promoters, we were able to obtain *in vivo* target

cell type transduction. Also, substituting the affinity peptide with a myocyte targeting one yielded increased transduction rates of cardiomyocytes.
Here, we demonstrate that retargeted AAV9s are an efficient and modular tool for transducing cardiac interstitial cell types. Accordingly, achieving gene transfer in this previously inaccessible set of cells will broaden the toolkit of cardiovascular community to better understand the intricacies of cardiovascular system, as well as eventually have tangible results in the clinic.

Oral Presentations

OR01

In vivo endothelial cell gene silencing by siRNA-LNPs tuned with lipoamino bundle chemical and ligand targeting

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Although small-interfering RNAs (siRNAs) are specific silencers for numerous disease-related genes, their clinical applications still require safe and effective means of delivery into target cells. Herein, we have developed highly efficient lipid nanoparticles (LNPs) for siRNA delivery, showcasing the advantages of novel pH-responsive lipoamino xenopeptide (XP) carriers. These sequence-defined XPs are assembled by branched lysine linkages between cationizable polar succinoyl tetraethylene pentamine (Stp) units and apolar lipoamino fatty acids (LAFs) at various ratios into bundle or U-shape topologies. Integrating of LAF_4-Stp_1 XPs into siRNA-LNPs at an optimized molar ratio led to robust cellular uptake, high endosomal escape, and successful *in vitro* gene silencing activity at extremely low (150 picogram) siRNA dose. Of significance is functional *in vivo* endothelium tropism of siRNA-LNPs with bundle LAF_4-Stp_1 XP after systemic injection into mice, demonstrated by superior knockdown of liver sinusoidal endothelial cell (LSEC)-derived FVIII in comparison with MC3-based LNPs, but moderate silencing of hepatocyte-derived FVII. Optimizing lipid composition following Click-modification of siRNA-LNPs with ligand cRGDfk efficiently silenced vascular endothelial growth factor receptor-2 (VEGFR-2) in xenograft tumor endothelial cells (TECs). Our findings shed light on the role of ionizable XPs in the LNP *in vivo* cell-type tropism, laying the groundwork for future therapeutic application.

OR02

Aerosolized gene and oligonucleotide therapy targeting microRNA-224 ameliorates pulmonary hypertension by orchestrating the BMP pathway

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Pulmonary arterial hypertension (PAH) is a severe vascular disease that leads to right heart failure and death. Currently there is no cure for PAH. We identified microRNA (miR)-224 as a lung

enriched miR and *in silico* approaches predicted miR-224 among the miRs that target PAH related genes. In this study we aimed to investigate the role of miR-224 in pulmonary vascular remodeling and to test the therapeutic effect of miR-224 gene- and oligonucleotide therapies in PAH.
We found pulmonary miR-224 levels to be increased in PH-diseased lungs and in PH human pulmonary artery smooth muscle cells (hPASMCs). *In vitro* studies revealed that miR-224 is necessary to induce hPASMCs proliferation.
We next tested the therapeutic effect of miR-224 inhibition using three different PAH animal models in mice and rats. In a first approach, we intra-tracheally aerosolized an AAV1-Tough Decoy-miR-224 (AAV1-TuD-224) to PH diseased mice. AAV-Control-treated mice displayed all the hallmarks of PAH, whereas AAV1-TuD-224-treated mice displayed a marked and significant decrease in those parameters. Next, we intra-tracheally aerosolized a chemically modified antisense oligonucleotide specific for miR-224 (LNA-224) to PH diseased mice and rats. LNA-224 significantly suppressed PAH. We finally delivered LNA-224 to monocrotaline-treated rats and found that miR-224 inhibition improves survival and ameliorates PAH. Mechanistically, we found that miR-224 represses BMP signaling by directly targeting four pathway factors.
Our data suggest that miR-224 plays a pivotal role in pulmonary vascular remodeling by targeting the BMP pathway. Aerosolized gene and oligonucleotide therapy targeting miR-224 may have therapeutic value for PAH.

OR03

Extracellular vaccine- or virus-derived SARS-CoV-2 spike protein: a potential link between reported pathologies

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Thromboses belong to the most serious complications in COVID-19. The trigger for rarely occurring thromboses associated with thrombocytopenia upon adenoviral vaccination against COVID-19 is unknown. As the spike protein could represent a pathogenetic link between the two conditions, we investigated the fate of spike protein, its processing to S1 and S2 subunits and its localization upon *de novo* expression from plasmids, approved COVID-19 vaccines and wildtype SARS-CoV-2.
Independent from the source, a significant proportion of the spike protein was found being not cell-associated. Rather, the spike protein was abundantly present in the medium. Depending on, whether or not the spike protein was proteolytically processed by furin, we found it either (i) anchored as full-length spike protein in extracellular vesicles (EVs), (ii) anchored as S2 subunit in EVs not associated with S1, or (iii) as free S1 subunit in the medium not associated with S2 or EVs.
We show that extracellular full-length spike protein and the S1 subunit attached to ACE2-expressing cells and mediated the binding of human plasma-derived spike protein-specific IgGs to the cell surface.
Similar results were obtained with another viral transmembrane protein, the glycoprotein of Vesicular Stomatitis Indiana Virus (VSV-G), suggesting a general applicable phenomenon for *de novo* expressed transmembrane proteins.

The extracellular presence of considerable amounts of functional spike protein might provide a possible explanation for reported pathological effects observed during COVID-19 or following genetic vaccination.

OR04

Enhanced safety in multiplex-edited T cells through combined use of distinct CRISPR enzymes for knock-in and base editing

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The development of potent, off-the-shelf T cell therapies often necessitates multiple genetic alterations. Traditional CRISPR-Cas nucleases, which induce DNA double-strand breaks (DSBs), can lead to genomic rearrangements, posing safety concerns for edited cells. We integrate a non-viral CRISPR-Cas nuclease-assisted method for gene knock-in with a SpCas9-derived base editing technique for DSB-free knock-outs. This combination allows for simultaneous and efficient gene modification within a single intervention.
Co-delivery of SpCas9 nuclease for knock-in and SpCas9 base editors enabled the insertion of a chimeric antigen receptor (CAR) into the T cell receptor alpha constant (*TRAC*) gene and the concurrent knock-out of major histocompatibility complexes (MHC) class I and II expression. While translocations between the targeted sites were reduced over conventional SpCas9 multiplexing, insertions and deletions at the base editing sites suggest significant frequency of DSB, presumably due to guide RNA interchange. By combining AsCas12a nuclease for CAR knock-in with a SpCas9-derived base editor, we efficiently produce triple-edited CAR T cells with significantly reduced translocation frequency, akin to that of unedited cells. These cells demonstrate resistance to allogeneic T cell targeting *in vitro*. The single-step method was also adapted to improve large-scale manufacturing of allogeneic MHC-I/II-silenced regulatory T cells overexpressing *HLA-E-B2M* fusion gene to evade NK cell lysis.
Our findings suggest a viable strategy for non-viral gene transfer and effective gene silencing or epitope masking through the use of distinct CRISPR enzymes for knock-in and base editing. This approach could pave the way for safer multiplex-edited cell products.

OR05

Peptide-assisted tethering of DNA repair effectors to Cas9 for precise genome editing

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The ability of CRISPR-Cas9 to induce dsDNA breaks can be exploited to revert a disease-causing mutation via the homology-directed repair (HDR) pathway. However, such strategies suffer from the intrinsically low frequency of HDR compared to the alternative non-homologous end-joining (NHEJ) repair pathway. Efforts to enhance HDR efficiency include the use of drugs or Cas9 fusion

proteins that promote HDR and/or inhibit NHEJ. While promising, the efficacy of these approaches varies greatly across cell types, possibly related to different expression profiles of DNA repair factors. To address cell type specificity, we introduce an advanced CRISPR toolkit leveraging the dimerization ability of synthetic peptides. The CRISPR Peptide-Assisted Localization (PAL) toolkit comprises two essential elements: 1) a Cas9 nuclease fused to a synthetic peptide, and 2) a library of DNA repair effectors fused to a complementary peptide. Here, we demonstrate how CRISPR-PAL can be used to identify the most effective combination of effectors tailored to a particular cell type. Distinct nuclease and effector combinations resulted in up to a 2.5-fold increase in HDR events compared to unmodified Cas9 across diverse cell types. Furthermore, dimerization in CRISPR-PAL enabled the use of novel combinations that were unamenable through mere overexpression of effectors. Ongoing experiments examine CRISPR-PAL's effectiveness in correcting Fanconi anemia-specific mutations by augmenting missing effectors at the target site. We anticipate that the CRISPR-PAL toolkit will be instrumental in the development of tailored genome editing solutions with broad therapeutic applications.

OR06

Translation of advanced cell therapies for HIV^{+} patients

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An increasing number of gene and cell therapies have been authorized for different types of cancer and non-malignant diseases. However, novel cell therapies with advanced therapy medicinal products (ATMPs) are difficult to implement, particularly for HIV^{+} patients. We aim to address this unmet medical need in the context of CAR-T cells by establishing dedicated processes for ATMP manufacture. In addition, to-be-infused T cells will be protected from de novo HIV infection by CCR5-gene editing. We previously established efficient production of (i) CD19-CAR-T cells and (ii) CCR5^{ko} T cells at the CliniMACS Prodigy. Now, we are developing a combined process for lentiviral CAR transfer and knockout of the HIV co-receptor CCR5 by mRNA electroporation of our TALE nuclease, CCR5-Uco-hetTALEN. We have successfully performed three manufacturing runs at the CliniMACS Prodigy to produce *CCR5*-edited CD19-CAR-T cells on a GMP-compliant, clinical scale. We produced up to 3.9x10^{9} viable T cells containing 37-55 % functional CD19-CAR-T cells, with an overall *CCR5*-editing rate of 42-54%. Furthermore, the third process was successfully performed under the addition of the antiretroviral substance saquinavir to supress viral replication during manufacturing of HIV^{+} cells. In summary, we have developed a GMP-compliant manufacturing process for *CCR5*-edited CAR-T cells under antiretroviral suppression, which might help to accelerate the translation of cellular immunotherapies, namely CAR-T cells for HIV^{+} patients in the future.

OR07

CD3-zeta gene editing to reprogram T or NK cells with chimeric antigen receptors

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Immune cells reprogrammed with Chimeric Antigen Receptor (CAR) technology offer substantial therapeutic promise in the fields of oncology, autoimmune disorders, transplant medicine, and infectious diseases. Traditional CAR-T cell therapies rely on autologous manufacturing using undirected viral gene transfer, resulting in logistical challenges and high costs per treatment. Further, viral vectors induce unphysiological regulation of CAR expression and increase the risk for malignant transformation through insertional mutagenesis of proviruses. To overcome these challenges, we developed non-viral gene editing of the *CD3ζ* (*CD247*) gene to reprogram immune cells for adoptive transfer applications.
By integrating truncated CAR-transgenes devoid of a primary activation domain into the *CD3ζ*-gene, we create functional CAR fusion-genes. This strategy harnesses the endogenous *CD3ζ*-gene as the CAR's activation domain, ensuring physiological regulation of CAR expression across various immune cell types including conventional T cells, TCRγδ T cells, regulatory T cells, and NK cells. Notably, this CD3ζ in-frame fusion approach also eradicates TCR surface expression in T cells, significantly reducing the risk of graft-versus-host disease in allogeneic off-the-shelf applications.
Our results demonstrate that *CD3ζ*-CD19-CAR-T cells exhibit leukemia control comparable to both *TRAC*-replaced and lentivirus-transduced CAR-T cells *in vivo*. Furthermore, tuning the expression level of the *CD3ζ*-CAR enhances its *in vivo* efficacy. Compared to CD19-specific TCR-like CAR architectures (HIT/STAR, eTruC receptor design), our *CD3ζ*-CAR T cells displayed superior functionality *in vivo*. Remarkably, *CD3ζ*-gene editing also successfully reprograms NK cells without compromising their intrinsic functions. In conclusion, *CD3ζ*-gene editing presents a promising and versatile platform for developing allogeneic off-the-shelf cell therapies utilizing redirected killer lymphocytes.

OR08

An oncolytic HAdV-5 with reduced surface charge combines diminished toxicity and improved tumour targeting

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Human adenovirus type 5 (HAdV-5)-based oncolytic viruses (OVs) hold promise as efficient anti-cancer therapy. Systemic OV administration is the preferable administration route as it allows to

target difficult-to-address tumours and metastases. Poor tumour targeting and off-target organ transduction, however, limit the therapeutic efficacy of HAdV-5-based oncolytic virotherapy. Especially the strong inherent liver tropism of HAdV-5-based vectors poses a risk of severe hepatotoxicity.
We generated a novel HAdV-5-based oncolytic vector with reduced overall net negative surface-charge by genetic modification of the major capsid protein Hexon (HexPos3). HAdV-5-HexPos3 exhibited superior and CAR-independent transduction efficiency in various cancer cell lines *in vitro*, further enhanced in the presence of HAdV-5 naïve murine plasma. Upon single i.v. administration into tumour-bearing NSG mice, replication-deficient HAdV-5-HexPos3 vector particles had a significantly reduced off-target organ tropism in all tissues analysed, including the liver. Moreover, we detected significantly increased intratumoural vector amounts for HAdV-5-HexPos3 after a single i.v. injection, leading to a 29-fold elevated tumour-to-liver ratio compared to a Hexon-unmodified control vector. Single i.v. injection of a conditionally replicating Hexon-unmodified control vector induced severe hepatotoxicity in tumour-bearing NSG mice. In contrast, i.v. injection of a conditionally replicating HAdV-5-HexPos3 vector was well tolerated, led to prolonged survival of respective animals and resulted in intratumoural vector amounts detectable for up to 56 days post treatment. Thus, the HAdV-5-HexPos3 represents a favourable oncolytic vector and a promising platform for the generation of novel HAdV-5-based oncolytic vectors with reduced systemic toxicity and improved therapeutic efficacy.

OR09

Base editing restore cellular phenotype of T cells of patients with Hyper-IgE-Syndrome

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Hyper-IgE-syndrome (HIES) is a rare primary immunodeficiency characterized by recurrent skin and pulmonary abscesses, elevated IgE serum levels, and a deteriorating quality of life. Disease-causing mutations in HIES patients are heterozygous and mainly found in the DNA-binding or the SH2-dimerization domain of *STAT3*. The mutations affecting DNA binding of STAT3 interfere with its function as a transcription factor and impede the activation of downstream target gene expression, such as *SOCS3*. In this study, we explored the feasibility of correcting the *STAT3* gene in patient T cells harbouring the heterozygous mutations K340E or R382W. Both mutations are located in the DNA binding domain, thus preventing downstream target gene activation. We designed a cytosine base editor to correct the K340E allele and an adenine base editor for R382W in order to convert the underlying point mutations back to wild type. Upon optimization of gRNA design and mRNA transfer to patient T cells, up to 98% and 86% of K340E and R382W alleles, respectively, were edited. Based on NGS analysis, our gene editing approach restored wild type STAT3 expression to up to 90% in patient cells. We verified functional rescue of the patient T cells by evaluating STAT3-mediated activation of a downstream target gene. Upon stimulation of base edited T cells with IL-21, STAT3 was phosphorylated and *SOCS3* expression upregulated to similar levels as observed in control T cells. In conclusion, our proof-of-principal study demonstrates the feasibility of using base editing in HIES patient T cells to restore physiological expression of functional STAT3.

OR10

Dual targeting of PD-L1 and ErbB2 by CAR-NK cells enables specific elimination of solid tumor cells and overcomes immune escape via antigen loss

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Retargeting natural killer (NK) cells with chimeric antigen receptors (CARs) can be a powerful approach to overcome NK cell resistance of tumor cells. However, for some tumors, targeting a single tumor-associated antigen may be insufficient to trigger effective NK cell activation or may lead to selection of antigen-loss variants and tumor immune escape. To overcome this hurdle, here we generated CAR-NK cells carrying two CARs targeting the tumor-associated antigens PD-L1 and ErbB2 (HER2), respectively. NK-92 cells were transduced with lentiviral CAR constructs, and their cytotoxicity against cancer cell lines of various solid tumor origins was compared to that of parental NK-92 and corresponding single target CAR variants. Dual targeting significantly increased *in vitro* cytotoxicity against PD-L1 and ErbB2 double-positive tumor cell lines including breast, ovarian, pancreatic, lung and gastric cancer cells compared to single-target CAR variants. These results were also confirmed in 3D spheroid tumor models and *in vivo* xenografts. No off-target cytotoxicity was observed. At the molecular level, this enhanced cell killing can be explained by the synergistic activation of PLCγ and MAPK pathways. Incubation of cancer cells with IFN-γ further enhanced killing efficacy by upregulating PD-L1 expression. Furthermore, blocking experiments revealed that dual PD-L1/ErbB2-CAR NK-92 cells can overcome immune escape based on the loss or inaccessibility of a single target antigen. In conclusion, we have shown that dual targeting of PD-L1 and ErbB2 enhances the efficacy of CAR-NK cells against otherwise difficult to treat tumors and counteracts potential resistance and immune escape mechanisms of cancer cells.

OR11

ITR instability in E. coli? The answer is 42 (°C) for improving quantity and quality of rAAV

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Recombinant adeno-associated virus (rAAV) gene therapies rely on inverted terminal repeats (ITRs) for effective vector production and long-term efficacy. However, ITRs in plasmids used for rAAV production degrade during standard propagation in *E. coli*. Typically, 130 bp ITRs or even shorter variants instead of the full 143 to 145 bp ITRs are found. The implication of these truncated ITRs for vector production and quality has so far remained elusive. To solve this mystery, we first developed an amplification-free sequencing technique to assess ITR degradation on individual *E. coli* plasmid molecules. We then recovered full-length ITRs from rAAV and investigated their degradation using different *E. coli* strains and cultivation parameters. We found that cultivating *E. coli* Stbl3, DH5alpha, BL21(DE3), or JM103 at 42°C, instead of traditional temperatures (37°C or 30°C), stabilized these full-length ITRs in all tested cases. Cultivation at 42°C additionally enabled us to assemble and clone full-length ITRs from synthetic oligonucleotides. We suspected the 'structural maintenance of chromosome' proteins SbcC or SbcD as possible culprits for ITR degradation. Indeed, a ΔSbcC strain, JW0387-KC, was best at maintaining full-length ITRs at 37°C, while still profiting from cultivation at 42°C, where degradation was not detected. Importantly, using plasmids with these intact ITRs for rAAV production significantly increased genomic titers by up to 7-fold compared to standard ITR plasmids, while reducing reverse-packaged plasmid backbone. Our findings underline the importance of maintaining full-length ITRs and open the possibility to further enhance rAAV gene therapies through increasing ITR "health".

OR12

Cell level imaging of Nucleic Acid Therapeutics in whole mouse bodies

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A major building block of the future landscape of precision medicine will be nucleic acid therapeutics. However, the performance assessment of mRNA carriers is limited by two major challenges: (1) a lack of imaging technologies to detect nanocarriers and subsequent protein expression in whole organisms at a cell-level resolution, (2) a lack of analysis methods to precisely quantify the delivery of cargo/nanocarriers in whole-body. Researchers either use low-resolution tools to assess the whole-body distribution (*e.g.*, bioluminescence, PET) or are forced to limit their

analysis to a few predetermined targets with histological techniques with cellular resolution, limiting the understanding of the precise biodistribution and the efficiency. Here, we address this problem by cell-level imaging of mRNA in whole tissue-cleared mice. Specifically, we administered lipid nanoparticles encapsulating an EGFP-encoding mRNA labeled with a fluorophore to mice, used an adapted 3DISCO clearing protocol for whole body clearing, and employed light sheet imaging to determine the mRNA biodistribution and the efficiency. For quantitative analysis, we used an AI-based image analysis pipeline automatically extracting body-wide distributions of the mRNA and the EGFP-expressing cells. We detected and quantified mRNA biodistribution at clinically used doses, which is 1000-fold lower than the existing whole-body imaging methods. Additionally, our LNP biodistribution analysis pipeline was found compatible with whole-body antibody staining, which allows for localization of target regions. With our method, it is possible to systematically screen the effects of injection routes or different nanocarriers at a whole-body scale, providing a way to develop more precise nucleic acid delivery platforms.

OR13

Scalable generation of functional human iPSC-derived CAR-macrophages that efficiently eradicate CD19-positive leukemia

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Macrophages are important innate immune cells and key regulators of the tumor microenvironment. Due to this pivotal role, they became attractive targets for novel cell & gene therapies, including the development of genetically-engineered macrophages modified with chimeric antigen receptors (CARs). However, the efficient manufacturing of CAR-macrophages from primary sources is challenging, due to low isolation yields of primary monocytes and their resistance to traditional gene editing methods. Thus, we here demonstrate the scalable and continuous production of functional CAR-macrophages from human induced pluripotent stem cells (CAR-iMacs), showing the potential use and therapeutic impact to target $CD19^{+}$ patient-derived cancer cells from patients with acute lymphoblastic leukemia. We successfully showed continuous CAR-iMac production of consistent quality using either small (3mL) or intermediate scale (40mL) devices showing an average yield of ~ 1×10^{7} cells/week. The generated CAR-iMacs exhibited a typical macrophage morphology and phenotype ($CD45^{+}$, $CD14^{+}$, $CD11b^{+}$, $CD163^{+}$) as well as stable CAR expression throughout the differentiation process. CAR-iMacs demonstrated enhanced phagocytosis and increased secretion of pro-inflammatory cytokines (IL-6, TNFα) against a lymphoma cancer cell line, in an antigen-dependent fashion, compared to control $eGFP^{+}$-iMacs. Furthermore, CAR iMacs exhibited enhanced phagocytosis against primary acute lymphocytic leukemia (ALL) patient samples. scRNA sequencing revealed distinct gene expression

in CAR-iMacs, compared to eGFP^{+}-iMacs, with upregulation of genes associated with an M1 polarization, adaptive immune cell recruitment and antigen presentation. In conclusion, we present a modern, scalable and robust platform for continuous generation of functional CAR-iMacs, which opens new avenues for modern off-the-shelf immune cell-based therapies.

OR15

Third-generation lentiviral gene therapy rescues function in a mouse model of Usher 1B

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Hearing loss (HL) is the most common sensory deficit in humans and a major global health problem, with severe consequences for affected individuals and society. Genetic causes account for >50% of sensorineural HL, which often presents as a HL syndrome in combination with further deficits. Usher syndrome type 1B (USH1B) is one of the most severe HL syndromes with congenital, profound deafness, vestibular areflexia leading to imbalance and vertigo, and progressive vision loss. Current treatment options are limited to cochlea implants, which can partially address HL, while vestibular hypofunction and blindness remain unaddressed. Pathogenic variants within the *MYO7A* gene are causative for USH1B, proposing gene therapy as a potentially curative treatment option. Therefore, a state-of-the-art lentiviral (LV) vector platform was used to deliver the large 6.6 kB *MYO7A* cDNA. Application of a dTomato reporter vector to normal-hearing mice demonstrated that our platform efficiently transduced inner ear cells, incl. cochlear and vestibular hair cells as the main USH1B target cells. A therapeutic LV vector expressing MYO7A and dTomato (LV.MYO7A) did not negatively affect hearing or balance function in normal-hearing mice. Strikingly, in homozygous Shaker-1 mice, an USH1B mouse model carrying a *Myo7a* point mutation, LV.MYO7A gene therapy strongly reduced the balance deficit and significantly improved hearing capacity. In heterozygous mutant mice, which were found to develop late-onset hearing loss, LV.MYO7A gene therapy completely rescued hearing to wild-type hearing thresholds. Thus, LV.MYO7A gene therapy constitutes the first treatment concept that addresses both the vestibular and the cochlear dysfunction in USH1B.

OR16

Modifying immune responses to adeno-associate virus vectors by capsid engineering

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Immune responses against AAV are considered a major challenge in AAV gene therapy. We aimed to reduce the innate immune recognition of AAV vectors by interfering with Toll-like receptor signaling. Using capsid engineering, we inserted a myeloid differentiation primary response 88 (MyD88)- derived peptide that is capable of blocking MyD88 dimerization. Insertion of the MyD88 peptide into the capsid did not hamper capsid formation or vector production yield. The new capsid variant, AAV2.MB453, demonstrated enhanced transduction compared to AAV2 in human monocyte-derived dendritic cells and in primary human hepatocyte cultures. Furthermore, in primary human cells reduced innate immune responses, including type I interferons, were observed compared to AAV2. *In vivo*, reduced CD8+ T cell responses against the transgene product and against the capsid were observed for AAV2.MB453. Furthermore, AAV2.MB453 treated mice showed delayed generation of AAV2-binding IgG2a antibodies and an increased Nab50. In summary, by inserting a MyD88-derived peptide into the AAV2 capsid we developed a novel variant with improved transduction efficiency and lower innate and adaptive immune responses.

Poster Presentations

P01

Gene therapy approaches to potentially rescue catecholaminergic polymorphic ventricular tachycardia (CPVT)

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CPVT is an autosomal dominant inherited cardiac arrhythmia. In this disease, the ryanodine receptor type 2 (RyR2) is leaky, leading to aberrant calcium release from the sarcoplasmic reticulum (SR) of cardiomyocytes during diastole, which manifests in delayed afterdepolarizations, potentially culminating in arrhythmia.
We are pursuing two potential gene therapies:
1. Concurrent knockout and transactivation (CONNACT) of functionally equivalent genes.
Here, we focus on knockout of dysfunctional Ryr2 gene and transactivation of a functionally equivalent counterpart i.e., Ryr1 or Ryr3. To this end we used, intraperitoneal injection of dual mRNA trans-splicing AAV9 vectors containing catalytically active Cas9-VPR and sgRNAs with different spacer lengths into WT mice. In qRT-PCR and Western blot experiments of cardiac tissue we show efficient Ryr2 knockdown and an increase in Ryr1 expression. RyR1 immunolabeling revealed the typical striated pattern of SR. However, surface ECG data displayed a reduction in QRS complex amplitude and widened P-waves in AAV-treated mice compared to control, indicating that RyR1 cannot rescue the depletion of RyR2. In further studies, we will test whether RyR3 can functionally replace RyR2 in cardiomyocytes.
2. Transactivation of Calsequestrin 2 (CASQ2).
Several in vitro studies have shown that an increase in CASQ2, the main Ca2+-buffering protein in the SR, promotes RyR2 closure. CASQ2 transactivation is therefore a promising approach to improve RyR2 function in CPVT. Using a catalytically inactive dCas9-VPR and 1 sgRNA resulted in approximately 4-fold overexpression of *Casq2* at the transcript level in WT mice, with no negative effects on surface ECG data.

P02

Prime editing strategies for modeling and treatment of dilated cardiomyopathy

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Dilated cardiomyopathy (DCM) is a heart muscle disease characterized by structural and functional myocardial abnormalities. Left ventricular or biventricular dilation results in impaired heart contraction. Up to 30% of DCMs are reported to have a monogenic etiology. Mutations in several genes can cause DCM, including genes encoding sarcomeric, nuclear envelope, or cytoskeletal proteins. As a result, decreased cardiac contractility and conduction defects occur, ultimately leading to arrhythmia, heart failure, and sudden cardiac death. To date, the only curative approach is heart transplant, and etiological treatments are lacking. Mutations in the *LMNA* gene are among the most prevalent candidates associated with severe genetic cardiomyopathy. We used prime editing (PE) to model and correct DCM-associated *LMNA* pathogenic variants in human cell lines and iPSC-derived cardiomyocytes. We compared different PE approaches to optimize editing frequencies, showing that the same pegRNAs can result in substantially different editing efficiencies across the cell types tested. We also compared adenine base editor (ABE) and PE for gene correction of a common *LMNA* mutation. Our work highlights the potential of PE for the treatment of genetic heart diseases.

P03

Prime editing for correcting familial mutation associated with cardiomyopathy

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Familial cardiomyopathies, particularly those linked to the deletion of Arginine 14 in Phospholamban (PLN R14del), remain formidable challenges in medical genetics, often leading to arrhythmogenesis and sudden cardiac death. Traditional therapies have shown limited efficacy in treating these genetic anomalies. This study aims to utilize the prime editing system to efficiently introduce the PLN R14del mutation into human cell lines and iPSC-derived cardiomyocytes. The goal is to develop a methodology for correcting this mutation, ultimately aiming to prevent cardiomyopathy features in animal models. We employed a prime editing system to generate the PLN R14del mutation in a human cell line and iPSC-derived cardiomyocytes as a disease model. A series of pegRNAs, in conjunction with nicking gRNAs, were tested for efficiently installing the mutation. Subsequently, we applied similar methodologies to correct the R14del mutation in engineered HEK cells and patient-derived iPSCs harboring the mutation. The study progresses with the development of an Adeno-associated virus, with an optimal pegRNA, as a delivery vehicle for the translational approach. We successfully identified pegRNAs capable of installing and correcting the PLN R14del mutation without high indel rates. The ongoing research focuses on the efficacy of the prime editing approach in correcting the R14del mutation in humanized PLNR14del mouse and pig models, with the anticipation of preventing arrhythmogenic and dilated cardiomyopathy features. This research demonstrates prime editing's potential in treating familial cardiomyopathies, potentially leading to innovative therapies and foundational advances in genetic medicine.

P04

Engineering miniature CRISPR-Cas proteins for gene editing in human cells

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CRISPR-guided Cas9 and Cas12 proteins enable RNA-targeted DNA binding and/or cleavage, as well as several “CRISPR 2.0” applications, such as epigenetic, base, and prime editing. However, the most commonly used Cas proteins are relatively large in size, complicating *in vivo* delivery. Here, we describe a combination of structure-guided and AI-based protein engineering strategies to increase the gene editing functionalities of a miniature type V system in human cells. Moreover, we use metagenomic databases to detect related CRISPR systems, in order to test the scalability of our engineering efforts across orthologues. Finally, we test our most potent mini-nucleases and base editors in human iPSC-derived cardiomyocytes and in mouse embryos. In sum, we show a new pipeline for scalable CRISPR protein engineering and provide a suite of miniature Cas12 proteins for gene editing in human cells.

P05

Establishing a targeted gene delivery platform for monogenic kidney disorders

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Gene therapies, along with genome editors based on the CRISPR-Cas systems, have become a new approach for treating diseases caused by pathogenic variants in various organs, including the liver, muscles, lungs, and the central nervous system. However, delivery to the kidney remains challenging. In this project, our goal is to establish a kidney delivery platform with the potential to correct monogenic kidney diseases using base editors. Due to the kidney's complex anatomy, we explore microsurgical methods for targeted renal delivery of adeno-associated viruses (AAVs). Local renal pelvis, artery, and vein injections, as well as systemic tail vein injection routes, were tested in Ai14 reporter mice to target different kidney cell types using the serotypes AAV8 and AAV9. In this mouse model, AAV-mediated delivery of a Cre recombinase leads to the excision of a loxP-flanked STOP cassette and consequently induces tdTomato expression. Flow cytometry (FC) results showed approximately 90% transduction in the liver, independent of the injection route, compared to up to 6% in the kidney with renal artery injections. Immunofluorescence revealed a delivery route-dependent distribution of tdTomato-expressing cells. The reporter system allows for the quantitative analysis of renal *in-vivo* delivery and the systematic optimization of transduction efficiency. Ongoing experiments include the use of cell-type specific promoters and testing of non-viral delivery approaches to enhance efficiency and specificity.

P06

Safety Support in (CAR)-T Cell Therapy Development: A qualitative qPCR assay as alternative approach for Replication Competent Lentivirus (RCL) monitoring

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Chimeric antigen receptor **(CAR)-T cell therapy** is an emerging field for treatment of hematologic malignancies and other cancer types. To introduce new or corrected genes into the activated T-cells, lentiviral vectors are commonly used. Although lentiviral vectors are engineered to be replication defective, they potentially pose risks to human health, such as generation of a Replication Competent Lentivirus **(RCL)** capable of infecting non-target cells. **qPCR assays for RCL monitoring** are a rapid method to detect and usually quantify lentiviral genes, such as the envelope gene VSV-G (vesicular stomatitis virus G glycoprotein). Since Food and Drug Administration **(FDA) guidance** requires that all RCL positive results should be pursued by direct culture assay to obtain and characterize the infectious viral isolate, we concluded that a quantitative assay is not necessarily needed to detect RCL. Therefore, we developed an alternative approach for a highly sensitive, cost-efficient **qualitative** qPCR assay to assess the presence of the VSV-G sequence for the purpose of RCL monitoring.

P07

Long-lasting antibody delivery by a myotropic Adeno-Associated Virus (AAV) Capsid Variant: high serum levels, favourable glycosylation profile and protection from SARS-CoV-2 challenge

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Delivery of monoclonal antibodies (mAb) by AAV-based vectors is a promising strategy for long-term protection from infectious diseases. Therefore, we designed a monocistronic AAV vector construct coding for TRES6, a neutralizing mAb against SARS-CoV-2. The AAV vector construct was packaged in two different AAV capsids: the hepatotropic AAV8 and the myotropic AAVMYO. Following either intravenous or intramuscular injection, both capsids led to high TRES6 serum concentrations persisting for the entire observation period of up to 52 weeks. TRES6 serum concentrations after AAVMYO delivery were approximately 3.7-fold higher. Despite finding a broad tissue distribution of vector DNA, transcriptionally active sites following AAVMYO transduction appeared to be restricted to skeletal muscle and heart. In AAV8 transduced mice, substantial vector RNA copies were only identified in the liver. The distinct tissue-specific transgene expression coincided with differences in the glycosylation pattern of the encoded antibody's Fc region: AAVMYO delivered TRES6 had a favourable glycosylation pattern, predicted to impact Fc-γ-receptor binding. SARS-CoV-2 challenge experiments in human ACE-2 transgenic mice confirmed efficient protection after delivery of the vector by both capsids.

P08

Development of a new therapeutic approach by genome editing of Tenascin-C expressed in breast tumor and microenvironment

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Tenascin-C (TN-C) silencing is a promising gene-based oncotherapy strategy because of its restricted tissue expression and its role in tumor survival, metastasis, angiogenesis and immune escape. In this study, we have used the powerful genome editing tool, CRISPR/Cas9 to study the effect of TN-C downregulation in human triple negative breast cancer cell line, MDA-MB-231, and then formulated a chitosan-based non-viral gene carrier vector to enable safe and efficient *in vivo* application of the CRISPR/Cas9 plasmids. A carrier system was prepared by grafting oxytocin to the cationic chitosan by using a polyethylene glycol heterobifunctional linker. The resultant nanoplex has been characterized by FT-IR, electron microscopy and dynamic light scattering, and it was shown that it carried CRISPR/Cas9 plasmids successfully into the cells. In vivo study, Oxy-PEG-CS-TN-C CRISPR/Cas9 nanoplexes were intratumorally administered twice a week to the xenograft breast cancer model in athymic mice. The most significant reduction in tumor diameter was detected in the Oxy-PEG-CS-TN-C CRISPR/Cas9 nanoplex group (42%). mRNA expression of TN-C was suppressed by approximately 48% in the nanoplex group. The expression of TN-C protein was markedly decreased. As a result, in our study a chitosan-oxytocin conjugate has been formulated for the first time, and a new therapeutic approach in triple negative breast cancer has been developed by editing TN-C using CRISPR/Cas9 carried by this non viral system. This study was funded by Inonu University, Department of Scientific Resarch Projects (TOA-2019-1543).

P09

DART-AAVs enable specific transduction of human CD8 T cells for *in vivo* gene transfer

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One of the biggest challenges of in vivo gene therapy is the lack of selective vector systems for a defined therapy-relevant cell population. Here we present a second generation of designed ankyrin repeat protein (DARPin)-targeted AAVs (DART-AAVs) exhibiting improved DARPin display and gene transfer activity. Insertion of the human CD8-specific DARPin 63A4 into the GH2/3 loop of the capsid protein 1 (VP1) of AAV2 and AAV6 resulted in vector stocks with unimpaired activities as compared to unmodified serotypes. The capsid core structure was unaltered with the DARPins protruding from the particle surface. When added to PBMC from healthy donors or from B-ALL patients, binding to 88 ± 8 % (n = 2) of all CD8+ cells in healthy, as well as samples, in which the high majority of cells wereCD8-negative. Reporter gene expression was detectable on 85 ± 5 % (n = 4) out of all CD8+ cells. On-target selectivity (percentage of CD8+ among GFP+ cells) reached 98.1 ± 0.8 % (n = 4). *In vivo* in humanized NSG mice, up to 40% of CD8+ T cells were hit upon a single vector injection. Selectivity for CD8+ T cells was close to absolute and liver burden was reduced 20-fold compared to unmodified AAV. These data demonstrate that capsid insertion of DARPins mediates highly selective and efficient gene transfer into mouse and human T lymphocytes. DART-AAVs have the potential to broaden in vivo gene therapy options substantially and to facilitate translatability between animal preclinical studies and human applications.

P10

Evaluation of a novel mRNA trans-splicing dual AAV vector strategy to treat Usher syndrome 1B

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Usher syndrome is the most common form of inherited deaf-blindness. Mutations in *MYO7A* are the predominant cause for USH1B, the most severe subtype of Usher syndrome. There is currently no therapy that can halt or mitigate retinal degeneration in USH1B patients. As the *MYO7A* coding sequence (6.7 kb) exceeds the packaging capacity of adeno-associated-viral

(AAV) vectors, alternative strategies including dual AAV approaches are required for gene supplementation. Here, we used a novel dual AAV vector strategy based on reconstitution via mRNA *trans*-splicing (REVeRT) to assemble *MYO7A* in mice, pigs and human retinal organoids. Upon subretinal injection, we show that our dual REVeRT AAV vectors lead to high *MYO7A* reconstitution efficiency (up to 56%) at protein level in the mouse and pig retina. A side-by-side comparison of REVeRT AAVs to a commonly used dual AAV approach based on reconstitution at the genomic level shows a 3-fold increase in *MYO7A* expression at transcript and 38% higher expression at protein level. The transgenic *MYO7A* protein was found to be mainly localized to the RPE and photoreceptor cells of mice, pigs and human retinal organoids. Our results provide compelling evidence that REVeRT leads to high expression of the full-length *MYO7A* protein in different species. A second strategy aiming at dCas9-VPR-mediated transactivation of *MYO7B* as a functional equivalent counterpart to *MYO7A* also shows promising data in *in-vitro* and *in-vivo* experiments. Further experiments are currently under investigation. After validation of our dual REVeRT-AAV vectors in Myo7a-deficient animals, we plan to initiate clinical trials in USH1B patients.

P11

Enhanced precision for *in vivo* gene delivery through bispecific AAV vectors towards targeting of HIV reservoir cells

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Precise delivery of therapeutic genes is one of the major requirements for *in vivo* gene therapy, with vector particles selectively transducing their target cells as essential tool. While current receptor-targeted vectors selectively enter cells via a single surface marker, many therapeutically relevant cell types, such as HIV reservoir cells, are defined by multiple cell surface markers. In the HIV context, elimination of proviral genomes in reservoir cells presents an attractive therapeutic strategy. Towards reaching this challenge, we present AAV vectors exhibiting an AND-gated binding behavior for the surface markers CD4 and CD32a.
A CD4- and a CD32a-specific DARPin were inserted into surface-exposed loops of the AAV2 capsid maintaining its structural integrity as determined by high-resolution cryo-electron microscopy. Remarkably, in contrast to their monospecific counterparts, these bispecific vectors exhibited a clear preference for CD4/CD32a double-positive cells. This preference was consistently observed in cell mixtures as well as in vivo after systemic vector administration into a mouse model harboring double-positive cells in bone marrow. When equipped with Cas9 nucleases targeted to the HIV provirus, these vectors inhibited HIV replication in vitro. Double-positive cells mixed into healthy donor blood were reproducibly hit by these vectors, even when making up only a small fraction of cells. Further investigations of the system demonstrated its flexibility in target receptor selection and the potential for improvements in selectivity.
These results suggest a novel avenue for receptor targeting by enabling gene transfer into cell subtypes defined by two cell surface markers.

P12

Next-generation sequencing-guided screening of AAV9 peptide display libraries yields novel neurotropic capsids

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Over the past decade, directed-evolution method was extensively used to generate novel AAV variants for targeted gene therapy in the central nervous system (CNS). These studies involved screening different peptide insertion libraries designs, considering serotype, insertion positioning, and peptide size. In this study, we employed NGS-guided in vivo screening to compare the performance of three AAV9 peptide insertion library designs: 7-mer peptide, 12-mer peptide, and liver-de-targeted 7-mer peptide. After each round of screening, a comprehensive bioinformatic analysis was conducted to monitor the process and compare CNS-derived peptides with off-target tissues.
These screenings revealed that candidates derived from the 12-mer insertion and liver-de-targeted 7-mer libraries exhibited improved performance compared to the unmodified 7-mer library. Subsequently, the CNS targeting properties of 28 selected mutant 7-mer and the 12-mer candidates were tested in mice using both a barcoded approach and as single variants. Among the 7-mer variants <50% showed higher CNS-expression compared to parental control vectors, while 100% of the 12-mer candidates outperformed AAV9 in terms of CNS expression and liver de-targeting. The two 12-mer candidates, AAV9-CNS_005 and AAV9-CNS_006, demonstrated enhanced CNS transduction, outperforming AAV9 by ~26 and 40-folds respectively, and AAV9-PHP.eB by ~4-5-folds. Immunohistochemistry analysis confirmed that both AAV9-CNS_005 and AAV9-CNS_006, like AAV9-PHP.eB, primarily targeted astrocytes, endothelial cells, and neurons in the CNS, while minimal expression was observed with AAV9.
Overall, this study emphasizes the importance of optimizing peptide library design, refining the screening process, and using stringent bioinformatic analysis to identify efficient and specific AAV variants.

P13

Pleiotropic effects of AAV.SERCA2a treatment for heart failure in DMD pigs

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Duchenne muscular dystrophy is a devastating muscle disease caused by loss-of-function mutations in the dystrophin (*DMD*) gene. Since pharmacologic treatment is ineffective over time, gene therapy including gene editing is rapidly evolving. In patients with advanced disease, who suffer from cardiomyopathy, locoregional AAV.SERCA2a gene therapy is a novel treatment option, which we tested in a porcine model of DMD.

We generated pigs lacking exon 52 of the *DMD* gene. Male offspring are characterized by heart failure (EF33±3%), arrhythmogenesis due to distinct regions of low or no-amplitude action potentials and by sudden cardiac death before 4 months of age. Using intracoronary delivery of AAV1.SERCA2a at a dose of $3x10^{13}$ virus genomes (vg) per pig by slow antegrade infusion of LAD & LCx improved left ventricular ejection fraction (EF 46±5% +AAV1.SERCA2a), although it didn’t significantly alter the amount of no-amplitude areas per LV.
Notably, the recruitment of CD45+ leukocytes significantly increased in DMD pigs, was reduced to wildtype levels after AAV.Serca2a treatment. Microvascular density was comparable to WT levels as opposed to severely reduced levels in DMD hearts. Finally, principal component analysis of the DMD proteome indicated, that a variety of components were moving towards WT levels, including inflammatory, mitochondrial and sarcomeric proteins.
AAV1.SERCA2a sufficed to normalize left ventricular function in DMD pigs, and initiated partial normalization of structural alterations of DMD hearts (microvessel density, inflammation). We conclude that although SERCA2a gene therapy does not correct the disease-causing mutation, it´s pleiotropic effects on the proteome help to partially rescue the DMD cardiomyopathy.

P14

Characterization of novel recombinant adeno-associated Virus (rAAV)-based capsid variants in the murine brain

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Gene therapy using recombinant adeno-associated virus (rAAV) is a common gene delivery tool for genetic diseases that is already used in clinic. rAAV vectors are non-pathogenic in humans, have no integrase activity and mediate long-term gene expression. It is known that different serotypes exhibit varying tropism, which is due to the various interaction with different receptors on the cell surface. AAV9 is known to successfully transduce cells of the CNS, such as neurons, and is also known to pass the blood-brain-barrier, making it a promising candidate for gene therapy in neurodegenerative disorders. Previous data from our lab showed widespread and high-level retinal transduction after intravitreal injection for the newly engineered vectors AAV2.NN and AAV2.GL, which have a 7-mer peptide insertion.
In this work, we tested these novel vectors with the AAV9 capsid encoding eGFP under the control of different promoters in the mouse brain. *In-vitro* studies revealed that all capsid variants using the CMV promoter led to eGFP expression in neurons and astrocytes. When using the neuron-specific hSyn promoter eGFP expression was only observed in neurons. Based on these results, we injected the viral capsids into the thalami of WT mice by stereotaxic surgery. After three weeks, eGFP expression was quantified in different brain regions by qRT-PCR and Western Blot. All capsid variants were most prominent in the thalamus, but could also be detected in efferent brain regions. Overall, this characterisation of AAV9.NN and AAV9.GL elucidates that these vectors are promising candidates for use in gene therapy targeting neurodegenerative diseases.

P15

T cells expressing HBV-specific chimeric antigen receptors harboring a Fab fragment control HBV infection in mice

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Chimeric-antigen-receptors (CARs) are synthetic receptors designed to drive antigen-specific activation of T cells upon binding to cognate antigen. CAR-T cells are used in cancer therapy but are also interesting for chronic viral infections. Our study aimed to generate novel CARs that target the hepatitis B virus envelope protein (HBVenv) using an antigen-binding fragment (Fab) consisting of heavy and light chains instead of a variable single-chain fragment. The aim of this study was to overcome functional alterations of the new format and study the antiviral efficacy of FabCAR-engrafted T cells in vitro and in vivo.We constructed novel CARs containing the Fab fragment of HBVenv-specific monoclonal antibodies as binding domains and CD3 as well as CD28 intracellular signaling domains. Multifunctional FabCAR-T cells could be induced via HBsAg stimulation. FabCAR-T cells specifically eliminated HBVenv transgenic cell lines Huh7S and HepG2SML. FabCAR-T cells showed antiviral activity by significantly decreasing the level of viral antigen, intracellular HBV DNA, and HBV cccDNA in HBV-infected HepG2-NTCP cells in vitro. To study in vivo efficacy, CD45.1 murine T cells expressing FabCAR were transferred to CD45.2 AAV-HBV infected, HBV-carrier Rag1 knock-out mice; after adoptive transfer, FabCAR-T cells proliferated and localized to the liver, resulting in target cell killing indicated by ALT flare and an antiviral effect by HBsAg and HBeAg reduction.T cells stably transduced with our FabCARs are polyfunctional and have antiviral effects in cell culture and in a preclinical animal model, which are promising candidates for treating chronic hepatitis B and HBV-associated hepatocellular carcinoma.

P16

Super-resolution microscopy reveals gene-transfer strategy-induced disparity of CAR expression affecting CAR-T cell function in an antigen density dependent manner

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The development of chimeric antigen receptor (CAR) T cell products with optimal safety and efficacy requires insights how distinct gene-transfer strategies affect CAR expression and ensuing T cell function.
We established a test platform that allows us to characterize and compare CAR-T cells engineered by either, lentiviral transduction (LV), Sleeping Beauty (SB) transposon-based gene

transfer or CRISPR-Cas-mediated targeted CAR insertion (KI), to express CAR constructs that cover a range of different target and epitope specificities in graded affinities.
We characterized the CAR-T cells phenotypically by flow cytometry, genomically by CAR copy number variation (CNV) determination via droplet digital (dd)PCR, and analysed CAR surface expression and organization by dSTORM super-resolution microscopy.
Further, we employed a library of target cells with distinct antigen densities to functionally characterize the CAR-T cells regarding short vs. long-term cytolytic activity, cytokine secretion, proliferation capacity, as well as the rate of antigen-induced cell death (AICD) *in vitro*.
We found that charachteristic CNV from highest (SB) to lowest (KI) manifested in pronounced differences in spatiotemporal CAR expression that translated to distinct anti-tumor functionality depending on the target-density. LV/SB CAR-T cells showed higher cytokine production and conferred stronger cytolytic activity compared to KI CAR-T cells in short-term (> 8 hours) in short term lysis assays.
LV/SB CAR-T cells showed higher cytokine production and stronger cytolytic activity compared to KI CAR-T cells in short-term assays. All CAR-T cells were equally effective with longer follow-up. We observed less AICD for KI CAR-T cells.
These results highlight the relevance of gene-transfer strategy for CAR-T cell function.

P17

mRNA technology for the development of CAR T cells targeting fibrotic diseases

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Engineered T cells expressing chimeric antigen receptors (CARs) have already been proven to be an effective treatment in cancer immunotherapy. For this reason, recent research approaches are investigating the use of CAR T cell therapies in non-malignant immune-mediated diseases such as infectious diseases, autoimmune or fibrotic diseases. Fibrotic diseases represent a number of different diseases characterized by the overgrowth, hardening, and/or scarring of various tissues caused by excessive deposition of extracellular matrix components. The main cellular mediators of fibrotic diseases are activated fibroblasts, which serve as the primary extracellular matrix-producing cells. While many surface antigens on activated fibroblasts have already been identified, the fibroblast activation protein (FAP) represents the most promising target. To date, the vast majority of CAR-T cells in development are based on stable genetic modification by viral vectors. However, the long-term consequences, for example, due to off-target effects, are still poorly understood. An alternative to stable genetic modification is the transient modification of cells by introducing an mRNA encoding the CAR protein into the cells. mRNA-based CAR cell therapies thus offer the possibility of a safe and pharmacokinetically controllable immunotherapy. Here, we present results for the reprogramming of T cells with CAR mRNA directed against FAP for the treatment of fibrotic diseases. The work includes the optimization of the anti-FAP CAR mRNA for prolonged CAR expression and reduced immunogenicity. In addition, novel image-based cytotoxicity assays using live cell microscopy have been developed, providing new insights into the kinetics of CAR T cell killing.

P18

PSMA-specific natural killer cells for prostate cancer immunotherapy

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Natural killer (NK) cells display built-in activity against tumor cells, which can be enhanced and rendered antigen-specific through expression of chimeric antigen receptors (CARs). Recently, CAR-expressing NK cells have been reported to confer promising safety and efficacy in patients with CD19-positive lymphoid neoplasms. However, CAR NK cell therapy has shown no clear benefit thus far against solid tumors. Aim of this project is the generation and preclinical evaluation of CAR NK cells for prostate cancer immunotherapy that recognize prostate-specific membrane antigen (PSMA). So far, we have developed robust and efficient protocols for the expansion and transduction of NK cells, as well as the functional evaluation of the resulting CAR NK cells. Primary NK cells were derived from healthy donors and expanded using feeder cells. Transduction with a gamma-retroviral vector encoding a second-generation PSMA-specific CAR, IL-15 and the dLNGFR marker was embedded into the expansion process. Upon transduction, some 60% of NK cells expressed the transgenes over a period of several weeks, during which the CAR NK cells maintained proliferative capacity. In order to assess anti-tumor activity in vitro, CAR-expressing NK cells were co-incubated with PSMA-positive or PSMA-negative tumor cells, and target cell killing was tracked over time using live cell imaging. Our results demonstrate that the CAR NK cells readily targeted tumor cells in a CAR-dependent and PSMA-specific manner, which was further enhanced by IL-15. Based on these promising results, in vivo evaluation of the PSMA-specific CAR NK cells in mouse tumor models is planned as the next step.

P19

Selective elimination of cancer cells in tissue culture by using the collateral activity of a novel CRISPR-Cas nuclease

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Since the discovery of the CRISPR-Cas9 system numerous new Cas variants have been identified with the ambition to create superior treatment approaches for various diseases including cancer. Our metagenomics screening revealed a novel Cas nuclease, termed G-daseE, which induces both collateral DNA and RNA degradation upon guide RNA-mediated recognition of a target RNA. Here we show that this RNA-inducible nuclease activity can be applied for selective elimination of cancer cells. G-daseE was assembled with target-specific gRNAs as RNP complexes and cell depletion was determined by quantitative fluorescence imaging and cytotoxicity assays. By directing RNPs against the mRNA of the housekeeping gene GAPDH, cell death could be triggered in all cell types tested including head neck and cervix cancer cell lines. Interestingly, cell elimination was also observed when the cancer-associated nuclear long non-coding RNA MALAT1 was targeted, proving the broad target RNA spectrum of G-daseE in human cells. To

address selectivity, we performed co-culture experiments and achieved selective depletion of GFP-positive cells from a mixed population consisting of GFP- and RFP-expressing HEK293 cells. Finally, we targeted the clinically relevant HPV18-E6/E7 oncogenes expressed in HeLa cervix carcinoma cells and succeeded in elimination of HeLa cells whereas HEK293 cells that do not carry the oncogenic mRNA remained viable. Remarkably, creating single mismatch mutations in the targeting gRNA abolished cell killing confirming high specificity of G-daseE. Altogether, we showed that G-daseE can be programmed to induce selective cell elimination upon recognition of user-defined marker RNAs providing an innovative strategy for targeted cancer therapies.

P20

Generation and analysis of cardiac progenitors expressing the DTPA-R PET-reporter gene for *in vivo* tracking of the cells in regenerative cell therapies

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Human heart regeneration is one of the most critical unmet clinical needs at a global level. The development of cell-based strategies for heart regeneration is an intense area of research. Recently, we demonstrated that the transplantation of human pluripotent stem cell (hPSC)-derived ventricular progenitors (HVPs) in injured pig hearts promotes remuscularization and prevents heart failure progression. Here, we introduce a novel reporter gene system (DTPA-R) to human pluripotent stem cell (hPSC) with the potential to quantitatively track the number and viability of transplanted cells *in vivo*. Tracking the grafted HVPs *in vivo* can provide useful dynamic information about their migration, viability, and functional effects within the heart. Importantly, the combination of PET and magnetic resonance imaging (MRI) would allow parallel assessment of heart function and its improvement upon HVP treatment.
The engineered hiPSCs were created by nucleofection and antibiotic selection. Correct gene knock-in, the absence of off-site mutations, and the absence of chromosomal abnormalities in the cells were determined. The DTPA-R reporter was expressed at the cell surface at levels of about 2 million copies for hiPSCs. Expression of the receptor was confirmed in HVPs and CMs at later time points. Uptake of the reporter probe [^{18}F]F-DTPA●^{nat}Tb of induced cells was measured in 2D cell culture and by *ex vivo* PET imaging of HVPs applied on native 3D pig heart slices. HVPs survival, differentiation, and ability of specific and efficient binding of the radioligand to the receptor was proven and the bound activity correlated with the number of applied cells. Non-specific binding to native pig heart tissue was negligible. Subsequent immunofluorescent analysis of the tissues overlayed with the obtained PET signals.
Using the [^{18}F]F-DTPA●^{nat}Tb system, life monitoring of cellular location and viability of transplanted HVPs and their regenerative effect after myocardial infarction, as well as potential off-target enrichments of cells outside the heart is possible. Further investigations in animal models are essential to get a better understanding of the dynamic behavior of the cells *in vivo*. This multimodal imaging could help improve preclinical research as well as clinical studies of regenerative cell therapies.

P21

Identification and modelling of patient-specific CD19 escape variants to CAR T cell therapy

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In the last five years, the FDA and EMA approval of four CD19-targeting chimeric antigen receptor T cell (CAR-T) products have revolutionized the treatment of patients with relapsed and/or refractory B cell malignancies. However, about 50% of patients treated with CD19-targeting CAR-T (CAR19) therapy don’t achieve durable disease control due to a lack of functional CAR-T persistence or tumor resistance. Resistance mechanisms are multifactorial (tumor intrinsic, immunosuppressive microenvironment, and CAR T cell dysfunction), and CD19 escape variants substantially contribute to it. Therefore, we hypothesize that CD19 escape variants must be present at a critical threshold in a heterogeneous tumor population to grow out under CAR19 treatment. We will employ a CRISPR-based editor tiling screen as an unbiased approach to discover CD19 escape variants to CAR19 therapy. We have designed a CD19 tiling screen library of 11,500 potential sgRNAs (targeting protein-coding sequences, promoters, enhancers, introns, and UTRs) to recruit cytokine and adenine base editors. Next, to verify the functional consequences of identified CD19 escape variants, we will perform confirmatory in vitro and in vivo assays. Our findings hold the potential for future clinical translation to predict patients' disease relapse and to guide the selection and design of personalized CAR-T products.

P22

Enhancing endosomal escape of siRNA through chemical electron transfer

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Drawing inspiration from reactive oxygen species capable of inducing lipid peroxidation, we explored chemical electron transfer (CET)-based self-activatable lipopolyplexes and lipid nanoparticles (LNPs) for producing singlet oxygen ($^1O_2$) in endosomes, disrupting endosomal membranes to enhance the escape of small interfering RNA (siRNA). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) was adopted as CET donor, and gold nanoparticle or, alternatively, hemin served as acceptor. Donor and acceptor were simultaneously encapsulated inside lipopolyplex or LNP. The donor can be specifically self-activated by transferred chemical energy between TCPO and hydrogen peroxide that is overexpressed in tumor microenvironment. Enhanced endosomal escape was proved by using a fluorescent galectin-8 (Gal8)-mRuby reporter; $^1O_2$ improved the recruitment of Gal8 by around 50-fold compared to the control group. Moreover, CET enabled a

reduction of the required siRNA dose. Ionizable LNPs were optimized by testing different lipids and a four-armed oligoaminoamide or a mini U-shaped tetra-oleoyl tri-lysino succinoyl tetraethylene pentamine as ionizable oligocations. Superior gene silencing at low siRNA dose was demonstrated with three targets, green fluorescent protein as fluorescent marker, the oxidative stress induced nuclear factor erythroid 2-related factor 2 (Nrf2) as oxidative stress protein, or eglin 5 (EG5/KSP) as mitotic spindle motor protein. Nrf2 and EG5 gene knock-down resulted in tumor cell killing in culture. This research introduces a new perspective for facilitating endosomal escape of nucleic acids.

P23

Base editing for monogenic kidney disease

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Autosomal Dominant Tubulointerstitial Kidney disease (ADTKD) caused by pathogenic variants in Uromodulin (UMOD) is the 3rd most common cause of monogenic kidney disease in adults. Dialysis and kidney transplantation are the only therapeutic options for subsequent end-stage-renal-disease. However, these are associated with adverse health effects, hence there is an urgent need for treatment alternatives. CRISPR/Cas base editors, through high efficacy and easy programmability, represent a promising novel treatment strategy. **This project aims to establish renal base editing for the treatment of ADTKD.** Systematic screening of suitable gRNAs and base editors for correction of pathogenic human UMOD variant C170Y in engineered HEK293T cells resulted in editing efficiencies of up to ~75%. Subsequent base editing in a mIMCD-3 cell line expressing the human C170Y variant attenuated the disease underlying ER-retention of Uromodulin and restored physiological trafficking to the cellular membrane. Two further approaches, independent of the causative pathogenic variant, are based on the observation that *Umod*-/- mice lack an obvious renal phenotype. Here, UMOD expression is inactivated by introduction of STOP codons or disruption of splice sites. In HEK293T cells, we achieved up to ~80% and up to ~50% on-target editing respectively. We will further validate our approach *in-vitro* in human renal organoids derived from patients affected by ADTKD-UMOD. We anticipate base editing to serve as an effective and innovative therapeutic approach for renal monogenic diseases.

P24

CAR-T cells: a new strategy to fight cytomegalovirus infections in the immunocompromised host

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Even today, human cytomegalovirus (CMV) infection or reactivation remains a life-threatening complication for immunocompromised patients, especially in stem cell transplant recipients. Inspired by cancer immunotherapy, we aim to establish and functionally investigate CMV-specific

chimeric antigen receptor (CAR)-T cells, with the goal of effective *in vitro* and *in vivo* CMV control. CMV glycoproteins are displayed on the surface of infected cells during the viral replication cycle. Here, multiple CAR constructs recognizing glycoproteins of murine CMV (MCMV) have been engineered in our group and MCMV-specific murine CAR-T cells are currently produced by retroviral transduction. Binding of a specific CAR-T cell to its (viral) antigen directly initiates T cell effector functions, via intracellular signalling domains of a T cell receptor. By performing *in vitro* killing assays, using two MCMV glycoprotein expressing reporter cell lines (Nano-luciferase, CRSTAL), we could proof antigen specific recognition by and activation of our murine CAR-T cells as well as granzyme B mediated cytotoxicity and target cell lysis. Currently, our CAR-T cells are investigated using MCMV infected murine fibroblasts. Initial results indicate weak direct lysis of infected cells but strong reduction of viral spread. In near future, a proof-of-principle *in vivo* study will be conducted in an established MCMV challenge model.

P25

Gene editing of MHC class I and II enhances allogeneic regulatory T cell therapy

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Regulatory T cells (Tregs) play a crucial role in balancing the immune system and show potential for treating autoimmune diseases and preventing transplant rejection. To date, clinical trials have primarily focused on adoptive cell transfer of patient-derived Treg. However, this autologous approach faces challenges including variability in Treg numbers among patients, impaired Treg function due to underlying pathology, lengthy and sometimes unsuccessful manufacturing processes as well as the related signficant costs. Here, we evaluated the effectiveness of unmatched, allogeneic Tregs as an 'off-the-shelf' immunomodulatory cell therapy. In a humanized mouse model with human skin transplants, we observed that allogeneic 'third-party' Tregs were significantly less effective in preventing graft rejection than autologous counterparts. Gene editing of *B2M* and *CIITA* in human Tregs allowed efficient silencing of MHC class I and II, respectively. A HLA-E-B2M fusion gene partially protected *B2M*-edited Tregs from NK cell lysis *in vitro*. Silencing of MHC class II increased the protection of Tregs from allo-specific T cell attack *in vitro*. Combined HLA-E-*B2M* knock-in and *CIITA* knockout significantly enhanced the suppressive activity of allogeneic, unmatched Treg in our humanized skin transplant model. In respect to long-term graft survival, allogeneic MHC-engineered Treg had comparable suppressive activity to autologous Treg. Peripheral blood analysis of humanized mice indicated circulating MHC-silenced Treg at day 20 of the experiment. We are conducting further in vivo tests to confirm these initial findings. Our study suggests that modifying MHC class I and II can facilitate successful allogeneic 'off-the-shelf' Treg therapy.

P26

Generation of enhanced CD19-specific CAR-T cells through identification and editing of exhaustion-associated genes

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Despite the success of CAR T cell-based immunotherapy, a sizable proportion of patients treated with CAR T cells experience disease relapse. This may be due to the low persistence or exhaustion of CAR T cells resulting from their continuous exposure to the tumor antigen. Several genes have been implicated in pathways that control T cell exhaustion, acting either as promoters or as antagonists of this mechanism. In an effort to generate CAR T cells that resist T cell exhaustion, we sought to establish a robust genome editing procedure for multiplexed editing of CD19-specific CAR T cells. First, we identified the optimal conditions for manufacturing large quantities of CD19-specific CAR T cells using lentiviral-mediated transgene delivery. We tested several protocols and identified the conditions that consistently yielded over 50% of CAR-positive T cells. We then established an assay to induce CAR T cell exhaustion *in vitro* through tonic CAR signaling, and used this system to identify genes that either promote or antagonize exhaustion using transcriptome analyses of CAR T cells collected at different time points. A variety of genome editing strategies are currently being tested to achieve our ultimate goal of manufacturing CAR T cells with reduced expression of genes that promote exhaustion while simultaneously increasing the expression of genes that prevent exhaustion. This study opens novel opportunities towards the generation of improved allogeneic cancer immunotherapies and might potentially extend the therapeutic use of this platform to more resilient tumor types in the future.

P27

Investigating the epigenetic regulation of the β-globin switch to identify novel curative opportunities for β-hemoglobinopathies via targeted epigenome editing

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Beta-hemoglobinopathies are severe genetic diseases caused by mutations in the β-globin locus. One option for novel treatment involves the transplantation of autologous, genetically corrected HSCs. Genome editing of the *BCL11A* erythroid-specific enhancers using site-specific nucleases shows efficient reactivation of foetal hemoglobin (HbF) expression. Yet, HSCs are highly sensitive to DNA damage, which might result in unpredictable genotoxic effects. The recent adoption of designer epigenome modifiers (DEMs) to deposit repressive marks in a targeted manner offers the

opportunity to achieve erythroid-specific *BCL11A* inactivation without altering the underlying genomic sequence. We anticipate that epigenetic inactivation of these *BCL11A* enhancers will counteract the developmental silencing of HbF and rescue the hematologic and pathologic features of β-hemoglobinopathies. We characterised the epigenetic marks decorating the erythroid-specific *BCL11A* enhancers during HSC differentiation toward the erythroid lineage using an established *in vitro* assay, and identified regions that differentially lose DNA methylation leading to enhancer activation. We produced DEMs targeting these regions to induce persistent inactivation of the erythroid-specific *BCL11A* enhancers. We will deliver *in vitro* transcribed mRNA encoding for selected DEMs into HSCs via nucleofection and will correlate the effects of epigenome editing with transcriptional changes of *BCL11A* and β-like globin genes. Furthermore, we will assess whether this approach leads to production of HbF and HbF+ cells after *in vitro* differentiation of epigenetically edited HSCs. We believe that this study will be instrumental to understand the key epigenetic features that control the γ-globin switch and will provide a novel therapeutic approach for patients with β-hemoglobinopathies.

P28

CRISPR prime editing in primary human T Cell subsets

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CRISPR prime editing (**PE**) is an evolution of CRISPR-Cas9 gene editing, allowing precision editing of T cell DNA with high versatility and accuracy. In previous work, we reported efficient PE in primary human T cells (*Petri, Zhang, Ma, Schmidts, et al., Nature Biotechnology 2021*). However, knowledge regarding PE efficiency in T cell subsets remains limited. Here, we assessed PE in primary human CD4 and CD8 T cells.
We acquired synthetic prime editing guide RNAs (**pegRNAs**) with stability-increasing modifications and produced PE mRNA through *in vitro* transcription. We sorted primary human CD4 and CD8 T cells and activated them with CD3/CD28 magnetic beads. We nucleofected pegRNAs and PE mRNA and incubated cells for 72h. We extracted genomic DNA and amplified target loci. We then assessed editing efficiency using sequencing.
We measured PE activity in multiple donors at two target sites. PE enabled the modification of CD4 and CD8 T cells with up to 40 % efficiency, installing G > T and G > C substitution edits that are difficult to install with conventional CRISPR-Cas9 and CRISPR base editing.
PE of T cell subsets might allow the production of differentially edited CAR-T subset mixtures with potentially enhanced properties. Differentially-edited CAR-T subsets could be deployed at varying ratios depending on tumor type and employed CAR. Here, we show efficient PE in CD4 and CD8 T cell subsets, potentially enabling the generation of differentially prime-edited CAR-T subset mixes with enhanced properties.

P29

Lipid-nanoparticle co-delivery of rAAV genomes and mRNA for AAV integrase Rep78 replicates wildtype AAV genome integration as assessed by targeted sequencing

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Recombinant AAV vector genome integration, occurring randomly at low frequency after rAAV gene therapy, is currently seen as a double-edged sword: It may ensure long-term gene expression but raises concerns about potential genotoxicity. In contrast, wildtype AAV integrates its genome mediated by its integrases Rep68/78, and AAV genetic safety stands the test of time. We, therefore, propose to embrace wildtype-like AAV vector genome integration by co-delivery of Rep78 mRNA. For a proof of concept, we transfected a cell culture model using a lipid nanoparticle (LNP)-based system with synthetic rAAV genomes and Rep78 mRNA at different ratios. We found that addition of Rep78 mRNA significantly increased transgene expression already at 0.2% molar fraction of total nucleic acids delivered, and the increase was highest with more than 2-fold more expression at 1.6% mRNA. Transgene expression from LNP-delivered AAV genomes sustained for the tested time periods of up to 17 days potentially through the formation of AAV genome-like episomes and integration. We then investigated vector genome integration by a Cas9-guided direct sequencing approach. 99.7% of recombinant AAV genomes remained episomal nine days after co-transfection. Still, 27 distinct integration events were observed, none of which located in known cancer genes. The integration pattern closely resembled published wildtype AAV patterns with integration at the known site AAVS1 and at other sites distant from assumed Rep recognition motifs. These findings provide an intriguing first step to a fully synthetic option for AAV-like gene therapy with LNP-based delivery of AAV genomes and, optionally, Rep78 mRNA.

P30

CRISPR associated substrate-linked directed evolution (CaSLiDE) for evolving highly efficient and specific miniature CRISPR-Cas systems

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The development of novel CRISPR–Cas genome editing tools continues to drive significant advances in the life sciences. Particularly, compact CRISPR–Cas such as Cas12f (composed of 400-600 amino acids) or Cas12j (composed of 700-800 amino acids) are promising candidates. Nevertheless, these CRISPR–Cas systems are hampered by their limited editing efficiencies in eukaryotic cells. To pave the way for broader applications in basic research and therapeutic

applications, it is important to improve their editing capabilities, specificity and expand their targeting scope.
In this study, we introduce the concept of CRISPR associated substrate-linked directed evolution (CaSLiDE) as a method to evolve CRISPR-Cas systems with increased efficiency and precision. We applied the CaSLiDE approach to evolve different Cas12f proteins, the ABE8e TadA domain and miniature base editors, thereby improving their utility. Our findings compellingly illustrate the potency of the CaSLiDE approach in the directed evolution of genome editing enzymes, underscoring its significance for future applications.

P31

Surveying sequence specificity of designer site-specific recombinases

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The specific targeting and editing of site-specific recombinases (SSRs) provide an appealing option for gene editing therapies where precision is crucial. Recent developments to engineer and evolve Cre/loxP-type SSRs, have made it possible to shift the specificity of Cre from its native target preference, the loxP site, to catalyze recombination of a range of DNA substrates. Effort in developing these designer-SSRs rely on the novel target selection and ability to incorporate mutations for successful retargeting of the SSR. In order to improve rational redesign of SSRs, it is critical to first understand Cre's specificity for DNA, allowing for a more strategic target selection and identify specificity-altering mutations.
Here we developed a high-throughput assay for assessing the DNA specificity of Cre and evolved Cre variants. This method measures the activity of natural and designer-SSRs on a large set of over 5,000 rationally designed, non-randomized DNA target sequences, to profile the recombinase/DNA specificity. The assay is carried out in *E. coli* to minimize experimentation and eliminate artifacts, and employs widely accessible Illumina sequencing as the final readout.
We demonstrated the capabilities of this assay by profiling specificity and relative tolerance to base substitutions of Cre and an evolved Cre variant. By comparing the changes in DNA specificity of the complexes, we identified specificity-altering mutations and used molecular modelling and dynamics simulations to elucidate the mechanism behind their specificity switch. Furthermore, we used the approach to investigate the therapeutic potential of these novel recombinases and characterize their general specificity to detect potential off-targets.

P32

A CRISPR-Cas9 double-hit strategy mitigates on-target aberrations and chromosomal translocations

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CRISPR-Cas nuclease-based genome editing aims to precisely modify the human genome. The introduced DNA double-strand breaks (DSBs) are typically repaired by non-homologous end-joining (NHEJ). It is well known that imperfect repair can lead to small insertions and deletions (indels) at the target site, and it has been speculated that large on-target aberrations are due to repeated cleavage of the target site by the programmable nuclease. Furthermore, DNA repair of multiple DSBs, e.g., a target site and an off-target site, may result in chromosomal translocations. We hypothesized that preventing repeated re-cleavage of the target site would reduce on-target aberrations as well as the frequency of translocations. To test this hypothesis, we targeted different loci in the human genome with a "double-hit strategy", i.e. we designed two CRISPR-Cas9 nucleases that targeted each locus (BCL11A, CD40L, CSF2) within 100 bp. To assess the extent and the frequency of chromosomal rearrangements, we used CAST-Seq and rhAmpSeq. Our data indicate that the use of two CRISPR-Cas9 nucleases to excise a ~80 bp DNA fragment reduced the frequency of large on-target deletions at e.g. the BCL11A locus by a factor of two and the average deletion size from 780 to 430 bp. Furthermore, the number of chromosomal translocation events between two target sites, e.g. CD40L and CSF2, was lowered 3-fold when each locus was hit twice instead of once. In conclusion, our study shows that large on-target aberrations and the frequency of chromosomal translocations can be mitigated by applying a CRISPR-Cas double-hit strategy.

P33

Lipo-xenopeptide polyplexes for CRISPR Cas9 based gene editing at ultra-low dose

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In this study, polyplexes formed with our previously described lipo-xenopeptide carriers were evaluated for CRISPR/Cas9-based genome editing. Consisting of lipo-amino fatty acids (LAF) linked to succinoyl tetraethylene pentamine (Stp) containing backbone, these double pH-responsive carriers enabled co-complexation of Cas9 mRNA and sgRNA. Carriers of different topologies, LAF/Stp ratios and LAF types were evaluated. Up to 3-fold higher gene editing efficacies than gold standard Lipofectamine were reached in multiple different cell lines and reporter models, namely $Hepa^{tdT:PCSK9}$, Hela GFPd2 and Hela mCherry-DMD_{Ex23}. Top performing U-shaped and bundle(B)-shaped carriers exhibited genome editing efficacies at sub-nanomolar EC_{50} concentration of 0.4 nM sgRNA and 0.1 nM sgRNA, respectively, even after incubation in full (≥ 90 %) serum. Co-delivery of Cas9 mRNA, sgRNA and ssDNA resulted in over 35% BFP/GFP conversion in Hela GFPd2 by homology directed repair. Importantly, intravenous administration of polyplexes mediated *in vivo* editing of the dystrophin gene, triggering mRNA exon 23 splicing modulation in dystrophin-expressing cardiac muscle, skeletal muscle, and brain tissue in BALB/c mice.

P34

Base editing of hematopoietic stem cells restores immune function in a mouse model of familial hemophagocytic lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) is an immunohematologic disorder caused by hyperactivated T cells and macrophages. Uncontrolled immune activation results from impaired lysis of antigen-presenting cells by T cells and NK cells, and is caused by mutations in the UNC13D locus. Although allogeneic hematopoietic stem cell (HSC) transplantation is curative, the high mortality in FHL patients urges the exploration of novel treatment options. Here, we developed a cytosine base editing (CBE) strategy to disrupt the disease-causing cryptic splice site in Unc13d intron 26 of Jinx mice, a preclinical model of FHL3. Electroporation of CBE-encoding mRNA and guide RNA into Jinx T cells and HSCs resulted in 61-71% of edited Unc13d alleles. Genetic and functional assays confirmed correct splicing of the Unc13d mRNA and restored cytolytic activity of the edited T cells. Furthermore, transplantation of Unc13d-edited HSCs into Jinx mice demonstrated functional restoration of lymphocyte cytotoxicity and protection against FHL. Unexpectedly, CAST-Seq and rhAmpSeq analyses revealed frequent CBE-induced chromosomal translocations and insertion/deletion mutations at on- and off-target sites. Off-target patterns and persistence of chromosomal translocations were however different in T cells and HSCs, proposing cell type-specific effects. Despite the high off-target activity, secondary transplantation of CBE-modified HSCs into 12 mice did not result in graft-related malignant transformation. In conclusion, our data demonstrate successful base editing to reverse the clinical phenotype in a preclinical FHL3 model, but also reveal cell type-specific off-target effects, highlighting that cell type-specific safety studies are vital to properly assess the risk-benefit ratio of these novel technologies.

P35

Intein-mediated *cis*- and *trans*-splicing enables tighter control of *Sleeping Beauty* transposase activity

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DNA transposon systems such as *Sleeping Beauty* (SB) are useful nonviral vectors mediating efficient and long-term transgene expression. In SB transposition, stable genomic integration of a gene of interest from a DNA donor molecule is obtained through a “cut-and-paste” reaction catalysed by the SB transposase. Conditional activity of the transposase can be achieved by using tissue-specific or drug-inducible promoters and can be an advantageous feature for more precise genome engineering and safer gene therapies. Leaky expression from these promoters, however, can lead to undesired and irreversible effects, as very little amounts of transposase can be sufficient for the generation of stable integrations. Here we report the use of intein-mediated protein splicing as a way to provide additional control over SB transposition. Based on secondary structures of the functional domains of the hyperactive SB100X transposase, we introduced both *cis*- or *trans*-splicing inteins at specific amino acid positions. We demonstrate that Npu DnaE split inteins enable efficient *trans*-splicing of the SB transposase yielding a reconstituted transposase that preserves highly efficient transposition activity. We combined this system with either inducible (Tet-On) or hepatocyte-specific promoters for conditional expression of each of the intein-tagged transposase domains. In addition, a ligand-dependent intein that undergoes *cis*-splicing only in the presence of 4-hydroxytamoxifen (4HT) provided tight post-translational control of the transposase activity with very low background in the absence of 4HT. These novel SB transposase variants permit tighter regulation of SB-mediated genome engineering and hold promise for advancing the field of gene therapy towards more targeted and controlled applications.

P36

Enhancement of *in vitro* AAV-mediated gene transfer for bone regeneration using a cationic poloxamer

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AAV is a promising candidate for gene therapy since it provides prolonged transgene expression without the risk of insertional mutagenesis. This can be used in the context of bone regeneration by packing genes into the AAV vector that promote the formation of new bone. In order to improve gene expression, enhancers which are compatible with *in vivo* and potential clinical use were developed and tested *in vitro*.
Different newly developed proprietary poloxamers were combined with AAV vectors and were tested for reporter gene expression of cell lines and primary cells *in vitro*. The mixes included thermo-responsive hydrogels. The polymer and serotype with the best results were selected for subsequent experiments using ovine bone marrow-derived mesenchymal progenitor cells. AAVs either coded for reporter gene eGFP or growth factor genes BMP-2 or VEGF. Transduction

efficiency was quantitated by fluorescence measurements (eGFP) or ELISA (growth factors). Cell viability was determined by XTT assay.
The experiments revealed a cationic poloxamer, which not only increased transduction efficiency in a dose dependent manner, but also promoted cell survival. Enhancement was seen with AAV2, AAV6 and AAV8 capsids, albeit at different rates. Cationic poloxamer additionally protected AAV from drying, potentially allowing for lyophylization. Thus, a cationic poloxamer with potential for *in vivo* application and clinical use was identified, which enhanced AAV-mediated gene transfer in general and in cells relevant to bone regeneration.

SBR has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement No. 874896.

P37

Universal CAST-Seq: Off-target detection of CRISPR nucleases and base editors

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Genome editing can be associated with genotoxicity, which must be carefully evaluated before gene-edited products can be used clinically. We have recently described CAST-Seq, a diagnostic assay for the genome-wide detection of CRISPR-Cas-induced chromosomal aberrations. The method is based on the detection of chromosomal rearrangements resulting from the simultaneous cleavage of the on-target and an off-target site. While CAST-Seq is able to identify CRISPR-Cas nuclease-induced chromosomal aberrations with high sensitivity, the sensitivity to detect off-target activity of base editors or prime editors is lower because these editing platforms do not rely on the formation of DNA double-strand breaks. In addition, in rare cases, designing effective CAST-Seq primers for a specific target site in GC-rich regions of the genome can be tedious. To overcome these limitations, we developed universal CAST-Seq (U-CAST), a method for detecting off-target activities of any CRISPR-based editing platform without the need for primer optimization. U-CAST is based on the fact that any DNA double-strand break can serve as an "anchor" to detect off-target events triggered by any genome/base/prime editor. Using U-CAST to identify the off-target activities of CRISPR-Cas9 nuclease and base editors, all targeting the same site in the EMX1 gene, revealed that the evoCDA1-BE4max base editor generated more off-target mutagenesis than Cas9 nuclease, while the next-generation CBE-T1.52 base editor effectively mitigated off-target effects. Because the method can be performed with already established U-CAST primers, U-CAST saves time and costs, and is particularly well suited for screening genome and base editors in early development.

P38

Targeting choroidal endothelial cells: Development of novel AAV variants for improved transduction

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Age-related macular degeneration (AMD) is a common degenerative disease affecting the central retina (macula). A key feature is the formation of new choroidal blood vessels growing into and damaging the central retina. Implementation of anti-angiogenic drugs like aflibercept or brolucizumab has improved the prognosis of neovascular AMD. However, around 15% of patients do not respond to these treatments. And even if the treatments are effective against neovascularization, they cannot halt retinal degeneration. In addition, the treatments have to be repeated every one to two months, which is a considerable burden for patients. A potential curative solution might be gene therapy with adeno-associated virus (AAV) vectors targeting choroidal endothelial cells (EC) to counteract neovascularization. However, successful gene transfer to choroidal ECs remains challenging. Here, we aimed to identify novel AAV variants with improved tropism and enhanced efficiency in transducing choroidal ECs. We found that modified AAV1 capsids can robustly transduce primary mouse choroidal ECs (transduction efficiency in the range of 60% and higher), in contrast to parental AAV1 or other naturally occurring serotypes. The engineered AAV1 variants carry the peptide insertions first described in AAV2.GL and AAV2.NN, previously identified AAV2 variants with enhanced transduction efficiency for retinal cells. The promising *in vitro* transduction properties were further evaluated in vivo in mice and *ex vivo* in human retinal explants. Future more, we started to test our variants *in vivo*. Overall, our study identified new AAV1-based capsid variants that could be used for the gene therapy of AMD in the future.

P39

Next round in chemical evolution of xenopeptides for pDNA and mRNA transfer: The spacer effect

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Chemical evolution of 2D sequences and 3D topologies within lipo-xenopeptides comprising succinyl tetraethylene pentamine (Stp) and lipoamino fatty acid (LAF) as the artificial amino acids revealed highly potent double pH-responsive carriers for pDNA, mRNA, siRNA or CRISPR Cas9 mRNA/sgRNA cargos when formulated as polyplexes or lipid nanoparticles (LNPs). Herein, in a next chemical evolution step the effects of spacer amino acids were investigated. In contrast to published molecules, the novel carriers were synthesized by connecting Stp and LAFs with L-ornithine instead of L-lysine. The introduction of ornithine resulted in shorter interspaces between the polar (Stp) and the apolar (LAF) cationizable domains. Original and new analogue structures were evaluated for both luciferase pDNA and mRNA delivery on Huh7 hepatocellular carcinoma and HeLa carcinoma cells. In direct comparison and for both investigated (U1 and B2) carrier topologies, the novel ornithine-spaced xenopeptides (ID 1827 and ID 1813) mediated high

transfection efficacies at low dose (50 ng/well), exceeding the previous lysine-spaced top-performing carriers (ID 1611 and 1621).

P40

Novel AAV variants for improved transduction of retinal microglia

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Recombinant adeno-associated virus (AAV) vectors have been widely used in gene therapy applications for central nervous system (CNS) diseases and have yielded favorable preclinical and clinical outcomes. These vectors can successfully transduce post-mitotic cells, such as neurons and astrocytes. Emerging evidence indicates that the microglia, the major resident immune cells in the CNS, play a central role in the pathobiology of neurodegenerative disorders. Consequently, these cells have recently become the focus of new treatment approaches. However, these approaches have been hindered by the difficulty in transducing microglia using viral and non-viral vectors. To address this, here we introduce a novel microglia culture protocol from mouse retina, which is reproducible and is characterized by high cell numbers and long *in vitro* viability. We used this model as a screening platform to evaluate CMV-mediated reporter transgene expression of engineered AAV6- or AAV1-derived capsids, the most used serotypes on glial cells. The variants carrying 12-mer peptide insertions, AAV-GL and AAV-GL.R, showed enhanced transduction efficiency of primary mouse retinal microglia cells compared with not only wild-type but also the recently published triple mutant AAV6 TM. This improved activity has been confirmed in both resting and chemically activated microglia, mimicking *in vivo* degenerating settings. Finally, we started to validate our variants in mouse models of retinal disorders. Overall, our study establishes a new *in vitro* platform for assessing microglial transduction and identifying novel AAV capsids with improved microglial transduction properties.

P41

Next-generation transfection reagent for large scale therapeutic lentiviral vector production

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Lentiviral vectors are the carrier of choice for allogenic or autologous cell therapies (such as CAR-T) because of its capacity to permanently integrate viral genome into host cell DNA. To produce those vectors, cell therapy producers generally use a transient transfection system that is scaled-up during process development phases. FectoVIR®-LV is the next generation of transfection reagent, free of animal component, designed to improve LV productivity in HEK-293 cell systems. FectoVIR®-LV is made for large scale manufacturing with reduced complexation volume and increased complex stability. On top of the performance in titers and the scalability, FectoVIR®-LV

is also compatible with standard expression booster such as sodium butyrate. These key benefits make FectoVIR®-LV transfection reagent a perfect match for lentiviral vector manufacturing.

P42

pPLUS® AAV-Helper, novel engineered pHelper plasmid to improve yield and quality of several AAV serotypes in suspension cell culture systems

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Harnessing rAAVs as viral vectors for therapeutic transgene delivery still requires improvements in yields and specificity to lower vector doses, and therefore manufacturing cost, as well as to improve patient safety. To this end, our research is focused on developing novel technologies to ensure manufacturing of high yielding rAAV particles using transient transfection, as well as enhancing features of rAAV vectors that act on the overall size of packaged material and specificity of delivery. Here we present our state-of-the art approach to design new helper plasmids (phelpers) with the aim of improving both the infectiosity (TU/mL) and the quality (full/empty ratio) of the viral particle obtained from suspension cultures. We took the opportunity to exploit our proprietary DNA assembly method technology to explore the synergies of multiple genetic features modularly assembled in synthetic plasmids. Comparison of the biological activity of several versions of rationally designed pHelpers led us to identify the optimal configuration able to outperform existing helper plasmids in every tested bioproduction conditions. Our expertise in DNA plasmid design and assembly together with our scalable transfection solutions for rAAV manufacturing gives us the potential to improve both productivity and specificity of gene therapy products.

P43

Transcriptome analysis supports specificity of multiplexed epigenome editing in primary T cells

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T cell inhibition is a major obstacle to the success of adoptive T cell immunotherapies. Mechanistic insights indicate that ligand-dependent activation of various inhibitory receptors coupled to different but synergistic intracellular pathways are associated to this ineffective state, creating an opportunity for novel therapeutic strategies. Genome editing has been used to selectively inactivate genes encoding for inhibitory receptors with encouraging results. However, when scaled up to address multiple targets simultaneously, side effects tend to be magnified due to the increase in genotoxicity related to multiple DNA breaks within the cell. Epigenome editing may be more suitable for multiplexing, as it allows to control gene expression by changing epigenetic marks needless of DNA sequence disruption. Despite this remarkable advantage,

genome and epigenome editing share the risk for unspecific interactions that could cause off-target effects. Here, we performed a thorough specificity study of designer epigenome modifiers (DEMs) used to silence two genes encoding key T cell inhibitory receptors (*PDCD1* and *LAG3)* with a multiplexed hit-and-run approach in T cells. Importantly, co-delivery of the two DEMs did not affect their silencing capabilities, resulting in a stable loss of expression of both PD-1 and LAG-3. A comparative transcriptome analysis confirmed the absence of DEM-mediated off-target silencing. Considering that the few significantly deregulated genes can be attributed to the engineering procedure itself and the *in silico* predicted DEM off-target binding sites were not within *cis*-regulatory regions. Our study confirms the safety of epigenome editing and encourages its exploitation to modulate multiple T cell inhibitory pathways.

P44

Epigenome editing results in successful transient reactivation of gamma globin gene expression and offers an innovative therapeutic option for β-Hemoglobinopathies

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β-hemoglobinopathies are severe genetic disorders caused by mutations affecting the production of the β-globin chain. The clinical-severity of these pathologies can be effectively mitigated by the reactivation of γ-globin (*HBG*) genes, resulting in elevated production of fetal hemoglobin. In this study, we aimed to investigate the epigenetic landscape of the γ-globin promoter in HUDEP-2 cells and utilize designer epigenetic modifiers (DEMs) to activate the *HBG* gene through precise epigenome editing.. We confirmed the presence of epigenetic marks indicative of gene inactivation at the *HBG* promoters, such as enriched H3K9me3 and H3K27me3 and high DNA methylation, via ChIP-qPCR and bisulfite sequencing, respectively. We then developed activating DEMs targeting three different positions within the proximal γ-globin promoter and tested their ability to reactivate *HBG* expression in HEK293T cells that typically do not express γ-globin. We delivered DEM to the cells in form of *in vitro* transcribed mRNA via lipofection. Two days later, we evaluated *HBG* expression levels using quantitative polymerase chain reaction (qPCR). We show that the three DEMs were highly efficient in reactivating *HBG* expression with the most effective exhibiting a remarkable 3 to 25-fold increase in γ-globin levels depending upon the targeted region. Current efforts aims at identifying more potent combinations of effectors and novel target sites to maximize long-term expression of the γ-globin gene, followed by validation in clinically relevant hematopoietic stem cells (HSCs). This approach holds great potential as a novel strategy for the treatment of β-Hemoglobinopathies and paves the way for a new era of therapeutics.

P45

siRNA CAPSULON Technology: Redefined siRNA Transfection

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The application of RNA interference (RNAi) in mammalian cells has brought about a paradigm shift in the field of functional genomics. This breakthrough enables scientists to manipulate gene expression with remarkable simplicity, efficiency, and precision within mammalian cells. Such a capability holds immense promise for driving scientific discoveries, commercial applications, and therapeutic innovations.
To ensure the successful attenuation of gene expression, the proficient transfection of small interfering RNA (siRNA) stands as a critical requirement, underscoring its pivotal role in achieving effective gene silencing outcomes.
The CAPSULON Technology developed by CapCoBIO GmbH empowers researchers with the ability to achieve remarkably effective siRNA transfection at varying concentrations, spanning from low to high. This flexibility grants researchers to select their preferred siRNA concentration for experimentation.
siRNA CAPSULON Kit is a straightforward and easy workflow for siRNA delivery with biodegradable polymer nanocapsules.
Key Features:

- **Highly efficient:** siRNA delivery without cytotoxic effects and off-target effects.
- **Cost effective:** low amounts of siRNA generate high knock-down effects.
- **Versatile:** applicable with any culture medium. No medium change needed.
- **Fast and easy:** streamlined three step protocol.

P46

Evaluation of the efficacy of Bcl-2-siRNA loaded NK cell-based exosomes in xenograft breast cancer model

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Developing strategies to induce apoptosis in cancer cells through its molecular players, such as Bcl-2, is of great importance in cancer treatment. Triggering apoptosis simultaneously both by extrinsic and intrinsic pathways can reverse apoptosis evasion in the cancer cells. NK-cell

exosomes (NKExos) contribute to cancer treatment by interacting with tumor and immune cells, and inducing tumor cell apoptosis by the extrinsic pathway. In this study, NKExos from NK-92MI cells loaded with siBcl-2 by lentivirally transducing the cells with Bcl-2 siRNA, were intratumorally administered twice a week to the xenograft breast cancer model in athymic mice. The most significant reduction in tumor diameter was detected in the group in which siBcl-2 loaded NKExos (65%) were applied. Immunohistochemical studies have shown that Bcl-2 protein in the breast tumor tissues were decreased upon exosomes administration. When degree of apoptosis in the tumor tissue was studied by TUNEL assay, it was observed that apoptosis was increased significantly in the group given exosomes loaded with Bcl-2 siRNA. Caspase 8, 9 and 10 mRNA expressions were found to be significantly increased in tumors treated in the siBcl-2 containing NKExo. It was determined that administering intravenously NKExos accumulated most in the liver and reached the tumor tissue. In our study, the antitumor effects of NK exosomes were combined with the use of exosomes as a gene carrier system and were successfully used for Bcl-2-based gene therapy in breast cancer. This research has been supported by The Scientific and Technological Research Council of Turkey, TÜBİTAK-1001 (217S455).

P47

Modeling delivery and efficiency of CRISPR systems in heart organoids

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Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. While some current CVD-focused CRISPR approaches, such as *PCSK9* or *TTR* knockout in the liver, are highly efficient, there is a lack of strategies that directly aim at editing heart cells in situ. Partially, this challenge arises from the absence of scalable and translationally relevant in vitro model systems for evaluating CRISPR delivery and editing techniques. In this study, we demonstrate that engineered human iPSC-derived heart organoids, referred to as epicardioids, serve as a viable platform for assessing LNP/mRNA delivery of CRISPR systems and conducting heart cell-specific gene editing within a human multi-cellular context. Epicardioids accurately mimic the morphological and functional characteristics of the ventricular wall, offering a pulsating, intricate 3D in vitro representation of the human heart. We show LNP-based delivery of mRNA specifically into the outer, epicardial layer of cardiac organoids and highlight strategies to rapidly test different LNP formulations, modified mRNAs, and CRISPR modalities in this scalable 3D system in vitro.

P48

Amphiphilic balance: fine-tuning of double pH-responsive mRNA carriers towards higher potency

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Highly potent double pH-responsive mRNA carriers were developed combining the polar cationizable succinoyl tetraethylene pentamine (Stp) artificial amino acid motif with apolar protonatable lipoamino fatty acid (LAF) domains. LAF units contain tertiary amines to induce a switch in polarity and conformational changes at endosomal pH. Different spatial arrangements (U-shape or bundle topologies) require distinct polar/apolar ratios. To further fine-tune this balance, two different strategies for hydrophobic modification of the Stp motif were applied. Either the succinoyl linker was replaced by dicarboxylic acids with bulky hydrophobic moieties, or the number of secondary amines was reduced. Thus, a library of U-shape and bundle structures with polar/apolar domain ratios of 1:2, 2:4 or 1:4 was synthesized and formulated as mRNA polyplexes. With carriers containing hydrophobically modified dicarboxylic acids, stable polyplex formation was achieved. For structures with reduced number of secondary amines, particle formation was more challenging. Strongest tunability was shown for U-shapes with higher ratios of the polar domain (1:2). In N2a, J774A.1 and DC2.4 cells, in comparison to previous top performers an up to ~10-fold further increase in luciferase transfection efficiency could be achieved with a cyclohexan-1,2-dicarboxylic acid linker. Furthermore, an increase in cell viability was detected in J774A.1 cells upon reduction of secondary amines. In contrast, structures with higher proportion of the apolar domain (2:4 and 1:4) were less affected by hydrophobic fine-tuning. Further investigation of octanol/water distribution (logD), lytic potential and performance in different cell lines will elucidate details of underlying mechanisms and reveal additional structure activity relations.

P49

Optimizing miR-146a mimics: Unleashing precision in Graft-versus-Host Disease treatment

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Loss of miR-146a is implicated in severe graft-versus-host-disease (GvHD) – a rate-limiting toxicity of allogeneic hematopoietic stem cell transplantation. We screened a panel of chemically modified cholesterol-conjugated miR-146a mimics in order to identify a clinically relevant chemical scaffold for GvHD therapy.
We tested the impact of the metabolic stability of the antisense strand as well and length and degree of complementarity of the sense strand of miR146a. A fully complementary sense strand impaired the silencing efficacy on an siRNA-like target reporter 147-fold ($p<0.0001$). However, a shorter sense strand left silencing efficacy largely unaffected in the siRNA-like target reporter assay. Silencing efficacy of the miR146a target IRAK1 was impaired 8.6-fold ($p=0.02$) by fully complementary sense strands but not by shorter sense strands. Silencing was completely abolished when combining the above sense strand modifications ($p<0.0001$). Interestingly, in an *in*

vitro model of GvHD, fully complementary sense strands rather improved the efficacy of inhibiting T cell proliferation in the context of both longer and shorter sense strands (1.7-fold and 1.5-fold, respectively, $p<0.0001$). The metabolic stability of the antisense strand did not significantly affect efficacy of miRNA mimics in either assay. We were able to inhibit T cell proliferation upon allogeneic stimulus up to 80% with an IC50=2.8 µM. Furthermore, we showed that chemically synthesized miR-146a mimics do not impair the T-cell-mediated anti-leukemia effect *in vitro*.
Collectively, we demonstrate that fully chemically modified miRNA mimics may be useful to treat GvHD and that the duplex structure of chemically synthesized miRNA mimics predicts miRNA efficacy.

P50

RNAi in macrophages mediated by efficient nanoparticles for siRNA delivery reveal complex effects on polarization markers upon knockdown of STAT3/STAT6

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The most abundant immune cell type in the tumor microenvironment are tumor associated macrophages (TAMs), affecting tumor progression, metastasis and therapeutic response. When exploring TAMs as targets in novel cancer immunotherapy approaches, their repolarization from the M2 (anti-inflammatory/pro-tumorigenic) into the M1 (pro-inflammatory/anti-tumorigenic) phenotype is an intriguing strategy.
Major bottlenecks of RNAi-based knockdown of M2 promoting genes include cellular siRNA delivery and correct intracellular processing in macrophages as a cell type notoriously hard-to-transfect. The cationic polymer polyethylenimine (PEI) is widely explored for delivering nucleic acids. Further advanced nanocarriers are tyrosine-modified polymers based on PEI or polypropylenimine dendrimers (PPI) for highly efficient siRNA delivery *in vitro* and *in vivo*.
Here, we explored a panel of PEI- or PPI-based nanoparticles for siRNA-mediated gene knockdown in macrophages and subsequent TAM repolarization. The tyrosine-modified linear 10 kDa PEI (LP10Y) or branched 5 kDa PEI (P5Y) as well as a tyrosine-modified PPI (PPI-Y) were most efficient for gene knockdown in macrophage cell lines or primary macrophages, independent of their polarization. Knockdown of STAT6 or STAT3 led to repolarization of M2 macrophages, as indicated by alterations in various M2 and M1 marker levels. This highly specific approach also demonstrated non-redundant functions of STAT3 and STAT6. Macrophage re-polarization from M2 to M1 upon PPI-Y/siRNA-mediated STAT6 knockdown increased tumor cell phagocytosis in a co-culture model.
In conclusion, we identify certain tyrosine-modified PEI- or PPI-based nanoparticles as efficient for macrophage transfection, and the specific, siRNA-mediated STAT6 knockdown as promising approach for macrophage repolarization and enhancement of their tumor suppressive role.

P51

Fine-tuning immunity – *ex vivo* siRNA treatment to enhance efficiency of cell-based therapies

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Allogeneic cell therapy stands out as a potent strategy for leukemia treatment. However, allogeneic T cells can trigger the desired Graft-versus-Leukemia (GvL) effect and the unwanted Graft-versus-Host (GvH) Disease. Conventional GvHD drugs are non-selective, simultaneously inhibiting both GvH and GvL effects, resulting in widespread toxicities and complications in patients. This study explores the potential of sequence-specific siRNAs to temporarily modify the phenotype of allogeneic T cells, thereby enhancing the efficacy of cell-based therapies.
We designed siRNAs targeting four genes (AURKA, WAPAL, KIF15, RAN) that were shown to be upregulated during GvHD. Through screening, we identified best performing siRNAs and subjected them to functional GvH and GvL assays to assess their ability to inhibit allogeneic T cell proliferation. Treatment with siRNA targeting RAN demonstrated dose-dependent inhibition of T cell proliferation in response to both allogeneic (activated by major-mismatched dendritic cells, up to 80%, $p=0.01$, $N=5$) and non-specific (activated by CD2/CD3/CD28 beads, 60%, $p<0.0001$, $N=3$) stimuli. Singular siRNAs against WAPAL, AURKA, and KIF15 showed no effect on T cell proliferation. Surprisingly, combinations of three siRNAs targeting different genes resulted in potent and specific T cell inhibition, achieving up to 75% inhibition of T cell proliferation upon allogeneic stimulus ($p= 0.007$, $N= 3$), while not impacting T cell proliferation upon non-specific stimulus. Treatment with siRNAs did not compromise therapeutically essential GvL effect of T cells. Furthermore, our siRNAs induced a shift in T cell phenotype towards regulatory T cells.
Our data provide proof-of-principle for siRNA mixes as a precision immunomodulatory therapy for GvHD.

P52

Control of bocavirus vector production by CRISPRa-VPR

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Parvoviruses have been widely repurposed as safe, versatile, and efficient gene delivery vectors with adeno-associated virus (AAV) among the most important in the gene therapy field. Yet, concerns over transduction efficiency, immune activation and limited cargo size must be addressed. Bocavirus vectors (BoV) have emerged as a promising alternative to AAV due to their expanded cargo capacity (up to 6.3 kb) and stringent cell tropism that is particularly relevant for treatment of lung diseases. The production of recombinant AAV/Bocavirus vectors (rAAV/BoV) depends on the combination of diverse viral genes encoded in numerous plasmids. However, the use of transient plasmid transfections poses significant hurdles for large-scale production such as plasmid preparation, transfection efficiency and the inherently constitutive expression of cytotoxic viral genes. We envision the creation of a cell line, where all genes required for

rAAV/BoV production are activated spatiotemporally. As initial proof-of-concept, we have modified the genome of the human bocavirus 1 (HBoV1) to be compatible with the CRISPR activation system (dCas9:VPR) for the controlled activation of capsid genes. The addition of a protospacer sequence downstream of the truncated HBoV1 p5 promoter allows for the tight control of the viral capsid gene by dCas9:VPR, resulting in 283-fold higher mRNA levels in HEK-293T cells. Furthermore, we adapted this novel system for large-scale rAAV/BoV production and measured a 1500-fold activation over the uninduced state, with vectors retaining their transduction potency. These findings pave the way for further reengineering of the rAAV/BoV genome to create a synthetic rAAV/BoV producer cell line.

P53

rAAV displaying an anti-EGFR affibody transduces neuroendocrine tumor cell lines and organoids and mediates efficient enzyme prodrug tumor cell killing

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Malignant tumors remain a leading cause of death and novel, specific, and effective therapies are urgently needed for several tumor types. Recombinant adeno-associated viruses (rAAV) provide well-characterized biological and structural features enabling tumor targeting at the capsid and at the genetic level. The epidermal growth factor receptor (EGFR) is a validated tumor marker addressed by several therapeutic antibodies. We explored the potential of rAAV for tumor therapy by rational insertion of a small antibody mimetic in the capsid and endowing them with a trans-gene encoding a prodrug-activating enzyme. The EGFR binding Affibody $Z_{EGFR:1907}$ was genetically incorporated within the variable region IV (453-loop) of rAAV-VP2 of the serotypes AAV2 or AAV9. The respective mosaic rAAVs were produced successfully. We demonstrated EGFR-dependent binding and transduction in model cell lines (MCF7, HeLa, A431). Ultimately, patient derived neuroendocrine tumor cells were transduced and killed mediated by thymidine kinase expression and ganciclovir activation.

P54

RNA-switches facilitate drug-inducible expression of therapeutic proteins by oncolytic viruses

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To promote the clinical translation of “armed” oncolytic viruses (OVs), there is a pressing need for new methods enabling the external control of therapeutic transgene expression to optimize manufacturing, safety and therapeutic outcomes. RNA-switches, consisting of a ligand binding RNA-domain (aptamer) connected to a regulatory RNA-sequence (expression platform), represent promising tools for gene regulation in size-limited DNA- and RNA-based vectors, without relying on the expression of potentially antigenic regulatory proteins. So far, mostly ON-switches with rather moderate induction folds were described and medically relevant inducible systems are still rare. In this work, distinct Tetracycline (Tet)-responsive RNA-ON-switches were assessed for their gene regulatory potential. Transient transfections of RNA-switch systems relying on various expression platforms identified lead RNA-switches for constructing oncolytic adenoviruses (oAds) that express a firefly luciferase in a Tet-dependent manner. Engineered oAds comprising a ribozyme- or a splicing-based RNA-switch showed significant induction upon Tet-addition with up to a 24-fold increase when combined. Importantly, these viruses retained oncolytic activity. Furthermore, the functionality of the designed regulated oAds was validated across various cell lines and distinct temporal schedules. Finally, therapeutic payloads (IL-2, IL-12 and IFN-β) were placed under the control of the most effective RNA-switch system, allowing us to show Tet-inducible cytokine expression from a plasmid (40-fold). In ongoing work, we explore regulated cytokine delivery by oAds and its therapeutic potential to predominantly activate anti-tumor immunity and as safety switch in case of emerging toxicities. The data highlight the transgene-independent functionality of the system, emphasising its broad applicability in gene- and virotherapy.

P55

Measurement of endothelial force in vivo

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Introduction: Vascular tone plays a crucial role in the regulation of endothelial biology as well as vascular flow and tissue perfusion. In vivo force measurements have thus far been difficult to perform although these in vivo measurements would be far more meaningful than cell culture studies. FRET-based tension sensors allow for the assessment of force across specific proteins. In our system, the fluorescent proteins mTFP1 and Venus are separated by a flageliform linker, which stretches under force, reducing FRET-efficacy. Here we utilize a VE-Cadherin and Vinculin tension sensors. To overcome the size-restrictions of the intended rAAV-vector, we used a dual vector system and an intein-split mechanism.
Methods: VE-Cadherin (CDH5-TS) and Vinculin (Vin-TS) tension sensors were split into an N-terminal and C-terminal part flanked by intein peptide sequences. This lead to the generation of pairs of tension sensor modules (CDH5-TS^{N} and CDH5-TS^{C} / Vin-TS^{N} and Vin-TS^{C}) able to realign inside the cell. rAAV-vectors of all constructs were generated, coated with dendrimer nanoparticles (PAMAM) and endothelial cell specific targeting peptides. These viruses were used in either cell culture or injected into mice via the tail-vein.
Results: In cultured cells we were able to demonstrate the successful recombination of both parts of the tension sensor module, their proper subcellular localization and function. In in vivo studies, we showed similarly the intein-split recombination as well as their function as tension sensors. We could also measure a decrease in endothelial force transduction after stimulation with Ang-2.