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RNA-therapeutics of gene haploinsufficiencies

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Artificial transactivation of endogenous genes ad libitum is a desirable goal for a number of basic and applied research purposes. We recently developed a fully synthetic ribonucleoproteic transactivator prototype. Kept together via an MS2 coat protein/RNA interface, it includes a fixed, polypeptidic transactivating domain and a variable RNA domain that recognizes the desired gene. Thanks to this device, we specifically upregulated five genes, in cell lines and primary cultures of murine pallial precursors. Even if gene upregulation is small, however it is sufficient to inhibit neuronal differentiation. Our transactivator activity was restricted to cells in which the target gene is normally transcribed. These features make our prototype a promising tool for clean rescue of gene haploinsufficiencies, since it could lead to a specific overstimulation of the spared gene allele in its physiological expression domain. On the other hand, we are interested in stimulating transcription of endogenous genes by small activating RNAs (saRNAs). These are miRNA/siRNA-like molecules, supposed to destabilize transcription-inhibiting ncRNAs or facilitate the recruitment of transcriptional complexes to chromatin. The transcription gain they elicit is small; however it may be sufficient to influence the behaviour of cells in a robust way. Moreover, silent genes generally do not respond to them. As such, also saRNAs are a promising tool for therapeutic stimulation of gene transcription. We selected a number of saRNAs able to stimulate haploinsufficient genes involved in CNS morphogenesis and physiology. We are studying their mechanism of action and exploiting saRNAs for in vivo correction of gene haploinsufficiency.

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CRISPR/Cas9-mediated gene correction of COL7A1 in dystrophic epidermolysis bullosa

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Mutations within the COL7A1 gene lead to the dystrophic form of epidermolysis bullosa (DEB) characterized by disturbed expression of type VII collagen and blister formation below the lamina densa. We exploited the recently discovered CRISPR/Cas9 system to correct a homozygous mutation within exon 80 of the COL7A1 gene by homology-directed repair (HDR). We have predicted protospacer adjacent motifs (PAMs) within intron 80 of the COL7A1 gene, enabling the specific cleavage of the DNA via the CRISPR/Cas9 system at the desired location. The guide RNA (gRNA) for the predicted PAM sequence was cloned either into a wild-type Cas9 dual vector system, aiming to induce double strand DNA breaks, or a D10A Cas9 dual vector system, aiming to cause single strand DNA breaks within intron 80. Homology COL7A1 arms for HDR were cloned into a donor vector, including a ruby/puro selection cassette. Transfected patient keratinocytes were selected either via antibiotic selection or fluorescent-activated cell sorting (FACS). PCR and sequence analysis of genomic DNA, isolated from puromycin selected cell populations and single cell clones, showed the genetic correction of the COL7A1 mutation. Restriction enzyme digestion of PCR products, employing a newly introduced restriction site, revealed the presence of 26% reverted alleles. Additionally, type VII collagen restoration was confirmed via Western blot analysis and immunofluorescence staining of puromycin selected cell populations and single cell clones. Our data suggest that genome editing using the CRISPR/Cas9 system can be used to repair genes involved in the severe skin disease epidermolysis bullosa.

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Developing an optimised TALEN-mediated *ex vivo* gene therapy for epidermolytic ichthyosis

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Epidermolytic ichthyosis (EI) is an inherited skin fragility disorder caused by dominant-negative mutations in either the keratin 1 (KRT1) or keratin 10 (KRT10) genes. As EI is difficult to treat and currently lacks a cure, there is an acute need for novel therapies. Keratin pairs specifically polymerise to build the intermediate filament cytoskeleton of epithelial cells. Dominant-negative mutant keratins integrate into this, resulting in fragility and collapse upon mild stress. This leads to cytolysis and blistering of the skin. Elimination of these mutant keratins is essential for curation of the disease. Our group previously demonstrated that zinc finger nucleases (ZFNs) can inactivate an EGFP transgene in murine keratinocyte stem cells efficiently without impairment of stem cell properties. Transcription activator-like effector nucleases (TALENs) are a new generation of highly sequence specific designer nucleases. They require few design parameters, while off-target effects are rare. We are developing an optimised *ex vivo* gene therapy for EI, using TALENs to knockout mutant KRT10 alleles in keratinocyte stem cells (KSCs). Optimised TALENs targeting KRT10 have been constructed. These cleave efficiently at the target site and induce frame-shift mutations known to knockout KRT10. TALEN-treated clones were seeded and screened, with 54% displaying successful modification without selection. A number of those identified were expanded and are being used in further biochemical studies to confirm phenotypic rescue. We aim to take a skin biopsy from an EI patient, isolate, grow and treat KSCs with TALENs to phenotypically cells prior to grafting these onto the patient's skin.

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Characterization of model for study of skeletal muscles regeneration in dysferlin-deficient mice

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Dysferlin is a membrane protein encoded by gene DYSF, it promotes membrane repair in striated muscle fibers (MF). Mutations in DYSF lead to loss of dysferlin, impaired membrane repair in muscle fibres and its destruction clinically manifesting as limb-girdle muscle dystrophy 2B. Preclinical studies of cell and gene therapy aiming to restore impaired muscle regeneration require well characterized small animal model. Gastrocnemius muscle of 5-months old dysferlin-deficient A/J and C57Bl/6 mice was injected by 100 μl of 5mg/ml bupivacaine (myotoxic agent). Paraffin sections of injected and contralateral calf muscles obtained 2, 4, 10 days after injection were stained with H&E, immunohistochemically with antibodies against macrophage antigen, myogenin, Ki-67. Necrotic MF with macrophage infiltration were found up to 10 days after injection in A/J mice, in C57Bl/6 mice on 2nd day after injection only, percentage of necrotic MF in A/J mice was significantly higher ($17,3 \pm 12,1\%$ vs $9,5 \pm 3,3\%$ in C57Bl/6 mice), reflecting more severe and continuous muscle damage in dysferlin deficiency. In C57Bl/6 mice injected muscle restores normal structure by 10th day after injection. Percentage of centrinucleated MF and proliferating myosatellites were significantly higher in all time points in A/J mice with maximum on 10th day after injection ($34,7 \pm 14,1\%$ vs $5,5 \pm 3.0\%$ and $18,6 \pm 12,9\%$ vs $3,3 \pm 1,6\%$ in C57Bl/6 mice, respectively) showing activated but incomplete MF regeneration associated with necrosis of newly formed MF. Modulation of regenerative response in dysferlin-deficient skeletal muscle is potential point of application for gene and cell therapy. This work was funded by a Russian Science Foundation grant (14-15-00916).

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Hit and go Cas9 delivered through a lentiviral based self-limiting circuit

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In vivo application of the CRISPR/Cas9 technology is still limited by unwanted Cas9 genomic cleavages. Long term expression of Cas9 increases the number of genomic loci non-specifically cleaved by the nuclease. We developed a Self-Limiting Cas9 circuitry for Enhanced Safety and specificity (SLiCES) which consists of an expression unit for the Streptococcus pyogenes Cas9 (SpCas9), a self-targeting sgRNA and a second sgRNA targeting a chosen genomic locus. The self limiting circuit by controlling Cas9 levels results in increased genome editing specificity. For its *in vivo* utilization, we next integrated SLiCES into a lentiviral delivery system (lentiSLiCES) via circuit inhibition to achieve viral particle production. Following its delivery into target cells, the lentiSLiCES circuit is switched on to edit the intended genomic locus while simultaneously stepping up its own neutralization through SpCas9 inactivation. By preserving target cells from residual nuclease activity, our hit and go system increases safety margins for genome editing.

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Expansion of human T regulatory cells by lentiviral vector mediated expression of STAT5B or BACH2 transcription factors

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It has been shown that HIV-1 insertions targeting transcription factors like BACH2 and MKL2 are enriched and persist for decades in patients under Anti-Retroviral-Therapy (ART), indicating that insertional mutagenesis provided a selective advantage to these cell clones. We identified that chimeric mRNA transcripts containing viral HIV-1 sequences fused by splicing to the first protein-coding exon of STAT5B or BACH2 are present in the hematopoietic cells of 30/87 patients under ART. These chimeric mRNAs, putatively encoding for the wild type forms of BACH2 or STAT5B, were found to be specifically enriched ($p < 0.001$) in T-regulatory (Treg) cells in all patients tested (N = 9), as the result of a viral driven selection mechanism known as promoter insertional mutagensis. LV-mediated overexpression of STAT5B and BACH2 in Treg cells purified from healthy donors significantly increased their proliferation rate ($p < 0.001$) without impacting on their immune-suppressive function as observed from specific *in vitro* and *in vivo* assay. These data suggest that HIV-mediated deregulation of STAT5B and BACH2 favor the maintenance and expansion of Treg cells in infected patients promoting long-term viral persistence. Beside its implication in HIV biology, the proliferative effect conferred by the STAT5B and/or BACH2 overexpression in Treg cells could represent a novel suitable approach for adoptive immunotherapy clinical application. Indeed, high number of cells are required for such clinical purposes, and this approach should led to higher yield of Treg cells over other protocols of expansion and should promote the persistence of the transduced Treg cells *in vivo*.

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CD4+ memory stem T cells: novel players in rheumatoid arthritis

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Memory Stem T cells (TSCM) are long living self-renewing memory T cells with long-term persistence capacity, which play a relevant role in immunological memory and protection against infectious diseases and cancer. The aim of this work is to investigate the potential role of TSCM as a reservoir of arthritogenic T cells in Rheumatoid arthritis (RA). We analysed the dynamics of TSCM (here identified as CD45RA+ CD62L+ CD95+ T cells) and other memory T-cell subpopulations by multiparametric 11-color flow cytometry in 27 patients