ID p1

p75 Neurotrophin Receptor Modulates Adult Neurogenesis Representing a novel therapeutic target in Alzheimer's Disease

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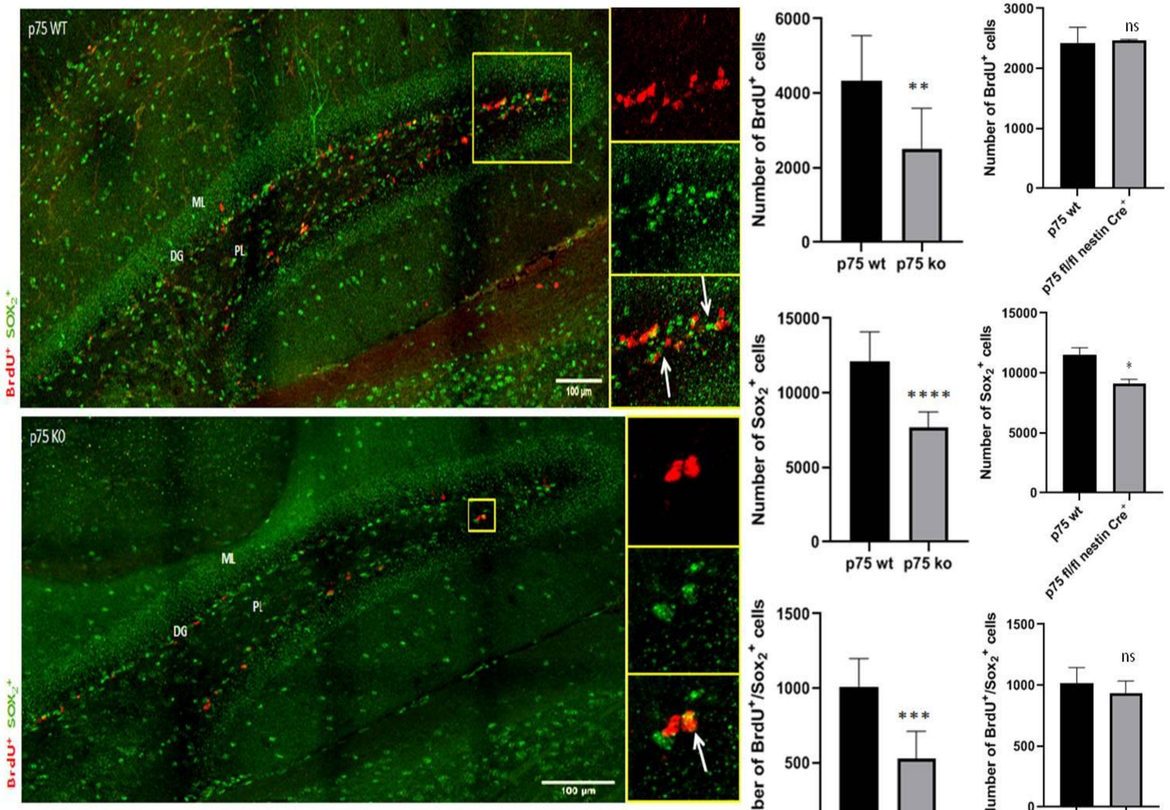
Introduction

The pan-neurotrophin p75 receptor (p75NTR) is a member of the TNF death receptor superfamily with pleiotropic expression in neural tissue. The altered expression profile combined with its controversial signaling in different cellular settings as pro-apoptotic and/or pro-survival mediator, makes p75NTR an appealing target in neurotherapeutics. p75NTR has been implicated in Alzheimer's Disease (AD) by demonstrating its ability to serve as a mediator of Amyloid beta induced degeneration. In parallel, its contribution to adult hippocampal neurogenesis, which drops sharply in AD, remains poorly understood. Here, we investigate the role of p75NTR in adult neurogenesis by addressing its function in rodent adult hippocampal and human induced pluripotent stem cells (hiPSCs) -derived NSCs under physiological and AD-related conditions.

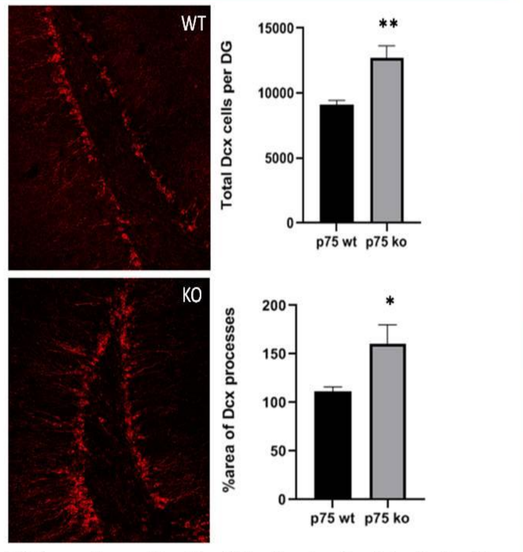
Proliferation assessment in dentate gyrus progenitor cells in p75NTR knock out mice and floxed/floxed NestinCre mice



Hippocampal Dentate Gyrus (DG) from 2-month-old p75wt *vs* p75ko and fl/fl NestinCre mice injected with BrdU for 5 and 7 days. Sections were immunostained for BrdU (red) and co-immunostained with Sox2 (green).

*(mean±s.d.; N=8 WT, N=8 KO, ** p 0,0067, *** p 0,0001, **** p < 0,0001; N=3 WT, N=3 fl/fl cre+, * p <0,05, ns = no significant)*

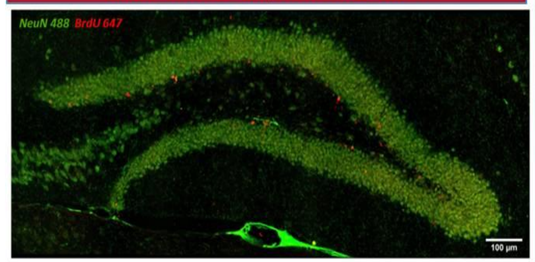
Increased production of new immature neurons in p75NTR ko mice



DG from 2-month-old p75ko & wt mice injected with BrdU for 7 days. Images depict DcX immunostained immature neurons.

*(mean±s.d.; n=5 ko vs. n=5 wt; *p<0.05; **p<0,005)*

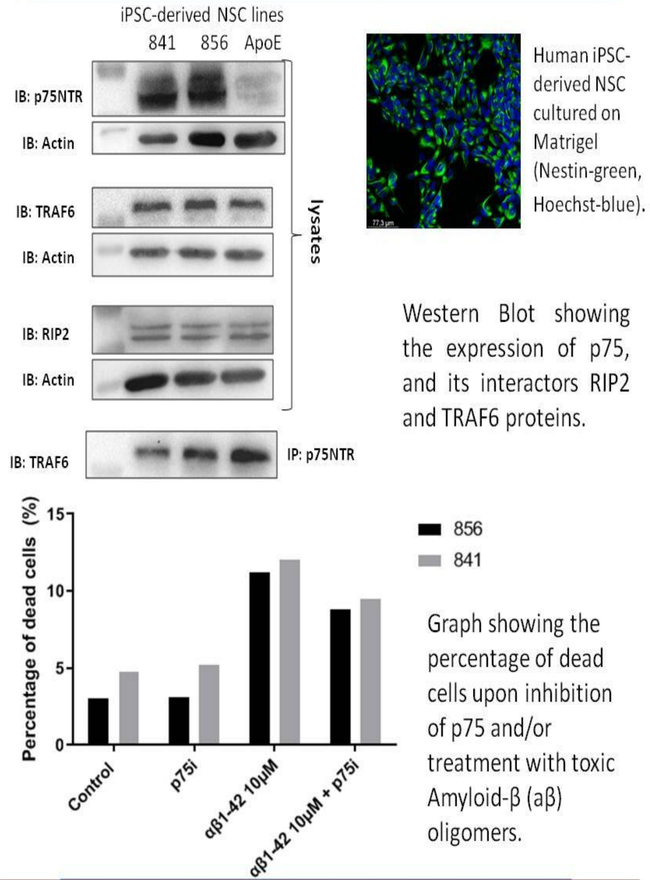
Reduction of NeuN expression and survival rates of NSCs in p75NTR ko mice



*(mean±s.d.; n=3 ko vs. n=2 wt; *p<0.05)*

DG from 2-month-old p75wt mouse. Sections were immunostained for BrdU (red) and co-immunostained with NeuN (green).

p75NTR signaling in hiPSCs-derived NSCs and its role in NSC survival



Human iPSC-derived NSC cultured on Matrigel (Nestin-green, Hoechst-blue).

Western Blot showing the expression of p75, and its interactors RIP2 and TRAF6 proteins.

Graph showing the percentage of dead cells upon inhibition of p75 and/or treatment with toxic Amyloid-β (aβ) oligomers.

Conclusions

- ↓BrdU⁺ cells in p75(-/-) mice – p75NTR-dependent effects on adult neurogenesis.
- No. of BrdU⁺ cells (p75 floxed) remains unchanged - cell-autonomous signaling pathways.
- ↑Dcx⁺ cells in ko mice - these immature neurons cannot proceed and differentiate into neurons.
- BrdU⁺/NeuN⁺ cells ↓ compared to wt mice - p75NTR is promoting survival and differentiation of cells.
- Expression of p75NTR, RIP2 and TRAF6 interacting proteins in hiPSCs.
- p75NTR negatively influences hNSC survival after treatment with aβ peptides - regulatory role in NSC pathology in human AD.

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ID p2

BLOCKING NANOBODIES AGAINST LCK AS POTENTIAL "SAFETY SWITCHES" IN T-CELL IMMUNOTHERAPIES

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Introduction

The selective and controllable inhibition of T-cell function comprises an invaluable tool in the field of T-cell immunotherapies (e.g.,CAR-T-cells) because CAR-T-cells besides their beneficial effects are often accompanied by severe side effects[1]. Based on that, the pharmaceutical industry is intensely seeking strategies to suppress T-cell activation and subsequently to manipulate CAR-T cell function[2]. Lck, a member of the Src family of protein tyrosine kinases (SFKs), is absolutely mandatory for T-cell and CAR-T-cell activation and function[3] and has become a very attractive target for the production of small molecule inhibitors. However, the development of highly selective and potent Lck inhibitors, suitable for clinical use, has not met with success, due to an astonishing structural homology shared by SFK members within their catalytic centers[4]. The current project aims to achieve selective downregulation of T-cell function and subsequently of CAR-T-cells via specific inhibition of the T-cell restricted kinase Lck, using intracellularly expressed nanobodies (Nbs).

Methods

Twenty four different Nbs, targeting a poorly conserved region of Lck were modified by the addition of an Endoplasmatic Reticulum (ER) retention signal, aiming to reduce the expression levels of newly synthesized Lck. An initial screen of different Nbs was accomplished by transfecting HEK293T cells with Lck in the presence or absence of Nbs. Lck expression levels were quantitated by Western Blot, whereas the ability of Nbs to recognize and bind Lck was tested with co-Immunoprecipitation (Co-IP) experiments.
Nbs were fused with an HA tag, in order to be detectable

Results

Nbs can be categorized into 3 groups based on their efficacy to decrease Lck levels (Figure1). In group A belong the most efficient Nbs with 40-60% Lck reduction whereas in group B belong less effective Nbs with 20-30% Lck reduction, compared to Lck levels in the absence of Nbs. In Group C belong Nbs with minimal impact on Lck levels.
The 4 most effective candidates Nbs were selected and tested for their ability to bind Lck (Figure 2). The Nb (NbS), which was able to bind Lck, was then examined for its specificity to recognize only Lck and not other SFK members (e.g. Lyn) (Figure 3).

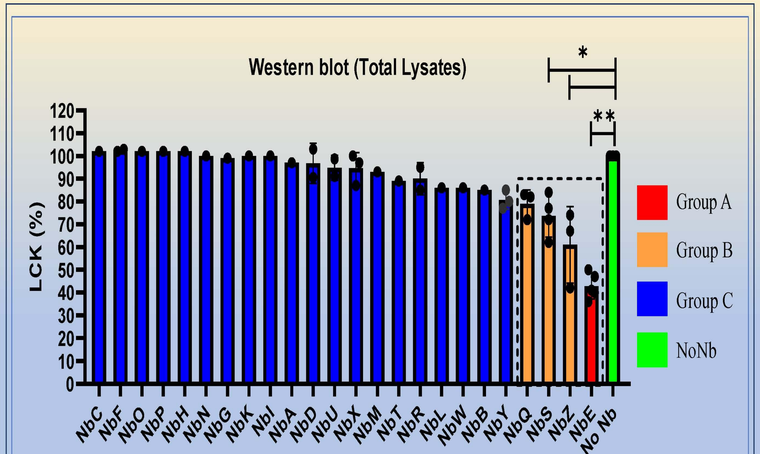

Figure 1. **Lck protein levels in the presence of different Nbs.** HEK293T cells were transfected with Lck with or without Nbs. The levels of Lck were quantitated by Western Blot and were normalized based on actin. The green bar represents control Lck expression in the absence of Nb. The dotted border includes Nbs selected for further evaluation. P-value <0,05

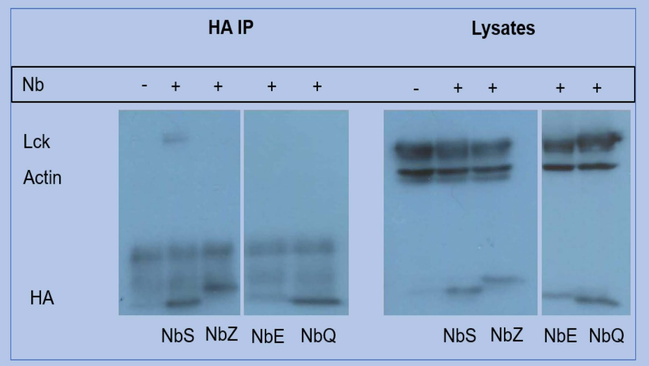

Figure 2. **Ability of the selected four Nbs to bind and decrease Lck.** HEK293T cells were transfected with Lck in the presence or absence of the 4 different Nbs. (Left). HA (Nb) was immunoprecipitated (IP) and then Lck was detected by Western blot, using anti-Lck antibody. (Right) Levels of Lck in the absence or presence of Nbs.

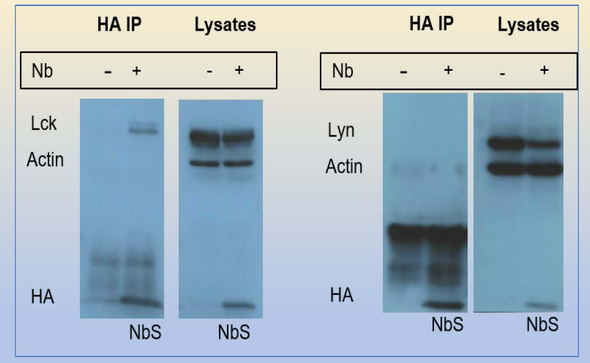

Figure 3. **Specificity assay of NbS to bind different SFK members.** HEK293T cells were transfected with Lck (left image) or Lyn (right image) in the presence or absence of NbS. HA (Nb) was immunoprecipitated and the presence of Lck or Lyn was detected by Western blot.

Discussion

We have identified a Nb (NbS) which could be an efficient and specific Lck-inhibitor. The next stage of our experiments includes the inducible expression of the top candidate Nb into model T-cell lines and evaluates its ability to downregulate their function. An inducibly expressed Lck-blocking Nb engineered into CAR-T could present a sophisticated "safety-switch" able to turn off T-cell activation thus restricting toxic side effects before they become life-threatening.

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ID p3

HBB$^{IVSI-110(G>A)}$-specific gene editing as advanced therapy for β-thalassemia

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Cyprus Institute of Neurology and Genetics, Molecular Genetics Thalassemia Department

Introduction

β-thalassemia is a disease affecting millions of patients worldwide, with an exceptionally high carrier rate in Cyprus in particular. The disease is brought about by defective β-globin (HBB) formation, and patients suffer from a wide array of symptoms, in the most severe cases with dependence on regular blood transfusions and iron chelating agents for survival. Current cures are limited to allogeneic hematopoietic stem cell (HSC) transplantation from compatible donors and to transplantation of autologous HSCs after gene addition, which are both marred by inaccessibility for most patients and, respectively, by high risks and prohibitive cost. Sequence-specific genome editors, utilized for the correction of genetic defects, have revolutionized the therapy of monogenic diseases. Of particular interest for clinical translation are base editors (BEs), which catalyse base transitions at a targeted base without reliance on potentially mutagenic double-strand breaks (DSBs), typical of the first generation of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and transcription activator-like effector nuclease (TALENs) editors. Cytosine BEs enable C>T transitions, whereas adenine BEs enable A>G transitions.

Methodology

Plasmids for ABEs and CBEs were obtained from Addgene (#139998/9, 140002/3) [3] and the T7 promoter added to CBE plasmids, before *in vitro* mRNA transcription and nucleofection of BEs as mRNA/gRNA into primary hematopoietic cells. To produce clonal cell models, HUDEP-2 cells [4] were nucleofected with Cas9/gRNA/donor mixtures and plate-sorted on a BD FACS Aria III. Efficiency of editing was assessed at the DNA level and, after erythroid differentiation, at the protein level. EditR software was used to assess base editing efficiency [5].

Objectives

I am evaluating four novel base editors with relaxed protospacer adjacent motif (PAM) requirements for their ability to functionally correct the common Cypriot *HBB*$^{IVSI-110(G>A)}$ splice mutation. The strategies considered aim to prevent aberrant splicing by (i) precise correction of the mutated A of the aberrant AG splice motif, (ii) mutation of the G of the same motif, and (iii) alteration of upstream sequence elements critical for aberrant splicing. Base editor designs are being evaluated using *in vitro* mRNA transcription for co-nucleofection with guide RNAs, which offers independence from commercially available Cas protein preparations and is far superior to plasmid-based transfection. Clones for *HBB*$^{IVSI-110(G>A)}$ based on the HUDEP-2 cell line [3] are being isolated to facilitate functional evaluation and clonal analyses of different editors. Future steps include functional evaluation in primary thalassemic cells and subsequent validation of shortlisted editors in long-term repopulating human HSCs by transplantation into immunodeficient mice to create chimeric mouse models.

Conclusions

This work has established efficiency of two novel nearly PAM-less ABEs and has demonstrated the impact of their editing power on improving the HBB/HBA ratio. Moreover, functionality of the two novel nearly PAM-less CBEs with the added T7-promoter was shown, which will allow for in vitro transcription and subsequent nucleofection. Finally, HDR-disruption and subsequent cell cloning of HUDEP-2 cells produced 16 clones with varying genotypes, two of which have the IVS110 mutation of interest and will facilitate future correctional work to be done on these cells.

Results

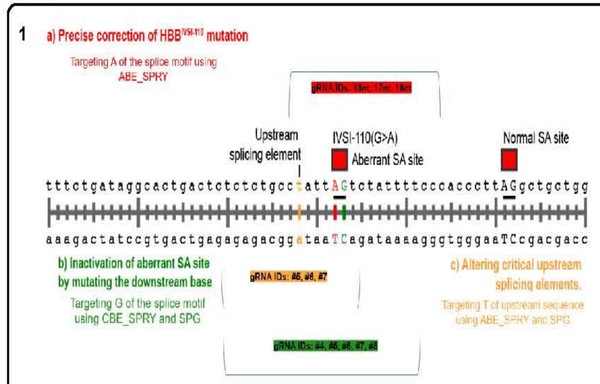

Figure 1. Guide RNA designs for the correction of HBB$^{IVSI-110(G>A)}$ β-thalassemia via base editing

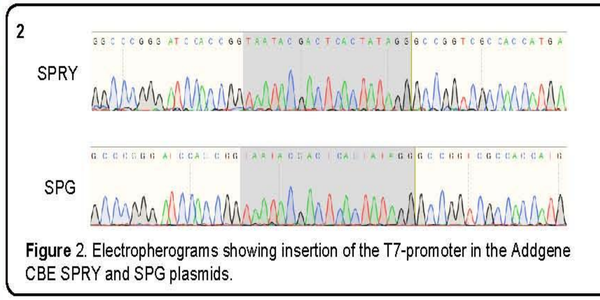

Figure 2. Electropherograms showing insertion of the T7-promoter in the Addgene CBE SPRY and SPG plasmids.

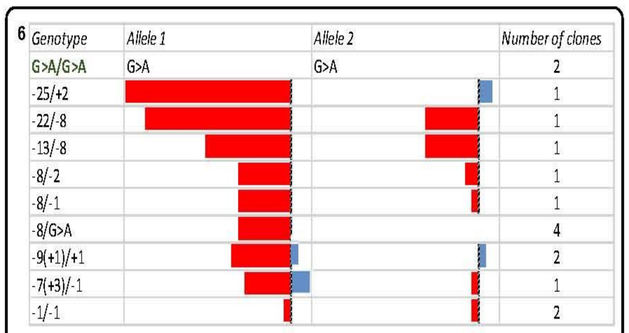

Figure 6. showing genotypes of the 16 HUDEP-2 clones resulting from disruption by homology directed repair (HDR) and following cell cloning and DNA-level analysis.

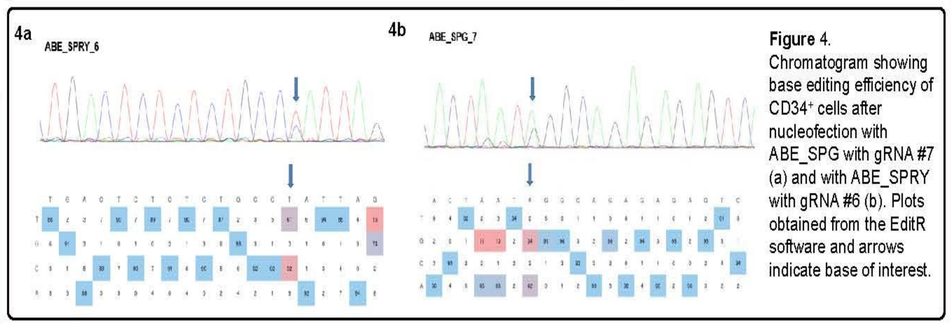

Figure 4. Chromatogram showing base editing efficiency of CD34^{+} cells after nucleofection with ABE_SPG with gRNA #7 (a) and with ABE_SPRY with gRNA #6 (b). Plots obtained from the EditR software and arrows indicate base of interest.

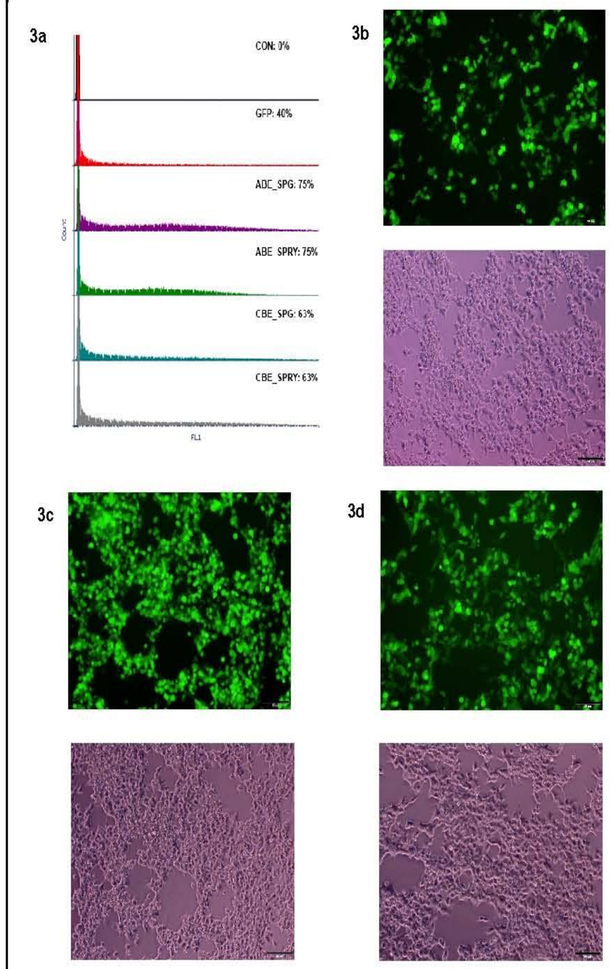

Figure 3a. Flow cytometry analysis of HEK293T cells transfected with GFP-containing, unaltered Addgene ABE SPG and SPRY plasmids and T7-cloned CBE SPG and SPRY plasmids with pmax-GFP + PUC as a positive control. 3b. Fluorescence microscope images of GFP-transfected cells (positive control; BF (top), F (bottom). 3c. Fluorescence microscope images of cells transfected with ABE_SPG plasmid (BF (top), F (bottom). 3c. Fluorescence microscope images of cells transfected with CBE_SPG_T7 plasmid (BF (top), F (bottom).

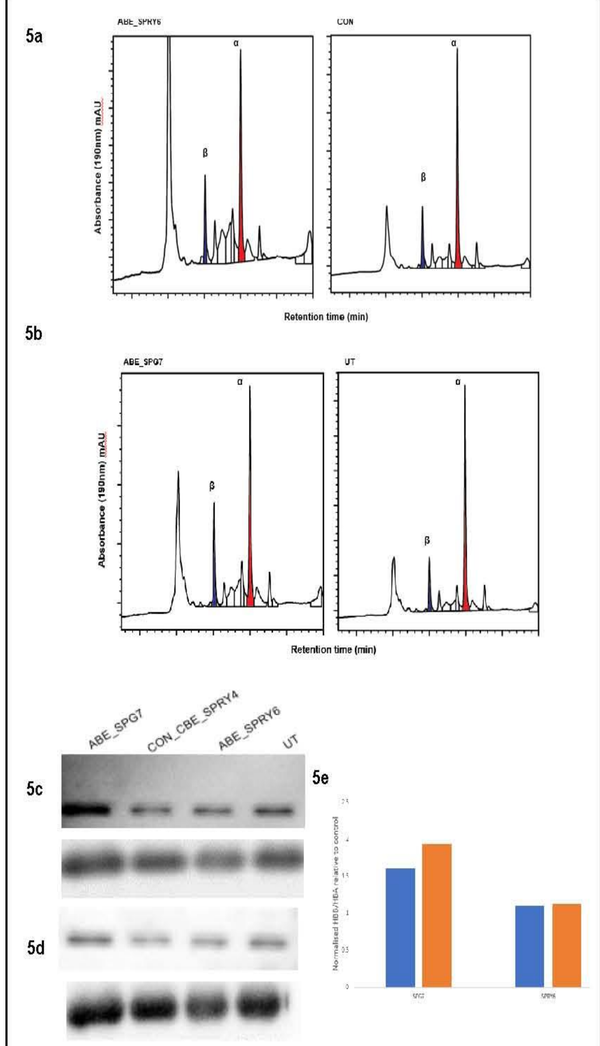

Figure 5. HPLC analysis of edited samples following differentiation: (a) ABE_SPRY_6 and (b) ABE_SPG_7. Immunoblot of edited samples following differentiation: (c) staining for HBA visualized at 780nm (bottom) with actin as a loading control (top, visualized at 680nm). (d) staining for HBB (bottom) and actin (top), both visualized at 680nm. The graph shows the change in the HBB/HBA ratio after normalization for both HPLC (blue) and immunoblot (orange) analyses.

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ID p4

Genome Editing for Beta-Haemoglobinopathies Without Double-Strand DNA Cleavage

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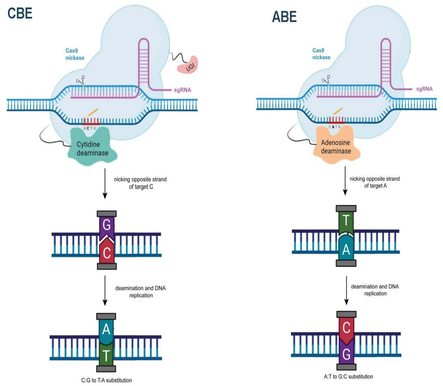
Introduction

Haemoglobinopathies, such as sickle-cell disease and β-thalassaemia, are the commonest monogenic diseases. Of these, β-thalassaemia has high prevalence in Cyprus and is marked by low adult haemoglobin ($\alpha_2\beta_2$), owing to defective β-globin (HBB) expression. Reactivation of foetal globin ($\alpha_2\gamma_2$/HbF) can ameliorate the severity of the disorder and may be achieved by erythroid reduction of γ-globin repressors, such as the transcription factor BCL11A.[1] This can be achieved by catalysing double-strand-break (DSB)-independent permanent transition of target nucleotides with base editors (BEs), which are safer and more efficient for precision edits than traditional DSB-dependent CRISPR/Cas technology. BEs typically catalyse transition edits, the cytosine base editors (CBEs) by C>T conversion,[2] and the adenine base editors (ABEs) by A>G conversion.[3]



Objectives

The objectives of our study are: (i) to adopt the newest generation of genome editors for application to targets of relevance of β-haemoglobinopathies, (ii) to investigate and edit disease modifiers such as the BCL11A erythroid enhancer, (iii) to compare different commercially available BEs against one another and against DSB-based technology and (iv) to evaluate multiplexed editing targets towards higher γ-globin levels and increased clinical potential of base editing.

Materials & Methods

The current project performed *in silico* design of target- and platform-specific guide RNAs to apply BE technology by nucleofection in erythroid cells. An *in vitro* mRNA synthesis for mRNA/gRNA-based delivery of BEs was established. The initial efficiency assessment at the DNA level and functional studies after erythroid differentiation at the protein level were conducted.

Results

1. Comparison of different BEs targeting the well-known BCL11A (γ-globin repressor)

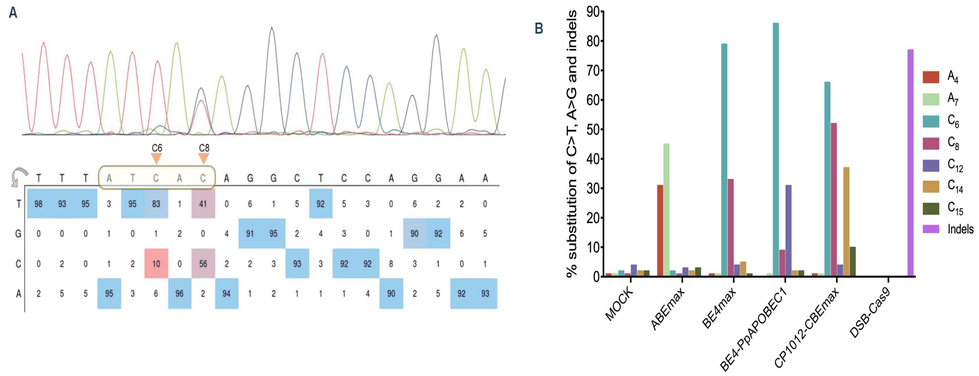


Figure 1. Efficiency assessment at DNA level. (A) The top plot is the chromatogram of the edited gRNA protospacer. The bottom plot illustrates the percent area of the signal for each base (ACGT) at the corresponding position through the gRNA. The highlighted shape shows the editing window and orange arrows show the edited C bases (C6,C8) within that window. Plots were obtained from EditR software. The C base at position 6 of the gRNA (C6) was 83% C>T edited, while C at position 8 (C8) was 41% C>T edited. (B) Editing efficiency of four base editors and Cas9 targeting the BCL11A. The graph illustrates the % substitution of C>T and A>G bases within the gRNA. The % of DSB-based indels is also presented. Each colour bar represents the corresponding base position within the gRNA.

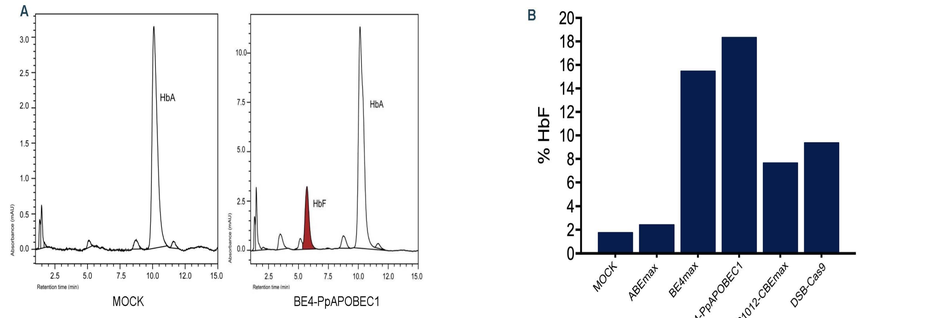


Figure 2. Functional analysis at protein level, based on HPLC. HPLC analysis of edited erythroid cells at day 9 of erythroid differentiation. (A) Chromatogram for last day of differentiated cells, coloured peaks indicate HbF induction. (B) Quantification of HPLC analysis. A chart showing the %HbF increase compare to mock. High HbF levels especially with BE4max (15.5%) and BE4-PpAPOBEC1 (18%) BEs.

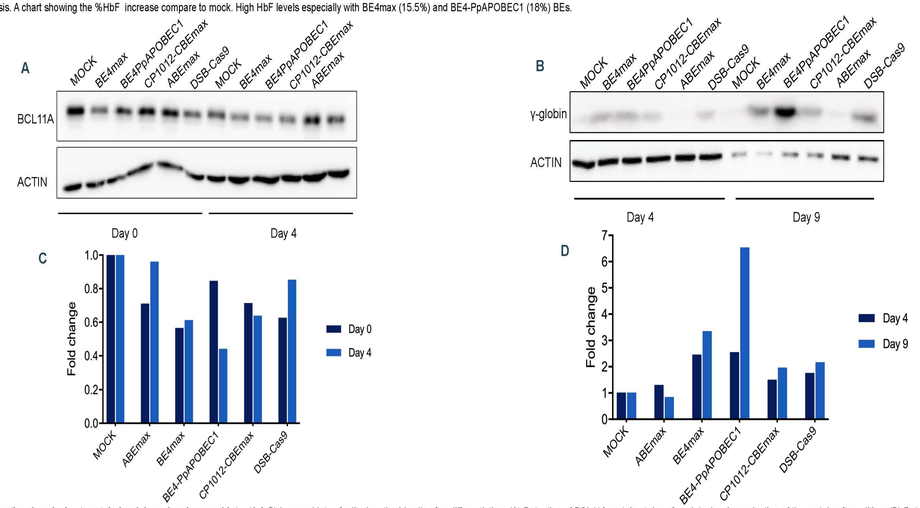


Figure 3. Functional analysis at protein level, based on immunoblots. (A & B) Immunoblots of edited erythroid cells after differentiation. (A) Detection of BCL11A protein at days 0 and 4, showing reduction of the protein after editing. (B) Detection of γ-globin at days 4 and 10 of differentiation, showing a discernible increase of γ-globin after editing, especially at day 10 of differentiation using the BE4-PpAPOBEC1 BE. (C & D) Quantification of the immunoblots shown in A and B, showing reduction of the levels of BCL11A protein after editing, and a comparably high increase of γ-globin, especially with the BE4-PpAPOBEC1 BE.

2. Multiplex base editing targeting *trans*-acting factors and corresponding *cis*-regulatory elements

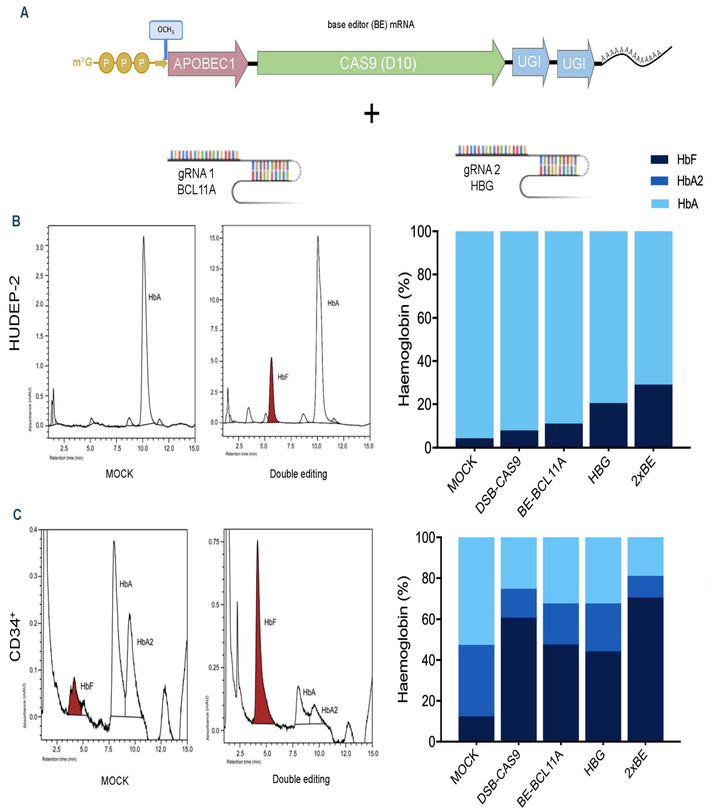


Figure 4. Multiplex base editing results. (A) Schematic diagram of the BE mRNA combined with two different gRNAs for two different targets, BCL11A and HBG. (B & C) HPLC quantification for HbF induction of double base editing in HUDEP-2 (B) and CD34+ (C) cells at days 8 and 6 of differentiation, respectively; (left) Exemplary chromatograms (red: HbF). (right) Quantification of HPLC analysis shown as % contribution of haemoglobins in stacked columns. At ≤29% HbF increase for HUDEP-2 cells (B) and ≤70% HbF increase in CD34+ cells, double BE for both systems has the highest HbF levels compared to single BE and DSB-based edits.

Conclusions

The present study demonstrates high efficiency and low toxicity of RNA-based delivery for base editing technology compared to the clinically applied RNP standard, superior editing outcomes based on BEs compared to DSB-based editing for a clinically relevant target, and superior, therapeutically relevant γ-globin induction by duplex compared to simplex BE and DSB-based applications.

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Acknowledgments

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Towards mutation-specific correction of *HBB*$^{IVSI-110}$-thalassemia using *HBB*$^{\beta AS3}$ GLOBE-based miR30-shRNA expression vectors for shRNA mono- and combination therapy

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BACKGROUND

Lentiviral-mediated transfer of the β-globin gene in hematopoietic stem cells (HSCs) represents a promising therapeutic approach for transfusion-dependent β-thalassemia patients for whom a compatible HSC donor is not available. However, results from ongoing clinical trials indicate successful outcome to be genotype-depended. For one of the most common β-thalassemia mutations in the Mediterranean population, the IVSI-110G>A (HBB:c.93-21G>A, rs35004220), an alternative splice acceptor site created 19 bp 5′ to the normal acceptor of intron 1 (IVSI) results in the generation of aberrant β-globin mRNA (*HBB*$^{IVSI-110}$) with an integrated 19-bp intronic segment and an in-phase premature stop codon, which is preferentially used by the splicing machinery.

Earlier work by our team demonstrated shRNA-mediated silencing of the aberrantly spliced HBB$^{IVSI-110}$ mRNA to be associated with a significant increase in normal β-globin levels and enhanced therapeutic effect of β-globin gene addition, but also with toxicity associated with constitutive shRNA expression (Patsali et al. 2018).

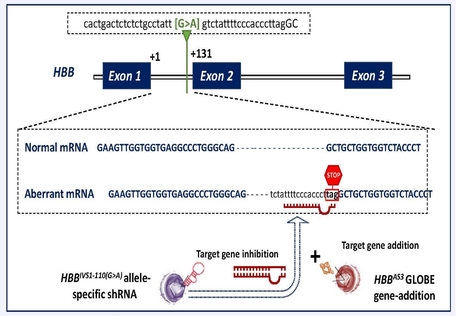

Figure 1: HBB$^{IVSI-110}$ The DNA sequence surrounding the IVS1 splice acceptor site is shown. Intron sequences are shown in lower case.

OBJECTIVES

- To express previously identified shRNAs targeting the aberrant HBB$^{IVSI-110}$ mRNA from the erythroid specific pol II- based β-globin promoter.
- To evaluate whether miR30-based shRNA-mediated silencing of the aberrant HBB$^{IVSI-110}$ mRNA could be used either as a monotherapy or in combination with *HBB*$^{\beta AS3}$ gene addition, using the GLOBE lentiviral vector (LV), for the treatment of HBB$^{IVSI-110}$ β-thalassemia.

METHODOLOGY

Generation of *HBB*$^{\beta AS3}$ GLOBE gene therapy vectors bearing a β-globin promoter-driven miR30-shRNA expression cassette alone or within the *HBB*$^{\beta AS3}$ IVSII

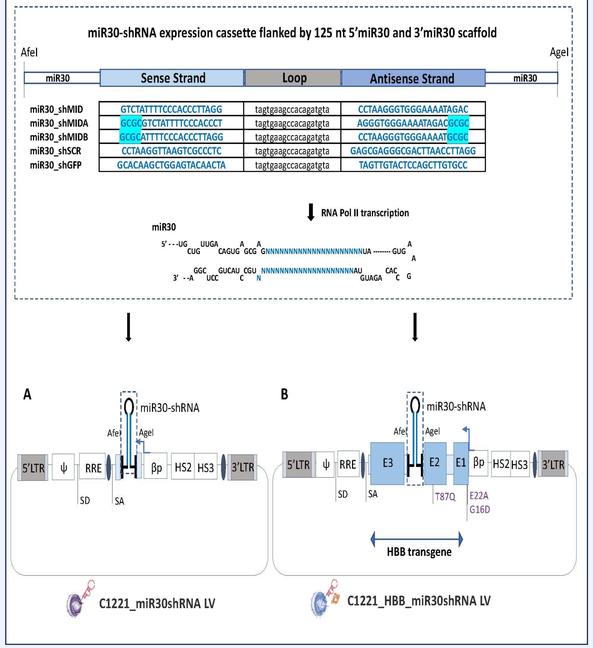

Figure 2: Design of RNApolII-driven miR30-based shRNAs targeting the aberrantly spliced *HBB*$^{IVSI-110}$ mRNA using the GLOBE *HBB*$^{\beta AS3}$ LV. Schematic representation of *HBB*$^{\beta AS3}$ GLOBE LV structure in its proviral form[2], modified to express the miR30-shRNA expression cassette **(A)** downstream of the *HBB* control elements in place of the *HBB*$^{\beta AS3}$ gene (→CMV1221_miR30shMID|MIDA|MIDB|SCR|GFP) to achieve erythroid-specific RNApol II-driven miR30shRNA production and **(B)** into intron 2 of the *HBB*$^{\beta AS3}$ gene of the GLOBE LV (→CMV1221_HBB_miR30shMID|MIDA|MIDB|SCR|GFP) to produce HBB/miR30shRNA co-expression vectors. Magnified above, shRNAs targeting the aberrant HBB$^{IVSI-110}$ mRNA consisting of two 21-bp stems linked by a 19-base loop were inserted into miR30 scaffold flanked by 125-bp miR30 flanking region on either side of the hairpin[3].

RESULTS

Evaluation of the CMV1221_miR30shRNA and CMV1221_HBB_miR30shRNA LVs bearing a miR30-shRNA expression cassette in MEL-GFP cells

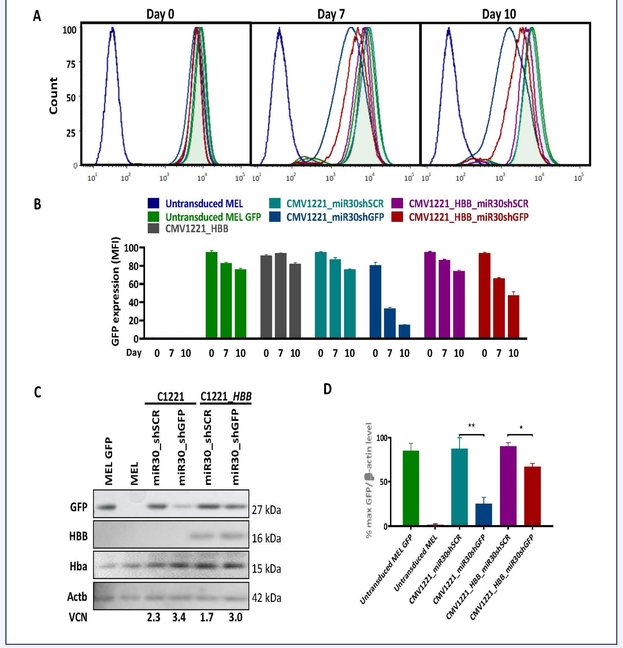

Figure 3: The CMV1221_miR30shRNA and CMV1221_HBB_miR30shRNA LVs containing miR30shGFP reduce GFP expression in erythroid cells. Functionality of the vectors carrying the miR30-shRNA expression cassette under the β-globin promoter alone or as part of intron 2 of the HBB transgene, was assessed upon transduction of MEL cells constitutively expressing GFP (MEL-GFP) at low multiplicity of infection (MOI: 3). **(A)** Representative histograms of GFP expression and **(B)** comparison of the fluorescence values (n=3) of MEL-GFP cells at days 0, 7 and 10 of erythroid differentiation. **(C)** Immunoblots of GFP, HBB, Hba protein levels in transduced MEL-GFP cells on day 7 of erythroid differentiation and **(D)** relative quantities of GFP (normalised to Actb) based on densitometry of immunoblots (ImageJ). Data represent mean ±SEM of three independent experiments from same LV preparation.

Validation of CMV1221_miR30shRNA and CMV1221_HBB_miR30shRNA targeting the aberrantly spliced *HBB*$^{IVSI-110}$ mRNA in MEL-HBB^{IVS} cells

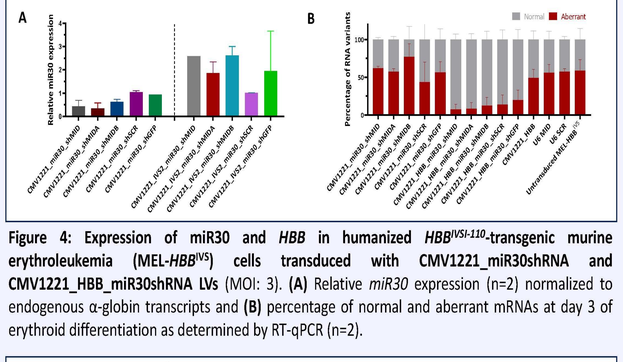

Figure 4: Expression of miR30 and *HBB* in humanized *HBB*$^{IVSI-110}$-transgenic murine erythroleukemia (MEL-*HBB*^{IVS}) cells transduced with CMV1221_miR30shRNA and CMV1221_HBB_miR30shRNA LVs (MOI: 3). **(A)** Relative *miR30* expression (n=2) normalized to endogenous α-globin transcripts and **(B)** percentage of normal and aberrant mRNAs at day 3 of erythroid differentiation as determined by RT-qPCR (n=2).

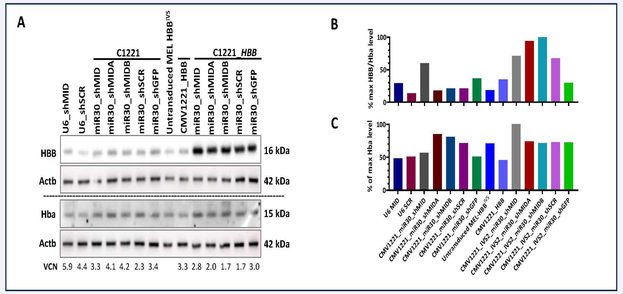

Figure 5: miR30shMID-encoding LVs mediate silencing of the aberrant *HBB*$^{IVSI-110}$ mRNA and enhance HBB levels. Comparison of miR30shRNA-encoding vectors for HBB production was assessed following transduction of MEL-HBB^{IVS} at low multiplicity of infection (MOI: 3). **(A)** Immunoblots of HBB and Hba protein levels in transduced MEL-HBB^{IVS} cells on day 7 of erythroid differentiation. Actb was used as a protein loading control. **(B)** Relative quantities of HBB (normalised to Hba) for MEL-HBB^{IVS}-transduced cells based on densitometry of immunoblots (ImageJ), shown as percentage of differentiation-normalized HBB chain levels relative to the highest value of the experiment and **(C)** Percentage of Actb-normalized Hba levels relative to the highest value of the experiment. Results are based on a single experiment.

CONCLUSIONS

This study provides further evidence supporting the role of the aberrant *HBB*$^{IVSI-110}$ mRNA as a partially dominant causative agent of disease severity in *HBB*$^{IVSI-110}$ thalassemia and as a potent target for mutation-specific gene therapy.

We show that the *HBB*$^{\beta AS3}$ GLOBE gene therapy vector bearing a functional miR30shRNA expression cassette can be used to knock down aberrantly spliced mRNA and enhance HBB expression and demonstrate the therapeutic relevance of combining RNA interference with same-vector *HBB*$^{\beta AS3}$ expression for *HBB*$^{IVSI-110}$ β-thalassemia.

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