Conversion of HbS to Hb G-Makassar by adenine base editing is compatible with normal hemoglobin function

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Introduction

Conversion of the pathogenic sickle allele to a naturally occurring, non-pathogenic hemoglobin variant, Hb G-Makassar, represents a long-term and durable treatment strategy for sickle cell disease (SCD). Using our engineered adenine base editor, we achieved highly efficient base editing in mobilized sickle trait (HbAS) and non-mobilized homozygous sickle (HbSS) CD34$^+$ cells. Although the Makassar variant is naturally occurring in human genetics and present in individuals in Southeast Asia with normal hematologic parameters in both heterozygous and homozygous states, we sought to further characterize Makassar hemoglobin and assess its biophysical and biochemical properties. Recombinant Makassar globin was co-expressed with alpha globin in *E. coli* and tetramers were purified to homogeneity. Recombinant tetramers were assessed for identity, purity, globin content, and heme content demonstrating comparability to hemoglobin tetramers isolated from primary sources (whole blood). Several characterization methods were employed, to assess size, molecular weight, oligomerization state, tetramer composition, and oxygen binding properties. These studies indicated Makassar globin could properly assemble into hemoglobin tetramers, displaying biochemical properties characteristic of hemoglobins. Furthermore, we assessed polymerization potential using a temperature jump method previously employed for kinetic measurements of sickle-fiber formation and found Makassar hemoglobin did not polymerize *in vitro* under conditions where sickle hemoglobin (HbS) readily polymerizes. Finally, a crystal structure of Hb G-Makassar has been determined at the 2.2 Å resolution and showed high similarity to the HbA (wildtype hemoglobin) structure with a RMSD of 0.385 Å for all the Cα atoms, which indicates that the glutamic acid to alanine (E6A) substitution in beta-hemoglobin does not seem to induce any significant conformational change in hemoglobin structures.

Altogether, our biophysical and biochemical characterization shows that the Makassar variant behaves as a functional hemoglobin. By replacing the pathogenic sickle globin with a benign hemoglobin variant with normal function, our base editing approach provides a promising autologous investigational cell therapy for the treatment of SCD.

Genome Editing with Adenine Base Editors (ABE)

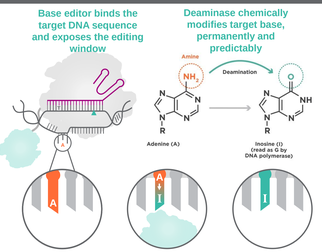


Figure 1. ABE is a fusion protein comprising an evolved TadA* deaminase (teal) connected to a CRISPR-Cas enzyme (grey). The base editor binds to a target sequence that is complementary to the guide-RNA (magenta) and exposes a stretch of single-stranded DNA. The deaminase converts the target adenine into inosine (which is read as guanine by DNA polymerases) and the Cas enzyme nicks the opposite strand. The nicked strand is repaired completing the conversion of an A:T to G:C base pair (1-2).

Makassar β-globin Variant Direct Editing SCD Strategy

Figure 2. Current base editing technologies cannot yet efficiently convert mutations like those that result from the A:T to T:A transversion in HbS. However, ABE variants were designed to recognize and edit the opposite stranded adenine residue of the valine codon. This results in the conversion of valine to alanine and the production of a naturally occurring variant in human genetics, Hb G-Makassar, which presents with normal hematological parameters and red blood cell morphology (3-5). Furthermore, alanine substitutions at this residue of the β–hemoglobin subunit does not contribute to polymer formation *in vitro* (6-8).

Highly efficient base editing in HSPCs to install Makassar variant

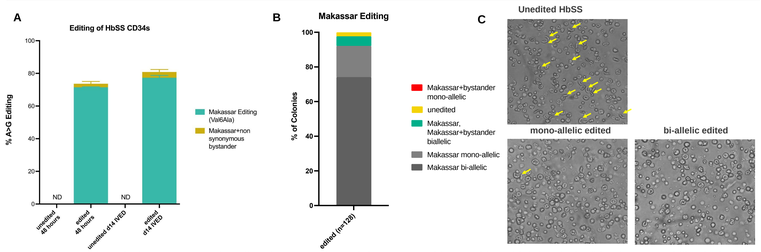


Figure 3. Editing of HbSS CD34$^+$ to install Makassar variant. **(A)** We were able to achieve high level of editing in non-mobilized whole blood CD34$^+$ cells isolated from several independent homozygous SCD patients (HbSS) with >75% A>G Makassar editing following in vitro erythroid differentiation (IVED). **(B)** single clonal analysis of editing frequency of IVED cells revealed >70% bi-allelic editing can be achieved. **(C)** mono-allelic Makassar edited cells exhibit reduced sickling in response to hypoxia (2% O_2) *in vitro*.

Recombinant Makassar globin exhibits normal hemoglobin biophysical/ biochemical properties and oxygen binding

Table 1. Summary of key protein attributes of rHbG, HbA, and HbS

Hemoglobin	Source	Purity (tetramer based)	N-terminal Met processing	Radius / Polydispersity	Heme content (heme : globin) normalized to HbA
HbA	RBC	> 90%	α 99% β 99%	2.6 nm / 11.9%	-
HbS	RBC	> 70%	α 99% β 99%	2.6 nm / 11.7%	1.1
rHbG	*E. coli* JM109	> 90%	α 99% βE6A 65%	2.4 nm / 9.7%	1.3

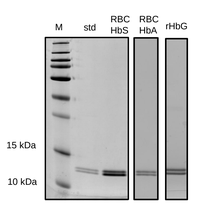


Figure 4. SDS-PAGE analysis of purified hemoglobins from red blood cells (HbA, HbS) or from *E. coli* JM109 (rHbG). The presence of two individual bands in the ~12 kDa range suggests the successful purification of α and β chains of the corresponding tetramer. Std (Standard HbA obtained commercially), RBC (red blood cells), HbA (wt hemoglobin), HbS (sickle hemoglobin), rHbG (recombinant Makassar hemoglobin)

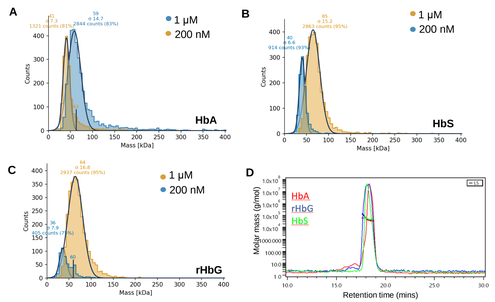
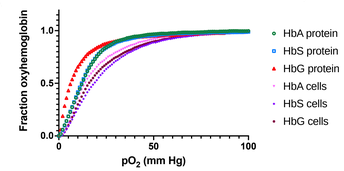


Figure 5. Oligomerization state analysis of rHbG. Mass photometry analyses of hemoglobin variants **(A)** HbA, **(B)** HbS, and **(C)** rHbG at either 1 µM or 200 nM. The distribution centered at ~64 kDa for the hemoglobins when analyzed at 1 µM suggests the presence of stable tetramers. The distribution centered at ~40 kDa when the same sample is analyzed at a lower concentration of 200 nM suggests the sample has dissociated to form stable dimers. **(D)** SEC-MALS analysis of purified HbA (red), HbS (green), and rHbG (blue). Samples eluted at a retention time characteristic of an Hb sample at equilibrium between dimer and tetramer under the conditions tested. The βE6A mutation does not alter the ability for Hb to form tetramers.



B

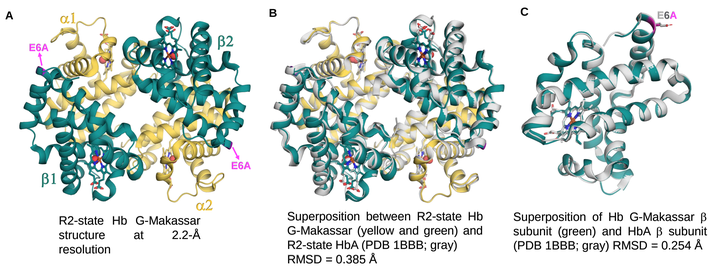
Sample	P_{50} (mm Hg)	n (Hill coefficient)
HbA IVED cells	17 ± 2	1.70 ± 0.05
HbS IVED cells	22 ± 3	1.8 ± 0.1
HbG IVED cells*	19 ± 2	1.75 ± 0.04

C

Sample	P_{50} (mm Hg)	n (Hill coefficient)
HbA protein	12.4 ± 0.6	2.1 ± 0.2
HbS protein	12 ± 1	2.1 ± 0.2
rHbG protein	6.5 ± 0.9	1.4 ± 0.1

Figure 7. Oxygen binding studies . **(A)** Representative oxygen binding curves and **(B,C)** P_{50} values and Hill coefficients for RBC cells containing HbA, HbS, or HbG (HbG, following IVED edited cells) and purified HbA, HbS, HbG proteins (labeled as protein in legend). Oxygen binding studies were performed using a HEMOX analyzer. Experimental P_{50} values and Hill coefficients were obtained from individual triplicate runs for each sample. *For HbG IVED cells, 92% of beta globins were identified as HbG and 8% HbS by a LC-MS-based peptide mapping method.

HbG is structurally similar to HbA



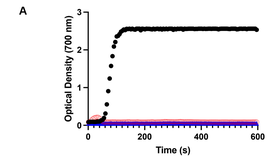
R2-state Hb G-Makassar structure at 2.2-Å resolution

Superposition between R2-state Hb G-Makassar (yellow and green) and R2-state HbA (PDB 1BBB; gray) RMSD = 0.385 Å

Superposition of Hb G-Makassar β subunit (green) and HbA β subunit (PDB 1BBB; gray) RMSD = 0.254 Å

Figure 8. Crystal structure of Hb G-Makassar. **(A)** Ribbon representation of the overall structure of the Hb G-Makassar functional tetramer (α2β2). The α globin subunits are shown in yellow, β globin subunits are shown in green, and the corresponding location of the βE6A substitution is shown in magenta. **(B, C)** Structural comparisons between Hb G-Makassar and HbA. **(B)** Superimposition between Hb G-Makassar (α in yellow, β in green) and HbA (grey) tetramers. **(C)** Superposition between Hb G-Makassar (green) and HbA (gray) β subunits. The Hb G-Makassar structure described here is highly similar to HbA, with a low RMSD (0.254 to 0.385 Å), suggesting that the βE6A substitution does not cause a significant conformational change under the conditions studied.

HbG does not polymerize *in vitro*

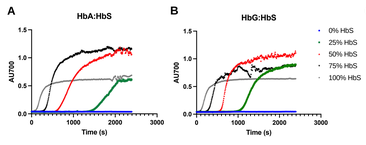


B

Sample	Concentration (mg/mL)	Delay Time (s)
HbS	2.8	56 s
HbA	2.8	-
rHbG	2.8	-

Figure 9. *In vitro* polymerization assays. **(A)** Polymerization assays were performed in 1.8 M PO_4 buffer pH 7.3 using a modified version of the temperature jump method described previously (10). A solution consisting of 2.8 mg/mL of either HbA (red open cicles), HbS (black circles), or rHbG (blue triangles) was preincubated with 1.8 M PO_4 pH 7.3 at 4 °C followed by an increase in temperature to 37 °C at which the optical density of the sample was recorded. Increase in optical density is attributed to the formation of polymers under tested conditions. **(B)** Polymerization delay times were calculated as described before (10). Under the experimental conditions tested no polymerization is detected for rHbG suggesting the βE6A mutation prevents polymer formation.

HbG does not exacerbate HbS polymerization when present in mixtures



C

Polymerizing Agent (%)	[HbS] (mg/mL)	Delay Time (s) HbA:HbS	Delay Time (s) HbG:HbS
0%	0	-	-
25%	0.5	1321	1039
50%	1.0	629	580
75%	1.5	323	281
100%	2.0	95	87

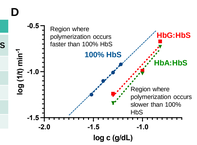


Figure 10. *In vitro* polymerization assays using mixtures of hemoglobins. Polymerization assays were performed as described above. A solution of 2.0 mg/mL total hemoglobin consisting of different ratios of **(A)** HbS:HbA or **(B)** HbS:HbG were preincubated with 1.8 M PO_4 pH 7.3 at 4 °C followed by an increase in temperature to 37 °C at which the optical density of the sample was recorded. **(C)** Polymerization delay times were calculated as described previously. **(D)** Delay times were plotted as a function of HbS concentration to assess concentration effects of either mixture on polymerization delay times. Results suggests HbG does not exacerbate HbS polymerization and behaves similarly to HbA:HbS mixtures under the conditions tested.

Low-frequency non-synonymous S9P bystander edits exhibit low biological risk

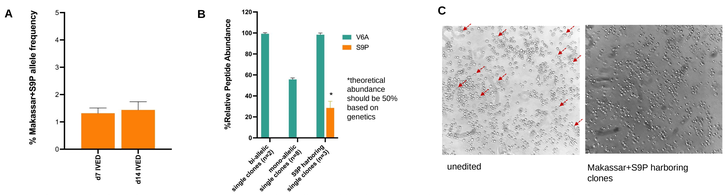


Figure 11. Characterization of non-synonymous bystander edit resulting in Ser9Pro (S9P) substitution **(A)** S9P bystander mutations co-occur with the Makassar V6A edit at stably low frequencies at d7 and d14 IVED (n>5 HbSS donors) **(B)** Measurement by LC-MS of V6A and S9P harboring peptides from single clonally derived IVED reveals lower than expected protein abundance in cells harboring the bystander **(C)** Isolated S9P harboring IVED cells do not have increased sickling in response to hypoxia *in vitro* (red arrows indicating sickled erythroid cells).

Conclusions

Our Hb G-Makassar direct editing strategy demonstrated high editing efficiency of single bases of CD34$^+$ cells after electroporation, which is sustained through erythroid differentiation. We demonstrate that at high editing efficiencies, high bi-allelic editing can be achieved with reduction of HbS globin levels to <15% and a reduction of *in vitro* sickling of edited cells exposed to hypoxic conditions. Through a comprehensive assessment of purified recombinant Makassar protein, we were able to demonstrate normal biochemical and biophysical properties, consistent with Makassar globin being compatible with normal hemoglobin function. We further demonstrated that Makassar globin does not polymerize *in vitro*, and cells co-expressing Makassar globin and sickle globin have properties similar to sickle trait cells. Finally, we de-risked a certain low frequency, non-synonymous bystander edit that result from on-target base editing. Coupled with autologous stem cell transplantation, the direct editing of the causative sickle cell mutation to the naturally occurring and asymptomatic Hb G-Makassar is a promising new treatment paradigm for patients with SCD.

References and Disclosures

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