

# Correction of $\beta^{654}$ -thalassaemia mice using direct intravenous injection of siRNA and antisense RNA vectors

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**Abstract** Although the therapeutic efficacy of  $\beta^{654}$ -thalassaemia treatment using a combination of RNAi and antisense RNA to balance the synthesis of  $\alpha$ - and  $\beta$ -globin chains has been demonstrated previously, and the safety of lentiviral delivery remains unclear. Herein, we used the same  $\beta^{654}$ -thalassaemia mouse model to develop a therapy involving direct delivery of siRNA and antisense RNA plasmids via intravenous injection to simultaneously knock down  $\alpha$ -globin transcript levels and restore correct  $\beta$ -globin splicing. The amount of  $\alpha$ -globin mRNAs in siRNA-treated MEL cells decreased significantly, and the properly spliced  $\beta$ -globin mRNA was restored in HeLa $\beta^{654}$  cells transfected with pcDNA-antisense plasmid. Furthermore, treatment of  $\beta^{654}$ -thalassaemic mice with siRNA and antisense RNA

plasmids resulted in significant reduction of poikilocytosis and reticulocyte counts in blood samples, decreased nucleated cell populations in bone marrow, and reduced intrasinusoidal extramedullary haematopoiesis loci and iron accumulation in liver. RT-PCR analysis revealed that treatment resulted in down-regulation of  $\alpha$ -globin mRNA synthesis by  $\sim 50\%$  along with an increase in the presence of normally spliced  $\beta$ -globin transcripts, indicating that the phenotypic changes observed in  $\beta^{654}$ -thalassaemic mice following treatment resulted from restoration of the balance of  $\alpha/\beta$ -globin biosynthesis.

**Keywords**  $\beta$ -Thalassaemia · Gene therapy · Small interfering RNA (siRNA) · Antisense oligonucleotide · Intravenous injection

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## 1 Introduction

$\beta$ -Thalassaemia is an inherited blood disorder in which  $\beta$ -globin chain synthesis is reduced or abolished [1].  $\beta$ -Thalassaemia is fairly common worldwide, affecting thousands of infants every year. Over 200 mutations causing  $\beta$ -thalassaemia have been described so far, including defects that alter gene expression at both the transcriptional and post-transcriptional levels [2].

$\beta^{654}$ -Thalassaemia is a particular mutation that results from a C  $\rightarrow$  T substitution at nucleotide position 654 in intervening sequence 2 (IVS-2-654, C  $\rightarrow$  T) of the  $\beta$ -globin gene. This is one of the most common mutations among Chinese patients with  $\beta$ -thalassaemia [3–5]. It results in a defect in RNA splicing, due to the introduction of a G–T dinucleotide that forms an apparent 50-nt splice donor site and simultaneously activates a cryptic 30-nt splice acceptor site at nt 579 in IVS-2. The result is formation of an aberrantly spliced mRNA

containing a 73-bp IVS-2 sequence, between exons 2 and 3, which acts as an “extra exon”. The thalassaemic pre-transcript is spliced almost exclusively via the aberrant sites, causing a deficiency of normally spliced  $\beta$ -globin transcripts. Many antisense oligonucleotides have been developed to not only inhibit gene expression but also reverse incorrect splicing of the pre-transcript [6, 7]. We previously constructed a mammalian expression vector producing an antisense fragment that targets the aberrant splice sites of the  $\beta^{654}$ -globin pre-transcript, restoring the correct splicing pattern in an in vitro transcription/splicing system and in cultured HeLa<sup>654</sup> and  $\beta^{654}$  erythroid cells [8–10]. Recently, Svasti et al. [11] delivered a splice-switching oligonucleotide (SSO) that blocked the aberrant splice site in a  $\beta^{654}$ -thalassaemia mouse model and restored significant amounts of  $\beta$ -globin in the peripheral blood of the diseased mouse.

In blood cells of  $\beta$ -thalassaemia patients, the synthesis of normal  $\alpha$ -globin chains is not affected, resulting in accumulation of excess unmatched  $\alpha$ -globin in erythroid precursors. Free  $\alpha$ -globin chains that do not form tetramers with  $\beta$ -globin precipitate in red cell precursors in the bone marrow to form so-called inclusion bodies, which are responsible for the extensive intramedullary destruction of the erythroid precursors and ineffective erythropoiesis [12]. Several studies have shown that the underlying mechanism for  $\beta$ -thalassaemia is  $\alpha/\beta$ -chain imbalance and the subsequent deleterious effects of the free  $\alpha$ -globin chains. The  $\alpha/\beta$  imbalance may be partially redressed by co-inheritance of  $\alpha$ -thalassaemia, suggesting that down-regulation of  $\alpha$ -globin genes may be a potential target for  $\beta$ -thalassaemia therapy [13–15]. In a previous study, we demonstrated effective treatment of mice with  $\beta^{654}$ -thalassaemia using a combination of RNAi and antisense RNA designed to balance  $\alpha/\beta$ -globin expression by introducing RNAi and antisense RNA into single-cell embryos in the offspring mice and the parents mediated with lentiviral vectors [16]. However, the ethics of germline therapy is still a subject of debate, and the safety of lentiviral mediation remains a concern. In this study, we report development of a feasible therapeutic strategy for  $\beta^{654}$ -thalassaemia using a mouse model (the *Hbb<sup>th-4</sup>/Hbb<sup>+</sup>* mouse) in which siRNA and antisense RNA were introduced to simultaneously knock down  $\alpha$ -globin mRNA levels and restore correct splicing of  $\beta$ -globin mRNA, respectively. This approach restored the balance of  $\alpha/\beta$ -globin chain biosynthesis and enhanced erythropoiesis.

## 2 Methods

### 2.1 siRNA and antisense RNA plasmid vectors

The polymerase III promoter (H1 promoter) was amplified by PCR from human genomic DNA and the Tc vector

(containing the H1 promoter) was constructed as described previously [17]. The pH1 vector was generated by cloning the H1 promoter into pREP4 vector (Invitrogen). Two pairs of complementary siRNA oligonucleotides for suppression of mouse  $\alpha$ -globin gene expression were designed as described [18]. The siRNA oligonucleotides were annealed and then cloned into pH1 using *Hind*III and *Nhe*I and were designated *mzi1* and *mzi2*. The siRNA coding sequences were: *mzi1*, 5'-GC ACC GTG CTG ACC TCC AAT TCA AGA GAT TGG AGG TCA GCA CGG TGC-3'; *mzi2*, 5'-GC CAC GGC TCT GCC CAG GTT TCA AGA GAA CCT GGG CAG AGC CGT GGC-3'. As a control, the siRNA fragment was substituted with the following coding sequence: 5'-GC CAC GGC TCT GCC CAG GTT TCA AGA GAA CCT GGG CAG AGC CGT GGC-3' (RNAi control).

The  $\beta$ -globin promoter and antisense RNA sequences were cloned into the pcDNA3.1 (+) vector (Invitrogen) using *Eco*RI and *Not*I to generate the pcDNA-antisense vector as described [16]. As a control, the antisense fragment was substituted with a nonspecific random sequence.

### 2.2 Cell culture and transfection

Murine erythroleukemia cells (MEL cells; ATCC) and HeLa $\beta^{654}$  cells (that stably express aberrantly spliced human  $\beta^{654}$ -globin pre-mRNA) were a kind gift from Professor R. Kole (Univ. North Carolina, Lineberger Comprehensive Cancer Cent., Dep. Pharmacology, Chapel Hill, NC 27599). The cells were cultivated in DMEM medium (GIBCO) supplemented with 10% foetal calf serum and penicillin/streptomycin at 37°C under 5% CO<sub>2</sub>.

The cells ( $5 \times 10^5$ ) were transfected for 48 h with 1  $\mu$ g plasmid and 2.5  $\mu$ l Lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected using hygromycin (300–600  $\mu$ g/ml, Sigma) or zeocin (100–200  $\mu$ g/ml, Sigma). Expression of  $\alpha$ -globin mRNA or normal and aberrant  $\beta$ -globin mRNA was measured after 3 weeks of selection.

### 2.3 Treatment of $\beta^{654}$ mice with siRNA and antisense RNA plasmids

$\beta^{654}$ -Thalassaemia mice (B6; 129P2-*Hbb<sup>tm2Unc</sup>*), obtained from the Jackson Laboratory, were heterozygotes (*Hbb<sup>th-4</sup>/Hbb<sup>+</sup>*) carrying a human  $\beta$ -globin gene with a splicing mutation generated by a C  $\rightarrow$  T substitution at nt 654 in IVS-2 OR  $\beta^{IVS-2-654}$  C  $\rightarrow$  T splicing mutation. These  $\beta^{654}$  mice manifested typical signs of a moderate form of  $\beta$ -thalassaemia, such as anaemia, splenomegaly, and abnormal haematologic indices [19]. The study was reviewed and approved by the Review Board of Shanghai Children's Hospital.  $\beta^{654}$  mice from the same pedigree were treated via tail vein injection with *mzi1* plasmid, pcDNA-antisense plasmid, or *mzi*

plasmid plus pcDNA-antisense plasmid (in a 1:1 M ratio). A total of 40–50  $\mu$ g plasmid ( $\sim 2$   $\mu$ g plasmid per gram weight of mouse) in 1 ml of saline were injected per day for 2 days. After 7 days, the plasmid was re-injected in the treated mice for another 2 days with the similar dosage. The control group was injected with the same total amount of control vectors (pH1 plus pcDNA3.1-con) in saline. The plasmid DNA injection procedure was described previously [20].

#### 2.4 Reverse transcription (RT) PCR analysis

Total RNA was isolated from cultured cells or blood cells of mice using an RNA isolation kit (U-gene) according to the manufacturer's instructions. Total RNA (1.5  $\mu$ g) was mixed with 2  $\mu$ l RT buffer, 4  $\mu$ l dNTPs (10 mmol/l), 0.5  $\mu$ l oligo(dT) (0.5 mg/ml), and 1  $\mu$ l reverse transcriptase (RT, New England Biolabs) in a 20  $\mu$ l reaction volume and incubated at 37°C for 1 h, then at 70°C for 10 min, and then left on ice for at least 2 min. PCR amplification of  $\alpha$ -globin and GAPDH cDNAs was performed in a thermocycler (Eppendorf, Germany) using 26 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. PCR amplification of normal  $\beta$ -globin and aberrant  $\beta^{654}$ -globin cDNA was performed using 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR primers are shown in Table 1.

#### 2.5 Real-time quantitative RT-PCR

$\alpha$ -Globin cDNA was generated as above and subjected to real-time PCR amplification using the RG3000 system (Corbett Research) and the QuantiTect SYBRGreen Kit (Qiagen, Hilden). PCR primers are shown in Table 1. Amplification reactions were performed at 95°C for 3 min followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Fluorescence (585 nm) was detected at 72°C. Each sample was analysed in triplicate. A standard dilution curve was obtained after the cDNA ( $\alpha$ -globin or  $\beta$ -globin) were cloned into PGEM T-VECTOR (Promega). GAPDH mRNA was analysed as an internal control.

#### 2.6 Haematological analysis

Mouse peripheral blood was collected in 40- $\mu$ l microhaematocrit tubes containing 2  $\mu$ l 0.5 mol/l EDTA (pH 8).

Red blood cell (RBC) counts, haemoglobin (Hb) levels, and reticulocyte counts were measured using a Hematology Analyzer (KX-21, Sysmex). Blood smears were stained using Wright's solution and visualized by microscopy.

#### 2.7 Histopathologic analysis

Small pieces of tissue (bone marrow, spleen, and liver) were embedded in paraffin wax, sectioned using a LEICA RM 2135 rotary microtome, and mounted on glass slides. The tissue sections were stained with haematoxylin and eosin or Gomori iron. Liver sections were stained with Pearl's Prussian blue to examine iron accumulation. Bone marrow smears were stained with Wright-Giemsa to calculate the proportion of nucleated cells. Slides were assessed in a blind manner.

#### 2.8 Statistical analysis

Statistical analysis was performed using SAS software. A Student's *t* test was used for intergroup comparisons, and a *P* value less than 0.05 was considered significant.

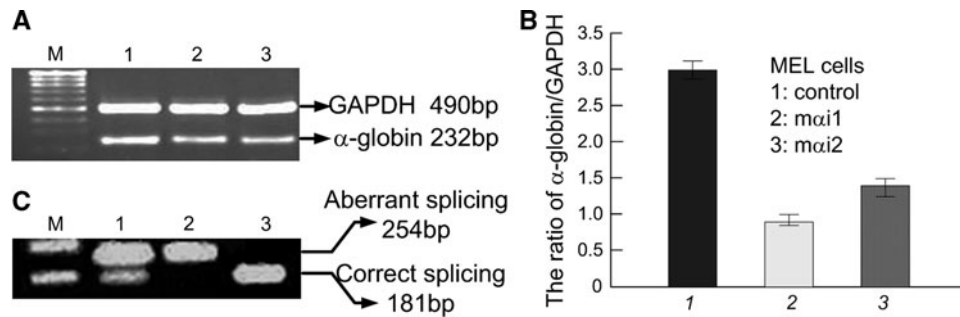
### 3 Results

#### 3.1 Plasmid vectors effectively reduce $\alpha$ -globin expression and restore $\beta$ -globin splicing in cultured cells

To knock down  $\alpha$ -globin transcription, MEL cells were treated with *mzi1* and *mzi2* siRNA plasmids with Lipofectamine mediation. After 3 weeks, total RNA was isolated and subjected to RT-PCR analysis. The amount of  $\alpha$ -globin mRNA in cells treated with *mzi1* and *mzi2* was lower than in cells treated with RNAi control plasmid (Fig. 1a). Real-time RT-PCR analysis demonstrated that the ratio of  $\alpha$ -globin mRNA to control GAPDH mRNA decreased dramatically in cells treated with *mzi1* ( $0.93 \pm 0.07$ ) or *mzi2* ( $1.37 \pm 0.13$ ) compared to cells treated with RNAi control vector ( $3.11 \pm 0.08$ ;  $p < 0.01$ ; Fig. 1b). In addition, the properly spliced  $\beta$ -globin transcript was restored in HeLa $\beta^{654}$  cells by treatment with the pcDNA-antisense plasmid, as demonstrated by real-time RT-PCR (Fig. 1c).

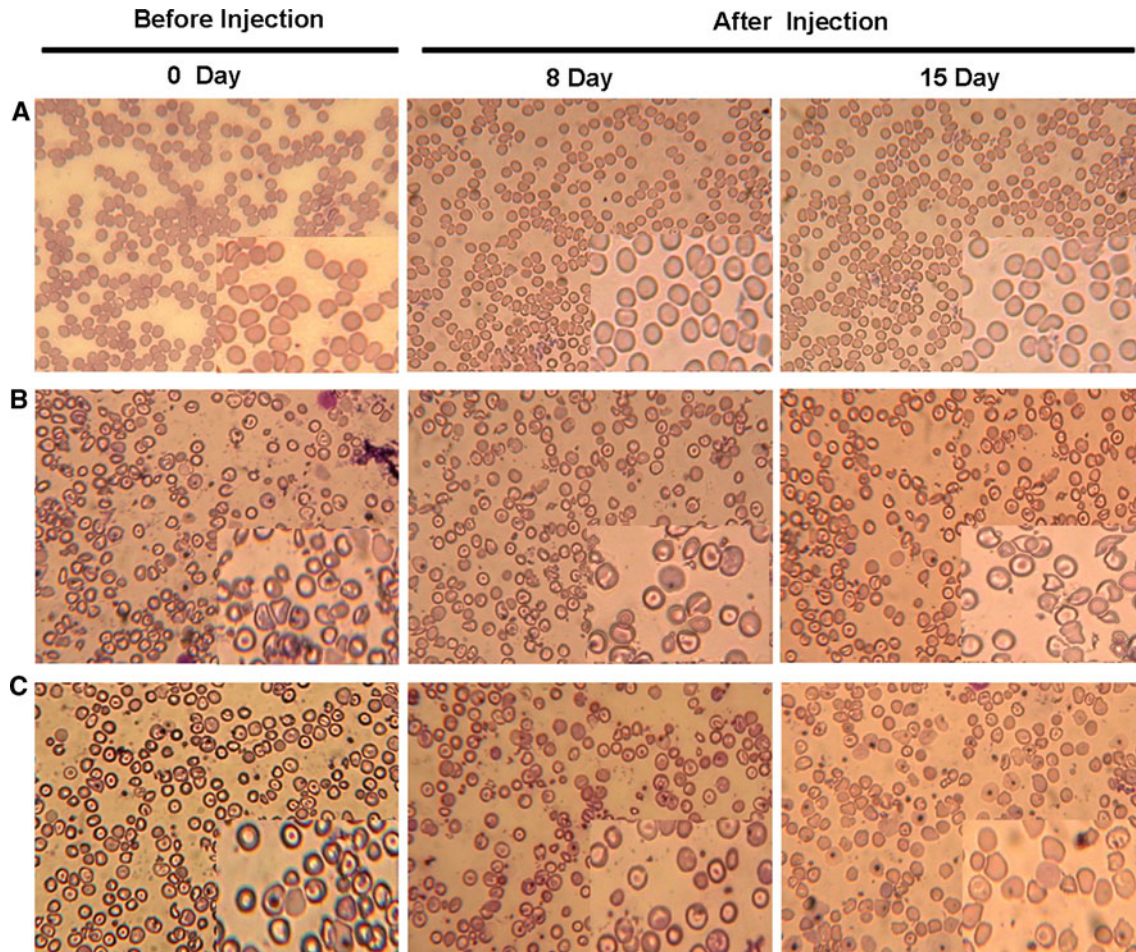
**Table 1** Oligonucleotide primers for PCR analysis

Primers	Oligo sequences	Notes
<i>mzi1</i>	5'-GGGTCACGGCAAGAAGGT-3'	<i>mzi1</i> and <i>mzi2</i> used to amplify cDNA from mouse $\alpha$ -globin mRNA (232 bp);
<i>mzi2</i>	5'-TGCTCACAGAGGCAAGGAAT-3'	
$\beta$ -5'	5'-CTCGGTGCCTTTAGTGATGG-3'	$\beta$ -5' and $\beta$ -3' used to amplify cDNA from aberrant (254 bp) and correct (181 bp) splicing variants $\beta$ -globin mRNA;
$\beta$ -3'	5'-AGCCTGCACTGGTGGGGTGAA-3'	
GAPDH1	5'-GTCTTACCACCATGGAGAAGG-3'	GAPDH1 and GAPDH2 used to amplify cDNA from GAPDH mRNA (control; 490 bp)
GAPDH2	5'-GCCTGCTTACCACCTTCTTGA-3'	



**Fig. 1** siRNA reduces  $\alpha$ -globin mRNA levels in MEL cells and antisense RNA restores correct splicing of  $\beta$ -globin mRNA in HeLa $\beta^{654}$  cells. **a** RT-PCR analysis of  $\alpha$ -globin mRNA in MEL cells treated with or without siRNA plasmid. Amplification products were subjected to 2% agarose gel electrophoresis. Lane 1 untreated control plasmid (PH) group, lanes 2 and 3 mzi1 and mzi2 siRNA-treated groups, respectively, M 100-bp DNA marker. GAPDH mRNA was analysed as an internal control. **b** Ratios of  $\alpha$ -globin/GAPDH mRNA in MEL cells treated with the indicated siRNA plasmid were

determined using quantitative real-time RT-PCR analysis.  $\alpha$ -Globin mRNA levels in siRNA-treated cells are decreased compared to the control ( $p < 0.01$ ). **c** Correction of aberrant splicing of  $\beta$ -globin mRNA using antisense RNA. Lane 1 HeLa $\beta^{654}$  cells treated with pcDNA-antisense plasmid, lane 2 HeLa $\beta^{654}$  cells treated with control plasmid (pcDNA3.1-con), lane 3 whole blood from healthy individual, M 100-bp DNA marker. The antisense RNA restored the correctly spliced  $\beta$ -globin mRNA in HeLa $\beta^{654}$  cells



**Fig. 2** Changes in peripheral blood morphology of  $\beta^{654}$  mice after treatment with plasmids (Wright staining,  $\times 400$ ). Wright's staining of peripheral blood smears from **a** a healthy mouse before and after treatment with saline, **b** a  $\beta^{654}$  mouse before and after treatment with

control plasmids, **c** a  $\beta^{654}$  mouse before and after combined treatment with antisense plus siRNA plasmids. Representative images are shown ( $\times 400$ )

### 3.2 Antisense RNA restores the correctly spliced $\beta$ -globin pre-mRNA in $\beta^{654}$ mice

$\beta^{654}$  mice from the same pedigree ( $n = 3$  per group) were injected with pcDNA-antisense plasmid or the appropriate control plasmids, and the changes in symptoms of anaemia were assessed. Prior to treatment, substantial amounts of poikilocytosis plus target cells were observed in peripheral blood smears prepared from  $\beta^{654}$  mice (Fig. 2). On day 17 after treatment, the amount of poikilocytosis plus target cells was reduced to nearly 40% in mice #6 and #7, respectively. The percentage was significantly lower than before treatment (day 0; approximately 48–58%;  $p < 0.05$ ; Table 2). The therapeutic effects of treatment lasted 27 days. In addition, the average Hb values and average RBC counts in treated  $\beta^{654}$  mice were significantly increased compared to untreated controls (Fig. 3a, b), whereas the reticulocyte counts were decreased after treatment (Fig. 3c). The reticulocytes in untreated  $\beta^{654}$  mice did not change over the course of the study (Fig. 3c). RT-PCR analysis of RNA isolated from blood cells (Fig. 4a) indicated that correctly spliced  $\beta$ -globin transcript

**Table 2** Changes in poikilocytosis plus target cells in blood smears following siRNA and/or antisense RNA treatment of  $\beta^{654}$  mice

Mouse	Poikilocytosis plus target cells (%)			
	Before injection	After injection		
	0 day	7 day	17 day	27 day
#1	N/A	N/A	N/A	N/A
#2	47.8 $\pm$ 2.2	45.3 $\pm$ 2.6	57.1 $\pm$ 3.5	50.9 $\pm$ 4.1
#3	47.8 $\pm$ 2.3	46.5 $\pm$ 3.0	47.5 $\pm$ 3.1	46.4 $\pm$ 5.3
#4	48.2 $\pm$ 2.6	50.5 $\pm$ 6.8	48.1 $\pm$ 0.4	Killed
#5	58.1 $\pm$ 3.4	55.0 $\pm$ 2.3 <sup>#</sup>	45.7 $\pm$ 2.0	44.0 $\pm$ 3.6
#6	47.7 $\pm$ 2.3	41.6 $\pm$ 1.5 <sup>a</sup>	41.1 $\pm$ 2.9 <sup>a</sup>	Killed
#7	52.0 $\pm$ 2.0	43.6 $\pm$ 3.1 <sup>a</sup>	41.3 $\pm$ 2.1 <sup>a</sup>	43.0 $\pm$ 2.6 <sup>a</sup>
#8	55.6 $\pm$ 3.5	53.3 $\pm$ 4.2	43.0 $\pm$ 2.1	48.4 $\pm$ 3.3
#9	48.3 $\pm$ 3.2	41.7 $\pm$ 2.1 <sup>a</sup>	42.7 $\pm$ 2.5 <sup>a</sup>	47.0 $\pm$ 5.3
#10	51.3 $\pm$ 1.1	42.0 $\pm$ 2.6 <sup>a</sup>	43.6 $\pm$ 1.6 <sup>a</sup>	43.0 $\pm$ 3.2 <sup>a</sup>
#11	59.0 $\pm$ 2.7	48.8 $\pm$ 1.8	48.1 $\pm$ 2.6	43.5 $\pm$ 4.1
#12	52.8 $\pm$ 3.2	40.6 $\pm$ 4.9 <sup>a</sup>	33.0 $\pm$ 5.2 <sup>a</sup>	Killed
#13	54.9 $\pm$ 3.3	35.7 $\pm$ 1.0 <sup>a</sup>	39.9 $\pm$ 6.9 <sup>a</sup>	42.1 $\pm$ 2.3 <sup>a</sup>

The proportion of poikilocytosis plus target cells per view-field ( $n = 10$ ) was determined under microscopy. All data represent mean  $\pm$  SD

#1 healthy control, #2–4  $\beta^{654}$  mice treated with control RNAi and control antisense plasmids, #5–7  $\beta^{654}$  mice treated with antisense plasmid, #8–10  $\beta^{654}$  mice treated with (mzi1 siRNA OR RNAi) plasmid, #11–13  $\beta^{654}$  mice treated with antisense plus mzi1 siRNA plasmids

<sup>a</sup> Significant difference ( $p < 0.05$ ) compared to values (on day 0 OR prior to treatment)

<sup>#</sup> No significant difference

was restored to 6% in the blood cells of the treated  $\beta^{654}$  mice, resulting in the amelioration of anaemia symptoms.

### 3.3 siRNA treatment restores the balance of $\alpha$ - and $\beta$ -globin transcripts in $\beta^{654}$ mice

Based on the results that the siRNA plasmids inhibited the expression of  $\alpha$ -globin mRNA in vitro, we further investigated the efficacy of the treatment of  $\beta^{654}$  mice with the mzi1 plasmid. After being treated with siRNA plasmids, poikilocytosis plus target cells reduced approximately 6–8% in treated  $\beta^{654}$ -mice (mice #8 and #9; Table 2). Haematological analysis indicated that the reticulocyte counts decreased after treatment, whereas reticulocyte counts in  $\beta^{654}$  mice treated with control RNAi were not affected (Fig. 3). The improved haematological parameters may be attributed to siRNA treatment because real-time RT-PCR analysis indicated a restoration of the balanced expression between  $\alpha$ - and  $\beta$ -globin genes in blood cells of the treated mice (Fig. 4b).

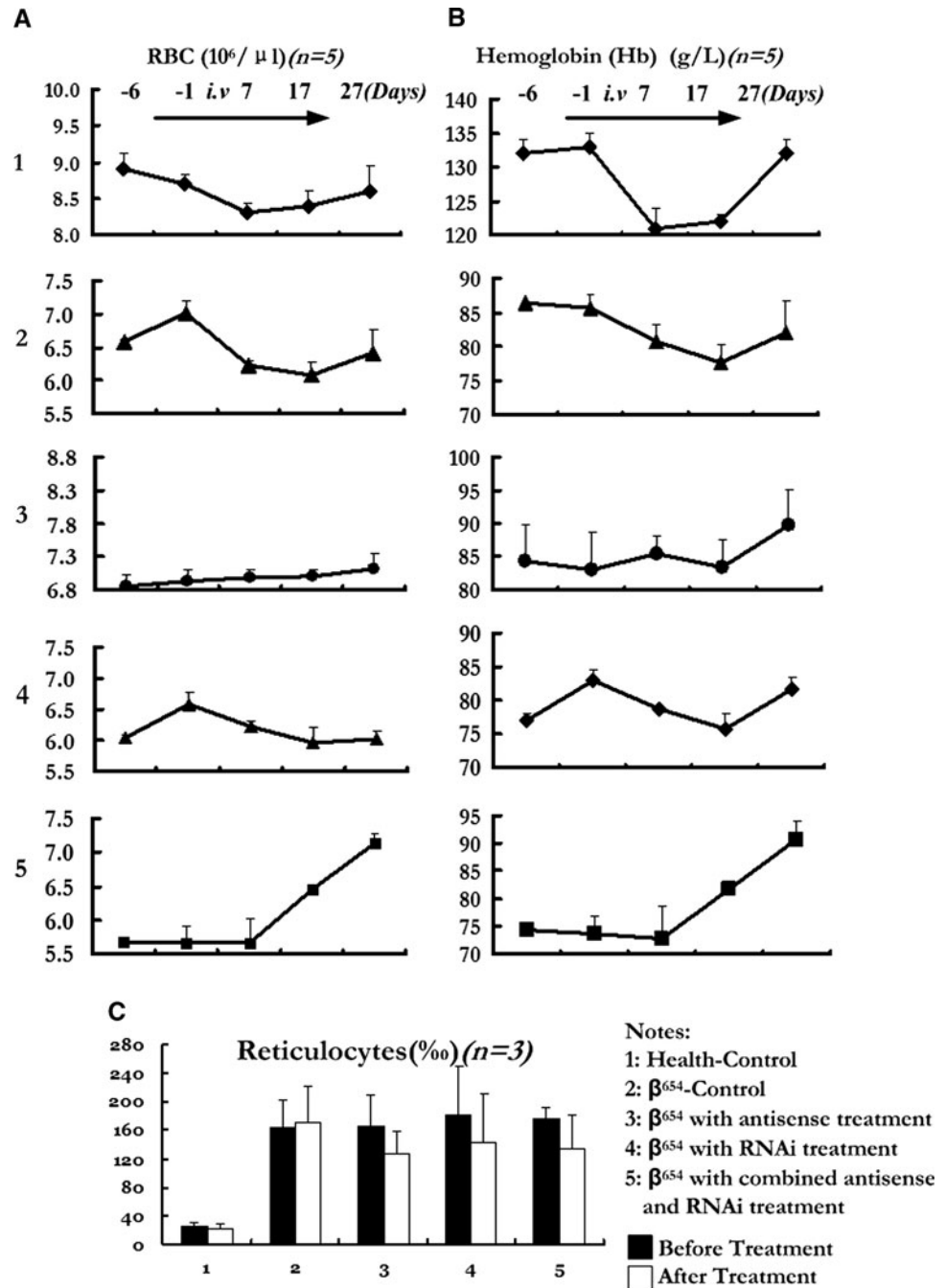
### 3.4 Moderate amelioration of $\beta^{654}$ -thalassaemia symptoms in mice using combined antisense RNA and siRNA treatment

We examined whether the treatment of  $\beta^{654}$  mice with both antisense RNA and siRNA plasmids would provide substantial alleviation of  $\beta$ -thalassaemia symptoms. The results showed that poikilocytosis plus target cells in the blood smears of  $\beta^{654}$  mice were sharply reduced after the combined treatment with antisense RNA plus siRNA plasmids (Fig. 2; Table 2). In addition, reticulocyte counts were decreased, and Hb values and RBC counts were elevated after the combined treatment compared to the control mice ( $p < 0.05$ ) (Fig. 3). These results indicated that the treatment with siRNA plus antisense plasmids provided a greater haematological benefit in  $\beta^{654}$  mice than treatment with only siRNA or antisense RNA. Visible abnormalities and toxicity effects such as significant weight loss and/or death were not observed in any treated groups.

### 3.5 Combined treatment induces morphological changes in nucleated cells of the bone marrow and spleen in $\beta^{654}$ mice

To compensate for anaemia, the proportion of nucleated cells in the bone marrow of  $\beta^{654}$  mice is significantly higher than in healthy mice. The number of nucleated cells per field of view in bone marrow from  $\beta^{654}$  mice was considerably decreased after the combined treatment (from  $50.8 \pm 2.8$  to  $30.0 \pm 2.7$ ), suggesting that the abnormal bone marrow proliferation in  $\beta^{654}$  mice was partially rescued (Fig. 5; Table 3). In spleens of untreated  $\beta^{654}$  mice,

**Fig. 3** Haematological analysis of  $\beta^{654}$  mice following individual or combined plasmid treatment. **a** Red blood cell (RBC) counts and **b** haemoglobin (Hb) levels were determined on days 7, 17, and 27 after injection with the indicated plasmid(s). RBC counts and Hb levels were increased in  $\beta^{654}$  mice following combined antisense and RNAi treatment (group 5). **c** Reticulocyte numbers were determined on day 27 after injection with the indicated plasmid(s)

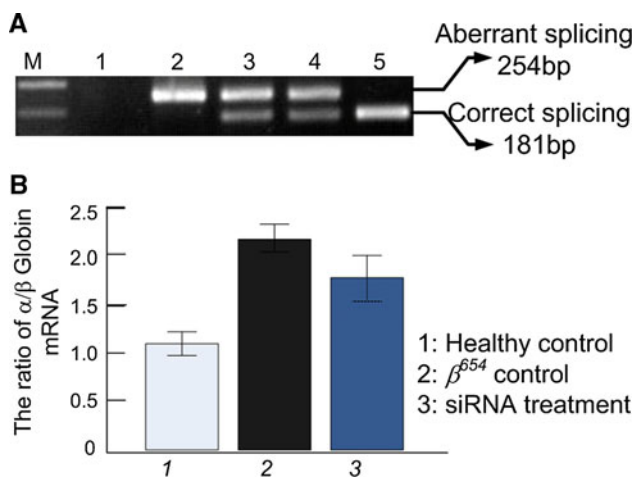


the amount of white pulp was decreased, while the amount of red pulp was substantially increased and contained more dense nucleated erythroid precursors compared to healthy mice. Other immature haematopoietic cells such as megakaryocytes were also present in the red pulp of untreated  $\beta^{654}$  mice. Following combined treatment, the number of nucleated erythroid precursors in red pulp was greatly reduced, and the amount of red pulp was decreased and contained fewer immature haematopoietic cells (Fig. 5). Because of the expansion of red pulp, splenic lymph nodules in untreated  $\beta^{654}$  mice were distinct from those of

healthy mice; almost no round splenic lymph nodules were observed in untreated  $\beta^{654}$  mice, but  $3.7 \pm 0.8$  round splenic lymph nodules per view-field were identified after treatment (Table 3).

### 3.6 Analysis of extramedullary haematopoiesis and iron accumulation in the liver following combined treatment of $\beta^{654}$ mice

Liver histopathology was examined to determine the effect of combined treatment on extramedullary haematopoiesis



**Fig. 4** RT-PCR and real-time RT-PCR analyses of  $\alpha$ - and  $\beta$ -globin gene expression in  $\beta^{654}$  mice following individual or combined plasmid treatment. **a** RT-PCR analysis of  $\beta$ -globin transcripts in peripheral blood samples. Amplification products were subjected to agarose gel electrophoresis. Lane 1 Healthy mouse, lane 2  $\beta^{654}$  mouse treated with control plasmid, lane 3  $\beta^{654}$  mouse treated with antisense RNA vector, lane 4  $\beta^{654}$  mouse treated with siRNA plus antisense RNA vectors, lane 5 healthy human, M 100-bp marker. Correctly spliced  $\beta$ -globin mRNA (181 bp) was identified in the blood of  $\beta^{654}$  mice treated with antisense RNA vector or with siRNA plus antisense RNA vector, but not in the control-treated  $\beta^{654}$  mouse. **b** Real-time RT-PCR analysis of the ratio of  $\alpha$ - and  $\beta$ -globin mRNAs in  $\beta^{654}$  mice following siRNA treatment compared to untreated control  $\beta^{654}$  mice ( $p < 0.05$ ). mRNA was isolated from the mouse nucleated cells on day 14 post-injection

(EMH) in  $\beta^{654}$  mice. In untreated  $\beta^{654}$  mice, EMH was prevalent, likely in response to the anaemia caused by RBC abnormalities. Moreover, the large quantity of abnormal RBCs correlated with profound iron accumulation in the liver. After treatment, the number of EMH foci was greatly reduced, and the number of cells exhibiting iron accumulation in the liver was reduced from  $108.2 \pm 7.8$  to  $58.6 \pm 6.1$  per field-view ( $p < 0.05$ ; Fig. 6). Decreased EMH and iron accumulation in treated mice reflect an amelioration of the anaemic phenotype.

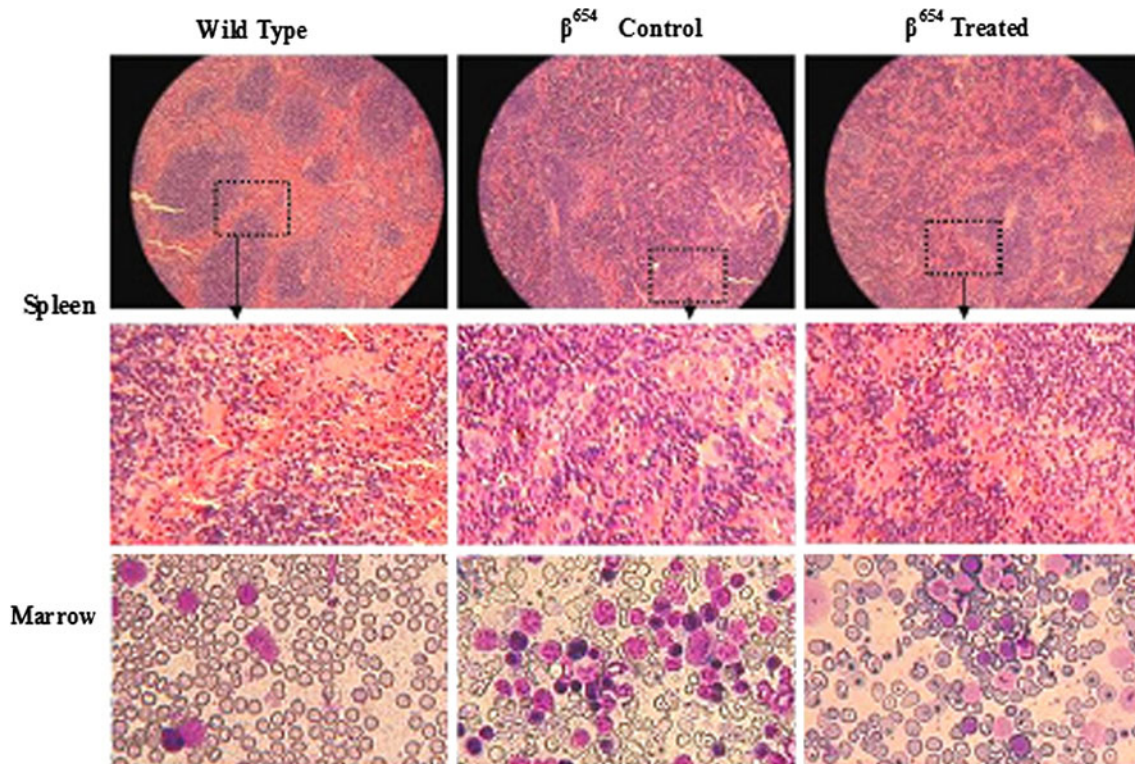
#### 4 Discussion

$\beta$ -Thalassaemia is caused by mutations in the  $\beta$ -globin gene that result in an imbalance of  $\alpha/\beta$ -globin synthesis [1]. Consequently, excess free  $\alpha$ -globin chains precipitate in the erythrocyte membrane and cause ineffective erythropoiesis [12]. Here, we report a strategy to restore the balance of  $\alpha/\beta$ -globin gene expression in  $\beta^{654}$ -thalassaemic mice. We previously demonstrated the therapeutic efficacy of simultaneously microinjecting lentiviral vectors containing a short hairpin RNA (shRNA) targeting  $\alpha$ -globin and an antisense RNA facilitating correct splicing of  $\beta$ -globin into single-cell embryos of  $\beta^{654}$  mice [16]. Although the

therapeutic efficacy of this approach for  $\beta^{654}$ -thalassaemia is well known, the introduction of genes into single-cell embryos using lentiviral mediation might reduce its feasibility in clinical practice, as the ethics and safety of such therapies are subjects of considerable debate. We were therefore interested in developing an alternative to germline therapy.

High levels of foreign gene expression can be achieved in mice by rapid tail vein injection of a large amount of naked plasmid DNA (pDNA), known as the hydrodynamics-based procedure. Liu et al. [21] demonstrated that the efficient gene transfer and expression can be achieved by a rapid injection of a large volume of DNA solution into animals via the tail vein after cDNA of luciferase and beta-galactosidase as a reporter gene was injection. Jiang et al. [22] also showed that hydrodynamics-based gene delivery into mice by intravenous administration of naked plasmid DNA is a more efficient procedure for expressing cytokines in vivo. Injection of naked plasmid DNA encoding TNF- $\alpha$  into the tail vein of mice following intravenous injection of B16 melanoma cells resulted in a profound reduction in lung metastasis [23]. When physiological regulation of gene expression is desirable, the delivery of a complete genomic DNA locus in vivo may prove advantageous for complementation gene therapy. Towards this end, using hydrodynamic tail vein injection, Hibbitt and colleagues [24] successfully demonstrated expression of large genomic DNA transgenes in mice. A similar approach was reported for the treatment of haemophilia A due to FVIII (factor VIII) deficiency [25]. For the treatment of blood disorders (such as thalassaemia) by direct in vivo gene-delivery method, one of the most important points is to ensure the successful introduction of DNA plasmid into erythroid cells. In our previous study, we have demonstrated that the eGFP-siRNA plasmid delivered by rapid tail vein injection into erythroid-specific eGFP transgenic mice could effectively suppress eGFP expression (over 20% reduction) in the recipient mice [17], indicating that the delivered DNA plasmid could introduce into the erythroid cells with effective dose. We also found that GFP could express in mouse marrow cells after treated with GFP plasmid by tail vein injection (see supplement 1). All the above studies showed that transgenic genes can express effectively in vivo by systemic administration of plasmid DNA through venous hydrodynamics-based gene delivery. We therefore examined a gene therapy approach in which mice with  $\beta^{654}$ -thalassaemia were subjected to tail vein injection of plasmid DNA encoding siRNA and antisense RNA designed to balance  $\alpha$ - and  $\beta$ -globin transcript levels.

The siRNA and antisense plasmids constructed in this study were specifically targeting to  $\alpha$ -globin or  $\beta^{654}$ -globin gene, respectively, and showed therapy effect after direct intravenous delivery. We demonstrated that the siRNA



**Fig. 5** Histopathologic analysis of nucleated cells from bone marrow and spleen of  $\beta^{654}$  mice after combined plasmid treatment.  $\beta^{654}$  mice were treated with or without siRNA plus antisense RNA vectors. On day 17 post-injection, spleen sections were removed and stained with H&E (upper panels  $\times 200$ , middle panels  $\times 400$ ). Wild-type mice were examined as an additional control. The number of megakaryocytes and

the red pulp area decreased, and a distinct white pulp marginal zone occurred in the  $\beta^{654}$  mouse following treatment. On day 17 post-injection, bone marrow cells were also removed and stained with Wright-Giemsa (lower panel  $\times 400$ ). The proportion of nucleated cells in bone marrow from  $\beta^{654}$  mice was considerably lower after treatment

**Table 3** Histopathology of haematopoietic cells in  $\beta^{654}$  mice following (treatment with siRNA plus antisense RNA OR combined treatment)

Mice	Rounded splenic lymph nodules in spleen (number per field view)	Nucleated cells in marrow (number per field view)	Liver cells with iron deposition (number per field view)
Wild type	$9.7 \pm 1.6$	$18.2 \pm 2.2$	$1.8 \pm 0.8$
$\beta^{654}$ control	$0.2 \pm 0.4$	$50.8 \pm 2.8$	$108.2 \pm 7.8$
$\beta^{654}$ treatment	$3.7 \pm 0.8^a$	$30.0 \pm 2.7^a$	$58.6 \pm 6.1^a$

All data represent mean  $\pm$  SD

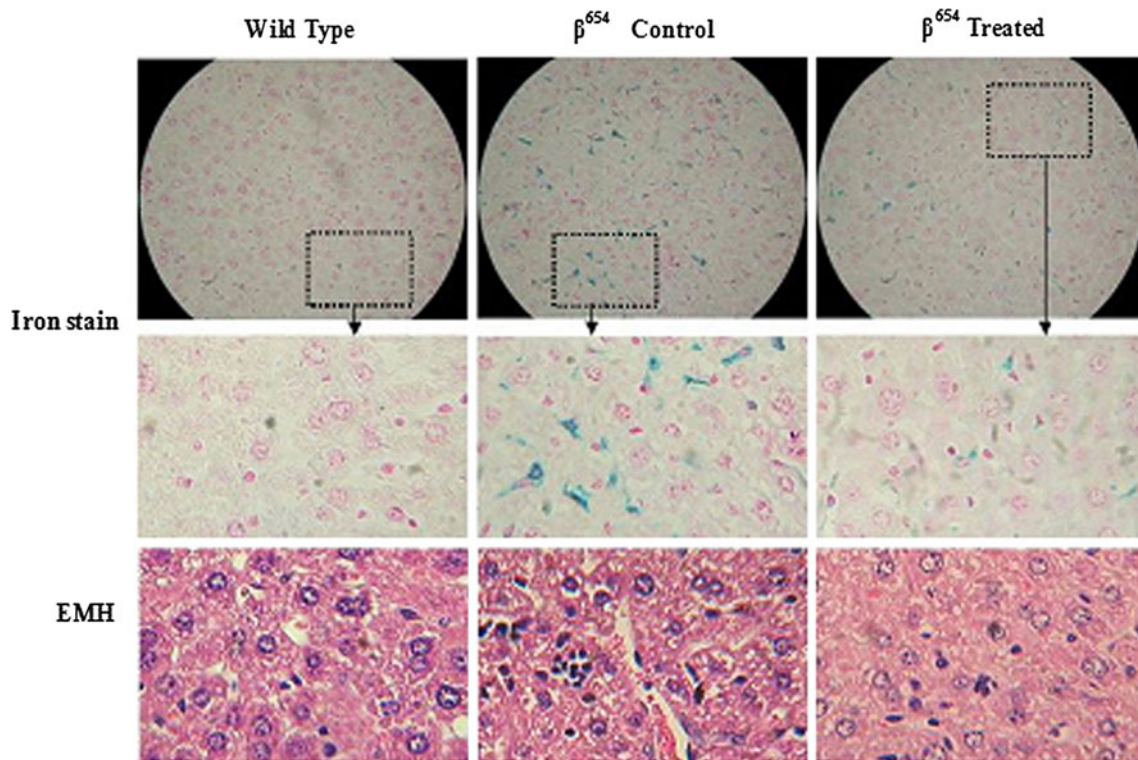
<sup>a</sup> A significant difference between the  $\beta^{654}$  treatment group compared to the  $\beta^{654}$  control ( $p = 0.05$ ) was observed

plasmid significantly suppressed  $\alpha$ -globin mRNA expression and that the antisense RNA plasmid effectively restored the correctly spliced  $\beta$ -globin mRNA, resulting in a more normal  $\alpha/\beta$  ratio and improved haematological parameters in  $\beta^{654}$  mice. In peripheral blood smears from  $\beta^{654}$  mice with classic  $\beta$ -thalassaemia, the percentage of poikilocytosis plus target cells, as well as reticulocyte counts, were clearly reduced following treatment with siRNA and antisense RNA. In comparison with the lentiviral approach [16, 26], the effects of plasmid injection were relatively transient. It is likely that lentiviral-mediated delivery results in integration of the transgene into the host chromosome, providing a longer-lasting effect. However,

the safety of lentiviral-mediated transgene therapy remains a concern. Here, we demonstrated that hydrodynamic tail vein injection of  $\beta^{654}$  mice with plasmid DNA encoding  $\alpha$ -globin siRNA and  $\beta^{654}$ -globin antisense RNA successfully ameliorated anaemia and provided therapeutic efficacy lasting for 27 days with no side effects. Even though direct injection of plasmid DNA has a relatively short-lived effect, likely because naked DNA cannot remain intact in the circulation, administration of additional doses to maintain efficacy is possible [17].

In summary, we demonstrate that direct intravenous injection of  $\beta^{654}$ -thalassaemia mice with plasmids encoding siRNA inhibited expression of  $\alpha$ -globin transcripts, and





**Fig. 6** Analysis of iron deposition and EMH in  $\beta^{654}$  mice after (combined plasmid treatment OR plasmid injection).  $\beta^{654}$  mice were treated with or without siRNA plus antisense RNA vectors. Wild-type mice were analysed as an additional control. On day 17 post-injection,

livers were removed and stained with Pearl's Prussian blue (*upper panel*  $\times 200$ , *middle panel*  $\times 400$ ) or H&E ( $\times 400$ ). Fewer erythroid precursors were observed in the sinusoids (H&E staining), and lower amounts of iron were detected in the liver of treated mice

that injection with plasmid encoding antisense RNA restored the correctly spliced  $\beta$ -globin transcript. Interestingly, injection with a combination of siRNA and antisense RNA plasmids provided effective correction of the  $\alpha/\beta$ -globin imbalance in these mice. The method described herein offers a promising approach for the treatment of patients with  $\beta$ -thalassaemia of intermediate severity OR treating intermedia  $\beta$ -thalassaemia. This study also demonstrates a potential new approach to gene therapy for a wide variety of genetic diseases.

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**Conflict of interest** The authors declare no conflicts of interest.

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