Citation: Molecular Therapy—Nucleic Acids (2016) 5, e293; doi:10.1038/mtna.2016.9
Official journal of the American Society of Gene & Cell Therapy
www.nature.com/mtna

An Efficient Method for Generation of Transgenic Rats Avoiding Embryo Manipulation

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Although rats are preferred over mice as an animal model, transgenic animals are generated predominantly using mouse embryos. There are limitations in the generation of transgenic rat by embryo manipulation. Unlike mouse embryos, most of the rat embryos do not survive after male pronuclear DNA injection which reduces the efficiency of generation of transgenic rat by this method. More importantly, this method requires hundreds of eggs collected by killing several females for insertion of transgene to generate transgenic rat. To this end, we developed a noninvasive and deathless technique for generation of transgenic rats by integrating transgene into the genome of the spermatogonial cells by testicular injection of DNA followed by electroporation. After standardization of this technique using *EGFP* as a transgene, a transgenic disease model displaying alpha thalassemia was successfully generated using rats. This efficient method will ease the generation of transgenic rats without killing the lives of rats while simultaneously reducing the number of rats used for generation of transgenic animal.
Molecular Therapy—Nucleic Acids (2016) **5**, e293; doi:10.1038/mtna.2016.9; published online 8 March 2016
Subject Category: Aptamers, ribozymes and DNAzymes

Introduction

Among rodents, rats are the most widely used animals for variety of experiments followed by mouse, rabbit, dog, pig, and primate.[1] For example, the areas of biomedical investigations where rat is particularly useful as a model are toxicology, teratology, experimental oncology, experimental gerontology, cardiovascular research, dental research, and experimental parasitology.[2] These uses of rats make them preferable to mouse as a model in biomedical research. This preference is sensible because rats have physiological and genomic similarity with human.[3–5] Rats are evolutionarily 5 million years closer to man than mouse.[5] Hence, rat models are relatively more important for biomedical research relevant to human.

Although rats are important in the biomedical research, an efficient method for the generation of transgenic rat is still awaited limiting their extensive use. Pronuclear microinjection technique is primarily used among various methods to generate transgenic rats.[6] The success rate is very less and unlike mouse embryos, most of the rat embryos do not survive after male pronuclear DNA injection[6] which reduces the efficiency of generation of transgenic rat by this method.[7] Also, this method requires hundreds of eggs collected by killing several females for insertion of transgene to generate transgenic rat. The efficiency of the standard male pronuclear DNA microinjection is enhanced by the sleeping beauty transposon-mediated transgenesis in rats which leads to a single-copy insertion.[8] However, this system has its own limitation, including, a phenomenon of overproduction inhibition (decreasing transposition phenomenon due to high concentration of transposase enzyme) and the necessity of embryo manipulation which is very difficult in rats.[8] Embryonic stem-cell–mediated transgenesis as an alternative to embryo manipulation in rats but was unexplored because until recently, attempts to isolate embryonic stem cells of rats had proven futile.[9] The method of viral transduction for transgenesis is complicated and requires higher degree of expertise.[3] These difficulties associated with rat transgenesis demanded an alternative, efficient method for production of transgenic rat. In the meanwhile, our group has achieved transgenesis in mice by exploiting the undifferentiated mouse spermatogonia to integrate foreign gene by the method of electroporation.[10,11] This principle may set the platform for the generation of transgenic rat using testicular approach. Since embryo manipulation was successful in mouse but not in rats, it was reasonable to apprehend whether testicular germ cells of rats will be amenable to transgenesis by electroporation. To generate transgenic rat by electroporating the male germ cell of testis, we used a strategy previously established for mouse transgenesis by our group.[11] Here, we tested the feasibility of transgene (linearized plasmid having enhanced green fluorescent protein (EGFP)) integration upon electroporation in germ cell of rat testis for efficient generation of transgenic rats. For proving the reliability of this technique, a pathophysiological model of alpha thalassemia (α thalassemia) was generated by overexpressing the beta (β) chain of globin in transgenic rats.

Results

Male germ-cell–mediated transgenesis in rat

The best result of electroporation was obtained by injecting 30–35 µl of linearized DNA of *pCX EGFP* construct (1 µg/µl) through three different sites into the testis of 40 ± 2 days old wistar male rat, followed by electroporation using eight square 90 V electric pulses in alternating direction which was achieved by changing pole of electrode after four pulses (**Figure 1b**). Each pulse was of 0.05 second with an interpulse interval of ~1 second delivered by an electric pulse generator while holding the injected testis in between tweezer-type electrodes. This electroporated rat

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Keywords: electroporation; embryo; spermatogonial germ cell; thalassemia; transgenic rat
Received 11 October 2015; accepted 7 January 2016; published online 8 March 2016. doi:10.1038/mtna.2016.9

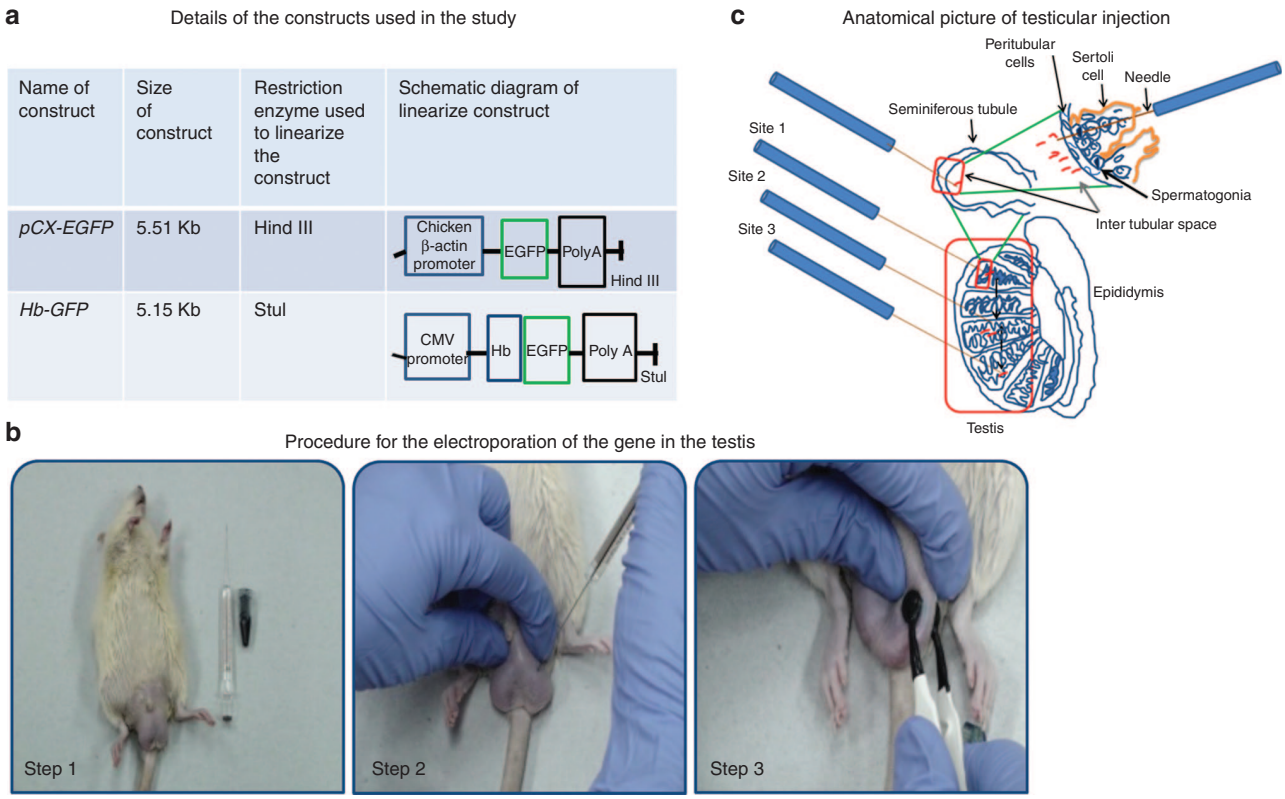

Figure 1 Standardization of electroporation conditions for generation of transgenic rat. (**a**) Schematics diagram of various constructs used for the study; the vector maps are given in **Supplementary Figure S6**. (**b**) Images of the procedure for the injection and electroporation of the gene in the testis using tweezer type electrode. (**c**) A cartoon depicting testicular injection leading to availability of DNA surrounding seminiferous tubule into intertubular spaces.

was designated as fore founder. A pictorial representation of the procedure was shown in **Figure 1b**,**c**. The whole procedure was accomplished in about 10 minutes.

pCX EGFP constructs 1 and 2 (used for standardization) both had the chicken β-actin promoter, an a ubiquitous promoter, cloned upstream of two different mutated forms (1 and 2) of green fluorescent protein (GFP) designated as *EGFP*. When the linearized *pCX EGFP* construct 1 was electroporated in the testis of fore founder, fluorescence for EGFP was observed in the testis even after 200 days of electroporation indicating that transgene was integrated into the genome (**Figure 2a**). The specific expression of EGFP in some of the germ cells of such testis was verified by coimmunolocalization of the EGFP and VASA, 15 days after electroporation (**Figure 2b**); immunohistochemical staining demonstrated presence of EGFP (green) in several VASA expressing (red) germ cells lying in the basement of the seminiferous tubule of electroporated rat. Flow cytometric analysis of the sperm isolated from the epididymis of fore founder rat showed the presence of EGFP-positive sperm confirming that the transgene was integrated into the germ cell from which EGFP-positive sperm were produced (**Figure 2c**).

When two such fore founder rats were separately mated with wild-type females, both of them produced transgenic progeny as detected by the PCR with the primer specific to the transgene (here, *EGFP*). To test whether the integrated transgene was transmitting to successive generation, a randomly selected transgenic founder was mated with a wild-type female. The PCR analysis of progeny showed that the founder produced the transgenic rat confirming that the transgene was transmitted to the next generation (**Figure 3a**). Gene integration and transmission using another construct, *pCX EGFP* (construct 2), suggested repeatability of the procedure (**Figure 3b**). Southern blot analysis was performed to validate the PCR analysis. For this, Southern blot was performed with genomic DNA isolated from randomly selected PCR positive rats from various founders and from the successive progeny of a selected founder. Southern blot analysis confirmed the genomic integration of the transgene (**Figure 4** and **Supplementary Figure S2a**). Southern blot data showed that the transgene was integrated in concatemer fashion in the genome and this phenomenon is commonly observed with the pronuclear DNA microinjection procedure.

Since the transgene (*pCX EGFP*) contains a mammalian ubiquitous promoter and *EGFP* as a marker, we evaluated the expression of the integrated transgene by live cell of hepatocyte isolated from the liver of randomly selected PCR positive rats (a total of 13 founders) and three wild-type rats by flow cytometry. The data showed that EGFP was expressed by hepatocytes of transgenic animals, indicating that the integrated transgene was functional (**Figure 3c**). Since multiple copies of transgene integrated in a concatemer fashion in the genome of transgenic

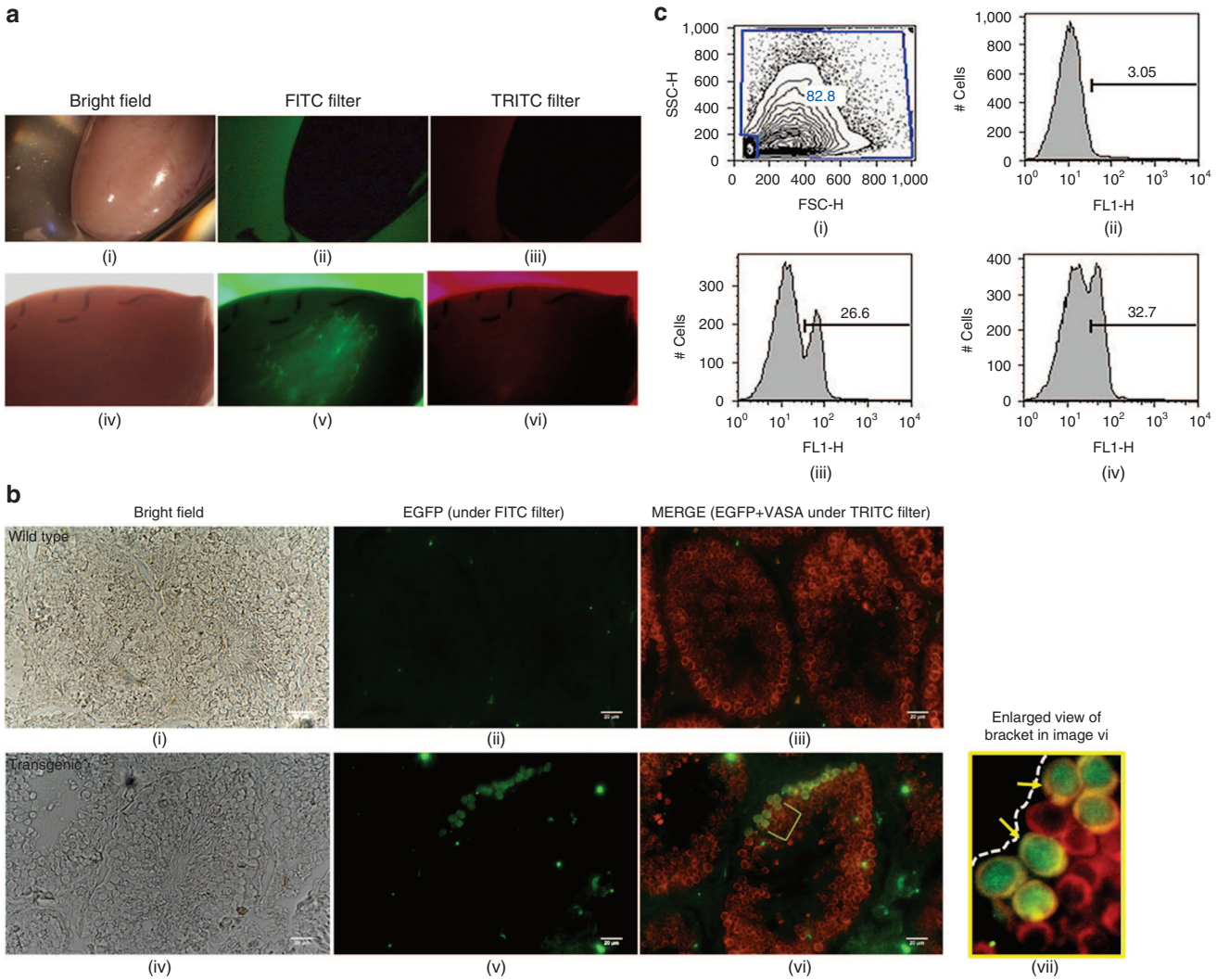

Figure 2 Evaluation of the testis of fore founder rat after electroporation for enhanced green fluorescent protein (EGFP) expression. (**a**) Testes was observed under stereomicroscope, 200 days after electroporation of construct carrying EGFP transgene. (**i**), (**ii**), and (**iii**) represented the nonelectroporated testis of wild-type rat, whereas (**iv**), (**v**), and (**vi**) represented electroporated testis of fore founder under bright field, fluorescein isothiocyanate (FITC) filter and tetramethylrhodamine isothiocyanate (TRITC) filter, respectively. (**b**) Immunostaining for EGFP in cross section of nonelectroporated testis under phase (**i**), FITC filter for EGFP staining (**ii**) and merge of FITC and TRITC filters for Vasa (**iii**), and same for electroporated testis under phase (**iv**), FITC filter for EGFP staining (**v**) and merge of FITC and TRITC filters for Vasa immunostaining (**vi**). (**vii**) An enlarged version of the selected area of image (**vi**) showing the EGFP as well as VASA expressing germ cells (yellow arrow) lying at the basement membrane (white dashed line) of the tubule. The EGFP and VASA were represented by green and red colors, respectively. The individual images for EGFP, VASA, and 4′,6-diamidino-2-phenylindole dihydrochloride are given in **Supplementary Figure S1**. Bar = 20μm. (**c**) Detection of EGFP-positive sperm in epididymis of fore founder rat electroporated with *pCX EGFP* construct 1 by flow cytometry. (**i**) Scatter plot of one of the representative sperm sample. (**ii**) Representative histogram of sperm collected from wild-type rat. (**iii**) Histogram of sperm collected from a fore founder after 200 days of electroporation using *pCX EGFP* construct 1. (**iv**) Histogram of sperm collected from a different fore founder after 200 days of electroporation using *pCX EGFP* construct 1. There was a remarkable shift in the forward scatter indicating a presence of EGFP expressing sperm in electroporated testes.

rat, we carefully analyzed the expression pattern of the integrated transgene as concatemerization has a chance of transgene silencing. The results showed that 46% (6 out of 13) of founder rats expressed EGFP in their hepatocytes. Some of the founders (founder number 6, 9, 10, and 13) displayed a higher expression of EGFP whereas some of them (founder number 8 and 11) have displayed relatively lower levels of EGFP expression in the hepatocytes.

Immunohistochemical analysis of the sections of the liver obtained from the founder transgenic rat showed uniform expression of EGFP, whereas no expression of EGFP was observed in the hepatic cells of wild-type animals (**Figure 3d**). We analyzed the transgene expression in various organs (liver, spleen, kidney, and testes) of the transgenic rat by western blot analysis and observed that the expression pattern was ubiquitous (**Figure 3e**).

Transgenic rat displaying α thalassemia

In order to generate α thalassemic rat mode using this method, the open reading frame (ORF) of β chain of hemoglobin (Hb)

from the rat was isolated and tagged with *GFP* and cloned downstream of cytomegalovirus (CMV), an ubiquitous promoter (see **Supplementary Figure S3a**). This construct was named as *HbGFP* construct. The functionality of *HbGFP* construct was confirmed *in vitro* by transfecting the HEK 293 cell line with *HbGFP* construct and visualizing for expression of EGFP (see **Supplementary Figure S3b**). We generated transgenic rat with *HbGFP* construct. The PCR and Southern blot analyses of genomic DNA obtained from progeny generated by rats electroporated with *HbGFP* construct showed that the transgene was propagated to successive (F1 and F2) generations (**Figures 4** and **5a**, and **Supplementary Figure S2b**). We detected the expression of EGFP tagged to β chain of Hb by western blot analyses of proteins isolated from liver, spleen, and kidney of the transgenic rat confirming that integrated transgene was functional (see **Supplementary Figure S4**). The Hb concentration in the blood of β globin chain overexpressing transgenic rat was found to be significantly ($P < 0.05$) lower (13.87±0.4308) as compared with that (16.54±0.1511) of control rats (**Table 1**). In the transgenic rats, a significant ($P < 0.05$) decrease in the mean corpuscular volume and mean corpuscular Hb was observed (**Table 1**) as compared with that of control (55.59±0.7938 versus 49.74±0.7554 and 17.79±0.1563 versus 16.50±0.1786, respectively). Presence of HbH inclusion bodies in some of the RBCs of transgenic rats was detected by Briliant Cresyl Blue (BCB) staining. The blood smear of transgenic rat showed the presence of microcytic and abnormal-shaped RBC indicating a milder form of α thalassemia (**Figure 5b**).The scanning electron microscopy of the blood of transgenic rat revealed the presence of microcytic RBCs with abnormal shapes (**Figure 5c**).

Efficiency of generating transgenic rats by electroporation

Electroporated male rats, referred to as fore-founder rats, were mated with wild-type females, 60 days postelectroporation. For this study, we have used three different constructs (with a total of eight fore founders) to evaluate the overall efficiency of this technique in generating transgenic rats. The results are summarized in **Table 2**. When fore founder was mated with wild-type female rats, the frequency of gene transmission in founder progeny ranged from 30 to 37.75% with an average frequency of 33.08% for three constructs used during this study. More than 50% of the progeny generated by mating of founder rats with wild-type rats carried the transgene; the frequency of transmission was even more when the two founders of the opposite sex were mated (**Figures 3a**,**b** and **5a**).

Discussion

Although mouse embryos are suitable for gene insertion and propagation, embryos of rats are fragile and fail to sustain embryo manipulation for transgenesis. The goal of this study was to increase efficiency of making transgenic rats by method of gene electroporation in male germ cells of testis, avoiding oocyte-mediated transgenesis.

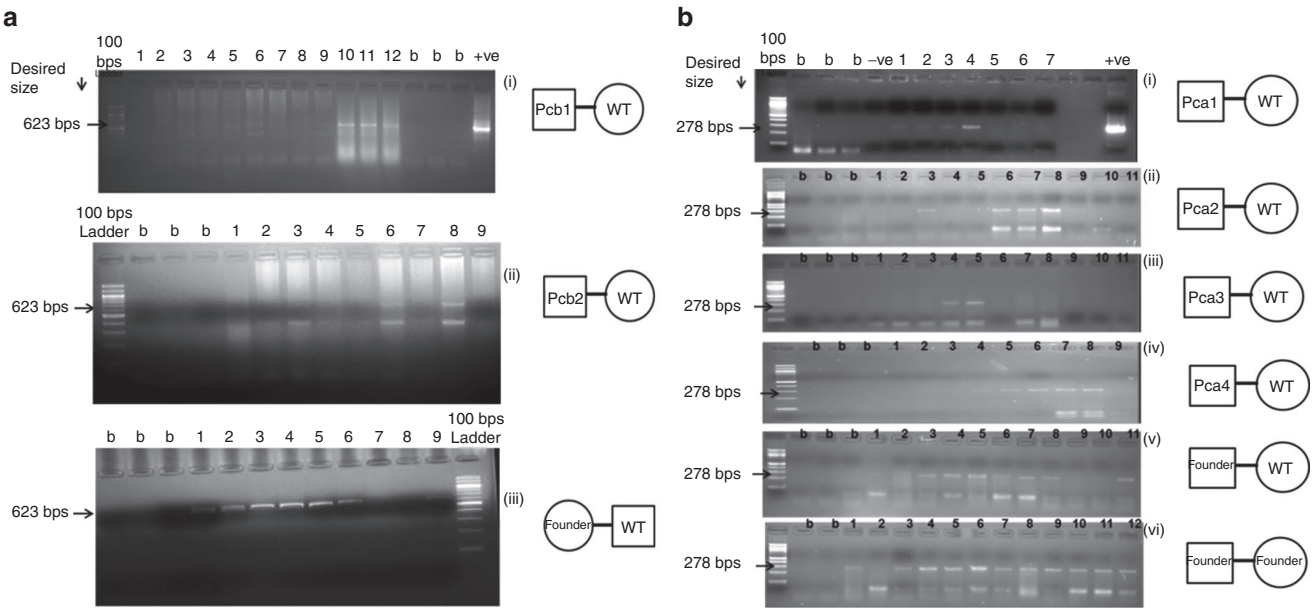

Figure 3 Generation of green fluorescent protein (GFP) transgenic rat by testicular electroporation. (**a**) Detection of transgene (*pCX* enhanced green fluorescent protein (EGFP) construct-1) by PCR. PCR screening of progeny generated from wild-type female rat mated with *pCX EGFP* (construct 1) electroporated fore founder Pcb1 (**i**) and Pcb2 (**ii**). (**iii**) PCR screening of progeny generated from the mating of a transgenic founder with wild-type female rat. A band size of 623 bp (arrow marked) represented the transgene positive rat. b = blank containing only PCR mastermix, +ve = *pCX-EGFP* construct 1, numerical indicated the progeny numbers in the gel. (**b**) Detection of transgene (*pCX EGFP construct-2*) by PCR. PCR screening of progeny generated from mating of wild-type female rat with *pCX EGFP* (construct 2) electroporated fore founder Pca1 (**i**), Pca2 (**ii**), Pca3 (**iii**), and Pca4 (**iv**). (**v**) PCR screening of progeny generated from a selected transgenic founder with wild-type female rat. (**vi**) PCR screening of progeny generated from the inbreeding of two founders. A band size of 278 bp (arrow) represented the transgene positive rat. b = blank containing only PCR mastermix, numerical indicated the progeny numbers in the gel, +ve = *pCX EGFP* plasmid construct 2, –ve = genomic DNA of wild-type rat.

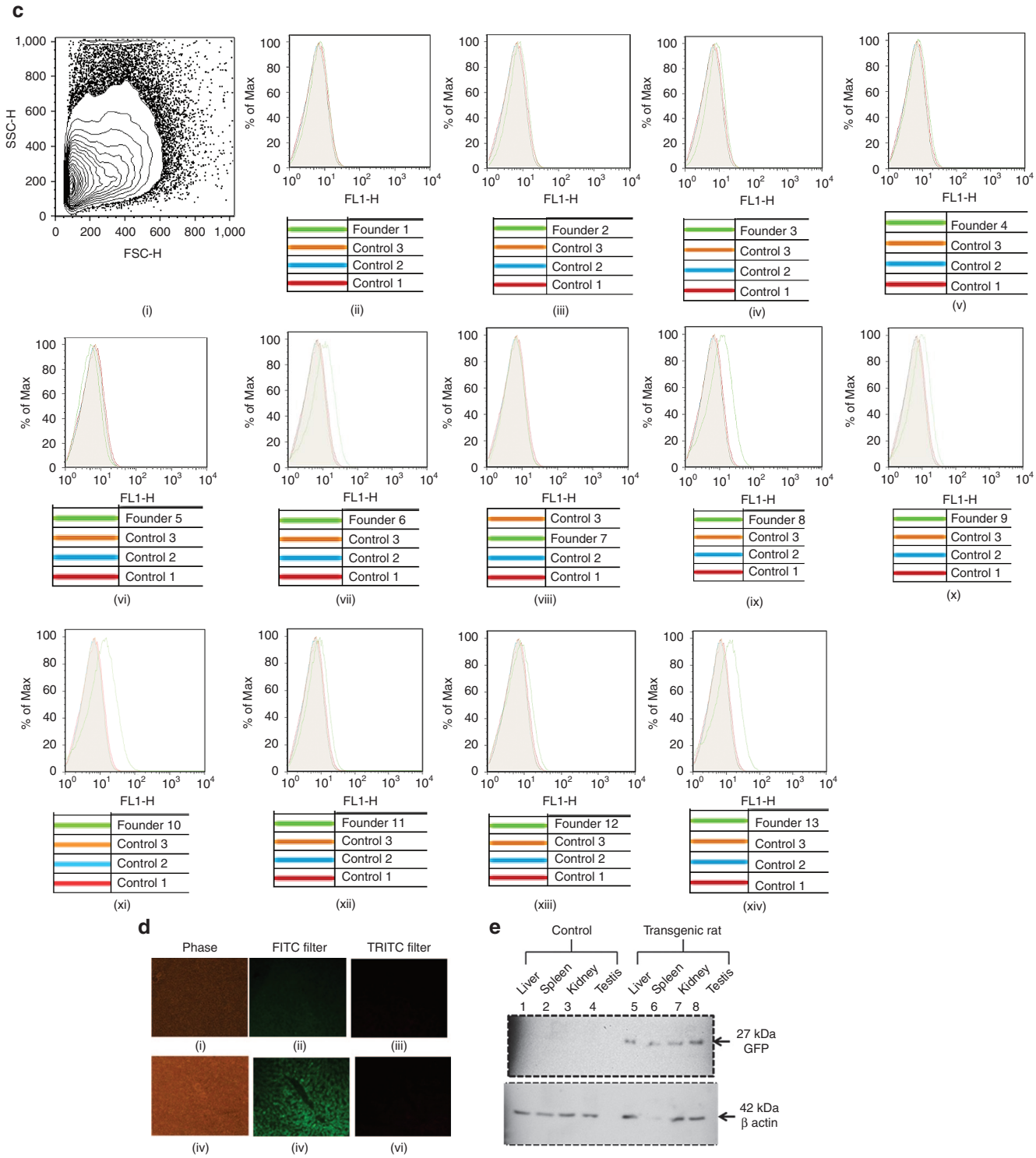

Figure 3 Continued (**c**) Flow cytometric analysis of hepatocytes isolated from various founder of *GFP* transgenic rats (founder number: 1–13) and control rats (number 1–3). (**i**) Scatter plot of one of the representative transgenic hepatocyte sample. (**ii**–**xiv**) Histogram of hepatocyte isolated from various founders and control rat. The hepatocytes were isolated from the liver of control and transgenic rats and analyzed with fluorescence activated cell sorting for the EGFP expression in the unfixed hepatocytes. (**d**) The immunostaining for EGFP in the cross section of liver of the control rat under phase (**i**), fluorescein isothiocyanate (FITC) filter (**ii**), and tetramethylrhodamine isothiocyanate (TRITC) filter (**iii**); and transgenic rat under phase (**iv**), FITC filter (**v**), and TRITC filter (**vi**), respectively. (**e**) Western blot analysis of EGFP in protein isolated from liver, spleen, kidney, and testes of transgenic (lanes 1–4) and control rats (lane 5–8). β-actin was used as internal control in the same blot. Blots were cropped to fit into the figure (see **Supplementary Figure S5** for full image).

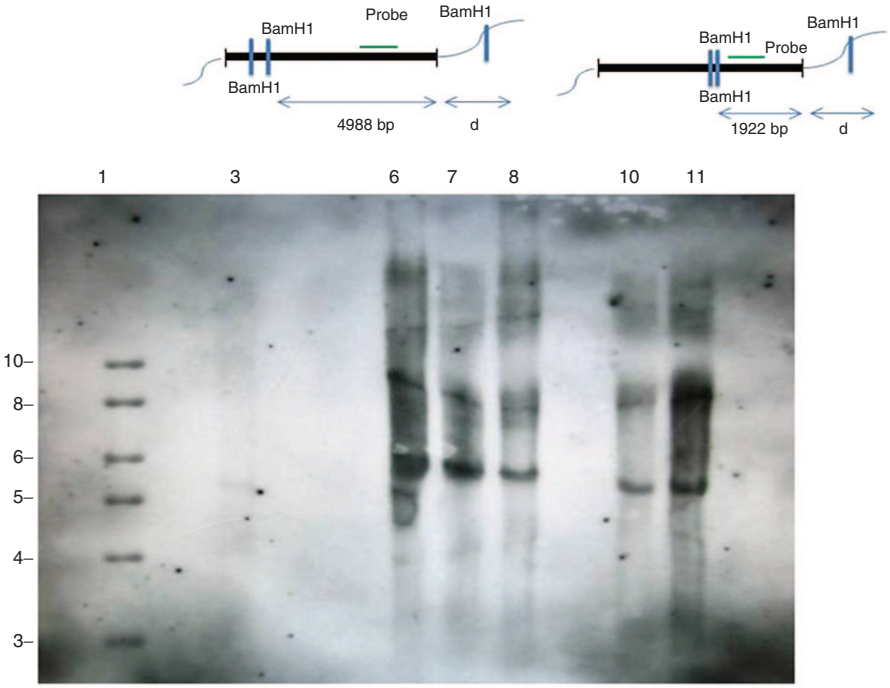

Figure 4 Southern blot analyses for determining genomic integration of transgene. Genomic DNA samples from *GFP* transgenic rats were digested with BamH1 and hybridized with a probe for *GFP*. Lane 1 represented 1-Kb ladder and lane 3 represented wild-type rat, lanes 6, 7, 8 represented transgenic rat from F_1, F_2, and F_3 generations. Band size of 4,988 bp or more were of desired size. Analysis of lanes 6–8 revealed multiple copy insertions and their genomic stability in the offspring. Genomic DNA samples from transgenic rats overexpressing β globin chain were digested with BamH1 and hybridized with a probe for *GFP*. Lanes 10 and 11 represented the transgenic rat from F_1 and F_2 generations. Desired band size of 1,922 bp or more were detected. Analysis of lanes 6–7 revealed multiple copy insertions with stable transmission of transgene.

Here, construct having *EGFP* was used for establishing gene integration by electroporation in male germ cells of rat, *in vivo*. Presence of fluorescence in the electroporated testis even after 200 days of elctroporation suggested that the linearized plasmid was permanently integrated into the rat genome because expression of genes beyond 14 days of gene delivery suggested genomic integration of the transgene.[12] Since we detected EGFP in the sperm obtained from epididymis, it was reasonable to assume that the transgene was integrated in germ cells using standardized parameters of electroporation. In transgenic rats having *EGFP*, 27 KDa protein of native EGFP was detected whereas in the β globin chain overexpressing transgenic rats, a fusion (β chain of Hb fused with GFP) protein of 44 kDa was detected using GFP antibody. This provided substantial evidence for the correct expression of the transgene as molecular weight of β chain of Hb is known to be around 17 kDa.[13]

About 5.6–11.4% of eggs transfected using lentiviral-based vectors are reported to produce transgenic progeny.[14,15] Success of intra cytoplasmic sperm injection-mediated transgenesis in rats is reported to vary between 0.7 and 6.8%,[16] that of sleeping beauty-mediated transgensis is about 14–65%[8] and the pronuclear microinjection efficiencies are about 2%.[2] In contrast to this, 33.08% of the progeny generated from single cohabitation of an electroporated fore founder (generated by us) with a wild-type female was transgenic. All (100%) electroporated males generated transgenic progeny. The procedure of electroporation can be performed within 10–15 minutes. Several founders can be generated within 3 months of electroporation (about 2 months required for development of transfected spermatogonia into sperm and 1 month for generation of progeny upon mating). This suggested that testicular gene electroporation method has the advantages over other currently available methods of rat transgenesis.

In the past, Zhou *et al.*[17] has elegantly demonstrated the feasibility of transgenesis in rats by *in vitro* transfection of female germ line stem cells with the desired transgene followed by ovarian transplantation, similar studies were reported for male germinal stem cells of mouse by Brinster *et al.*[18] Unlike these, our procedure did not require isolation, culture, and expertise for transplantation of germ cells using survival surgeries. The conventional embryonic stem cell-mediated transgenesis for generation of transgenic rat require the germ line competency of the stem cell line.[9] In our approach, we exploited the germinal stem cells of testis without their isolation from testis or culture, *in vitro*, before transfection. Previously, we have reported the generation of transgenic mice by electroporation of male germ cell *in vivo* which does not require any surgery.[11] Here, we used a similar technique for generation of transgenic rats. The efficiency of generation of transgenic mice is 100% as all the mice (fore founder electroporated with various transgene) used in the study generated the transgenic progeny. Procedure of rat transgenesis is also equally (100%) efficient. Percentage of progeny (generated by the electroporated fore founder) carrying the transgene was about 57–62% in case of mice,[11]

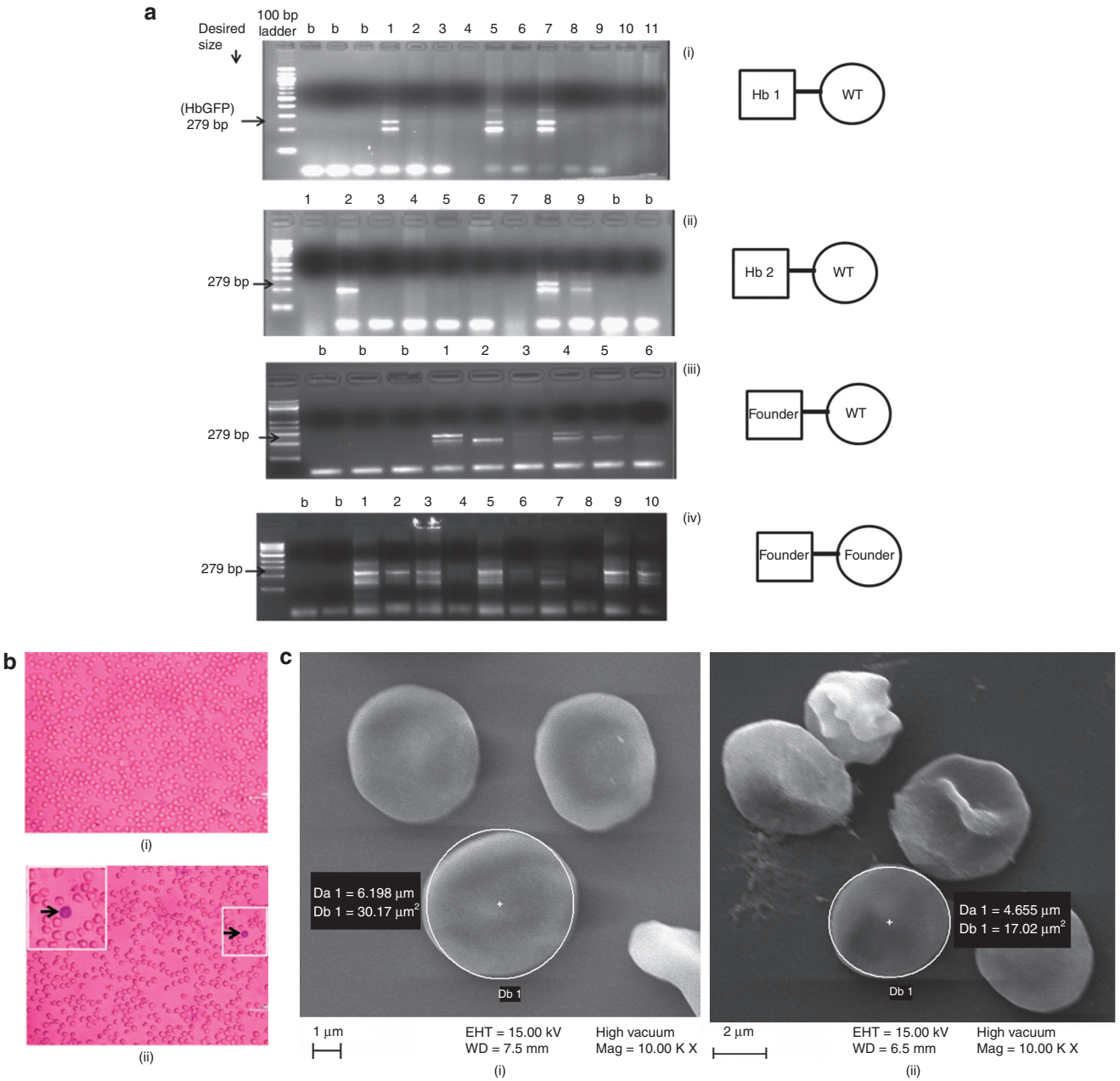

Figure 5 Generation of α thalassemic rat. (**a**) Detection of transgene (*Hb GFP* construct) in progeny of fore founder rat by PCR. (**i**) and (**ii**) PCR screening of progeny generated from two fore founders (Hb1 and Hb2) electroporated with *Hb GFP* construct. (**iii**) PCR screening of progeny generated from one male founder mated with wild-type female. (**iv**) PCR screening of progeny generated from the inbreeding of two founders (PCR positive male and female). A band size of 279 bp (arrow marked) represented the transgene positive rat. b = blank containing only PCR master mix. Numerical indicate the progeny numbers in the gel. (**b**) Briliant Cresyl Blue (BCB) staining of whole blood of β globin chain overexpressing transgenic rat. (**i**) Whole blood smear of control rat (8 weeks). (**ii**) Whole blood smear of β globin chain overexpressing transgenic rat showing BCB positive RBC (black arrow) along with several microcytic erythrocytes. A magnified image of the selected area (marked white) was shown in the upper left hand corner. Figures shown were representative of images taken from ten random fields out of ten independent experiments for each group. Images were taken at 20× original magnification. (**c**) Evaluation of the sizes and shape of RBC of wild-type (**i**) and β globin chain overexpressing transgenic (**ii**) rat by scanning electron microscopy. The images revealed the difference in the shape of RBC between the two. The shape of RBC was distorted in the transgenic rat and the sizes of some of the RBCs in the transgenic rats were smaller (17.02 μm^2) than that of control (30.17 μm^2).

whereas it was 33% in case of rats. It should be noted that in case of transgenic founders generated by oocyte-mediated technique, the choice for founders for generating transgenic lines is limited because only a few of progeny turn out to be transgenic.[19] However, in the technique developed by us, mating of electroporated fore founder male with several wild-type females can increase the yield of founder progeny.

Table 1 Blood parameters of 6-month-old control and β globin chain overexpressing transgenic rat

Animals/blood parameters	Level of hemoglobin (Hb) in g/dl	Mean corpuscular volume (MCV) in fl	Mean corpuscular hemoglobin (MCH) in pg
Control rat ($n = 8$)	16.54 ± 0.1511	55.59 ± 0.7938	17.79 ± 0.1563
Transgenic rat ($n = 7$)	13.87 ± 0.4308	49.74 ± 0.7554	16.50 ± 0.1786

Table 2 Efficiency of generation of founders by electroporation

Construct used	Name of electroporated fore founder	Total number of transgenic progeny per litter	% Transgenic progeny	Average % of transgenic progeny for each construct	Overall % of transgenic progeny for all constructs	Figure number for reference
pcx-EGFP (*construct 1*)	Pcb1	5/12	41	—	—	Figure 3a(i)
pcx-EGFP (*construct 1*)	Pcb2	2/9	22	—	—	Figure 3a(ii)
				31.5		
pcx-EGFP (*construct 2*)	Pca1	3/7	42	—	—	Figure 3b(i)
pcx-EGFP (*construct 2*)	Pca2	4/11	0 36	—	—	Figure 3b(ii)
pcx-EGFP (*construct 2*)	Pca3	2/11	18	—	—	Figure 3b(iii)
pcx-EGFP (*construct 2*)	Pca4	5/9	0 55	—	—	Figure 3b(iv)
				37.75		
Hb GFP	Hb1	3/11	27	—	—	Figure 5a(i)
Hb GFP	Hb2	3/9	33	—	—	Figure 5a(ii)
				30		
					33.08	

Since linearized plasmid was used for generation of transgenic rat using our technique, the integration occurred in concatemer fashion in the genome similar to that observed with the pronuclear DNA microinjection procedure. This phenomenon has a demerit of transgene silencing.[8,20] About 46% (6 out of 13) of founders expressed EGFP as detected by the fluorescence activated cell sorting analysis of the hepatocytes obtained from various founders indicating that our method has the ability to generate transgenic rat with optimal gene expression. Immunohistochemical analysis of the section of the liver also confirmed EGFP expression by hepatic cells. Because of transgene silencing due to concatemer formation, several founders are needed to be screened for identifying useful founders, providing a proper expression of the transgene which then can be used to generate transgenic lines. There are methods like the transposon-based transgenesis using the embryo that delivers monomeric transgene units in the genome that rarely get silenced in transgenic rat.[8] However, the generation of various transgenic lines of rat by our method is fast and cost effective. Multiple founders with different level of gene integrations can be easily generated by the researchers because various spermatogonial cells, which eventually make sperm, may incorporate transgene to various extent and patterns. Final selection of appropriate founder where expression of transgene is ideal for desired physiological studies may be done for creating transgenic line with desired expression.

The generation of knockout rat using CRISPR-CAS9 technology by exploiting the male germ line cell transfection *in vitro*, followed by their transplantation, has been reported previously.[21] Since our technique of *in vivo* testicular electroporation of rat testis to deliver foreign gene has a high efficiency, this method has the ability to deliver guide RNA (gRNA) and Cas9 RNA directly into the male germ cell to generate knockout rats. This will be user friendly, less cumbersome, and more cost effective as it does not involve the germ cell transplantation.

Using the above technique, we generated α thalassemic rat model by overexpressing the β chain of Hb. Reduction in concentration of Hb and levels of mean corpuscular volume and mean corpuscular Hb in transgenic rats overexpressing β chain of Hb was similar to that found in α thalassemic patient.[22] The electron microscopic observation of RBCs of transgenic rats revealed deformation similar to that found in cases of human α thalassemia.[23] The appearance of α thalassemia phenotype due to the abundant expression of the β globin chain in transgenic mice was reported previously by Srinoun *et al.*[24] and was also found by us in the transgenic rat. This suggested that using this technique, transgene was stably integrated and transmitted to the next generation with a visible pathophysiological phenotype.

In conclusion, this study confirmed that transgenesis in rat is possible through the testicular route. More importantly, this efficient method will ease the generation of transgenic rats without killing the lives of rats while simultaneously reducing the number of rats used for generation of transgenic animals. These two aspects of our method of generation of transgenic rat followed the principles of the 3Rs concept proposed by Russel and Burch.[25] A disease model of α thalassemia phenotype generated by this technique showing pathophysiologies associated with α thalassemia validated authenticity of this technique. It can be easily adopted by researchers replacing difficult to perform oocyte-mediated male pronuclear DNA injection in rats. This simple, less time consuming, and less complicated method of rat transgenesis will help in generating large number of relevant animal models for biomedical research.

Materials and methods

Animals. Wistar rats (*Rattus norvegicus*) were obtained from the Small Animal Facility of the National Institute of Immunology (New Delhi, India). Animals were kept in a hygienic air condition with suitable humidity and handled by trained personnel. The animals were housed in groups of maximum four animals in cages with their free access to dry pellets and

sterile water. The animals were anesthetized with approved anesthesia before giving mild electric current for standardization of rat transgenesis and also, before blood sampling. Eye ointment was applied in both eyes after anesthetizing the animals. The animals were killed individually in a separate room by carbon dioxide inhalation in an in-house built carbon-dioxide device and by cervical dislocation.

Transgene constructs. A total of three construct were used in this study. The *pCX EGFP* construct 1 promoter (a kind gift from Dr. Y. Junming of the University of Tennesse, Memphis, United States) were used in the detailed study involving standardization of the *in vivo* electroporation technique. The *pCX EGFP* construct 2 (a kind gift from Dr. Masaru Okabe, Osaka University, Japan) having the *EGFP* gene cloned under chicken β-actin was used only for reconfirmation of gene integration and transfer of the transgene to progeny by this method. Further studies involving *pCX EGFP* construct were performed using construct 1 unless otherwise mentioned in the text. These two constructs differ only in their level of EGFP expression because of certain amino acid substitution in the coding region of EGFP; the rest of the plasmid backbone is quite similar. The schematic diagram of *pCX EGFP* construct was given in the **Figure 1a**.

Hb GFP construct was generated by fusing the ORF of the β chain of Hb of rat with GFP which was driven by CMV promoter. For this, the ORF for the β chain of Hb was amplified without a stop codon from the RNA isolated from bone marrow of adult rat. The ORF for the β chain of Hb was fused with the N terminus of *EGFP* of separated from *EGFPN*$_2$ vector with restriction enzymes Sac I and Sac II (*Hb-GFP* construct). The primer sequence for amplifying ORF of the β chain of Hb of rat was given in **Supplementary Table S1**.

Preparation of DNA for in vivo electroporation. The *pCX EGFP* (constructs 1 and 2) and *Hb-GFP* construct were linearized with HindIII and Stu I, respectively, without disturbing their promoter element and gel purified using Qiagen kit (Hilden, Germany).

Electroporation condition standardization using pCX EGFP. Wistar male rats of 40±2 days old were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (8 mg/kg). Hair was removed from the lower abdominal area of rat. Solution of DNA containing trypan blue (0.04%), used for monitoring the accuracy of the injection, was injected slowly into the testis. A 30-gauge needle was used to puncture testicular tissue prior to insertion of Hamilton syringe through the punctured site. Around 30 µl suspension of linearized *pCX EGFP* construct (1 µg/µl) was injected in each testes of 40±2 days old wistar male rat through three different sites. The injections were given on the contralateral side of the epididymis, which is a side of the testis, away from the epididymis as shown in **Figure 1c**. The needle was injected upto a length so that it crossed the outer covering of the testis and skin to enter in the middle axis of the testis. Opening the testis after injection of trypan blue solution, we found that this procedure leads to the presence of trypan blue solution predominantly in the interseminiferous tubular area surrounding the site of injection. The distance between each injection site was kept constant and injections were given along the whole length of the testis at three different places, one of which included the midpoint of testis. Injections at the extreme ends of testis were avoided as these are the regions close to rete testis and cauda epididymis. A time lapse of 30 seconds was given before pulling out the Hamilton syringe to stop the back flow of the DNA solution. This was followed by electroporation by an electric pulse generator (Electroporator-ECM2001 from BTX Instrument Division, Harvard Apparatus, Holliston, MA) using eight square pulses in alternating direction (changing pole of electrode after four pulses). Each pulse was of 0.05 second with an interpulse interval of ~1 second delivered by an electric pulse generator while holding the injected testis in between tweezer-type electrodes. For standardization, electric pulses ranging from 40 to 140 V were given keeping all other parameters (above mentioned) constant. The fore founders (male electroporated either with *pCX EGFP* construct 1 or *pCX EGFP* construct 2) were mated with wild-type females after 60 days of electroporation. The progeny generated from these fore founders were analyzed for presence of transgene.

The electroporated fore founder (electroporated with 90-V electric pulses with a time constant of 0.05 second) was killed 200 days after electroporation and whole testis was observed under a stereo-zoom microscope for visualization of EGFP in testis of rat electroporated with pCX EGFP as described by us before.[11] The sperm were isolated from the epididymis of these fore founder and were analyzed by a flow cytometer for EGFP expression (FACS caliber, BD Biosciences, San Jose, CA). For immuohistochemical localization of EGFP, the testes of electroporated rats were isolated after 15 days of electroporation and fixed in 4% paraformaldehyde for 20 hours before they were processed and embedded in paraffin.[11] After deparafinization, sections were stained for EGFP with mouse anti-GFP antibody (Catalogue#632381, Clontech, Mountain View, CA), rabbit anti-VASA (DDX 4) antibody (Catalogue#ab13840, Abcam, Cambridge, MA) as described by us previously.[11] 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was used to stain the DNA for nuclear signal localization.

The fore founders (male electroporated either with *pCX EGFP* construct 1 or *pCX EGFP* construct 2) were mated with wild-type females after 60 days of electroporation. Analysis of genome from the tail biopsy of the progeny generated from these fore founders with primers specific for transgene (*EGFP*) was perfomed as described by us before.[11] Details of primer were given in **Supplementary Table S2**.

The randomly selected PCR-positive progeny (founders) as well as progeny from a selected line of rat bearing *EGFP as a transgene* was further evaluated by Southern blot analysis for the authenticity of genomic integration. The gDNA was digested by BamH1 and Southern blot was performed as per the standard protocol and was described by us previously.[10] Probe was prepared by amplifying a fragment that contained *EGFP* gene (633 bp) using the P1: *GACGTAAACGGCCACAAGTT*, P2: *GGCGGTCACGAACTCCAG* primers by PCR and was labeled either with DIG or with radioactive P^{32}.

Western blot analysis was performed using protein isolated from various organs (liver, kidney, spleen, and testis)

of rat bearing *EGFP as a transgene* and control rats using the standard protocol described by us before.[10] Probe for β-actin was used as endogenous control in the same blot as described before.[10]

For visualization of EGFP, the PCR-positive F_1 progeny of *GFP* transgenic rat and control rat were killed and the hepatocytes were isolated from the liver and analyzed by a flow cytometer (FACS caliber, BD Biosciences).

A portion of liver from the founder rats as well as control rats were fixed in Bouins solution and immunohistochemistry for EGFP was done. To stain for GFP protein, primary antibody used was mouse monoclonal anti-GFP antibody (Catalogue#632381, Clontech, Mountain View, CA) at a dilution of 1:250 and the secondary antibody of goat antimouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Invitrogen, Grand Island, NY) was used at a dilution of 1:500.

Generation of α thalassemic transgenic rat. The schematic diagram of *HB GFP* construct was given in the **Figure 1a**. The HEK 293 cells were transfected with the *HB GFP* construct using Turbofect transfection reagent (Catalogue#R0531, Fermentas, Grand Island, NY) as per manufacturer recommendation to evaluate the integrity of construct. The expression for EGFP was analyzed under microscope in the presence of UV light using fluorescein isothiocyanate filter. Nontransfected HEK 293 cells were used as the control.

The linearized *Hb-GFP* construct (a total of 30 µg DNA with a concentration of 1 µg/µl) was injected into the testis of 40±2 days old wistar rat and electroporated using eight square 90V electric pulses in alternating direction with a time constant of 0.05 second and an interpulse interval of ~1 second via an electric pulse generator. The electroporated male rats were allowed to mate with wild-type female rats 60 days post electroporation. The pups generated were screened by PCR as described before in the text. For Southern blot analysis, the gDNA obtained from PCR-positive β globin chain overexpressing transgenic rat (various founders) was digested with BamH1 and separated on agarose gel. Southern blot analysis, either with DIG probe or P^{32} probe for *GFP*, was performed for determining propagation of transgene in successive generations (F_1 and F_2) as described before in the text.

Western blot analysis was performed on proteins from various organs (liver, spleen, and kidney) of β globin chain overexpressing transgenic and control rats using antibody against GFP to detect the fusion protein as described before in the text. Probe for β-actin was used as endogenous control in the same blot as described before.

Blood samples were collected from 6 months old β globin chain overexpressing transgenic rats and control rats in tubes coated with dipotassium ethylenediaminetetraacetic acid (K_2EDTA). All tests were done within 2 hours of obtaining the blood samples. The Hb, mean corpuscular volume, and mean corpuscular Hb of blood cells were determined using automated analyzer for hematology.

The blood was collected from the 8 weeks old rats in K_2-EDTA coated tube. The BCB staining of blood was done as described previously.[26,27] A 1% BCB staining solution was made by dissolving 1.0 g of BCB (Sigma-Aldrich, St Louis, MO) in 100 ml of 0.85% normal saline solution. K_2-EDTA blood and 1% BCB staining solution were added to Eppendorf tube at a ratio of 1:1. The mixture was incubated in a 37°C water bath for 1 hour before blood smear was prepared and stained. About 10,000 RBCs were observed for HbH inclusions.

Scanning electron microscopy of the cells from freshly drawn blood samples from 2 months old β globin chain overexpressing transgenic rats and control rats were performed. 250 µl blood collected in K_2EDTA coated tube was washed three times with 0.1 mol/l sodium cacodylate buffer and the pellet was fixed with 2% glutaraldehyde for 30 minutes at room temperature. It was again washed three times with 0.1 mol/l sodium cacodylate buffer and the pellet was suspended in 1% osmium tetroxide for 90 minutes at RT. This was followed by three washes with distilled water. The pellet was suspended in distilled water and allowed to settle on polylysine-coated glass coverslips. The cells were then dehydrated in a graded series of ethanol and dried (critical-point drying). The samples for scanning electron microscopy analysis were mounted on metal grids using double-sided adhesive tape and coated with gold under vacuum before observation. The shapes of RBC were studied by scanning electron microscope (Leo 435VP, Cambridge, UK).

All animals were housed and used as per the national guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals. Protocols for the experiments were approved by the Institutional Animal Ethics Committee and animals were kept according to guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals.

Supplementary material

Figure S1. Immunohistochemical localization of EGFP in the germ cells of electroporated testis of rat.
Figure S2. Southern blot analysis of various founders of GFP rat and HbGFP rat.
Figure S3. Generation of HbGFP construct.
Figure S4. Detection of fusion protein in HbGFP rat.
Figure S5. Expression of GFP in the various organs of GFP transgenic rat.
Figure S6. Vectors map of HbGFP construct.
Table S1. Cloning primers for fishing out beta chain of Hemoglobin from mRNA (of bone marrow).
Table S2. Screening primers for different transgenic rat lines.

Acknowledgments We are thankful to all the staff of the Small Animal Facility. Thanks are due to Ram Singh, Dharamvir Singh and Birendar Roy for the technical assistance. We thank Y. Junming (University of Tennesse, Memphis, United States) for providing the pCX EGFP (construct 1) used in this study. We thank Masaru Okabe (Osaka University, Japan)for providing the pCX EGFP (construct 2) used in this study. We are thankful to P. Nagrajan for providing facilities and expertise during the course of the study. We are thankful to Ms Rekha Rani for the technical help during electron microscopy. We are grateful to the Director, NII for valuable support. We are thankful to Neerja Wadhwa and Hironmoy Sarkar for the help during tissue preparation and microscopy. We are grateful to Department of Biotechnology, Govt. of India, for providing the financial assistance under grants BT/PR11313/

AAQ/01/376/2008, BT/HRD/35/01/01/2010 (TATA Innovation Award) and BT/PR10805/AAQ/1/576/2013. The authors declare that they have no competing interests.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)