Transgenesis via permanent integration of genes in repopulating spermatogonial cells *in vivo*

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Current techniques for making transgenic mice are cumbersome, requiring trained personnel, costly infrastructure and collection of many zygotes from mice that are then killed. We developed a reproducible nonterminal technique for transfecting genes in undifferentiated spermatogonia through *in vivo* electroporation of the testis; about 94% of male mice electroporated with different transgenes successfully sired transgenic pups. Such electroporated males provide a valuable resource for continuous production of transgenic founders for more than a year.

Pronuclear DNA microinjection in the oocyte is the most frequently used technique for generating transgenic mice[1]. This technology is expensive, labor-intensive, time-consuming and requires hundreds of eggs collected from several females. Other methods for generating transgenic mice, such as viral transduction or cloning are also complicated and require coordination of many experimental steps[1]. The complexities of the existing technology and need for several donor females have restricted generation of transgenic cattle for biopharming and transgenic nonhuman primates for their use as surrogates of human diseases[2]. With the availability of complete human and mouse genomes, use of transgenic mice as a model is bound to increase. Hence, there is an urgent need to develop an alternate, cost-effective and more rapid approach for obtaining desired transgenic founders, preferably with minimum or no loss of animal lives.

Recent pioneering studies provided evidence that male germ cells can readily incorporate exogenous DNA in their genome by *in vitro* transfection[3,4]. Although transgenic mice have been generated by heterologus spermatogonial cell transplantation after viral and other modes of transfection *in vitro*, the technique is inefficient and sometimes requires up to 9 months for generation of first progeny in mice[3–5]. Some of the recipient mice reject donor spermatogonial cells[4]. This may also restrict use of this technique in higher mammals where immunocompatible recipients are not readily available. Other attempts at genomic integration of transgenes in male germ cells including those of *in vivo* electroporation of transgenes in the testis yielded limited success[6,7]. Loss of fertility has been observed in some mice after i*n vivo* transfection of testicular germ cells with retroviral constructs carrying a *lacZ* gene at 5–10 d of age, and only 26% of the fertile males sired transgenic mice[8]. This approach did not allow transfection of spermatogonial cells of mature (28–42 d old) mice. The majority of such studies used reporter genes such as *lacZ* and *Gfp* for making transgenic mice[4,5,7]. Virus-mediated gene delivery systems[4,8] provide moderate gene transfer efficiency, but they can induce harmful effects such as uncontrolled inflammation and infection[9]. Therefore, development of an *in vivo* technique for propagation of genes via nonviral transfection of germ cells and long-term maintenance of these cells *in vivo* may lead to important advances in the field of transgenesis.

We describe here a user-friendly, less time-consuming and relatively inexpensive technique for generation of transgenic mice by *in vivo* electroporation of the desired gene into undifferentiated spermatogonia of the testis. In most of the previous studies exploring male germ cells, genes had been delivered into the retetestis or seminiferous tubular lumen, and assisted reproductive techniques were frequently used[10,11]; results were mixed[12]. We delivered genes to the interstitium of the testes because undifferentiated spermatogonial cells are located outside the blood-testis barrier at the basement of the tubules and therefore have direct access to interstitial fluid. We used FVB mice for our study, and protocols were approved by the Institutional animal ethics committee of the National Institute of Immunology (India). We injected various amounts of DNA in different locations and used various conditions of testicular electroporation to standardize the procedure using a linearized *IRES2-Egfp* construct (**Supplementary Methods** online). After injecting linearized DNA interstitially at 3 different sites, we electroporated the testis using a tweezers electrode (**Supplementary Protocol** online). We isolated the seminiferous tubules and cultured them immediately after electroporation. The seminiferous tubules expressed EGFP *in vitro* up to 22 d after electroporation, suggesting that the expression was not episomal (**Fig. 1**). We obtained optimal transfection efficiency by injecting 20–35 μl of linearized DNA (0.5 μg/μl) into the testis of 30 ± 2 d old FVB male mice and then electroporating using 8 square 40-V electric pulses in alternating direction with a time constant of 0.05 s and an inter-pulse interval of ~1 s. Expression of EGFP in the testis of a mouse 50 d after electroporation indicated that the transgene was integrated in the genome (**Supplementary Fig. 1** online).

Production of spermatozoa from undifferentiated spermatogonial cells in mice requires 35 d (ref. 3). We mated mice electroporated with *IRES2-Egfp* with wild-type females, 35 d or more after electroporation. We refer to such electroporated males as

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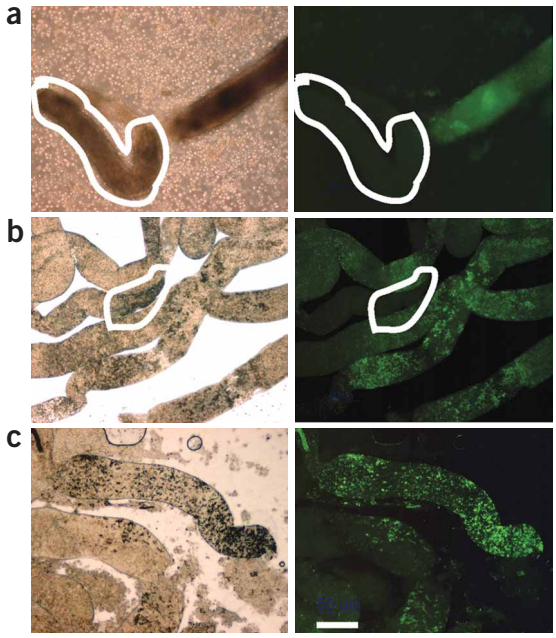

Figure 1 | EGFP expression in the seminiferous tubules after electroporation with a transgene carrying *Egfp*. (**a**–**c**) Phase-contrast images (left) and fluorescence images (right) of the seminiferous tubules, cultured immediately after electroporation with *IRES2-Egfp* show expression of EGFP on day 4 (**a**) day 18 (**b**) and day 22 (**c**) of the culture. Note areas outlined in white are dense in the phase contrast but do not display any fluorescence, confirming specificity of the fluorescence. Scale bar, 50 μm.

forefounders because we used them to generate the founders for propagating the transgene. Amplification of genomic DNA (gDNA) by PCR from the progeny of one of the forefounders, which we designated as 13M, suggested that the transgene was successfully propagated (**Supplementary Fig. 2** online). Southern blot analysis of gDNA from several randomly selected mice from each generation confirmed the PCR results. We detected multiple copies of the transgene by Southern blot of gDNA from the F1 generation. We also observed this banding pattern in Southern blots of gDNA of corresponding F2 and F3 generations (**Supplementary Fig. 3** online).

To validate the procedure of *in vivo* testicular electroporation we tested 3 more transgene constructs, a 5′ *Egfp* fusion of the gene encoding scaffold-matrix attachment region binding protein 1 (*Smar1*, also known as *Banp*) under the control of a *CMV* promoter (*Egfp-Smar1*), *Egfp* driven by a promoter fragment of the gonadal germ cell–specific transcription factor ALF (*Alf-Egfp*; *Alf* is also known as *Gtf2a1l*) and the gene encoding human insulin-like growth factor binding protein 1 driven by the glial fibrillary acidic protein promoter (*Gfap-Igfbp6*). In each case, we electroporated the left testes of mice and surgically removed the right testes to prevent dilution of transgenic sperm with normal sperm. We generated transgenic pups by natural mating. We screened the gDNA of offspring for the presence of the transgene by PCR using transgene-specific primers. The PCR results for all transgenic lines confirmed genomic integration of the transgenes (**Supplementary Figs. 4–6** online). Hemicastration was probably responsible for the high frequency of PCR-positive transgenic pups in the F1 generation of these lines.

We confirmed the PCR amplification results by Southern blot analysis (**Supplementary Fig. 3**). Southern blot analysis of two *Alf-Egfp* transgenic mice (A31 and A33), sired by forefounder designated as 86M, yielded banding patterns different from each other, which were maintained in their respective progeny. This suggested differential gene integration in various spermatogonial cells at the time of electroporation. Hence, our procedure may generate several founders with varying gene expression from a single forefounder.

RT-PCR analysis of mRNA isolated from various organs from F_3 generation of mice revealed that the transgenes were transcriptionally active (**Supplementary Fig. 7** online). Expression of *Smar1* mRNA in lymph nodes, thymus and spleen was higher in *Egfp-Smar1* transgenic mice as compared to wild-type mice (**Supplementary Fig. 8a** online). Mice transgenic for *Egfp-Smar1* also showed a specific cytoplasmic staining for EGFP in hepatocytes (**Supplementary Fig. 8b**), confirming expression of the integrated transgene.

In *Alf-Egfp* transgenic mice, expression of *Egfp* mRNA was limited to the gonads (**Supplementary Fig. 7c**) and was not detected before day 14 postpartum, corresponding to the age at which endogenous *Alf* is transcriptionally active[13] (**Fig. 2a**).

In *Gfap-Igfbp6* transgenic mice, *Igfbp6* mRNA was specifically expressed in the brain (**Fig. 2b**). The hIGFBP6 protein is known to sequester IGF-II from the milieu of astrocytes affecting differentiation of GFAP-expressing cells in the brain[14]. We noticed a decline in *Gfap* mRNA in the brain of these transgenic mice (**Fig. 2b**) similar to that reported for transgenic mice generated by traditional methods using the same construct[14]. Furthermore,

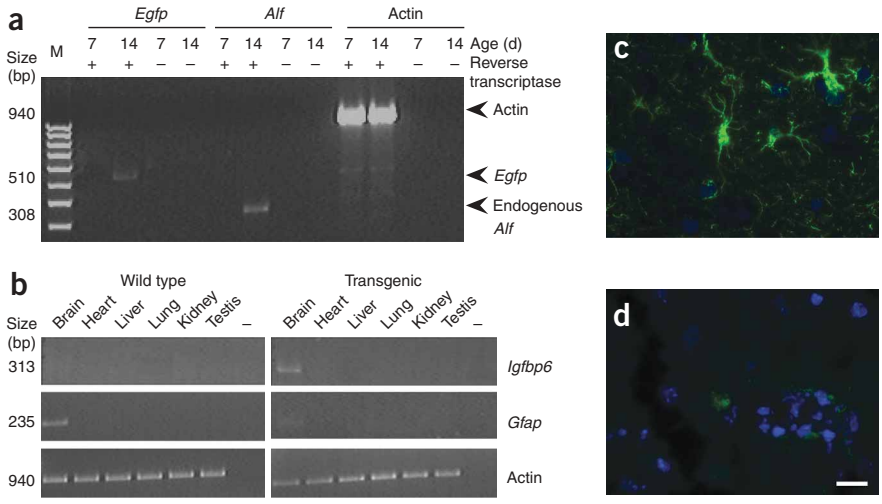

Figure 2 | Analysis of transgene expression in mice. (**a**) RT-PCR analysis of *Egfp*, endogenous *Alf* and actin mRNA in testes of *Alf-Egfp* transgenic mice on days 7 and 14 postpartum. M, marker. (**b**) RT-PCR analysis of *Igfbp6*, endogenous *Gfap* and actin mRNA in the wild-type and *Gfap-IgFBP6* transgenic mice. –, RT-PCR in absence of reverse transcriptase. (**c**,**d**) Immunological staining for GFAP in the cerebella of wild-type mice (**c**) and *Gfap-Igfbp6* transgenic mice (**d**). Scale bar, 10 μm.

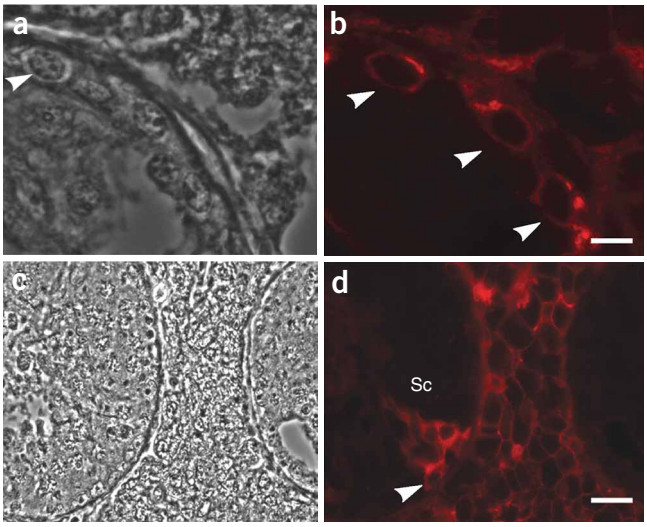

Figure 3 | Immunohistochemical localization of EGFP expression in the testes of forefounders. (**a**–**d**) Phase-contrast images (**a**,**c**) and fluorescence images of the same areas (**b**,**d**) of the testicular sections. The electroporated testes from forefounders (electroporated with *Egfp-Smar1*) were surgically removed 395 d after electroporation, fixed and stained (secondary antibody was conjugated to Texas red). Arrowheads, EGFP-expressing germ cells at the basal compartment of tubule. Sc, a rare EGFP-expressing cytoplasmic extension of an electroporated Sertoli cell found in the seminiferous tubule of the forefounder. Scale bars, 5 μm (**a**,**b**), 10 μm (**c**,**d**).

immunohistochemical analysis revealed that the expression of GFAP in the cerebella of transgenic mice was meager compared to that in wild-type mice (**Fig. 2c**,**d**). Only 10–20% of the brain tissue of transgenic mice expressed GFAP as compared to about 50–75% in that of wild-type mice (**Supplementary Fig. 9** online). This finding of a direct pathophysiological effect of the product of transgene (*Igfbp6*) on the mouse brain[14] greatly strengthens the integrity of our technique.

Notably, one of the forefounders produced transgenic offspring as long as 384 d after electroporation (**Supplementary Table 1** online), indicating that the transgene was integrated in stem and/or transit amplifying undifferentiated spermatogonia at the time of electroporation. Immunohistochemical analysis of the testis removed 395 d after electroporation with the gene carrying *Egfp* displayed EGFP expression in germ cells lying at the basement of a seminiferous tubule (**Fig. 3a**,**b**). Cytoplasmic extensions of one Sertoli cell also expressed EGFP, indicating occasional transfection of this somatic cell (**Fig. 3c**,**d** and **Supplementary Fig. 10a** online). We found several EGFP-expressing germ cells in testes of other forefounders, 300 or more days after electroporation (**Supplementary Fig. 10b**).

To determine whether spermatogonial stem cells (SSCs) were electroporated by our *in vivo* electroporation procedure, we isolated cells from the testes electroporated with the *Egfp* gene, enriched the cells for SSCs using major histocompatibility complex (MHC) class I^- and $Thy1.2^+$ selection by immunomagnetic cell sorting (Miltenyi Biotec) and cultured them on STO feeder cells or mouse embryonic fibroblast (MEF) feeder layer[15] (**Supplementary Note** online). Formation of stem cell–like clusters that expressed EGFP and OCT-4 suggested that the integration of the transgene might have occurred in SSCs at the time of electroporation in these forefounders.

In summary, we electroporated 17 mice using four different constructs and all except one produced transgenic pups, indicating a success rate of >94% for our method (**Supplementary Table 2** online). This and the ability of an electroporated testis to generate founders with differing extent of gene integration will substantially decrease the amount of mice necessary to make transgenic lines. Additionally, this procedure may also facilitate transgenesis in large animals whose gestation period is protracted. Limited use of animals and the potential to produce large numbers of transgenic animals in a short duration are the two major benefits underlying such a proposition. With our technique, first transgenic progeny can be generated within 60 d of electroporation as compared to 82–177 d after *in vivo* viral transfection[8]. To our knowledge, this is the first electroporation-mediated technique for transfection of undifferentiated repopulating spermatogonial cells *in vivo* that resulted in integration and long-term maintenance of the transgene in the germ cell and its transmission via mating. In the past, *in vivo* electroporation either did not result in generation of transgenic animals or required assisted reproductive techniques (intracytoplasmic sperm injection and embryo transfer) to achieve a live birth of transgenic progeny[7,10]. Unlike other *in vivo* transfection procedures[7,8], this method contributes to the prolonged ability, in some cases more than a year, of electroporated founders to sire transgenic progeny by natural mating. The method requires neither assisted reproductive techniques nor sophisticated laboratory setup and highly trained personnel. In addition to contributing to developing an ethically superior (deathless) and easily adaptable time-saving procedure, our new spermatogonia–mediated technique also broadens the potential scope of transgenesis by potentially extending the technology to animals of economic and pathophysiological importance.

Note: Supplementary information is available on the Nature Methods website.

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