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Note

Gene Suppression of Mouse Testis *In Vivo* Using Small Interfering RNA Derived from Plasmid Vectors

Takami Takizawa[1], Tomoko Ishikawa[1], Takuji Kosuge[1], Yoshiaki Mizuguchi[2], Yoko Sato[3], Takehiko Koji[3], Yoshihiko Araki[4] and Toshihiro Takizawa[1]

[1]Department of Molecular Medicine and Anatomy, Nippon Medical School, Tokyo, Japan, [2]Department of Surgery for Organ Function and Biological Regulation, Nippon Medical School, Tokyo, Japan, [3]Department of Histology and Cell Biology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan and [4]Institute for Environmental and Gender-Specific Medicine, Juntendo University Graduate School of Medicine, Chiba, Japan

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We evaluated whether inhibiting gene expression by small interfering RNA (siRNA) can be used for an *in vivo* model using a germ cell-specific gene (*Tex101*) as a model target in mouse testis. We generated plasmid-based expression vectors of siRNA targeting the *Tex101* gene and transfected them into postnatal day 10 mouse testes by *in vivo* electroporation. After optimizing the electroporation conditions using a vector transfected into the mouse testis, a combination of high- and low-voltage pulses showed excellent transfection efficiency for the vectors with minimal tissue damage, but gene suppression was transient. Gene suppression by *in vivo* electroporation may be helpful as an alternative approach when designing experiments to unravel the basic role of testicular molecules.

Key words: siRNA, electroporation, Tex101, mouse testis

I. Introduction

Generating mice through homologous recombination in embryonic stem (ES) cells by disrupting a specific gene is the most potent method to clarify the role of the gene product and to assess its protein function under physiological conditions [1, 5, 8, 11]. The molecular functions of tremendous numbers of gene products have been reported in the past two decades using this methodology. To date, working groups and databases of knock-out (KO) mice such as the International Knockout Mouse Consortium (IKMC; http://www.knockoutmouse.org) are expanding, with the members of the IKMC working together to mutate all protein-coding genes in mice using a combination of gene trapping and gene targeting of C57BL/6 mouse ES cells. Creating KO mice is thus becoming a general experimental approach worldwide, although considerable technical concerns still exist for the gene disruption process to produce KO mice. For example, artificial bacterial chromosomes, which are generally used for gene cloning and targeting vector construction, occasionally contain an unexpected random sequence deletion or mutation that may affect the physiology of the gene-targeted mice. Thus, knock-in mice must be generated with the targeted gene to observe recovery of the gene product function(s) to confirm the actual phenotype of the KO mice, which is both time-consuming and expensive. Additionally, although other species such as cattle can be used to generate genetically null animals [7], the majority of reported KO animals use the mouse ES cell system, which limits the methodology.

To compensate for these disadvantages, gene suppression using synthesized small interfering RNA (siRNA) [2] is widely applied for functional studies. Although the inhibition of gene expression by siRNA is partial and transient in most cases and is applicable mostly to cultured cells, this method has advantages when using cells isolated from various species of animals and plants. Thus, RNA interference (RNAi) by siRNA is quite effective to study cellular function in human medicine.

The aim of the present study was to evaluate whether

Correspondence to: Toshihiro Takizawa, M.D., Ph.D., Department of Molecular Medicine and Anatomy, Nippon Medical School, 1–1–5 Sendagi, Tokyo 113–8602, Japan. E-mail: t-takizawa@nms.ac.jp

inhibiting gene expression by siRNA can be used in an *in vivo* model with a germ cell-specific gene (*Tex101*) [4, 10] as a model target in mouse testis. TEX101 is a germ-cell marker that is expressed during gametogenesis and shows sexually dimorphic expression in developing gonadal tissues [4, 10], suggesting that it might play an important role in germ-cell development and differentiation. However, its reproductive functions remain to be elucidated. We generated plasmid-based expression vectors of siRNA targeting the *Tex101* gene and transfected them into mouse testes using *in vivo* electroporation.

II. Materials and Methods

Animals

Postnatal day 10 (P10) BALB/c male mice were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, Nippon Medical School.

Plasmid-based RNAi

Coding sequences for mouse *Tex101* (MGI: 1930791) were targeted by 21mer siRNA sequences designed using the BLOCK-iT RNAi Designer (Invitrogen, Carlsbad, CA, USA). The obtained target sequences, (*Tex101*–251) 5'-AC GATCCAGGCCGTACCTTTA-3', (*Tex101*–399) 5'-GGC GGTGACTTTCATCCAGTA-3', and (*Tex101*–761) 5'-AG ACCTGTAGTTACCAGTCAT-3', were not significantly homologous to other mouse proteins as determined by a Blast analysis. The sequences were used to generate oligonucleotides targeting *Tex101*. The oligonucleotides were inserted into the pcDNA6.2-GW/EmGFP-miR (Invitrogen), which has co-cistronic expression of emerald green fluorescent protein (EmGFP), allowing for the determination of transfection efficiency by fluorescence microscopy. Three RNAi vectors targeting the *Tex101* gene (designated RNAi *Tex101*–251, RNAi *Tex101*–399, and RNAi *Tex101*–761) were sequence-verified. The pcDNA6.2-GW/EmGFP-miR-negative control plasmid contains an oligonucleotide targeting *lacZ*, which served as a negative control (designated RNAi *lacZ*). The function of these RNAi vectors was tested by quantitative RT-PCR and Western blot to verify knockdown of COS-7 cells transfected with the TEX101 expression vector (designated pCD-TEX101) [3]. The oligonucleotides were also subcloned into a pCAG vector [6], which was kindly provided by Prof. Jun-ichi Miyazaki of Osaka University.

In vivo electroporation

Plasmid vectors for injection were dissolved in Tris-EDTA buffer (pH 8.0) or phosphate-buffered saline (PBS) at 1–20 mg/ml with 0.04% trypan blue to monitor needle positioning and injection accuracy. Approximately 2–5 μl of vector solution was directly injected into P10 testis using a 50-μl syringe (part no. 80920, Hamilton, Reno, NV, USA) attached to a 27-gauge needle (part no. 7750-18, Hamilton). The right testis was injected with RNAi *Tex101*, and the left testis was injected with the control vector (RNAi *lacZ*). Electric pulses were generated with an electric pulse generator (Electroporator CUY21EDIT, NEPA GENE, Chiba, Japan). Testes were held between a pair of tweezer-type electrodes (CUY650P3, NEPA GENE), and square electric pulses were applied.

Immunohistochemistry analysis

Bilateral testes were removed from mice at 1, 3, 7, and 14 days after the RNAi-vector injection and electroporation. The number of mice killed on each day (at 1, 3, 7, and 14 days after injection) was 3, 15, 25, and 14, respectively. Testes were fixed for 2 hr at room temperature in 4% paraformaldehyde in PBS (pH 7.4). Tissue sections (5 μm thick) were cut with a cryostat (HYRAX C 50, Carl Zeiss, Jena, Germany), mounted on 3-aminopropyltriethoxy-silane-coated round glass coverslips, allowed to air-dry, and then washed three times in PBS). The sections were incubated first with 1% bovine serum albumin in PBS for 1 hr at room temperature to block nonspecific protein-binding sites and then with a mouse anti-TEX101 monoclonal antibody (0.03 μg/ml) [4] and a rabbit anti-GFP polyclonal antibody (5 μg/ml; Invitrogen) for 30 min at 37°C, followed by five washes in PBS. The sections were then incubated for 30 min at 37°C with Alexa Fluor-594-labeled goat anti-mouse IgG (10 μg/ml) and Alexa Fluor-488-labeled goat anti-rabbit IgG (10 μg/ml) (Molecular Probes, Eugene, OR, USA), washed three times in PBS, and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (Molecular Probes). After five additional washes in PBS, the sections were mounted in ProLong Gold Antifade Reagent Medium (Molecular Probes) on glass microscope slides and examined under a microscope (BX60, Olympus, Tokyo, Japan) equipped with a Spot RT SE6 CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Control sections received the same treatment, except that the primary antibody was either omitted or replaced with purified nonimmune mouse IgG.

III. Results and Discussion

Tex101 mRNA is expressed in spermatocytes and spermatids with the first wave of spermatogenesis from P12 onward but is undetectable in spermatogonia [10]. Thus, we investigated the effect of RNAi *Tex101* just before the first wave of spermatogenesis (i.e., P10). Because the suppression ability of single RNAi *Tex101* was satisfactory in most trials (Fig. 1), we used RNAi *Tex101*–399 for subsequent experiments. We optimized the electroporation conditions for transfection with a plasmid-based siRNA expression vector in the mouse testis (Table 1). A square electric-pulse application in two different directions at a 40 V pulse of 10 ms duration with two subsequent 20 V pulses of 50 ms duration comprised the most efficient method for *in vivo* electroporation of the P10 mouse testis. At 3 days post-transfection, many GFP-positive cells including germ cells

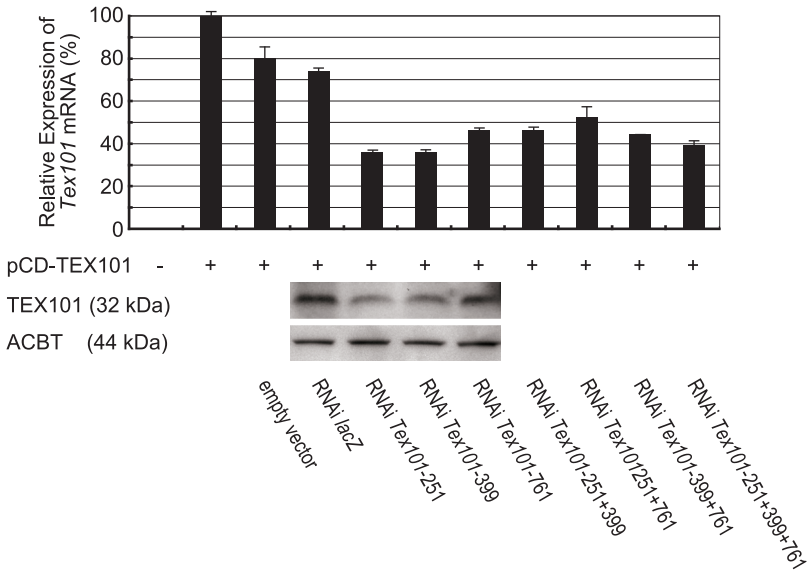

Fig. 1. Suppression of *Tex101* expression by plasmid-based small interfering RNA (siRNA) in COS-7 cells transected with pCD-TEX101. The relative expression level of *Tex101* mRNA was determined by real-time PCR. The mean *Tex101* expression value in the pCD-TEX101-transfected cells was "100%". TEX101 protein expression level was determined by Western blot. ACBT was used as loading control.

Table 1. *Summary of electric pulse conditions for electroporation in P10 mouse testis*

Voltage (V) of Electric Pulse (EP)	Number of EP-Applications (per direction)	EP Duration (ms)	Number of EP Direction Changes	Total EP Duration (ms)
20V-EP	1~10	50	2	100~1000
30V-EP	6~10	50~80	2	960~1000
40V-EP	2~6	10~80	2	40~960
40V-EP with subsequent 20V-EP	1 (40V-EP) → 2~10 (20V-EP)	5~50 (40V-EP) → 10~999 (20V-EP)	2	10~100 (40V-EP) → 200~19980 (20V-EP)
60V-EP with subsequent 20V-EP	1 (60V-EP) → 4 (20V-EP)	5~20 (60V-EP) → 50 (20V-EP)	2	10~40 (60V-EP) → 400 (20V-EP)
80V-EP with subsequent 20V-EP	1 (80V-EP) → 4 (20V-EP)	5~50 (80V-EP) → 50 (20V-EP)	2	10~100 (80V-EP) → 400 (20V-EP)

(spermatogonia, young spermatocytes), Sertoli cells, and stromal cells appeared in the RNAi vector-transfected testes (Fig. 2A, B). At this time, GFP-positive and TEX101-negative young spermatocytes (e.g., zygotene/pachytene spermatocytes) remaining in the basal compartment of the seminiferous epithelium were observable in RNAi *Tex101*-transfected testes (Fig. 2C–E). It should be noted that RNAi *Tex101* suppressed the expression of TEX101 in the spermatocytes. In contrast, GFP-positive and TEX101-positive young spermatocytes (e.g., pachytene spermatocytes) were visible in the adluminal compartment of the seminiferous epithelium in RNAi *lacZ*-transfected testes (controls; Fig. 2F–H). Unfortunately, GFP-positive germ cells decreased markedly 7 days after transfection (data not shown). In addition, the best transfection efficiency (ratio of seminiferous tubular cross-sections having more than one GFP-positive cell to total number of cross-sections counted) was approximately 50%, although only a few cells (germ cells and/or Sertoli cells) were detected as GFP-positive cells in some cross-sections. These results suggest that *in vivo* gene suppression using plasmid-based siRNA expression vectors targeting *Tex101* gene was transient, and that a functional analysis of the gene product would be complicated compared to studies using conventional KO mice. *Tex101* mRNA is expressed in spermatocytes and spermatids of the testis [9]. Moreover, TEX101 protein remains on the cell surfaces of testicular sperm [9, 10]. For *in vivo* analysis of *Tex101* suppression during spermatogenesis, RNAi *Tex101* should be stably expressed in germ cells at least for 1 month after transfection since testicular sperm appears at P28. Thus, we must reconsider and reevaluate the vector characteristics. Other systems such as virus vectors may be more suitable for *in vivo* gene suppression by siRNA.

At present, we cannot confirm the physiological function(s) of TEX101, as we have no definitive experimental data obtained from *Tex101* KO mice. However, our results indicate that *in vivo* electroporation is a suitable method to

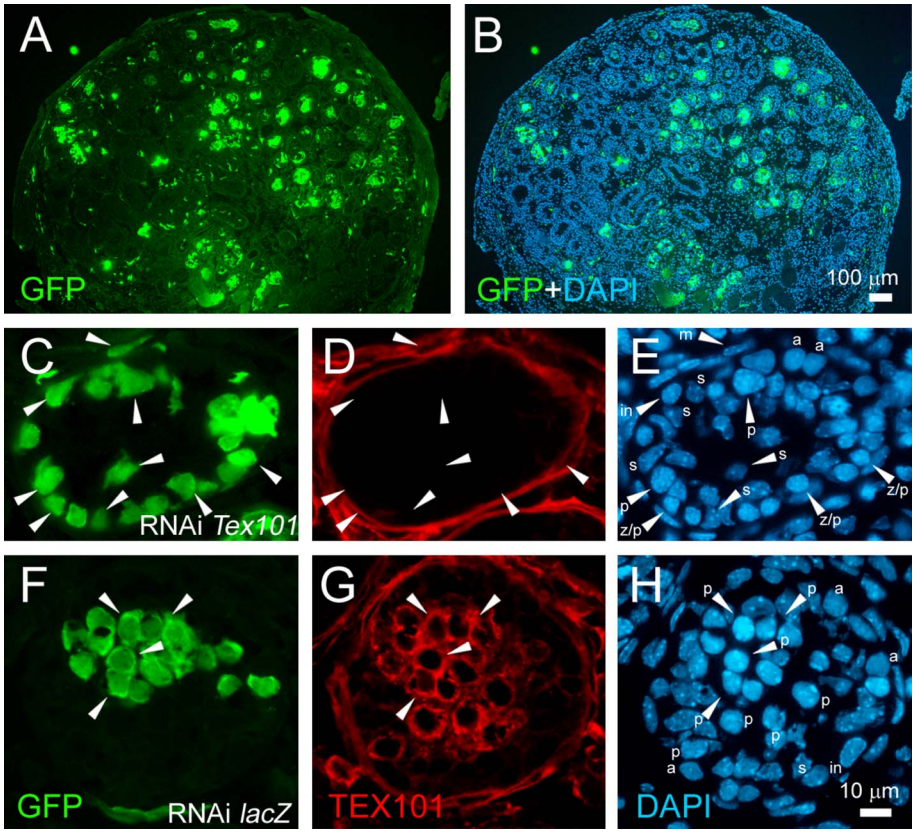

Fig. 2. Immunohistochemical analysis of the RNAi *Tex101*-transfected P10 mouse testis. (**A**) Low magnification view of green fluorescent protein (GFP) immunostaining of mouse testis 3 days after transfection. (**B**) DAPI-stained image of the same section shown in **A**. Seminiferous tubules transfected with plasmid-based RNAi vectors were co-cistronically expressed with GFP. Images in **A** and **B** are at the same magnification. (**C–E**) Higher magnification views of the RNAi *Tex101*-transfected testis. GFP-positive (**C**) and TEX101-negative (**D**) zygotene/pachytene spermatocytes (z/p) were present in the basal compartment of the seminiferous epithelium in the RNAi *Tex101*-transfected testis. The same section with DAPI-stained nuclei is shown in **E**. (**F–H**) Higher magnification views of the RNAi *lacZ*-transfected testis. In contrast, GFP-positive (**F**) and TEX101-positive (**G**) pachytene spermatocytes (p) were visible in the adluminal compartment of the seminiferous epithelium in the RNAi *lacZ*-transfected testes. The same section with DAPI-stained nuclei is shown in **H**. Some GFP-positive cells are indicated with arrowheads. Type A (a) and intermediate (in) spermatogonia, Sertoli cells (s), and myoid cells (m) are evident. Images in **C–H** are at the same magnification.

deliver siRNA to study cellular functions of gene products in the testis, when the pulse conditions are optimized appropriately. In this study, 40-V electric pulses of relatively long duration often caused tissue damage (e.g., degeneration of seminiferous tubules) at the pulse-applied sites. In contrast, relatively low-voltage pulse applications (i.e., 20 V) showed low transfection efficiency of the plasmid-based RNAi vector in the P10 mouse testis. A combination of high- and low-voltage pulses resulted in excellent transfection efficiency of the vectors with minimal tissue damage (Fig. 2A, B). Compared to other siRNA delivery systems, such as viral vectors or chemical transfection, electroporation has some advantages because it is noninvasive and can be performed without specific immune stimulation. Although electroporation has the disadvantage of causing tissue damage [12], the testis is an organ with an ability to resist damage because the seminiferous tubules are surrounded by tunica albuginea, a physically stable connective tissue. Therefore, this methodology would be useful for filling-up in the study between *in vitro* and *in vivo* models when using specific organs like testis. Our data appear to be applicable to investigate testicular molecules in general, and may facilitate our understanding of the function of gene products before *in vivo* functional analyses, such as loss-of-function assays in mice by targeted gene disruption. Although more precise optimization is necessary, this method would be helpful for designing experiments to unravel the basic role of testicular molecules.

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