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# Pronuclear microinjection is not suitable for RNA polymerase III promoter driven constitutive RNAi transgenesis in mice for XY male-to-female sex reversal by *Sry* gene knockdown

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## ABSTRACT

**Silencing of gene expression by RNA interference (RNAi) has become a widely used tool. For the study of mammalian gene function expression vectors for short hairpin RNA (shRNA) were developed. However the standard methods of shRNA transgenic (Tg) mice production have not been established. *Sry* (sex-determining region on the Y chromosome) is a mammalian sex-determining gene on the Y chromosome. In mice, the transient expression of *Sry* in supporting cell precursor cells between 10.5 and 12.5 days post-coitus (dpc) triggers the differentiation of Sertoli cells from granulosa cells. Then high efficiency of *Sry* gene silencing in Tg mice should induce XY male-to-female sex reversal. An shRNA Tg mouse targeting *Sry* gene was attempted to be generated by pronuclear microinjection. A low rate (Tg pups/all pups born after microinjection = 2/154 to 7/178) of Tg pups was observed. These Tg mice showed no XY male-to-female sex reversal. The results suggest that exogenous expression of small RNA might exert a negative effect on embryonic development and another approach should be needed for RNAi transgenesis in mice.**

**Keywords:** Transgenic Mice; Pronuclear Microinjection; RNA Interference

## 1. INTRODUCTION

RNA interference (RNAi) has become a routine genetic tool to study gene function in mammalian cells including cancer cells [1]. Short hairpin RNA (shRNA) expression vectors have been developed which can be designed for knockdown of specific gene expression [2]. However the standard methods of shRNA transgenic (Tg) mice production have not been established. As RNA polymerase III (polIII) promoter such as U6, H1 and tRNA is ubiquitous promoter that is commonly used for shRNA expression, the phenotype of shRNA Tg mice should be similar to the phenotype of the knockout mice of the target gene. *Sry* (Sex determining region on the Y chromosome) is a transcription factor with a DNA-binding domain referred to as the high mobility group (HMG), which triggers a gene expression cascade required for initiating male sex differentiation in the bipotential indifferent gonads of mammals [3]. Mouse *Sry* is expressed for a brief period between 10.5 and 12.5 days post-coitus (dpc) in the supporting cells of undifferentiated gonads that differentiate into Sertoli cells instead of granulosa cells [4-6]. For specific purposes, shRNA targeting *Sry* was constructed [7] and the corresponding Tg mice were then attempted to be generated. As the pronuclear microinjection method is standard method for production of Tg mice, shRNA Tg mice were produced by pronuclear microinjection method in this study. If *Sry* knockdown in mice would be success, XY female-to-male sex reversal should be occurred. Then shRNA targeting *Sry* gene will be useful tool to establish the methods of RNAi transgenesis in mice.

## 2. MATERIALS AND METHODS

### 2.1. Animals

The following strains of mice were purchased from a commercial animal breeder (Sankyo Labo-Service Corporation, Inc., Tokyo, Japan): B6C3F1 (C57BL/6NxC3H/He), C57BL/6J, and ICR. The mice were kept in an environment with regulated temperature (22˚C - 25˚C), humidity (40% - 50%), and illumination cycles (14-h light, 10-h dark), and were provided with food and water *ad libitum*. The present study was conducted according to the committee guidelines of the National Institute of Advanced Industrial Science and Technology for animal

experiments and the Law for Prevention of Cruelty to Animals and the Guide for Care and Use of Laboratory Animals in Japan. The experiments complied with current Japanese laws.

### 2.2. Tg Mouse Generation

Tg mice were generated by microinjecting DNA into the pronuclei of zygotes collected from the oviducts of superovulated B6C3F1 females that were mated with B6C3F1 males. All methods for generating the Tg mice used here have been described in the protocol reported by Hogan *et al.* [8]. The construction of U6-shRNA1 (containing one shRNA expressing construct) and tRNA-shRNA1-8 + Rz3 (eight shRNA and one ribozyme expressing constructs were connected) targeting *Sry* gene were described previously [7]. The *Eco*RI/*Hin*dIII DNA fragment for U6-shRNA1 and the *Eco*RI/*Mfe*I DNA fragment for tRNA-shRNA1-8 + Rz3 were excised and separated by electrophoresis through 1% agarose gel; the fragment was then purified by CsCl ultra-centrifugation. The purified DNA fragment was dissolved in a solution containing 10 mM Tris-HCl (pH 7.4) and 0.25 mM EDTA (pH 7.4) and was used for pronuclear microinjection. To identify Tg animals and sex chromosome karyotypes (XX or XY), genomic DNA was isolated from the tip of the tail, and the genomic DNA was screened by polymerase chain reaction (PCR) amplification as previously described [9]. Transcripts of the *Sry* in the XY gonads at 11.5 dpc were determined by reverse transcription polymerase chain reaction (RT-PCR) as previously described [9].

### 2.3. Histology

For the histological analysis, tissues were fixed in 4% paraformaldehyde. After fixation, the tissues were processed for paraffin embedding as previously described [6]. The tissues were then sectioned at 6 μm, and the sections were used for hematoxylin and eosin staining.

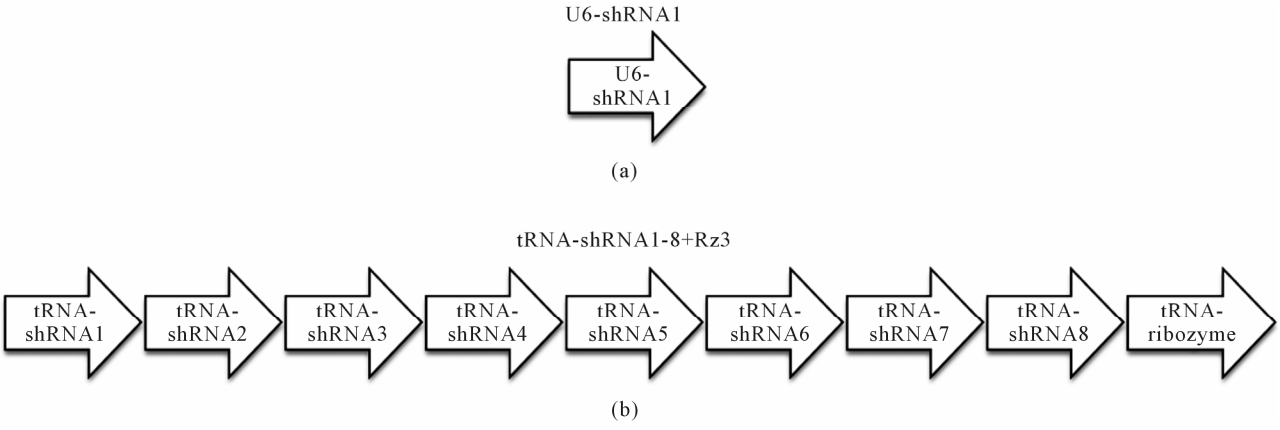
## 3. RESULTS

### Production of Tg Mice

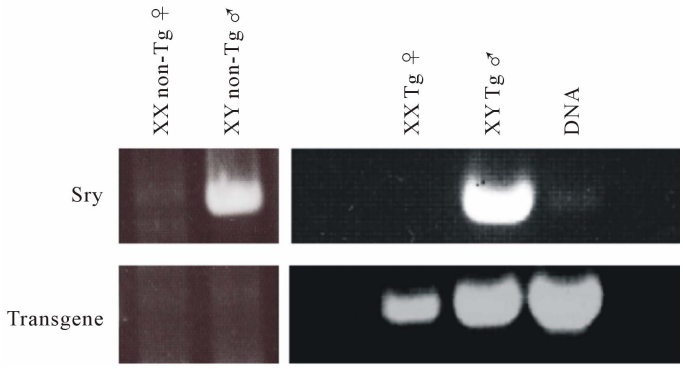
The constructs of shRNA targeting *Sry* gene (U6-shRNA1 or tRNA-shRNA1-8 + Rz3) were used for DNA microinjection to produce Tg mice (**Figures 1(a)** and **(b)**). The constructs were selected since these constructs were exhibited the highest silencing effect toward *Sry* [7]. In the analyses of DNA pups born, the percentages of Tg mice were 3.9% (Tg pups/all pups born after microinjection = 7/178) for U6-shRNA1 and 1.3% (Tg pups/all pups born after microinjection = 2/154) for tRNA-shRNA1-8 + Rz3. Seven U6-shRNA1 Tg lines (5 female and 2 male) were analyzed. All Tg mice were fertile. The female karyotype was XX, and that of the male was XY. $F_1$ XY Tg mice from breeding with the all five $F_0$ XX female Tg mice were male (**Figure 1(c)**). Two tRNA-shRNA1-8 + Rz3 Tg lines (female and male) were analyzed. The female karyotype was XX, and that of the male was XY. All Tg mice were fertile. $F_1$ XY Tg mice from breeding with the $F_0$ XX female Tg mouse were male. There was no histological abnormality in the testis of the Tg mice (**Figures 1(e)** and **(f)**). *Sry* expression levels of Tg XY gonads at 11.5 dpc were not lower than that of wild-type littermates (**Figure 1(d)**). Thus, shRNA was silenced at the inserted locus and not expressed in Tg tissues or *Sry* expression was not suppressed by the shRNA. Then, male-to-female sex reversal could not occur with the polIII promoter driven constitutive shRNA expressing transgene by pronuclear microinjection method.

## 4. DISCUSSION

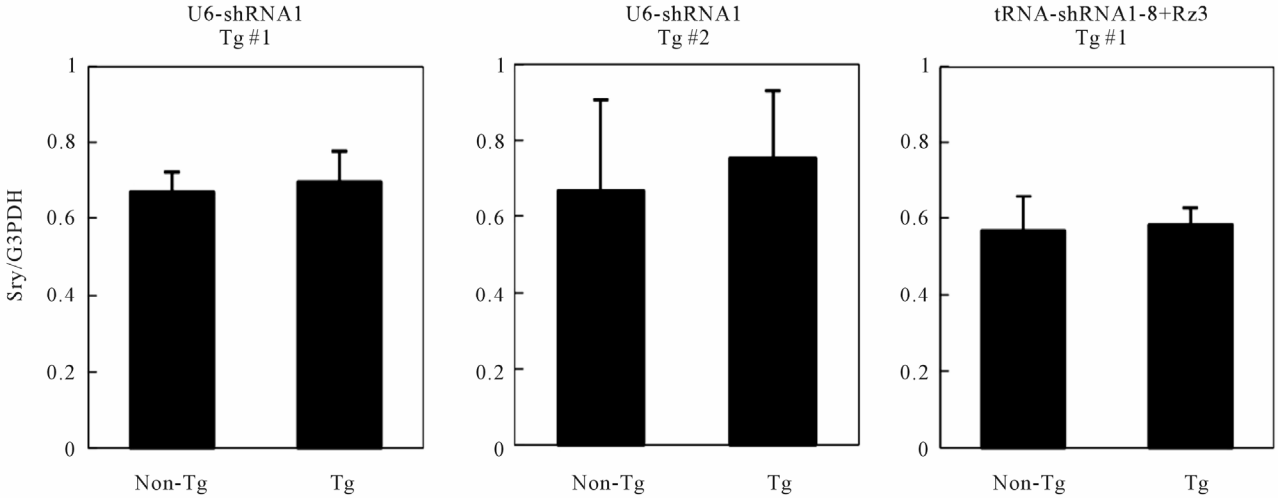
In this study, an shRNA Tg mouse production approach was used to repress the expression of *Sry*. Foreign mouse *Sry* has been shown to induce XX female-to-male sex reversal [3]. Then it was expected that transgenesis of shRNA targeting *Sry* would induce XY male-to-female sex reversal. Tg mice expressing shRNA targeting *Sry*



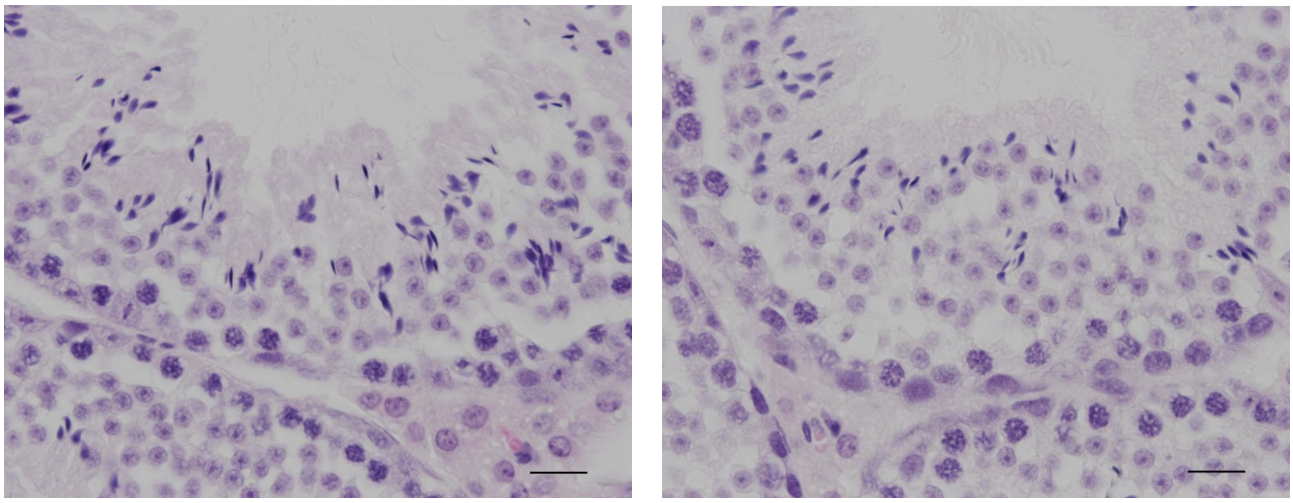

 




(c)




(d)



(e) (f)

**Figure 1.** Production and analysis of transgenic (Tg) mice. (a), (b) A schematic representation of the transgene construct used in the microinjection; U6-shRNA1 and tRNA-shRNA1-8 + Rz3; (c) PCR analysis with the genomic DNA to identify the U6-shRNA1 Tg mice and the presence of the Y chromosome. Endogenous *Sry* was used to identify the presence of the Y chromosome. Tg, transgenic; non-Tg, non-transgenic; DNA, Injection DNA control; XX, XX karyotype; XY, XY karyotype; ♀, female phenotype; ♂, male phenotype; (d) *Sry* expression levels relative to those of *G3PDH* in the XY gonads of each Tg line at 11.5 dpc. Expression levels of *Sry* in the Tg mice were not reduced. Values are expressed as the mean ± S.E.M. (n = 3); (e), (f) Hematoxylin and eosin staining of testis from tRNA-shRNA1-8 + Rz3 Tg mice (e) and wild-type littermates (f) bar, 20 μm.

were attempted to be generated. The efficiency of transgenesis was remarkably low (1.3% to 3.9%) compared with the efficiency of usual Tg experiments [10-13]. Furthermore, no XY male-to-female sex reversal occurred with the transgene. All the female Tg mice were characterized as XX and the male Tg mice were characterized as XY. This result might suggest that ubiquitous and strong expression of shRNA yields embryonic lethality; the integration of the transgene at the locus where the transgene is silenced could explain why the XY sex reversal was not occurred in the Tg mice. It is also possible that the design of shRNA sequences was not suitable for suppression of *Sry* expression. However the shRNA constructs exhibited the strong silencing effect toward *Sry* gene in the cultured cells [7]. The knockdown efficiency of the shRNA constructs *in vivo* may be confirmed by organ culture on agar blocks and magnetically induced transfection using embryonic genital ridges [14] or by injection into tail vein of pregnant mice [15].

For transgenic RNAi in mice, one-cell embryos [16-29] or embryonic stem (ES) cells [30-37] have been used as the target (**Table 1**). The methods of ES cells need several months for many steps such as vector construction, clone isolation, chimera mouse production and breeding. However ES cells have made possible a ubiquitously active locus (e.g., *ColA*1 and *Rosa*26) targeting transgene integration [34-37]. One-cell embryos have been used for pronuclear microinjection [16-25] or lentivirus infection [17,26-29]. Long double strand RNA (dsRNA) (around 500bp) expressing vectors were constructed for microinjection [19-23]. However these constructs were effective only oocytes or early embryos as expression of dsRNA induces apoptosis by dsRNA-dependent protein kinase (PKR) in most mammalian cell types [38]. In the case of shRNA expressing constructs, Carmell *et al.* reported that Tg mice produced by pronuclear microinjection did not show a phenotype that was expected for the targeted genes such as tyrosinase (albino), myosin VIIa (shaker), Bmp-5 (crinkled ears), Hox a10 (limb defects), homogentisate 1,2-dioxygenase (urine turns black upon exposure to air), Hairless (hair loss) and melanocortin 1 receptor (yellow) [17]. The report agrees with the present study in that pronuclear microinjection is not suitable for RNAi transgenesis. On the other hand Peng *et al.* reported that pronuclear microinjection of shRNA-expressing constructs was used for genetic screening in mice. In the study one of the transgene targeting *p*57 was effective and the knockdown effect was transmitted for many generations [18]. Then it might depend on the targeting gene whether the shRNA-expressing constructs have knockdown effect in the Tg mice produced by pronuclear microinjection. The method of lentivirus infection has some advantages over the pronuclear microinjection method [26]. First, the injection of the lentivirus into the perivitelline space is less invasive than the pronuclear microinjection. Second, the efficiency of transgenesis is much higher than that of pronuclear microinjection. Third, single copy insertions are occurred by lentivirus infection. On the other hand multiple copies are inserted into one locus in the most cases of transgenesis by the pronuclear microinjection method. Single copy insertion is attractive for RNAi transgenesis since it is expected that too high expression of shRNA should induce embryonic lethality in this study. Moreover high efficiency of transgenesis would make it easy to analyze $F_0$ Tg embryos and it should be useful tool when the

**Table 1.** Application of RNAi for genetics of mice.

| Target | Method | Expression system | Reference |
|---|---|---|---|
| One-cell embryos | microinjection | constitutive polIII | [16-18] |
| | | constitutive polII | [19] |
| | | oocyte specific polII | [20-23] |
| | | Cre/*loxP* | [24] |
| | | tetracycline-responsive polII | [25] |
| | lentiviral infection | constitutive polIII | [17,26-29] |
| ES cells | electroporation | constitutive polIII | [30,31] |
| | lentiviral infection | constitutive polIII | [28] |
| | | Cre/*loxP* | [32,33] |
| | site specific recombination | tetracycline-responsive polIII | [34] |
| | | tetracycline-responsive polII | [35-37] |

gene knockdown causes embryonic lethality. $F_0$ Tg embryos also can be analyzed using tetraploid aggregation technique and ES cells containing shRNA-expressing construct [30-33]. Kirilov *et al.* reported that lentivirus mediated $F_0$ Tg mice were genetically mosaic and the mosaicism led to a reduced rate of germ line transmission [29]. It is possible that the overexpression of shRNA might have interfered with the processing of endogenous micro RNA (miRNA) and should be cause of the high mosaicism and the low degree of germ line transmission. To overcome the problem, inducible shRNA expression system should be useful such as Cre/*loxP* system [24,32, 33] and tetracycline system [25,34-37]. In the Cre/*loxP* system, a *loxP*-flanked stuffer sequence should be inserted into the shRNA expression construct and can be removed by Cre. After the recombination, shRNA can be expressed and work for target gene knockdown [24,32, 33]. Tissue and stage specific promoter can be used for the control of Cre expression and the Cre Tg mouse will be used for the spatiotemporally controlled shRNA expression. Chemical inducible Cre recombination system also has been developed using doxycycline (a member of the tetracycline antibiotics) dependent transcription or tamoxifen (an agonist/antagonist of the estrogen receptor) dependent nuclear translocation. The tetracycline system should need two Tg lines—the tetracycline transactivator (tTA; for Tet-Off) or reverse tTA (rtTA; for Tet-On) expressing line and the line containing the construct of shRNA under the control of tetracycline responsive promoter. Dickins *et al.* established Tg lines expressing miRNA based shRNA controlled by tetracycline responsive–cytomegalovirus promoter using pronuclear microinjection method, and the doxycycline-dependent reversible induction was worked effectively [25]. More-

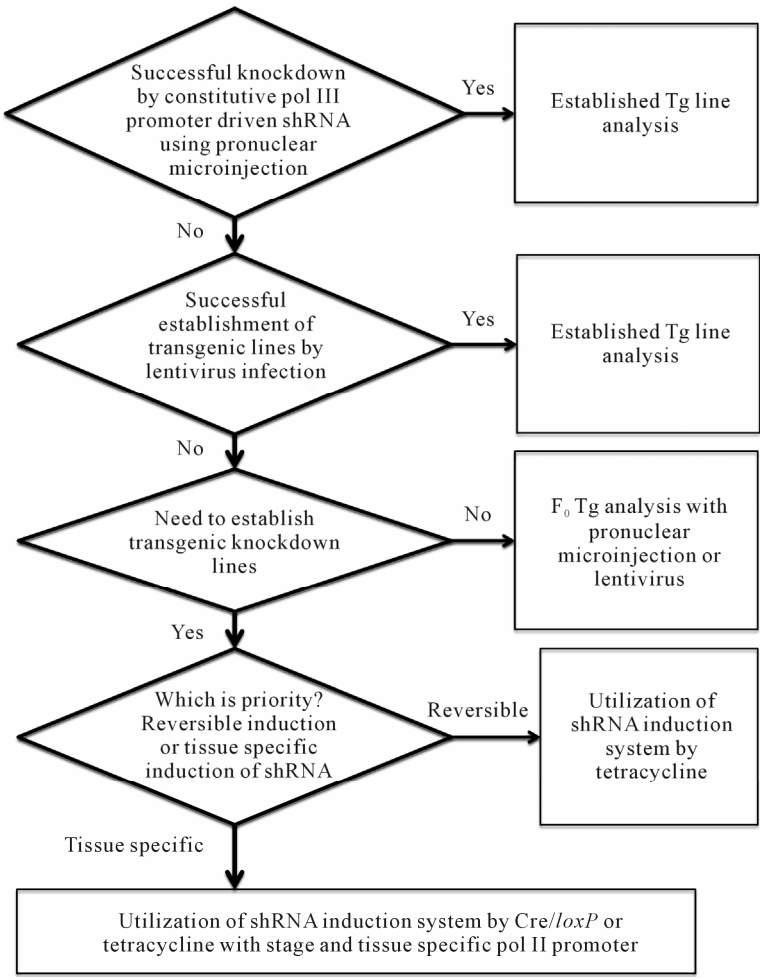



**Figure 2.** Flowchart to assist selecting the most appropriate method for RNAi transgenesis. As summarized in the text, strong ubiquitous expression of small RNA might induce embryonic lethality. It makes it impossible to establish constitutive shRNA expressing Tg lines. When establishment of Tg lines were failed for such a reason inducible shRNA system should be tried. The tetracycline system and Cre/*loxP* system should be suitable.

over, tissue specific polII promoter would be applied for the tetracycline-dependent shRNA expression system [25]. However, Cre/*loxP* system might be more useful than tetracycline system since strong transgene expression can be switched on by Cre/*loxP* system when weak polII promoter such as *Sry* promoter would be used [6, 39,40]. As blood-brain barrier and blood-testis barrier have low permeability to doxycycline, the tetracycline inducible system may not be applicable for the expression in brain and testis [41]. As for the knockdown of *Sry* gene at the embryonic stage the tetracycline inducible system should be applicable since shRNA expression was induced in Tg embryos when the water or food pellets containing doxycycline were supplied to the pregnant mothers [35,36]. In conclusion the pronuclear microinjection method is not suitable for the generation of constitutive RNAi Tg mice for *Sry* gene knockdown. Recently, methods of conditional RNAi transgenesis in mice have been developed [42,43]. **Figure 2** shows flowchart for selection of an appropriate RNAi transgenesis method. As male-to-female sex reversal is one of the most obvious phenotype, *Sry* is useful as a target gene to test the RNAi transgenesis method.

## 5. ACKNOWLEDGEMENTS

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