—Original Article—

Transgenic mouse offspring generated by ROSI

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Abstract. The production of transgenic animals is an important tool for experimental and applied biology. Over the years, many approaches for the production of transgenic animals have been tried, including pronuclear microinjection, sperm-mediated gene transfer, transfection of male germ cells, somatic cell nuclear transfer and the use of lentiviral vectors. In the present study, we developed a new transgene delivery approach, and we report for the first time the production of transgenic animals by co-injection of DNA and round spermatid nuclei into non-fertilized mouse oocytes (ROSI). The transgene used was a construct containing the human CMV immediate early promoter and the enhanced GFP gene. With this procedure, 12% of the live offspring we obtained carried the transgene. This efficiency of transgenic production by ROSI was similar to the efficiency by pronuclear injection or intracytoplasmic injection of male gamete nuclei (ICSI). However, ICSI required fewer embryos to produce the same number of transgenic animals. The expression of *Egfp* mRNA and fluorescence of EGFP were found in the majority of the organs examined in 4 transgenic lines generated by ROSI. Tissue morphology and transgene expression were not distinguishable between transgenic animals produced by ROSI or pronuclear injection. Furthermore, our results are of particular interest because they indicate that the transgene incorporation mediated by intracytoplasmic injection of male gamete nuclei is not an exclusive property of mature sperm cell nuclei with compact chromatin but it can be accomplished with immature sperm cell nuclei with decondensed chromatin as well. The present study also provides alternative procedures for transgene delivery into embryos or reconstituted oocytes.
Key words: Intracytoplasmic sperm injection (ICSI), Round spermatid nucleus injection (ROSI), Transgenesis
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The production of transgenic animals is an important tool for experimental and applied biology [1]. The possibility of generating transgenic organisms able of expressing foreign genes allows the study of gene function and regulation *in vivo*. Transgenic animals have many diverse applications (reviewed in [2] and [3]). They can be used in toxicology as test animals, in mammalian developmental genetics, for the introduction of human genes associated with disease processes and comprehension of molecular mechanisms, for the analysis of the regulation of gene expression, for the production of pharmaceutical proteins in farm animals and non-pharmaceutical proteins in biotechnology, for genetic engineering of livestock in agriculture; for accelerated introduction of genetic characters into a strain/breed and for the production of animals with an antigenic make-up compatible with humans so that their tissues and organs can be used in transfusions and transplants [4–7].

Over the years, many approaches for the production of transgenic animals have been tried. One of the first to be shown effective in

mammals and still the most common and widely used across species is pronuclear microinjection [8]. This procedure, used to over- or under-express certain genes or to express genes entirely new to the host organism, involves the direct microinjection of DNA (a single gene or a combination of genes from the same or another species; designated a transgene) into the pronucleus of a fertilized oocyte. When occurring, DNA insertion in the host genome is random, and its expression is dependent on the site of integration [9]. An alternative method of transgenesis that has been attempted is sperm-mediated gene transfer. With this method, transgenesis is facilitated by performing *in vitro* embryo fertilization [10] or intracytoplasmic sperm injection (ICSI) with mature sperm cells previously incubated with exogenous DNA molecules [11, 12]. In ICSI-mediated transgenesis, sperm cells are previously demembranated by a freeze-thaw procedure or by a detergent treatment in order to expose their nuclei to the DNA of interest. ICSI-mediated gene transfer (ICSI-MGT) has reached the practical level in pigs [13], and it has been demonstrated that ICSI-MGT is a more efficient technique for generating transgenic porcine embryos than pronuclear injection [14, 15].

Other procedures such as transfection of male germ cells [16–18], somatic cell nuclear transfer [19–21] and lentiviral vectors [22] have also been attempted. However, the application of most of these procedures remains limited by the technical difficulty. Low survivability after microinjection, impaired *in vitro* embryo development, pre- and postimplantation embryo loss, reduced rates of transgene integration and unpredictable copy numbers, variable transgene

expression and germ line transmission, are frequently responsible for poor results (reviewed in [23]).

In the present study, we tested a new transgene delivery approach, and we report for the first time the production of transgenic animals by co-injection of DNA and round spermatid nuclei into non-fertilized mouse oocytes. Round spermatid nucleus injection (ROSI) is a well-established assisted reproductive procedure that has been shown to generate viable offspring in the mouse [24]. Our intention with this approach was to evaluate the potential of ROSI to induce transgenesis, and to analyze its efficiency compared with pronuclear injection and ICSI-MGT. ROSI offers unique possibilities for transgenesis and genetic rescue of azoospermic animals that do not produce the spermatozoa needed for normal sexual or ICSI-mediated fertilization. Round spermatids are spermatogenic haploid cells that, through a process termed spermiogenesis (reviewed in [25]) develop into mature spermatozoa. Spermiogenesis is a differentiation process involving chromatin condensation, loss of cytoplasm, acquisition of oocyte activating factors and formation of a flagellum. Differences between spermatids and mature sperm, in particular differences in chromatin structure [26], justify an evaluation of their potential to promote transgenesis and serve as vectors for exogenous DNA molecules. In this study, we determined that transgene transport and incorporation accomplished by intracytoplasmic injection do not occur exclusively in mature spermatozoa but that immature sperm cell nuclei with decondensed chromatin such as round spermatid nuclei can also be effective in promoting transgenesis.

Material and Methods

Reagents and media

All chemicals and media were purchased from Sigma Chemical (Alcobendas, Madrid, Spain) unless otherwise stated.

Animals

Experiments were done with hybrid B6D2F1 mice (Harlan Iberica SL, Barcelona, Spain). This mouse strain was used as the donor of oocytes, spermatozoa for ICSI experiments, spermatids for ROSI experiments and as the donor of embryos for pronuclear injection experiments. Females were 6–8 weeks old at the time of the experiments, and males were at least 3 months old. CD1 females were used as surrogate mothers for embryo-transfer experiments and mated with vasectomized CD1 males. Lactating CD1 foster mothers were occasionally used to raise pups. Mice were fed *ad libitum* with a standard diet and maintained in a temperature- and light-controlled room (23 C, 10 h dark: 14 h light). Animal experiments were carried out in strict accordance with the recommendations in the guidelines of European Community Council Directive 2010/63/EU. Experiments were approved by the Committee on the Ethics of Animal Experiments of INIA (Madrid, Spain).

Transgene preparation

The enhanced green fluorescent protein (EGFP) plasmid construct (4.7 kb, pEGFP-N1, Clontech Laboratories, Palo Alto, CA, USA) used for our experiments contained the human CMV immediate early promoter and the enhanced GFP gene. This construct was linearized with *Afl* II prior to use. The transgene was purified using an Elu-Quik DNA Purification Kit (Schleicher & Schuell, Dassel, Germany) according to the manufacture's instructions. DNA was resuspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8). The concentration of EDTA in the TE buffer was reduced from 1 mM to 0.1 mM, since it has been reported that the use of ion chelators (EGTA or EDTA) significantly reduces the efficiency of ICSI-mediated transgenesis in mouse embryos [27].

Gamete collection, zygote collection and spermatid-transgene mixing

Metaphase II (MII) oocytes from 6- to 8-week-old female mice, which had been superovulated with 5 IU of equine chorionic gonadotropin followed 48 h later by an equivalent dose of hCG, were collected 14 h post human chorionic gonadotropin (hCG) administration. Cumulus cells were dispersed by 3–5 min incubation in M2 medium containing 350 IU/ml hyaluronidase, and oocytes were washed and maintained in potassium modified simplex optimized medium (KSOM) medium at 37 C in a 5% CO_2 air atmosphere until use.

Zygotes for pronuclear microinjection were collected from superovulated 6- to 8-week-old female mice, which had previously mated with B6D2F1 males, 19 h after hCG administration by procedure similar to that above. Females with a vaginal plug were culled, and zygotes were recovered from the ampulae. Cumulus cells were dispersed by 3–5 min incubation in M2 medium containing 350 IU/ml hyaluronidase, and embryos were washed and maintained in KSOM medium at 37 C in a 5% CO_2 air atmosphere until use.

Fresh spermatid cells were collected from mature (3–6 months old) male mice. Mice were euthanized, and the caudae epididymides were removed using a pair of fine scissors. To collect spermatogenic cells for ROSI, the seminiferous tubules of the testes were minced as described previously [28, 29], except the cells were suspended in HEPES-buffered CZB medium. Briefly, testes were placed in erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA, pH 7.2), and the tunica albuginea was removed. Seminiferous tubule masses were transferred into cold (4 C) Dulbecco's phosphate-buffered saline (PBS) supplemented with 5.6 mM glucose, 5.4 mM sodium lactate and 0.1 mg/ml of polyvinyl alcohol (GL-PBS) and then cut into small pieces and pipetted gently to disperse the spermatogenic cells. Then, the cell suspension was filtered through a 38-mm nylon mesh and washed three times by centrifugation (200 *g* for 4 min). After centrifugation, the pellet was resuspended in GL-PBS and stored at 4 C. Phase contrast microscopy was used to distinguish haploid round spermatid cells from diploid spermatogenic precursors and somatic cells [30]. The round spermatid was characterized by its small size (between 8–10 μm) and by a dense, smooth dark nucleus positioned centrally or inclining towards the cell membrane. In some of these cells, the early acrosomal vesicle (Sa) or acrosomal cap (Sb 1) was clearly visible as a bright white spot or sickle-shape adjacent to the nucleus. For transgenesis experiments, mixtures of equal volumes of 10 μl of the fresh spermatid cell suspension in M2 and plasmid EGFP-DNA were kept on ice for 2 min. Final EGFP concentrations of 2.5–10 ng/μl in this blend were used in our experiments.

Embryo micromanipulation, culture and transfer

ROSI-mediated transgenesis (ROSI-MGT) was performed into metaphase II oocytes at room temperature using spermatid-DNA

mixing. One volume of spermatid cell suspension-DNA solution was mixed with five volumes of M2 medium containing 10% polyvinylpyrrolidone (PVP; Mr 360,000) in M2 solution to decrease stickiness. The microinjection dish contained an injection drop (BSA-free M2 medium), a spermatid-DNA drop (sperm cell suspension-DNA solution in M2/10% PVP) and an M2/10% PVP needle-cleaning drop. Injections were performed under an inverted microscope equipped with Eppendorf micromanipulators (Hamburg, Germany) and a PMM-150 FU piezoimpact unit (Prime Tech, Japan) using a blunt-ended Fluorinert FC-770-containing pipette with an inner diameter of 6–7 μm. Round spermatids, characterized by a centrally located chromatin mass, were selected for injection into oocytes. Spermatid nuclei were individualized by pipetting in and out each round spermatid in the spermatid-DNA PVP containing drop. Oocytes were injected in groups of ten. After an injection recovery period of 15 min at room temperature in M2 medium, surviving oocytes were placed in mineral oil-covered KSOM and maintained at 37 C in a 5% CO_2 air atmosphere until chemical activation. ROSI-fertilized oocytes were activated by a 20 min treatment in calcium-free CZB medium containing 10 mM $SrCl_2$. Embryos were extensively washed to remove the activation medium and returned to KSOM culture medium at 37 C in a 5% CO_2 air atmosphere. Pronuclear microinjection of zygotes was carried out as previously described [31]. Microinjected embryos were placed in mineral oil-covered KSOM and maintained at 37 C in a 5% CO_2 air atmosphere for 24 h. Embryos that progressed to the 2-cell stage were transferred into oviducts of pseudopregnant CD1 females. Embryo transfer of ROSI-fertilized and pronuclear-injected embryos was performed according to the standard methodology, which has been described previously [32]. ICSI-MGT was performed as previous described [7].

Analysis of genomic DNA and EGFP expression

Genomic DNA was prepared from tail biopsies following standard procedures [33] and used for polymerase chain reaction (PCR). The oligonucleotides used for detecting the specific 340 bp PCR product of EGFP were 5'-TGAACCGCATCGAGCTGAAGG-3', GFP1F, and 5'-TCCAGCAGGACCATGTGATCG-3', GFP2R. The PCR conditions were as follows: Taq polymerase (Promega, Madison, WI, USA); 2 min at 93 C; 30 cycles of 30 sec at 93 C, 45 sec at 60 C and 35 sec at 72 C; and a final extension step of 10 min at 72 C [34].

Immunohistochemistry

Analysis of EGFP expression in transgenic animals was performed by immunocytochemistry as described previously [31, 35]. EGFP expression was analyzed in samples from the testis, kidney, heart, lung, liver, spleen, striated muscle, endothelium and pancreas. Tissues were fixed in Bouin's fluid, embedded in paraffin and sectioned (5 mm). Slides were deparaffinized with xylene, rehydrated (100%, 96% and 70% ethanol, water; 4 min each) and antigens retrieved by heating in trisodium citrate buffer (10 mM). Endogenous peroxidase was blocked in 0.3% hydrogen peroxide, and the tissue sections were permeabilized with PBS-0.1% Tween 20 (MERCK-Schuchardt, Steinheim, Germany), blocked in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 30 min and treated with an Avidin/Biotin Blocking Kit (Vector Laboratories). Then, slides were incubated overnight at 48 C with rabbit anti-GFP antibody (GeneTex, San Antonio, TX, USA), followed by incubation for 1 h at room temperature with a biotinylated goat anti-rabbit IgG secondary antibody (BA-1000; Vector Laboratories), 30 min with a Vectastain Elite ABC Kit (Vector Laboratories) and 10 min with a Vector NovaRED Substrate Kit (Vector Laboratories). Coverslips were mounted using VectaMount medium (Vector Laboratories) and observed by bright-field microscopy (OPTISHOT-2; Nikon, Kanagawa, Japan). Negative controls were performed in the same way using sections from wild-type mice with anti-GFP antibody and by omission of the primary antibody before the addition of the secondary antibody.

Qualitative analysis of EGFP mRNA expression

The quantification of EGFP mRNA transcripts was performed by real-time quantitative PCR. The PCR conditions were 94 C for 3 min, followed by 40 cycles of 94 C for 10 sec, 56 C for 30 sec and 72 C for 10 sec, with 10 sec of fluorescence acquisition (SYBR channel; Rotor-Gene 2000; Corbett Research, Qiagen, Madrid, Spain). The comparative cycle threshold (CT) method was used to quantify expression levels. The ΔCT value was determined by subtracting the CT value for the endogenous control (*Gapdh*) in each sample from the CT values for each gene in the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e., the sample with the lowest target expression) as a constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ [36]. The relative transcript abundance generated through qRT-PCR was analyzed using one-way ANOVA with post hoc multiple pairwise comparisons using the Student-Newman-Keuls method in SigmaStat (Jandel Scientific, San Rafael, CA, USA).

Statistical analysis

Differences in the efficiency of transgenesis were evaluated by a Z-test analysis. Chi-square analysis was used for all other comparisons. SigmaStat statistical software version 3.11 (Jandel Scientific) was used for the statistical analysis. Differences of $P < 0.05$ were considered significant.

Results

In order to test if mouse ROSI-MGT could be feasible, round spermatid nuclei were co-injected with a 5.4 kb EGFP construct into non-fertilized metaphase II oocytes, and the resulting offspring were analyzed for tissue integration and expression of the EGFP transgene. ROSI assays with co-injection of EGFP (used in concentrations of 2.5–10 ng/ul) were compared with experimental assays performed in the absence of it. The outcome of these assays is presented on Table 1. In total, 748 oocytes were injected, 65% (484) of which survived injection, and 443 (92%) developed to the 2-cell stage. These embryos were subsequently transferred into pseudopregnant females. Out of these embryo, 35 (8%) developed to term, and 4 (12% of live offspring) were transgenic for EGFP. As expected, the transgene was not detected in any of the 20 live offspring generated by ROSI in the absence of the transgene. In this control experiment, 622 oocytes were ROSI fertilized, 424 (68%) survived injection, and 387 (91%) developed to two-cell stage embryos and were transferred

Table 1. *In vitro* development and development to term of B6D2F1 mouse embryos generated by ROSI (R) with and without EGFP, ICSI (I) and pronuclear injection (PI)

[EGFP] (ng/μl)	Injected oocytes/ embryos	Surviving oocytes (% of the injected oocytes/embryos)	2-cell embryos (% of the surviving oocytes)	Embryos transferred (recipients)	Live offspring (% of the embryo transferred)	Transgenic offspring (% of the live offspring)
R 0	622	424 (68) [a]	387 (91)	387 (17)	20 (5) [a]	0 (0)
R 2.5–10	748	484 (65) [a]	443 (92)	443 (20)	35 (8) [ab]	4 (12) [a]
I 2.5–10	119	93 (78) [ab]	80 (86)	80 (4)	11 (14) [bc]	4 (36) [a]
PI 2.5–10	269	226 (84) [b]	206 (91)	200 (10)	40 (20) [c]	9 (22) [a]

[a–e] Values with different superscripts are significantly different (Z-test; P < 0.05).

into pseudopregnant females. It was interesting to observe that co-injection of the exogenous DNA molecule at the concentrations used did not have a significant impact on the oocyte survivability, embryo progression to the 2-cell stage or development to term. The efficiency of live transgenic animals produced by ROSI-MGT was similar to both pronuclear injection and ICSI-MGT (Table 1). However, fewer embryos were used to produce the same number of transgenic animals when ISCI-MGT was used (Table 1).

We examined the tissue morphology and fluorescence signals in various organs of transgenic mice by fluorescence stereomicroscopic observation of autopsy samples (Fig. 1) and found that size and morphology were not distinguishable between EGFP transgenic mice produced by ROSI and wild-type mice. EGFP fluorescence was observed in the majority of the organs examined. When we compared the levels of EGFP expression evaluated by real-time PCR and immunohistochemistry in different tissues of transgenic mice generated by pronuclear microinjection and ROSI (Fig. 2), significant differences were not detected for most tissues. For two of the transgenic lines generated by pronuclear injection, slightly higher EGFP expression was detected in the pancreatic tissue. On the other hand, two of the transgenic lines generated by ROSI displayed a low level of transgene expression in the endothelium and liver, which remained undetected in the transgenic lines generated by pronuclear injection. As expected, the expression of the transgene did not affect reproductive, developmental, or other important physiological processes.

Discussion

The results observed from our microinjection experiments lead to the conclusion that transgenesis can be induced by co-injection of exogenous DNA molecules and round spermatid nuclei into metaphase II oocytes. This is particularly interesting because it indicates that the transgene incorporation mediated by intracytoplasmic injection of male gamete nuclei is not an exclusive property of mature sperm cell nuclei with compact chromatin. This study shows that microinjection of immature sperm nuclei with decondensed chromatin such as those of round spermatids can also mediate transgenesis by a similar method. Many factors have been appointed to explain the efficiency of ICSI-mediated transgenesis, one of these factors being the extensive reprogramming that the sperm nucleus undergoes after microinjection into the oocyte. Such nuclear reprogramming, involving extensive chromatin remodeling including decondensation, exchange of protamines by histones and other chromatin-associated

proteins before and during male pronucleus formation (reviewed [37]), provides an opportunity for the integration of exogenous DNA molecules when co-injected. The fact that transgenesis can be induced with decondensed immature sperm cell nuclei seems to suggest that incorporation in the host genome of co-injected exogenous DNA molecules can occur independent of the level of chromatin condensation in the injected nucleus. Another factor that has been indicated as an explanation for the efficiency of ICSI-mediated transgenesis is the possible involvement of the DNA-repair machinery of the oocyte in inserting transgene template molecules to resolve nicks generated on the sperm cell chromatin by the required preinjection treatment for sperm cell fragmentation, which is related to paternal genome demethylation. Although this may still be valid for ICSI, ROSI-mediated transgenesis was accomplished in this study without any particular pretreatment of donor cell nuclei. In this case, transgene incorporation was not facilitated by any pretreatment of the donor nuclei. Moreover, active demethylation of paternal genome, or more exactly, conversion to 5-hydroxymethylcytosine, is related to protamine replacement by histones. Polanski [38] described differential demethylation dynamics among ICSI and ROSI zygotes due to the lack of protamines in the round spermatid genome, since histones are replaced in the elongated spermatid. The histone-associated DNA of round spermatids showed resistance to the characteristic global demethylation of the protamin-associated DNA of the mature sperm, being more similar to female genome [38]. Whether or not transgene incorporation mediated by ICSI and ROSI share similar or completely different mechanisms still requires further investigation.

The results of this study indicate that the usage of round spermatids for production of transgenic animals still remains a relatively inefficient process, as only 4 of the 748 oocytes injected gave rise to transgenic offspring. However, the proportion of transgenic offspring among live offspring (12%) does not discourage the use of round spermatid injection for transgenesis purposes. Our results indicated few if any significant differences in EGFP expression between transgenic animals generated by ROSI and PN injection. The main difficulty seemed to be development to term after ROSI. In our experiments, development to term was not significantly reduced by co-injection of exogenous DNA, at least at the concentrations used. A significant difference in the number of live offspring obtained after ROSI with and without co-injection of EGFP was not observed. This poor development to term observed as a consequence of the use of immature male gametes for assisted reproduction, in particular round spermatids, has been associated with interference with the mouse embryo preimplantation epigenetic program, inefficient genome
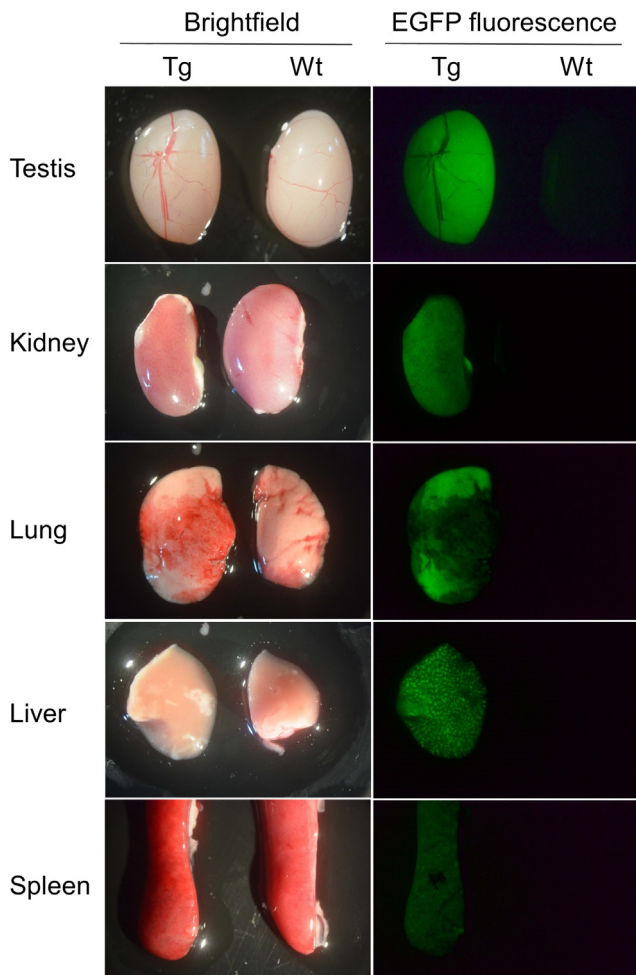
Fig. 1. EGFP is expressed in different tissues of transgenic mice generated by ROSI-mediated transgenesis. Bright-field (left) and EGFP fluorescence images (right) of the testis, kidney, lung, liver and spleen of transgenic (Tg) and wild-type (Wt) mice are shown.

activation and aberrant spermatid transcription [39–41]. In agreement with these observations, Kurotaki *et al.* (2015) [42] showed in a recent study that ROSI-derived embryos failed to undergo active DNA demethylation. Interestingly, these demethylation failures led to more abnormally sized fetuses. These results, even though they are not conclusive, could also explain the low efficiencies obtained with ROSI; that is, they could be the result of a deleterious effect in embryo development produced by inefficient demethylation of the male genome. Future research could investigate the possibility that the use of preactivated oocytes instead MII oocytes could perhaps improve the efficiency of ROSI-MGT by increasing embryo survival [29]. Another interesting research topic encouraged by the results of this study would be examination of whether or not somatic cell nuclei co-injected into enucleated oocytes with exogenous DNA could also promote transgenesis. Somatic cell nuclear transfer (SCNT) without *in vitro* culture and drug selection of transformed donor nuclei, which frequently decreases the development of reconstituted embryos, could be a viable transgenesis procedure for those species in which the efficiency of PN injection remains poor and ES cell lines are not available. Aside from this and the other interesting questions that the results of this study may raise, we believe that the main observation reported in this study is by itself of great interest, as it constitutes the first report of transgenic animal generation mediated by ROSI and suggests further areas of research regarding an alternative transgene delivery procedure that has been previously investigated.
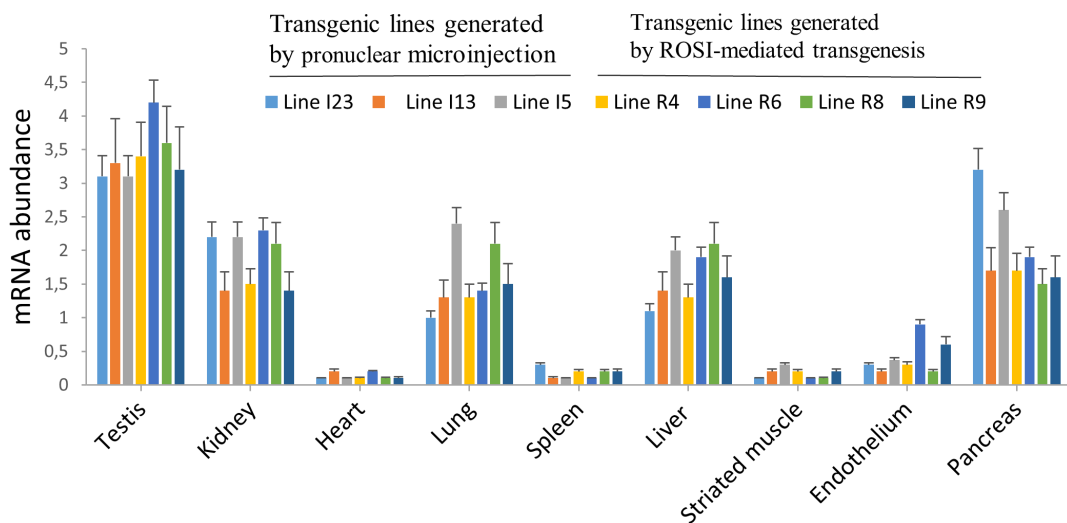
Acknowledgments

Fig. 2. EGFP expression (mRNA abundance relative to *Gapdh* expression) evaluated by real-time PCR in different tissues of transgenic mice generated by pronuclear microinjection and ROSI-mediated transgenesis.

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