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Cryopreservation of *Xenopus* Transgenic Lines

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ABSTRACT *Xenopus laevis* has been widely used for molecular, cellular, and developmental studies. With the development of the sperm-mediated transgenic method, it is now possible to study gene function during vertebrate development by using this popular model. On the other hand, like other animal species, it is labor intensive, and the maintenance of transgenic lines is expensive. In this article, we investigated the possibility of using sperm-cryopreservation as a means to preserve transgenic frog lines. We demonstrated that cryopreserved sperms are viable but not fertile under our in vitro fertilization (IVF) conditions. However, by microinjecting cryopreserved sperm nuclei, we successfully regenerated a transgenic line carrying a double promoter transgene construct, where the marker gene encoding the green fluorescent protein (GFP) is driven by the γ-crystallin gene promoter and a gene of interest, encoding a fusion protein of GFP with the matrix metalloproteinase stromelysin-3 (ST3-GFP), is driven by a heat shock-inducible promoter. We demonstrated the functional transmission of the ST3-GFP transgene by analyzing the phenotype of the F1 animals after heat-shock to induce its expression. Our method thus provides an inexpensive means to preserve transgenic frog lines and a convenient way for distribution of transgenic lines. Furthermore, the ease with which to microinject nuclei compared to the technically demanding transgenesis procedure with variable outcome should facilitate more laboratories to use transgenic *Xenopus laevis* for functional studies in vivo. *Mol. Reprod. Dev. 67: 65–69, 2004.*

Key Words: *Xenopus laevis*; sperm cryopreservation; transgenesis

INTRODUCTION

Transgenesis is an invaluable approach for studying gene function in frogs in vivo, especially during postembryonic development, in part due to the inability to knockout genes in amphibians. Transgenesis is now an established technique for *Xenopus laevis* (Kroll and Amaya, 1996; Huang et al., 1999; Offield et al., 2000; Damjanovski et al., 2001). Sperm nuclei are mixed with linearized plasmid DNA and restriction enzyme and then injected into thousands of eggs on a single day. The efficiency of producing normally developing tadpoles is up to 8% of the number of eggs injected, but egg quality is a variable and limiting aspect of the procedure (Amaya and Kroll, 1999; Offield et al., 2000; Sparrow et al., 2000). Unlike mouse transgenesis, the long generation times in frogs makes it more attractive to re-make transgenic animals for each experiment rather than use transgenic frog lines. In addition, maintenance of frog lines is costly in terms of space and money. However, because of random insertion (number and location of insertion sites and copies per insertion), each transgenic embryo or tadpole will be unique, potentially increasing the variability of the experimental results. Furthermore, technical demand and variability in outcome from the transgenic procedure make it impractical for different laboratories to repeat some experiments and/or to study the effects of the same transgene on different biological process. Thus, it is highly desirable to have a reliable method to preserve and easily distribute transgenic frog lines for different and/or future studies. Here, we present methods to allow long-term preservation of transgenic frog lines and to improve efficiency and reduce variability in producing transgenic animals through cryopreservation of sperm nuclei of transgenic animals.

Mouse sperm cryopreservation methods have been used for many years in part due to similar concerns of overcrowding in mouse rooms encountered in mouse transgenic systems (Bath, 2003). Different methods have various levels of efficiency and most mouse facilities now use the cryopreservation method of Nakagata (Nakagata, 2000). Improvements to the use of cryopreserved sperm in in-vitro fertilization (IVF) have also been made (Bath, 2003), and in some cases intracytoplasmic sperm injection is more efficient than IVF (Szczygiel et al., 2002). Evidence that these methods are not directly transferable across species comes from species-specific techniques across different mammals (Thurston et al., 2002). In fact, despite the success in obtaining mobile and/or fertile sperms after cryopreservation in a few species of frogs (Beesley et al., 1998; Browne et al., 1998), there has been no report on *Xenopus laevis*, the most widely used amphibian for molecular, cellular, and developmental studies.

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We are interested in studying gene function through transgenesis during frog metamorphosis, a postembryonic process that involves diverse changes in various organs/tissues but is controlled by a single hormone, thyroid hormone (Dodd and Dodd, 1976; Yoshizato, 1989; Gilbert et al., 1996; Shi, 1999; Shi et al., 2001). We have previously developed a double promoter construct carrying two expression cassettes in one plasmid for noninvasive detection of transgenesis (Fu et al., 2002). We verified the functionality of this approach based on stromelysin-3 (ST3) as a transgene (Damjanovski et al., 2001). Constitutive over-expression of ST3 during late embryogenesis causes characteristic developmental malformations and in most cases death. This overexpression produces the same phenotype when controlled by the heat shock promoter (Fu et al., 2002). In the current study, we used this methodology to evaluate the effects produced by ST3 transgenes in F1 animals generated through IVF and sperm cryopreservation.

MATERIALS AND METHODS

Animals and Transgenesis

Wild-type *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI). Transgenesis was carried out as described (Kroll and Amaya, 1996; Damjanovski et al., 2001). Transgenic animals receiving the CGHSwG double promoter construct (Fu et al., 2002) were reared to adulthood.

Nuclei Freezing and Injection

Sperm nuclei from mature transgenic frogs were prepared for transgenesis using the same procedure as for wild-type transgenesis (Kroll and Amaya, 1996; Damjanovski et al., 2001). For sperm nuclei microinjection, 2 μl of sperm nuclei was diluted directly into 150 μl sperm dilution buffer (SDB buffer, (Kroll and Amaya, 1996)) and injected into dejellied individual eggs under the same settings as transgenesis.

Sperm Freezing and Fertilization

Three cryopreservation solutions (CPS) were used: 0.5 M DMSO, 50% FBS in 1× MMR (Beesley et al., 1998), 15% v/v DMSO, 10% sucrose w/v in 1× MMR (Browne et al., 1998), and 80% glycerol in 1× MMR. Testis pieces were macerated with forceps in an equal volume of CPS on ice, aliquoted, and frozen at −80°C. Sperm thawed at 37°C until the slush point were put on ice and activated by adding 2 vol. distilled water. Activated sperm was measured for motility using a hemocytometer. Solutions with motile sperm were added to eggs for IVF. IVF using wild-type eggs and transgenic testis was carried out as described (Sive et al., 2000).

Analysis of Transgenic Animals

When embryos reached stage 20 (Nieuwkoop and Faber, 1956), normal embryos were selected and heat shocked for 15 min three times in a row with 15 min spaces at room temperature (Wheeler et al., 2000; Fu et al., 2002). At stages 35, 40, and 48, tadpoles were scored for normal development.

RESULTS

We have previously generated transgenic *Xenopus laevis* animals with a double promoter construct carrying the GFP under the control of γ1-crystallin gene promoter and GFP-stromelysin-3 (ST3) fusion protein under the control of the heat shock-inducible promoter (Fu et al., 2002). To investigate the possibility of preserving the transgenic line through frozen sperms of the transgenic animals, we isolated and froze sperm nuclei at the −80°C from a male transgenic frog. The frozen nuclei were resuspended and microinjected into wild-type eggs. For comparison, we carried out the standard transgenic procedure on the same batch of eggs by using the same double promoter construct originally used to generate the transgenic male. Under our experimental conditions, about 20% of the injected eggs from both normal transgenesis and direct microinjection of germ line sperm nuclei developed into normal four cell embryos. There is no significant difference between transgenesis and transgenic sperm nuclei injection at this point. At stage 20, just after gastrulation, the development of 7% of the total injected eggs from transgenesis and 12% of those from sperm nuclei microinjection was still normal. At stage 35 when the eyes are fully developed in embryos, the tadpoles were sorted into transgenic and nontransgenic ones based on GFP expression in the eye (Fig. 1). Overall, 5% from transgenesis vs. 9% from sperm nuclei microinjection survived the procedure to this stage. However, only 25% of the stage 35 embryos from transgenesis were transgenic while 50% from the sperm nuclei microinjection carried the transgenes. Based on several independent experiments, we found that $1.3 \pm 0.4\%$ out of the injected eggs from transgenesis developed into transgenic embryos at stage 35 compared to $4.1 \pm 0.7\%$ out of sperm nuclei microinjection.

From an IVF with fresh sperms from the same F0 transgenic frog, we found that 50% of the F1 animals were transgenic. Thus, the results from both the IVF and sperm nuclei microinjection suggest that there was only one transgene insertion site or a few transgene insertion sites located very close to each other on a single chromosome (thus not segregated during recombination in the F1 animals). Similar analysis on a second transgenic male frog suggests that it had two insertion sites on two separate chromosomes.

To verify that the transgenes in the F1 animals were functional, we carried out a heat shock experiment to study the expression and function of the transgenic ST3-GFP fusion protein. We selected normal embryos at stage 20 (including both transgenic and nontransgenic animals) and gave them one heat shock treatment. After heat shock, tadpoles expressing GFP in the lens under the control of the γ1-crystallin promoter (Offield et al., 2000) also expressed ST3-GFP in the body due to the heat shock promoter on the same construct used for the transgenesis that produced the F0 male (Fig. 2). Wild-type siblings, i.e., those lacking GFP expression in the lens, did not turn green under UV after heat shock. In F0

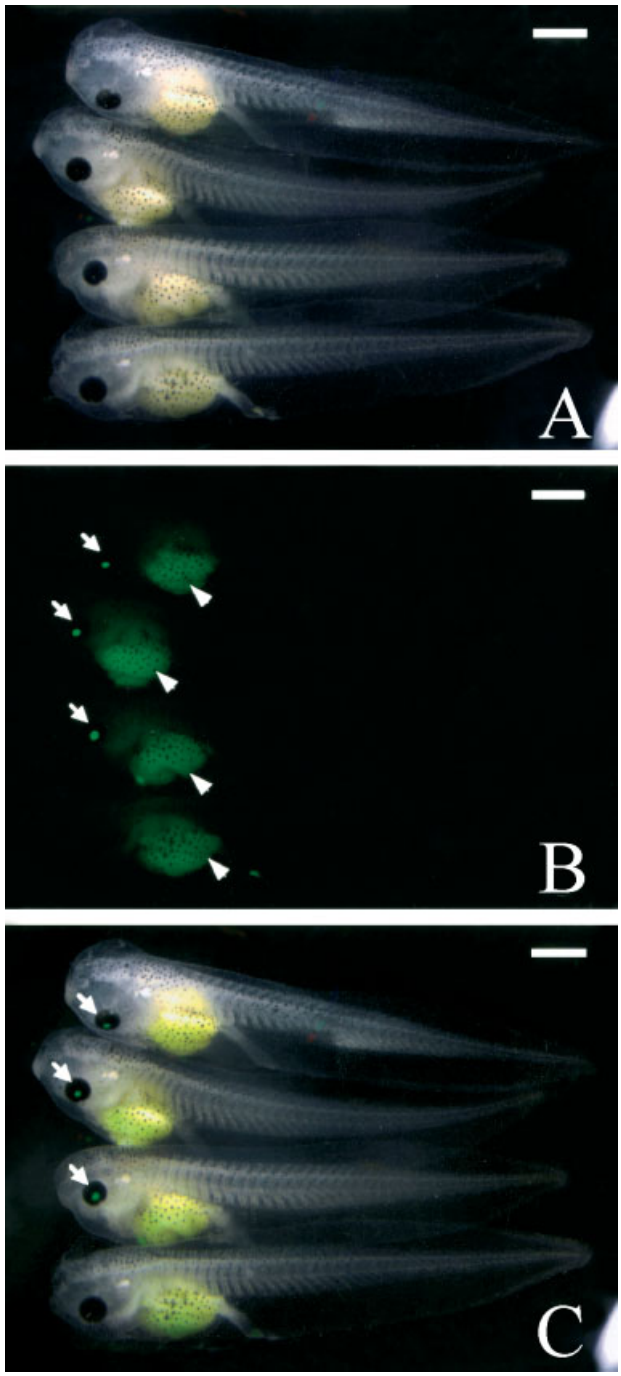

Fig. 1. Large population of F1 tadpoles are transgenic. F1 tadpoles from sperm nuclei microinjection developed normally in the absence of heat shock (i.e., stage 40, **A**). Under a UV microscope, a nontransgenic tadpole was not green anywhere except the auto-florescence from yolk in the abdomen (**B** and **C**, the bottom one), while transgenic ones had green eyes (B and C, the upper three). Merged picture (C) highlights the location of GFP expression from γ1-crystallin promoter within the double promoter construct used for transgenesis that generated the F0 male. Showing here are representative F1 animals, 50% of which were transgenic. Arrows indicate GFP expression, arrow heads indicate auto-florescence from yolk. Bar: 1 mm.

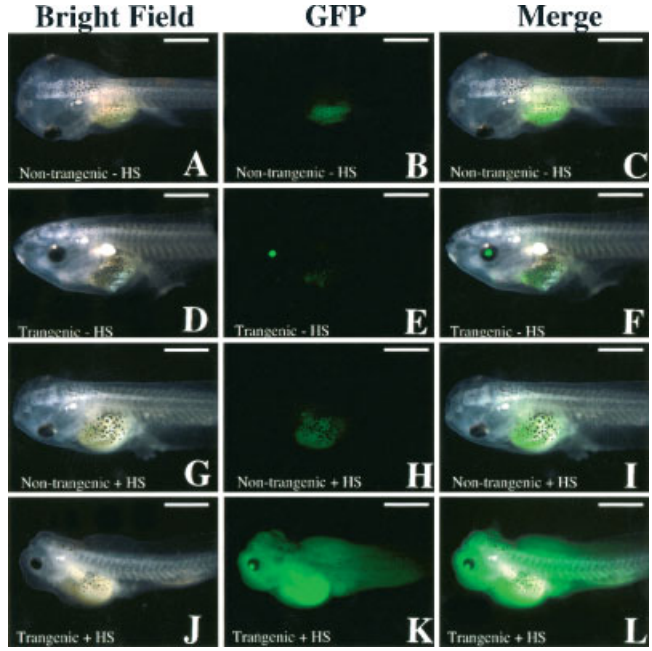

Fig. 2. Overexpression of ST3 in F1 tadpoles at early stage induces anomalies in survived ones. F1 embryos were produced by injecting sperm nuclei into dejellied eggs. Normal embryos at stage 20 were selected and subjected to heat shock. Pictures were taken 4 days later under a microscope at bright field (**A**, **D**, **G**, **H**) or under UV light for GFP fluorescence (**B**, **E**, H, **K**), and merged (**C**, **F**, **I**, **L**). Nontransgenic tadpoles developed normally with (G, I) or without (A, C) heat shock, and they had no GFP fluorescence (B, H). Transgenic tadpoles were only green in the eyes (E) and developed normally without heat shock (D, F). Most of the transgenic tadpoles died at early stages after being heat shocked initially at stage 20, and the survived ones had GFP fluorescence throughout the body (K) and showed serve anomalies (**J**, K, L). HS, heat shock. Bar: 1 mm.

ST3-GFP transgenic animals, constitutive (Damjanovski et al., 2001) or induced (Fu et al., 2002) embryonic expression of the transgene causes abnormal development and death. The F1 transgenic animals that were given heat shock also developed abnormally (Fig. 2). In addition, F1 transgenic tadpoles showed similar mortality as the F0 (Fu et al., 2002). Half the number of heat shocked ST3-GFP tadpoles survived compared to heat-shocked wild-type animals or transgenic and wild-type animals not given heat shock (Table 1).

A further increase in the convenience of cryopreservation of transgenic frog lines would be the use of frozen sperm in IVF. Cryopreservation of *Xenopus laevis* sperm has not been reported in the literature so we used published procedures for other species (Beesley et al., 1998; Browne et al., 1998). We tested three cryopreservation solutions (CPS) for their ability to allow for motility after thawing. The 80% glycerol CPS gave zero motility before freezing. The DMSO/FBS and DMSO/sucrose CPS gave 50% motility before freezing. After freezing, the DMSO/FBS CPS and DMSO/sucrose CPS resulted in 25–50% motility (percent number of sperms actively moving in the hemocytometer). Measurements were performed in triplicate and the experiment was

TABLE 1. Percentages and Numbers (in Parenthesis) of Embryos With or Without Transgenic Overexpression of ST3 That Developed Normally to a Given Stage

	Heat shock at stage 20		No heat shock	
Stage	Transgenic	Nontransgenic	Transgenic	Nontransgenic
20	100% (50)	100% (33)	100% (50)	100% (33)
35	62% (31)	91% (30)	92% (46)	91% (30)
40	52% (26)	82% (27)	74% (37)	73% (24)
47/48	18% (9)	36% (12)	34% (17)	33% (11)

Only the embryos that were normal at stage 20 were selected for further analysis. Same number of tadpoles at stage 20 were used for the heat shock group and no heat shock control group.

performed twice. Sperm cryopreserved in all three CPS were tested for their ability to fertilize eggs in vitro. In no case were we successful at obtaining fertilized embryos, even after trying with eggs from six females.

DISCUSSION

As frog transgenesis becomes a more commonly used procedure, there will be a greater need for long-term storage of transgenic lines that are not being used and/or an easy method of distributing transgenic lines to other laboratories for multiple studies of the same transgene on different biological processes. Cryopreservation of transgenic sperm nuclei is shown here to be feasible. Using wild-type females does not dilute the transgene copy number for transgenic males with only 1 or 2 transgene insertion sites as 50% or 25%, respectively, of the F1 animals would carry the same number of the transgene as in the F0 males.

A more convenient technique would be IVF using cryopreserved transgenic sperm. In addition, developmental abnormalities due to damaged nuclei (from nuclei microinjection) are expected to be dramatically reduced. This procedure has been successful in many mammal species and one frog species (Browne et al., 1998), but has not been reported for *Xenopus laevis*. In many other frog species, recovery of sperm motility is achieved but IVF ability has been assessed in only one case (Browne et al., 1998). We assayed motility of sperm cryopreserved in three CPS solutions. No motility was observed in one of the CPS, but in the DMSO/sucrose and DMSO/FPS solutions, we found sperm motility comparable with published reports on other species. However, we were not successful at obtaining embryos from cryopreserved sperm, even though fresh sperm gave high numbers of embryos. Thus, sperm motility after freezing was not predictive of fertilization success.

Regardless, by combining sperm cryopreservation and IVF through nuclei microinjection, we have shown here that it is possible to preserve transgenic frog lines avoiding the use of large amounts of precious animal facilities. Furthermore, injection of sperm nuclei from F0 animals with even only a single site of transgene insertion was several times more efficient in generating transgenic animals than fresh transgenesis with the same construct. This makes it much more cost effective for different laboratories to share the same transgenic lines for studying different biological processes. Furthermore, nuclei injection can be done much more easily than transgenesis itself. This should allow many laboratories currently not utilizing this powerful method, in part due to high technical demand and variability in outcome from the transgenic procedure, to use transgenic animals for in vivo function studies by simply obtaining cryopreserved sperm nuclei from other laboratories. Conceivably, in the near future, improved conditions for cryopreservation and/or IVF will be developed for *Xenopus laevis* to allow in vitro fertilization (IVF) with cryopreserved sperm. This will make cryopreservation much more convenient and valuable for function studies in *Xenopus laevis*.

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REFERENCES

Amaya E, Kroll KL. 1999. A method for generating transgenic frog embryos. Mol Embryol: Methods Protoc 97:393–414.

Bath ML. 2003. Simple and efficient in vitro fertilization with cryopreserved C57BL/6J mouse sperm. Biol Reprod 68:19–23.

Beesley SG, Costanzo JP, Lee RE. 1998. Cryopreservation of spermatozoa from freeze-tolerant and -intolerant anurans. Cryobiology 37: 155–162.

Browne RK, Clulow J, Mahony M, Clark A. 1998. Successful recovery of motility and fertility of cryopreserved cane toad (*Bufo marinus*) sperm. Cryobiology 37:339–345.

Damjanovski S, Amano T, Li Q, Pei D, Shi Y-B. 2001. Overexpression of matrix metalloproteinases leads to lethality in transgenic *Xenopus laevis*: Implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dynamics 221:37–47.

Dodd MHI, Dodd JM. 1976. The biology of metamorphosis. In: Lofts B, editor. The biology of metamorphosis. New York, NY: Academic Press. pp 467–599.

Fu L, Buchholz D, Shi Y-B. 2002. A novel double promoter approach for identification of transgenic animals: A tool for in vivo analysis of gene function and development of gene-based therapies. Mol Reprod Dev 62:470–476.

Gilbert LI, Tata JR, Atkinson BG. 1996. Metamorphosis: Postembryonic reprogramming of gene expression in amphibian and insect cells. New York, NY: Academic Press.

Huang H, Marsh-Armstrong N, Brown DD. 1999. Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that overexpress type III deiodinase. Proc Natl Acad Sci USA 2;96(3):962–967.

Kroll KL, Amaya E. 1996. Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. Development 122:3173–3183.

Nakagata N. 2000. Cryopreservation of mouse spermatozoa. Mamm Genome 11:572–576.
Nieuwkoop PD, Faber J. 1956. Normal table of *Xenopus laevis*, 1st Edn. Amsterdam, The Netherlands: North Holland Publishing. 252 p.
Offield MF, Hirsch N, Grainger RM. 2000. The development of *Xenopus tropicalis* transgenic lines and their use in studying lens developmental timing in living embryos. Development 127:1789–1797.
Shi Y-B. 1999. Amphibian metamorphosis: From morphology to molecular biology. New York, NY: John Wiley & Sons, Inc. 288 p.
Shi YB, Fu L, Hsia SC, Tomita A, Buchholz D. 2001. Thyroid hormone regulation of apoptotic tissue remodeling during anuran metamorphosis. Cell Res 11:245–252.
Sive HL, Grainger RM, Harland RM. 2000. Early development of *Xenopus laevis*: A laboratory manual. New York, NY: Cold Spring Harbor Laboratory Press.
Sparrow DB, Latinkic B, Mohun TJ. 2000. A simplified method of generating transgenic *Xenopus*. Nucleic Acids Res 28:e12.
Szczygiel MA, Kusakabe H, Yanagimachi R, Whittingham DG. 2002. Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. Biol Reprod 67:1278–1284.
Thurston LM, Watson PF, Holt WV. 2002. Semen cryopreservation: A genetic explanation for species and individual variation? Cryoletters 23:255–262.
Wheeler GN, Hamilton FS, Hoppler S. 2000. Inducible gene expression in transgenic *Xenopus* embryos. Curr Biol 10:849–852.
Yoshizato K. 1989. Biochemistry and cell biology of amphibian metamorphosis with a special emphasis on the mechanism of removal of larval organs. Int Rev Cytol 119:97–149.