Foreign DNA transmission by ICSI: injection of spermatozoa bound with exogenous DNA results in embryonic GFP expression and live Rhesus monkey births

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Exogenous DNA transfer, mediated by intracytoplasmic sperm injection (ICSI) with plasmid-bound spermatozoa, results in the production of transgene expressing embryos in rhesus macaques (Macaca mulatta, mean = 34.6%; n = 81). Rhodamine-tagged DNA encoding the green fluorescent protein (GFP) gene binds avidly to spermatozoa. The rhodamine signal, while lost at the egg surface during in-vitro fertilization (IVF), is traced by dynamic imaging during ICSI and remains as a brilliant marker on the microinjected spermatozoa within the oocyte cytoplasm. The transgene is expressed in preimplantation embryos produced by ICSI, but not IVF, as early as the 4-cell stage with the number of expressing cells and the percentage of expressing embryos increasing during embryogenesis to the blastocyst stage. The three offspring that resulted from seven embryo transfers (a set of anatomically normal twins, one male and one female, stillborn 35 days premature, and a healthy male born at term) demonstrate that primate spermatozoa with exogenously bound DNA retain their full reproductive capacity in ICSI, but raise the concern that, theoretically, ICSI could transmit infectious material as well.

Key words: GFP/ICSI/rhesus/sperm vector/transgenesis

Introduction

The role of the spermatozoon during fertilization includes the transfer of a haploid genome to the resultant zygote. This capacity has been exploited as an innovative strategy for the delivery of exogenous DNA for the production of transgenic animals (Lauria et al., 1993; Kim et al., 1997; Perry et al., 1999), though the strategy is of debatable reproducibility. The production of transgenic non-human primates might well be vital, not only for creating the most clinically relevant models for human diseases, but also for understanding the molecular basis of human reproduction as well. Furthermore, the promise of safe and effective gene therapy protocols cannot be fully realized until an appropriate system for investigation is found to fill the gap between knock-out mice and seriously ill patients.

Consequently, there are strong justifications for developing reliable and effective reproductive protocols for producing genetically modified non-human primates. The use of intracytoplasmic sperm injection (ICSI) for transmission of exogenous DNA represents a new approach in producing genetically modified non-human primates as research specimens. However, the discovery of sperm-mediated exogenous DNA transfer during ICSI raises serious concerns that this clinical and animal protocol might inadvertently transmit pathogenic material, e.g. infectious viruses and bacteria.

Materials and methods

Sperm collection, preparation and handling

Commercially available frozen bull semen, ejaculated rhesus monkey (Macaca mulatta) semen, and spermatozoa collected from hamster and mouse epididymis were used for plasmid DNA labelling. Male rhesus monkeys of proven fertility have been trained to routinely produce acceptable semen samples by penile electroejaculation (Bavister et al., 1983). After liquefaction of the coagulated ejaculate, the liquid semen was washed in 5 ml of Tyrode’s albumin lactate pyruvate medium (TALP–HEPES) by centrifugation at 400 g for 5 min. After resuspension of the pellet in 1 ml TALP–HEPES, a small sample was removed for structural analysis, while the remainder was counted and diluted to a concentration of 2×10⁷ spermatozoa/ml in 1 ml equilibrated TALP in a 15 ml conical tube.

Bull semen was separated on a Percoll gradient and rhesus monkey spermatozoa were washed in a TALP–HEPES buffer (Bavister et al., 1983). Spermatozoa (1×10⁹/14 µl) were mixed with 500 ng (1 µl) of plasmid DNA (Rh-CMV-GFP) and incubated at 39°C for 30 min. Labelled spermatozoa were washed and centrifuged three times in TALP–HEPES buffer, followed by fluorescent microscopic imaging before being used in in-vitro fertilization (IVF) and ICSI. Trypsin treatment of labelled spermatozoa was carried out by incubation with 2 mg/ml of trypsin (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) for 30 min prior to washing.

Follicle stimulation regimen

Ovulation stimulation in female rhesus monkeys exhibiting regular menstrual cycles was induced by exogenous gonadotrophins (Zelinski-
of pronuclei was assessed 12 days after fertilization. Oocytes were washed in equilibrated TALP and returned to culture. Culture of injected oocytes and embryos was maintained in CMRL + 10% fetal calf serum (FCS; Hyclone Laboratories Inc, Logan, UT, USA) on Buffalo rat liver cell monolayers (BRL 1442; ATCC, Rockville, MD, USA) seeded in 10 µl drops overlaid with oil. Embryos were selected at the 3–16-cell stage for transfer into staged recipients.

Plasmid construction
The plasmid DNA (pGeneGrip–Rhodamine/GFP) included a green fluorescent protein (GFP) cDNA and a rhodamine-binding site under the control of cytomegalovirus (CMV) promoter (Gene Therapy System, San Diego, CA, USA). Rhodamine labelling of the plasmid DNA was carried out according to the manufacturer’s instructions.

The CMV promoter was selected since it is a strong viral promoter widely used in transgenic studies. Although it lacks specificity, its constitutive expression pattern is an advantage during evaluation of gene delivery efficiency. The use of the GFP transgenic reporter is a powerful tool for determining successful delivery of exogenous DNA into oocytes and embryos. Although fluorescent microscopy is required, successful production of transgenic mice after GFP selection (Takada et al., 1997; Perry et al., 1999) suggests limited or no effect on embryo and fetal development.

The utility of a fluorescent DNA marker (rhodamine-conjugation to DNA), is invaluable. It permits live imaging of the DNA dynamics during both IVF and ICSI. Confocal and conventional digital imaging verify the binding of the DNA to the spermatozoon, as well as the fate of the exogenous DNA after the spermatozoon enters the oocyte cytoplasm. Rhodamine was chosen for several reasons including its excitation by long wavelength (and hence less damaging, lower energy) red light, and avoidance of any confusion between the rhodamine DNA fluorescence and the anticipated green fluorescence from GFP transgene expression.

Binding of exogenous DNA to spermatozoa
Spermatozoa (1x10⁶/14 µl) were mixed with 500 ng (1 µl) of plasmid DNA and incubated at 37°C for 30 min. Labelled spermatozoa were washed and centrifuged in TALP–HEPES buffer and examined by epifluorescence before being used for ICSI.

Dynamic imaging of DNA-bound spermatozoa during ICSI
Images were captured on an inverted TE-300 Nikon microscope equipped with a Princeton CCD camera and Metamorph software (Universal Imaging, West Chester, PA, USA). Final images were prepared using Adobe Photoshop (Adobe Systems Inc, MountainView, CA, USA).

Detection of embryonic GFP-transgene expression by live, digital low light level epifluorescence imaging
Live embryos were photographed with a Nikon TE-300 inverted microscope equipped with fluorescent isothiocyanate (FITC) filters and a Princeton CCD camera. Images were captured and analysed by Metamorph software (Universal Imaging, West Chester, PA, USA).

Detection of embryonic GFP-transgene expression by immunocytochemistry
To detect GFP expression, selected embryos were fixed and immunostained with a polyclonal rabbit anti-GFP antibody (ClonTech, Palo Alto, CA, USA). After zona pellucida removal with 0.5% pronase, embryos were attached to polylysine-coated coverslips and fixed for 1 h in 2% formaldehyde in TALP–HEPES. Fixed embryos were permeabilized in 0.1 mol/L PBS containing 2% Triton X-100 detergent for 40 min, followed by incubation for 30 min in a PBS blocking solution containing 150 mmol/l glycine and 3 mg/ml BSA. The
primary GFP antibody was diluted 1:100 in PBS and applied for 1 h at 37°C. After a 30 min wash in PBS with 0.1% Triton detergent, GFP primary antibody was detected using rhodamine-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody. DNA was labelled with 5 μg/ml Hoechst 33342 added to the penultimate rinse and embryos. They were then mounted in Vectashield antifade (Vector Labs, Burlingame, CA, USA) and examined with a Zeiss Axioshot epifluorescent microscope equipped with appropriate filters and high numerical aperture objectives.

**Embryo transfer**

Female rhesus monkeys with normal menstrual cycles synchronous with those of the egg donors were screened as potential embryo recipients. Screening was performed by collecting daily blood samples beginning on day 8 of the menstrual cycle (with first day of menses as day 1) and analysing the concentrations of serum progesterone and oestrogen. Timing of ovulation was detected by a significant decrease in serum oestrogen and an increase in serum progesterone to >1 ng/ml. Surgical embryo transfers were carried out on days 2 or 3 into the oviduct of the recipient, by mid-ventral laparotomy. The oviduct was cannulated and two 4–8-cell stage embryos were transferred via a small catheter.

To confirm implantation, blood samples were collected daily and analysed for serum oestrogen and progesterone (Lanzendorf et al., 1990) and pregnancies confirmed by ultrasonography on day 35 post-transfer. During ultrasound, measurements were taken of total fetal length, femur length, head circumference, fetal cardiac activity and size of yolk sac. Ultrasound was performed once more, during the second trimester, to determine developmental normality. In recipients that maintained adequate oestrogen and progesterone concentrations, but who were deemed not pregnant by ultrasound examination, blood samples were analysed for serum monkey chorionic gonadotropin (mCG) measured by an LH bioassay (Ellinwood and Resko, 1980).

**Detection of transgenes using polymerase chain reaction (PCR)**

Tissues from the two stillborn rhesus monkeys were collected and maintained at −20°C until DNA extraction. Buffy-coats collected from blood were isolated by centrifugation using PMN isolation medium (Robbins Scientific Corporation, Sunnyvale, CA, USA). DNA was extracted by proteinase K digestion followed by phenol–chloroform extraction. After ethanol precipitation, the DNA pellet was resuspended in Tris–EDTA buffer (pH 8.0). Genomic DNA (1 mg) was used for PCR analysis using GFP-specific primer set GFP-#1: TGAACCGCATCGAGCTGAAG, and GFP-#2 CGATGTTGTGGC-.

**Results**

Rhodamine-tagged plasmid DNA serves as a dynamic fluorescence marker that demonstrates the avid binding of DNA to the surface of the sperm head, as shown by confocal microscopy (Figure 1A, mouse; Figure 1B, bovine; Figure 1C, rhesus monkey. Hamster, ram and rabbit, data not shown). This signal is retained after thorough washing, though its trypsin lability suggests that the adherence at the sperm cell surface is protein-mediated (Lavitrano et al., 1992; Zani et al., 1995).

Laser-scanning confocal and digital epifluorescence imaging of fertilization in rhesus monkeys by ICSI demonstrates the preservation of the rhodamine–plasmid fluorescence in association with the microinjected spermatozoa throughout the ICSI procedure and during the early stages of pronuclear development (Figure 1D–E; Figure 2). The brightness of the signal, which might have been quenched by the deeper focus through the cytoplasm, indicates that most of the exogenously-bound DNA is retained after ICSI. Furthermore, the plasmid remains associated with the sperm nucleus and does not disperse (Figure 1D–E). Dynamic, live, high resolution imaging (plan Apo ×100; 1.4 NA; oil immersion) demonstrates the persistent binding of the rhodamine-tagged plasmid DNA to the spermatozoon during sperm selection (Figure 2A), during sperm microinjection (Figure 2B), and after successful ICSI (Figure 2C). The signal of the rhodamine-labelled plasmid is lost as the oocyte enters the first cell cycle since the dim image becomes undetectable as it expands and may also be quenched by the egg cytoplasm. Microinjected free plasmid disperses swiftly throughout the oocyte cytoplasm (data not shown).

In contrast to ICSI, IVF with rhodamine-tagged, plasmid-bound spermatozoa demonstrates that most of the signal is lost at the oocyte surface during sperm incorporation (Figure 1F1 and 1F2). Comparisons of Figure 1F1 (dynamic rhodamine-labelled plasmid DNA detected by laser scanning confocal microscopy) and 1F2 (transmitted optical imaging at the same plane focused at the egg surface) reveal that the lower spermatozoon, which has already penetrated the egg cytoplasm (white arrows), fluoresces at a barely detectable level. Figure 1F1 and 1F2 shows the upper, partially-penetrated, spermatozoon during sperm incorporation: the basal region fluoresces brilliantly and optical sectioning demonstrates that it remains outside the oocyte (yellow arrow), whereas the apical region (green arrow) has entered the oocyte and has lost the rhodamine fluorescence. The sharp demarcation at the equatorial region of the sperm head is the site of sperm–oocyte membrane fusion (Yanagimachi, 1994). The contrast between the regions of bright and lost fluorescence suggests that the rhodamine-tagged DNA is removed quickly at this specific zone. The signal cannot be traced as diffusing within the now hybrid sperm–oocyte plasma membrane. Perhaps the rhodamine-tagged DNA is mechanically or enzymatically removed, or perhaps its dispersion at the surface is faster than our dynamic imaging can assess.

GFP expressing embryos were created by ICSI, though not through IVF, with these DNA-bound spermatozoa (Figure 1D). Rhesus monkey spermatozoa, which have bound rhodamine-tagged circular plasmid DNA encoding the GFP gene under the control of CMV promoter (Rh-CMV-GFP), retain the plasmid after microinjection into mature rhesus monkey (Figure 1D), or bovine (Figure 1E) oocytes. Mosaic GFP expression is detected as early as the 4-cell stage (Figure 1H: one of four blastomeres fluoresces green with GFP superimposed on the Hoffman modulation contrast image); the number of blastomeres and the percentage of expressing embryos increase at least until the blastocyst stage, in which both the inner cell mass and trophectoderm exhibit GFP fluorescence (Figure 1I).
Figure 1. Plasmid transfer by intracytoplasmic sperm injection (ICSI). Rhodamine-labelled plasmid DNA binds avidly to (A) mouse, (B) bovine, and (C) rhesus monkey spermatozoa. Rhodamine-tagged DNA remains on the surface of microinjected spermatozoa after ICSI: rhesus monkey spermatozoa microinjected into (D) a rhesus monkey oocyte or (E) into a bovine oocyte. (A–D) Blue = Hoechst DNA imaging. (F1 and F2) Labelled rhesus monkey spermatozoa during in-vitro fertilization (IVF). F1, rhodamine-tagged plasmid DNA is lost at the egg surface during IVF (arrows). The partially penetrated spermatozoon demonstrates the loss of exogenous DNA in the penetrated half (green arrow). F2, imaging of the same focal plane. (G) Detection of green fluorescent protein (GFP) expression in a 16-cell stage rhesus monkey embryo using anti-GFP monoclonal antibody (red) and Hoechst DNA staining (blue). (H) Live 4-cell and (I) blastocyst stage rhesus monkey embryos expressing GFP after transgenesis by ICSI using rhodamine-labelled plasmid DNA encoding the GFP gene bound to the injected spermatozoon. (A–F) laser scanning confocal microscopy. (A, B and C) produced by overlaying images of 14 labelled spermatozoa and each individual image of a spermatozoon is an overlay of 16 images taken at different focal planes. (G) was collected by digital low light level fluorescence imaging (Princeton CCD, differential interference contrast, Zeiss Axiophot).
Direct GFP fluorescence detection is not the most sensitive indicator of GFP expression. In the embryo shown in Figure 1G, fluorescence was undetectable by direct GFP imaging. Therefore, the embryo was fixed and labelled with anti-GFP antibody (anti-GFP, red; blastomere nuclei, blue). A single blastomere with a detectable signal under fluorescent microscopy was observed, indicating that GFP expression of the transgene is only detectable using anti-GFP immunocytochemistry. Undetectable direct GFP fluorescence may be due to values of GFP expression that are below threshold, to protein misfolding or to partial translation of the peptide containing the recognized epitope.

The GFP expression observed here could reflect the maintenance of the plasmid DNA in an episomal, non-integrated state. Experiments in which the GFP plasmid was microinjected directly into the oocyte cytoplasm (not with the sperm DNA), have been performed. GFP expression results, but with earlier onset. In addition, rhodamine–DNA remains associated with the microinjected sperm nucleus unless they are bound prior to injection. Most oocytes expressed GFP, usually at the 1–4-cell stages, probably due to the expression of episomal, non-integrated plasmid DNA before the maternal–embryonic gene expression transition. In contrast, in most embryos the onset of expression using microinjected DNA-bound spermatozoa occurs after the maternal–embryonic gene expression transition, thought to occur in monkeys at the 4–8-cell stages, and can be mosaic and spatially restricted to as few as single blastomeres in a morula. Furthermore, dynamic low-light level imaging of the rhodamine-labelled DNA on the microinjected spermatozoon demonstrates that DNA remains associated with the injected spermatozoon within the oocyte cytoplasm (Figure 2).

GFP expression is efficient in rhesus monkey embryos produced by the injection of modified sperm (34.6 ± 4%, n = 81). In contrast with murine transgenic protocols, in which blastocysts selected for developmental competency and transgene marker expression are implanted (Perry et al., 1999), pregnancies in rhesus monkeys are routinely successful only when 4–8-cell embryos are transferred (Hewitson et al., 1999). For this reason, the growth of GFP-expressing blastocysts is an important experimental achievement, although it has not led directly to the production of transgenic offspring.

Three pregnancies in rhesus monkeys (with GFP-expressing embryos transferred at the 4–8-cell stage) have resulted from the seven transfers carried out during the last rhesus monkey breeding season (October–February). A healthy male, named ‘George,’ was born at term (Figure 3), and a set of anatomically normal twins (male and female) was stillborn at 35 days premature. GFP epifluorescence detection in the stillborn tissues and in the newborn did not indicate fluorescence above the background level. PCR analysis of stillborn tissues and white blood cells collected from the newborn were negative for the GFP gene.

**Figure 2.** Live, digital low light level epifluorescence imaging of intracytoplasmic sperm injection (ICSI) in rhesus monkeys using spermatozoa bound with rhodamine-labelled plasmid DNA. A single spermatozoon, suspended in 10% polyvinylpyrrolidone (PVP) and displaying rhodamine labelling, is aspirated tail-first into an injection pipette (A). The pipette is inserted through the zona and oolemma membrane of an oocyte, immobilized with a second suction pipette, and the spermatozoon is placed deep within the oocyte cytoplasm (B). A brief aspiration of cytoplasm ensures the correct positioning of the spermatozoon within the oocyte prior to its release (C). All procedures were performed at ×100 magnification using digital low light level fluorescence imaging to ensure continued rhodamine visualization.
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Figure 3. George: the world’s first rhesus monkey infant conceived by intracytoplasmic sperm injection (ICSI), using spermatozoa with bound rhodamine-labelled plasmid DNA.

Discussion

The delivery of exogenous genetic material into primate oocytes during ICSI results in both embryonic transgene expression, as well as live births and demonstrates the feasibility of this new procedure. This report also demonstrates fundamental differences between fertilization by ICSI and IVF. In so doing, it highlights molecular variations in the choreography of this assisted reproductive technology (ART) protocol from the norm, and elicits disturbing predictions that microinjected spermatozoa could deliver contagion. We emphasize that these theoretical risks are just that, and furthermore we propose strategies for helping to ensure that the microinjected spermatozoa are germ-free so that ICSI remains a safe and effective procedure.

Fertilization by ICSI, unlike IVF, bypasses the normal plasma membrane interactions that have been shown to exclude foreign genes adhering to the spermatozoa. Consequently, the delivery of genetic material into an oocyte during ICSI raises concerns of an alternative pathogen entryway and consequent infection of the embryo. A recent report (Brossfield et al., 1999) demonstrates the tenacity with which exogenous human papillomavirus DNA binds to human spermatozoa and shows that this virus is resistant even after extensive sperm washing, and hence underscores this issue. Equally troublesome is the observation that the viral DNA enhanced sperm motility. There are potential ramifications for infertility clinics and their patients, as well as for the colony management of endangered species and biomedical research animals.

Since ICSI violates the natural route of fertilization and the natural defence mechanism of an oocyte, several strategies are proposed to reduce or eliminate the potential pathogens adhering to the exterior of spermatozoa chosen for ICSI. Ideally, these sanitizing treatments should employ both physical removal and chemical decontamination to ensure that only germ-free spermatozoa are introduced. These chemical treatments must also not influence normal reproduction and therefore must either be removed or neutralized prior to or during ICSI. The elimination of bacterial and viral pathogens could be accomplished by enzymatic hygienic treatments, particularly if the enzymes are physically bound so that they are removed prior to ICSI, or if they have a pH or other ion sensitivity such that they are neutralized within the cytoplasm. For example, proteinases and/or nucleases (DNases, RNases) could be used. One author (Gagne et al., 1991) has demonstrated the usefulness of DNase treatment of bovine spermatozoa which carry foreign DNA. Since even substantial washes are unlikely to result in complete decontamination, physical binding to an exogenous substrate is proposed. Polystyrene or magnetic beads, with brilliantly fluorescing dyes, are commercially available for enzyme cross-linkage.

An innovation for both the sanitizing of spermatozoa for ICSI and for sperm selection criteria can only now be proposed.
Among the vexing problems of ICSI, perhaps the foremost is sperm selection. Since we do not yet understand Nature’s selection criteria for choosing the successful spermatozoon, ICSI clinicians and researchers have few unambiguous measures. A recent report (Berkovitz et al., 1999) was greeted with enthusiasm as an important non-invasive assay for selecting among the myriad of potentially viable spermatozoa. The binding of decontaminating enzymes to the zona pellucida is proposed as a simple and feasible approach to: (i) physically eliminate the exogenous material bound on the spermatozoon since it is the first barrier during fertilization; (ii) destroy foreign infectious particles without interfering with the viability of the spermatozoon for reproduction, since the spermatozoon retains its intact plasma membrane; and (iii) select spermatozoon inside the perivitelline space after penetration through the zona pellucida, since this relies on a non-invasive and natural method for choosing the spermatozoon for ICSI.

In this study, we show that primate spermatozoa with bound DNA retain their full reproductive potential for full-term offspring normal by every measurable criteria. The biomedical rationales for producing transgenic primates are clear – to fill the gap between transgenic mice and patients. However, this undertaking differs considerably from the production of transgenic rodents, which requires only weeks to achieve live births and is a more permissive system for analysing results following humane euthanasia. Murine transgenesis by ICSI has been recently reported (Perry et al., 1999) as a successful alternative to pronuclear microinjection, and demonstrates 2–2.8% DNA integration. Breaching the sperm plasma membrane was shown to enhance transgenesis efficiency, perhaps due to increased DNA binding, internalization and/or integration.

Although we have not demonstrated the integration of exogenous DNA in the offspring, the delivery of exogenous DNA into oocytes by ICSI has been shown by GFP expression during embryonic development. Transgenic frogs have been successfully produced after microinjection of spermatozoa, partially decondensed by incubation in cell-free extracts together with restriction enzymes and exogenous DNA (Kroll and Amaya, 1996). Transgenic primates promise unrivalled relevance for clinical investigations, but differ fundamentally from transgenic mice in many ways: (i) rodent oocytes are unlimited, whereas rhesus monkeys provide oocytes with maximal development only during the first hormonal stimulations; (ii) murine surrogates are limitless, while rhesus monkey surrogates are fewer and more precious; (iii) embryo transfers in rhesus monkeys may each need to be limited to a single prime embryo to avoid high-risk multiple pregnancies and the rearing of premature babies, whereas mice can have up to 10 offspring; (iv) gestation is eight times longer in rhesus monkeys than in rodents (~165 days versus ~20 days in mice); (v) fecundity in rhesus monkeys is seasonal, with pregnancies optimally established for only 5 months of the year; (vi) primates merit specialized care and treatment, precluding many invasive or terminal analyses; (vii) primates reach reproductive age at ~5 years versus a few months in mice; (viii) rodent reproductive protocols have been optimized for embryo culture conditions permitting transfer of blastocysts in embryos selected on the basis of transgene expression, versus cleavage-stage embryos in primates; (ix) transgenic mice can be reared comparatively inexpensively; (x) inbred murine strains have been characterized for optimal molecular manipulations, whereas primate colonies have been deliberately managed to avoid inbreeding; and (xi) murine genetics are unrivalled among mammals, but the human genome project will extrapolate more directly to primates. While primates require greater and longer-term investments and dedication, the births reported here, along with the <3% murine DNA integration rates, provide justification for pursuing the imperative goal of producing primate models for both serious human diseases and for assessing the efficacy of gene and cell therapeutic strategies.

We caution that extrapolations from this work to clinical applications are dangerous. While the scientific literature now includes the thankfully still fictional discussion of human ‘genetic enhancement,’ (Gordon, 1999) the sole intention of our exploration on genetic modifications of non-human primates is for the production of human disease models. As with other ART and molecular medical innovations, full and frank dialogues between ethicists, clinicians, scientists, lay people, and regulatory authorities will be necessary for the consideration of where science could go and, perhaps, more importantly, where science should go.

Transgenesis by ICSI represents an unorthodox but promising approach for exogenous DNA transmission, and could be particularly valuable in systems in which oocytes, surrogate mothers, and the number of embryos transferred are precious and limiting, as in primates. Transgenesis by ICSI would eliminate the problem of locating the male pronucleus for subsequent microinjection within the nearly opaque cytoplasm in oocytes from domestic species (i.e. pigs and cows) or when both pronuclei are indistinguishable (e.g. primates). Ironically, sperm-mediated exogenous DNA transfer during ICSI raises the worry that this globally adopted clinical protocol might inadvertently transfer infectious materials to the next generation (Brossfield et al., 1999), thus affecting infertility patients, their families and clinicians, as well as animal colonies propagated by ICSI. Nevertheless transgenesis by ICSI applications include the production of models for investigating the molecular basis of hereditary diseases, demonstration of the safety and efficacy of gene, stem or somatic cell therapy prior to clinical trials, endangered species preservation, and perhaps even a new approach for sperm-mediated gene therapy.

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