TABLE II
Efficiency of Methods for Microinsemination and Nuclear Transfer Using Male Germ Cells

Male germ cells	Intracytoplamic injection	Electrofusion	Virus (HVJ)-mediated fusion
Spermatozoa	High	No	No
Round spermatids	High	Low	No
Primary spermatocytes	Moderate	Moderate	No
Primordial germ cells (G1 phase)	High	Moderate	Moderate
Primordial germ cells (M phase)	Low	Moderate	Moderate

impossible to fertilize oocytes with mature spermatozoa by membrane fusion methods (Table II).

The key to successful ICSI in mice by direct injection was the use of a piezo pulse–driven micromanipulator (Fig. 2). The piezo crystal impact unit of the micromanipulator propels the injection pipette in short stabbing motions to make a deep resealable hole in the oocyte membrane (Ediz and Olgac, 2004). This enables the injection of a spermatozoon without damage to the oocyte. Kimura and Yanagimachi (1995b,c) mastered this technique and soon were able to produce live offspring very effectively using round spermatids and secondary spermatocytes. Successful mouse ICSI without the piezo system has been reported, but it seems to require a very high level of skill (Rybouchkin *et al.*, 1995; Suzuki and Yanagimachi, 1997).

In addition to mice, this technology is now being applied to livestock and other laboratory animals. Piezo micromanipulators are indispensable to successful microinsemination in rodents, whose oocytes are particularly fragile. These include rats (Hirabayashi *et al.*, 2002a,b), the golden hamster (Haigo *et al.*, 2004; Yamauchi *et al.*, 2002), and mastomys (*Praomys coucha*) (Ogonuki *et al.*, 2003a). This technology is widely used for injection of mature sperm as well as for many other types of microinsemination (Ogura and Yanagimachi, 1999) and cloning procedures (Ogura *et al.*, 2001; Wakayama *et al.*, 1998a).

The combination of piezo micromanipulators and mouse oocytes can now be used to investigate the oocyte-activating factors of sperm and spermatogenic cells from a variety of species, because piezo-assisted injection alone does not activate mouse oocytes (Yazawa *et al.*, 2000). As these are interspecies experiments, the results do not always accurately reflect intraspecies microinsemination results (e.g., see ROSI in rabbits, which will be discussed later). The piezo-driven technique has also provided excellent experimental models by which to evaluate patterns of intracellular calcium oscillations and transfer of oocyte activation factors to pronuclei (Ogonuki *et al.*, 2001).

III. Microinsemination with Spermatogenic Cells

The history of microinsemination of spermatogenic cells, like that of ICSI, began with hamsters (Ogura and Yanagimachi, 1993; Ogura *et al.*, 1993), but the first live offspring were produced using round spermatids in mice (Ogura *et al.*, 1994). However, as mentioned above, experiments in this series were undertaken by electrofusion, and therefore the efficiency of production of live pups was low. We obtained only 22 pups from 662 transferred embryos during the electrofusion study (Ogura *et al.*, 1995), but we soon shifted to the technique of piezo-assisted microinsemination developed by Kimura and Yanagimachi (1995b) to achieve much better efficiency. As shown in Table I, live pups in several animal species have been produced by this method, not only with mature sperm cells but also with immature spermatogenic cells (Fig. 3).

A haploid set of paternal chromosomes is required in the fertilized oocyte, so even with the use of spermatogenic cells that have not completed meiosis, a haploid set of chromosomes is ultimately required in the oocyte. Because primary spermatocytes are premeiotic and secondary spermatocytes are undergoing meiosis, their nuclei must complete meiosis within the oocyte. Therefore, oocytes in first meiosis or second meiosis must be used. Spermatids have already completed meiosis and have the same haploid number of chromosomes as spermatozoa, so by synchronization with oocyte chromosomes, a normal diploid embryo can be constructed. Figure 4 depicts a

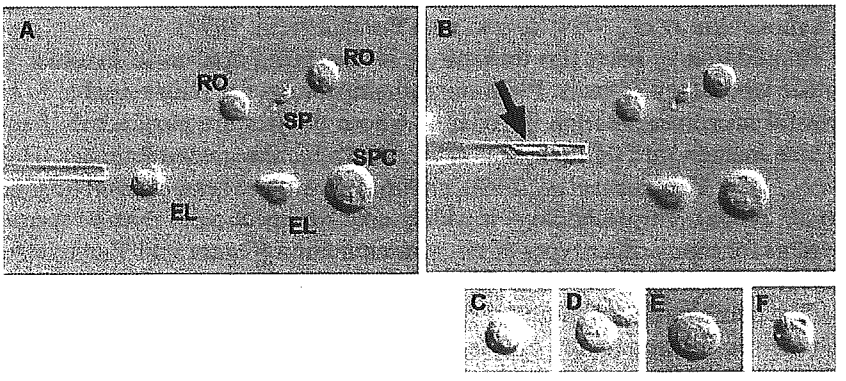

FIG. 3 Spermatogenic cells of different species. (A) Mouse cells. SP, spermatozoon; EL, elongated spermatid; RO, round spermatid; SPC, primary spermatocyte. (B) An elongated spermatid is drawn into an injection pipette. The condensing nucleus is clearly visible (*arrow*). (C–F) Round spermatids from a golden hamster, mastomys, rabbit, and cynomolgus monkey, respectively. Round spermatids can usually be identified by their low nucleus:cytoplasm ratio and round nuclei. Bar = 10 μm.

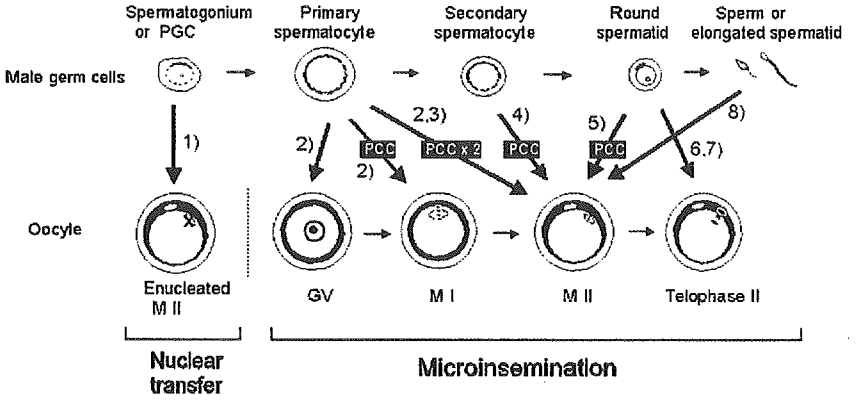

FIG. 4 Combinations of oocytes and male germ cells used to construct diploid embryos. Male germ cells, at stages ranging from late-pachytene primary spermatocytes to mature spermatozoa, can participate in the formation of diploid embryos and full-term development following microinsemination using different stages of oocytes. Primary and secondary spermatocytes that have not completed meiosis can undergo meiosis to form a haploid set of chromosomes within oocytes. By contrast, younger male germ cells—primordial germ cells and gonocytes—can participate in the formation of diploid embryos through nuclear transfer into enucleated MII oocytes, that is, by nuclear-transfer cloning. Whether these reconstructed embryos develop into offspring depends on the status of genomic imprinting of the donor-cell genome. Day-10.5 primordial germ cells have been demonstrated to support full-term development. PCC, premature chromosome condensation. References in the schema: (1) Miki *et al.*, 2005; (2) Ogura *et al.*, 1998; (3) Kimura *et al.*, 1998b; (4) Kimura and Yanagimachi, 1995c; (5) Ogura *et al.*, 1999; (6) Ogura *et al.*, 1994; (7) Kimura and Yanagimachi, 1995b; (8) Kimura and Yanagimachi, 1995a.

combination of these spermatogenic cells and oocytes. However, the ability to form a haploid set of chromosomes does not necessarily result in a spermatogenic cell's functioning normally as a gamete.

For a diploid embryo to develop into a normal live offspring, one other important condition is required, namely, resetting of genomic imprinting by DNA methylation and other mechanisms (Kaneko-Ishino *et al.*, 2003; Reik and Walter, 2001). Genomic imprinting confers functional differences on paternal (sperm) and maternal (oocyte) gene alleles, and genes with these differences are termed "imprinted genes." Studies in mice have confirmed that paternal genomic imprinting is complete before birth. In mouse fetuses produced by ROSI, Shamanski *et al.* (1999) confirmed normal allelic expression of paternal (*Snrpn, Igf2, Peg1*) and maternal (*Igf2r, Mash2, H19*) imprinted genes.

The rate of development of fertilized oocytes varies greatly with the type of spermatogenic cells used (Table III). A number of factors may be involved, including the lower yields caused by repetitive micromanipulation and

TABLE III
Efficiencies of Microinsemination and Nuclear Transfer in the Mouse[a]

Germ cells	Constructed/ used eggs	Morula and blastocyst/ constructed	Implant/ transferred	Offspring/ transferred
Mature spermatozoa	90%	80%	70%	40%
Elongated spermatids	90%	70%	60%	40%
Round spermatids	90%	70%	50%	30%
Primary spermatocytes	50%[b]	70%	40%	5%
Primordial germ cells (day 12.5)	70%	80%	40%	5%[c]
Primordial germ cells (day 10.5)	70%	Not examined	50%	1%

[a]Data from experiments using B6D2F1 oocytes.
[b]Survival at M II.
[c]Live fetus at day 10.5.

complex procedures, the physical resistance of the nuclei to handling, and the efficiency of synchronization between male and female nuclei.

Spermatogenic cells can be collected by mechanical procedures, including mincing and pipetting of the seminiferous tubules, or by enzymatic processing (Ogura and Yanagimachi, 1999). The former is easier for collection of spermatogenic cells in relatively advanced spermatogenesis after the spermatocyte stage, whereas the latter causes less damage to cells and is suitable for younger cells, such as early primary spermatocytes and spermatogonia. Figure 3 shows a photograph of mouse spermatogenic cells collected from the seminiferous tubules using a mechanical procedure.

A. ELSI

Spermatids are haploid spermatogenic cells that have completed meiosis, and they are roughly divided—based on their morphology—into earlier round spermatids and later elongated spermatids. These two types differ both morphologically and biochemically. The protocol of microinsemination and its outcome reflects these differences. In general, ELSI is based on ICSI and is relatively efficient in producing live offspring. This is because elongated spermatids have oocyte activation factors and have undergone nuclear condensation (histones have been replaced by transition nuclear proteins or by protamines [Fig. 3]). From these characteristics, synchronization with oocytes can be achieved with injection alone. ICSI in Muridae (especially

rats and mastomys) is difficult because the sperm have large, falciform heads. However, ELSI can be performed even with relatively thin pipettes. In mastomys, live offspring have been produced with elongated spermatids, but not with spermatozoa or round spermatids (Ogonuki *et al.*, 2003a). The advantages of ELSI are summarized in Table IV. ELSI has not been attempted, but it may be well suited to many other animal species.

B. ROSI

ROSI's effectiveness in producing viable offspring is quite low, due to various factors (Table IV). In addition, testicular cell suspensions contain a mixture of spermatogenic and somatic cells at various stages. Thus, considerable experience is required to accurately identify the round spermatids. Figure 3 shows examples of round spermatids from mice, hamsters, rabbits, and cynomolgus monkeys. These can usually be identified because of the low nuclear/cytoplasm ratio (high in lymphocytes and spermatogonia) and round nuclei. The final confirmation can be achieved by observation of the haploid chromosomes, which are prematurely condensed following injection into mouse oocytes (Ogura and Yanagimachi, 1999). The accuracy of identifying round spermatids also depends on the composition of the sperm suspension. For example, there are very few first-wave round spermatids in the testis of 17-day-old male mice. When ROSI was performed using a suspension of such cells, the birth rate per embryo transfer decreased to 0.9% (Miki *et al.*, 2004b) (Fig. 5). The accuracy of cell identification confirmed by chromosomal analysis was 60% (usually 90% or higher [Miki *et al.*, 2004b]). Thus, to properly interpret experimental results, the accuracy of identification of round spermatids should be known beforehand.

Unlike ICSI or ELSI using spermatozoa or elongated spermatids with their adequate oocyte activation ability, ROSI often requires artificial oocyte activation. Spermatids from humans, cynomolgus monkeys, and golden hamsters usually contain sufficient amounts of SOAF, and ROSI-derived embryos may develop to fetuses or live offspring (Table I) (Haigo *et al.*, 2004; Ogonuki *et al.*, 2003b; Tesarik *et al.*, 1995). Spermatids from mice, rats, and mastomys contain almost no oocyte-activating factors, so artificial activation is necessary (Hirabayashi, *et al.*, 2002b; Kimura and Yanagimachi, 1995b; Ogonuki *et al.*, 2003a). Pigs and rabbits fall somewhere in between. For example, with ROSI in rabbits, about half of the oocytes are activated, and embryos divide, but very few of those develop to blastocysts. However, with artificial activation, the development of rabbit ROSI-derived embryos can be improved to the same extent as in ICSI. This suggests that rabbit round spermatids contain insufficient SOAF (N. Ogonuki, unpublished data). Rabbit oocytes, in particular, require repeated stimulation (to increase

TABLE IV
Comparison of Spermatozoa and Spermatids as Male Germ Cells for Microinsemination

	Physical stability of the nucleus	Oocyte-activating capacity[a]	Population in testicular cells	Tolerance to cryopreservation	Pipette for injection
Mature spermatozoa	High	Full	Large	Good	Large
Elongated spermatid	High	Moderate	Small	Good	Small or intermediate
Round spermatid	Low	None or little	Large	Poor	Small

[a]Typical cases for rodents.

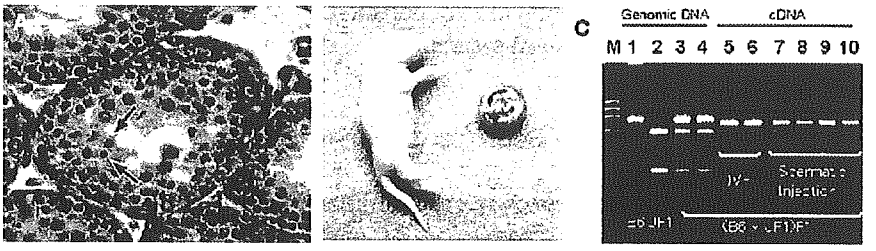

FIG. 5 ROSI using the first-wave cells in mice. Round spermatids can be retrieved from 17-day-old males at the youngest (*arrows* in A), and they supported full-term development following ROSI (fetus and its placenta in B). The first-wave spermatogenic cells have completed male-germ-cell-specific genomic imprinting as far as we examined for H19 (C), Igf2, and Meg3 in ROSI fetuses at day 9.5 of pregnancy (Miki *et al.*, 2004b).

intracellular calcium levels) for sufficient activation (Ozil, 1990). Proper oocyte activation in this species can thus be difficult.

The timing of artificial oocyte activation is also important if ROSI is to succeed. Meiosis stage II (M II) oocytes contain sufficient amounts of metaphase (or maturation)-promoting factor (MPF) for condensation of the introduced nucleus. This chromosome-condensing ability gradually disappears after oocyte activation. In round spermatids with DNA-binding proteins composed of histones, chromosome condensation in the presence of MPF occurs rapidly. Thus, the kinetics differ depending on when the nucleus is incorporated relative to oocyte activation (Ogura and Yanagimachi, 1999). As with fertilization using spermatozoa, synchronization by microinjection of the round spermatid nucleus into the oocyte should ideally be done during telophase II. However, Kishigami *et al.* (2004) reported efficient embryo development and production of live offspring using activation immediately after ROSI. Construction of normal diploid embryos is also reported to have occurred by induction of premature chromosome condensation (PCC), activation of oocytes while preventing polar body extrusion, and finally removal of one of the female pronuclei (Ogura *et al.*, 1999).

As mentioned above regarding ICSI, the MTOCs originate from sperm during fertilization in most animals. What happens following ROSI? In pigs, MTOC-like structures form near the injected round spermatid nucleus, but these do not form extensions, unlike the MTOCs in ICSI. Rather, a microtubule network arises from the ooplasm (Lee *et al.*, 1998). The kinetics are similar to those for microtubules after ICSI using only sperm heads without centrioles (Kim *et al.*, 1998). These observations suggest that there must be a mechanism by which oocytes recognize centrioles in mature sperm, analogous to the paternal mitochondrial-recognition system described

previously. If sperm centrioles are not detected, then the MTOCs are formed by the oocyte using redundant systems.

C. Microinsemination of Secondary Spermatocytes

There are only a few reports on microinsemination using secondary spermatocytes, and the reliable production of viable offspring has only been reported for mice (Kimura and Yanagimachi, 1995c). Secondary spermatocyte chromosomes, like M II oocytes, have a 2n haploid structure, so through induction of PCC in M II oocytes, there is complete synchronization with oocyte chromosomes. Subsequent stimulation of oocyte activation results in production of a normal diploid embryo with 1n haploid male and female pronuclei (Fig. 4). Although micromanipulation itself is not difficult, advanced skill is required to identify the secondary spermatocytes. In mice, these are about 14 m in diameter, roughly the same size as developing primary spermatocytes. Kimura and Yanagimachi (1995c) isolated cell nuclei in a glass injection pipette and defined cells with nuclei twice the size of round spermatid nuclei as secondary spermatocytes. Observations in mouse oocytes after induction of PCC confirmed an 86% accuracy rate of identification. This is surprisingly high, considering that secondary spermatocytes account for less than 1% of spermatogenic cells. The birth rate per embryo transfer was also very high, 24%.

D. Microinsemination of Primary Spermatocytes

Primary spermatocytes are spermatogenic cells in meiotic prophase I. This phase is broadly divided into the leptotene, zygotene, pachytene, and diplotene phases, based on the status of homologous chromosome pairing. With microinsemination, the chromosomes of the primary spermatocytes are forced into meiosis I (homologous chromosome segregation), so the chromosomes must be completely ready. Handel (1998) showed that, using induction of chromosome condensation with okadaic acid, there was normal transition to meiosis only with later-phase pachytene and diplotene chromosomes. These types of primary spermatocytes must be used for microinsemination. Primary spermatocytes are in the G2 phase of the cell cycle. Therefore, for cell-cycle synchronization, the oocytes should be in the G2 or M phase—specifically, the germinal vesicle (GV), metaphase of meiosis I (M I), or M II stage (Fig. 4).

We have obtained normal offspring from M I oocytes that were subjected to cytochalasin to arrest the cell cycle at M I (Ogura *et al.*, 1998). Arresting these oocytes at M I allows maternal and paternal chromosomes to be

completely synchronized at M I. Offspring cannot be obtained by this method without such treatment, perhaps because spermatocytes in prophase I cannot synchronize with oocytes in prometaphase I (Ogura *et al.*, 1997a). Examination of chromosomes in the M II stage with each method has shown a high rate of chromosome abnormalities using GV and M II oocytes. In many cases, there was premature separation of sister chromatids during meiosis I (Ogura *et al.*, 1998, 2002). With M I oocytes, there was a normal haploid set.

In our own laboratory, we have produced live offspring only using M I oocytes, but Kimura *et al.* (1998b) have also reported live offspring using M II oocytes. These findings indicate complete genomic imprinting in male germ cells before meiosis I. In addition, male-germ-cell chromosomes can undergo all stages of meiosis within oocytes.

IV. Practical Applications of Microinsemination

Microinsemination has many practical applications. In mice, in particular, it is highly efficient, and there is a wide range of applications for inbred and genetically engineered animals.

A. Production of Transgenic Animals

Ever since transgenic (TG) mice were first produced in the 1980s (Palmiter *et al.*, 1982), the main method of production of transgenic animals has been injection of genes into the pronuclei of zygotes shortly before syngamy. More recently, viral vectors, especially lentiviral vectors, have been shown to be effective for gene transduction in mammalian embryos (Hofmann *et al.*, 2003; Lois *et al.*, 2002). There is potential for a wide variety of applications in the future. Meanwhile, microinsemination involves injection of sperm into oocytes, and if genes are introduced at the same time, TG animals can also be produced.

Perry *et al.* (1999b) investigated this idea in a mouse ICSI model and produced TG animals with high efficiency. Interestingly, when DNA was attached to undamaged sperm, there was almost no transgenesis. Successful transgenesis occurred only when the sperm membranes were disrupted by freeze–thawing or treatment with detergent (Triton). The reason for this is not clear, but one explanation is that membrane disruption permits closer binding of the DNA to the nucleus. Introduction of bacterial artificial chromosome (BAC) DNA up to 170 kb in size was possible (Perry *et al.*, 2001). However, it may be difficult to reach a balance between the degree

of membrane disruption of male germ cells necessary to increase gene transfer efficiency and that needed to possibly decrease embryo development efficiency (Szczygiel *et al.*, 2003). Experience is required to achieve reliable results. In addition to mice, reproducible results have also been reported in rats (Hirabayashi *et al.*, 2005a; Kato *et al.*, 2004) and pigs (H. Nagashima, personal communication). We have also used this technique successfully with ROSI to produce transgenic mice (A. Ogura *et al.*, unpublished data) (Fig. 6).

Furthermore, genes can be introduced *in situ* in spermatogenic cells in the testicles. Then, sperm containing the introduced gene can be selected and used for microinsemination to produce transgenic mice (Huang *et al.*, 2000). Genes have been introduced, as also reported by Yamazaki *et al.* (1998), by injection of DNA into the seminiferous tubules, followed by electroporation with the testicles placed between the electrodes. The key to the success of Huang *et al.* was the use of a marker protein (an enhanced yellow fluorescent protein, or EYFP) and an EYFP-carrying mitochondrial localization signal. For some unknown reason, when an enhanced green fluorescent protein (EGFP) gene—which is ubiquitously expressed in the cell cytoplasm—was used, the spermatogenic cells with introduced genes tended to die. After gene introduction, sperm with the EYFP fluorescence in the midpiece were used for microinsemination to produce transgenic mice with high efficiency.

Microinsemination can also be applied to promote germ-line transmission of the genome present in chimeric mice. We produced chimeric mice using EGFP gene-transfected embryonic stem (ES)-like cells, and their round spermatids with EGFP fluorescence were selected for microinsemination. One of the two offspring obtained was positive for EGFP and confirmed germ-line transmission of the ES-like cell line (Kanatsu-Shinohara *et al.*, 2004). In such experiments, using fluorescent protein genes with a ubiquitous cytoplasmic expression promoter (e.g., "green mice" [Okabe *et al.*, 1997]),

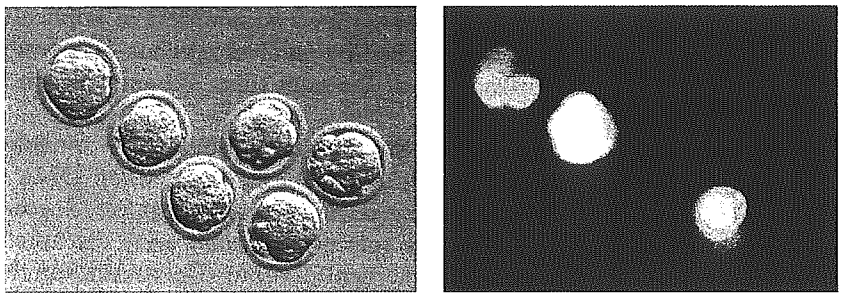

FIG. 6 Mouse morulae produced by round spermatid nuclei injection (ROSI) carrying the gene for enhanced green fluorescent protein (EGFP). Some embryos showed GFP-specific fluorescence (right) because of the gene integration and transcription. Thus, ROSI and ICSI can be applied to transgenic experiments.

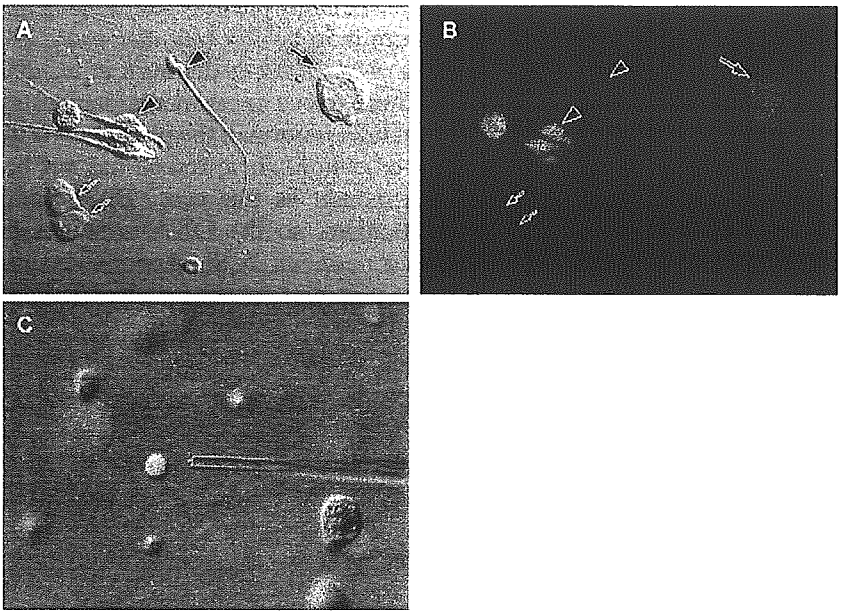

FIG. 7 (A, B) EGFP expression in spermatozoa and spermatogenic cells from a "green mouse." Round spermatogenic cells, such as primary spermatocytes (*large arrow*) and round spermatids (*small arrows*), show easily discernible fluorescence, but spermatozoa do not, because of the paucity of their cytoplasm. Only occasional cytoplasmic residues are positive for fluorescence (*arrowheads*). (C) Spermatogenic cells from a chimeric mouse produced from EGFP-transfected embryonic stem cells. Germ-line transmission can be ensured by microinsemination using fluorescent-positive spermatids.

use of round spermatids rather than mature spermatozoa is necessary to select transgenic cells because of the lack of cytoplasmic fluorescence in the mature spermatozoa (Fig. 7).

B. Research in Mutant Mice

Many abnormalities in fertilization have been reported in mice associated with spontaneous mutations, gene targeting, and random mutagenesis (Ward *et al.*, 2003). The most effective use of microinsemination may be for reproductive rescue in mice with spermiogenesis failure. Even with infertility caused by incomplete spermiogenesis, if the male genome's chromosomes can undergo meiosis to produce a haploid set, then by incorporation of the male genome into an oocyte by microinsemination, the chances of producing a normal fertilized oocyte are good.

Reproductive rescue has been reported in many phenotypes, ranging from spermatogenic arrest to abnormal sperm morphology (Akutsu *et al.*, 2001; Baart *et al.*, 2004; Kai *et al.*, 2004; Li *et al.*, 2003; Nakamura *et al.*, 2004; Yanagimachi *et al.*, 2004) (Fig. 8). These rescue experiments demonstrate that the gamete genome in these mutant mice is normal; the phenotype is thus limited to spermiogenesis failure. An exception where rescue has not been achieved is the protamine knockout mouse. In this case, DNA-binding proteins cannot be replaced by protamines, so the DNA is irreversibly damaged (Cho *et al.*, 2003). However, even in ICSI experiments using semen from double-knockout mice for transition nuclear proteins 1 and 2,

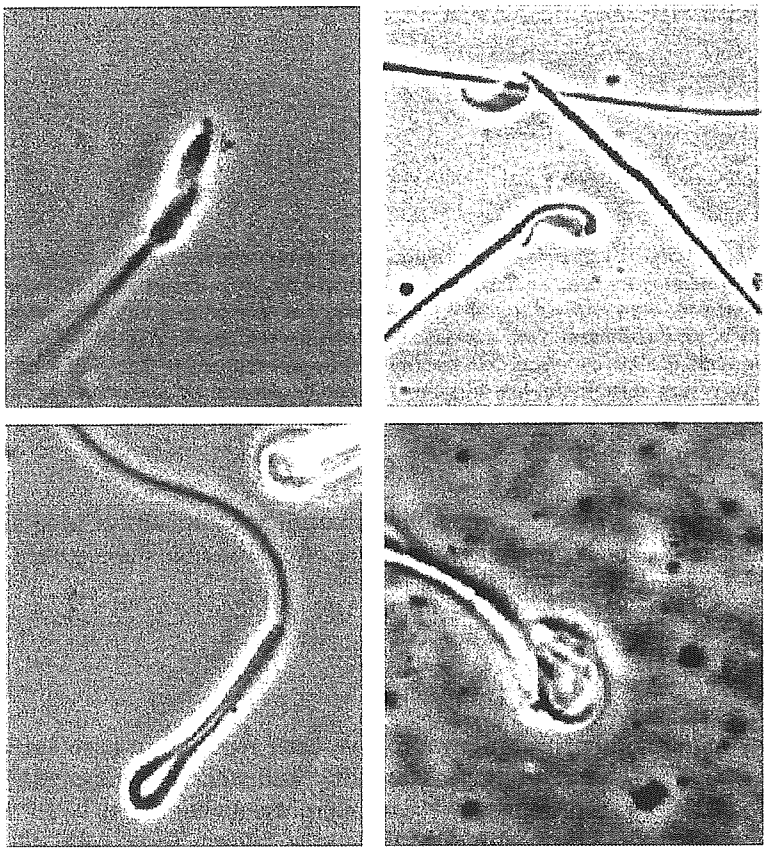

FIG. 8 Misshapen sperm heads from spontaneous or gene-modified mutant mice. Although their morphology is clearly abnormal, all such types of sperm can support full-term development by conventional ICSI at practical rates (birth rate 10–30% per embryos transferred).

live offspring were produced even though the DNA was unstable (Zhao *et al.*, 2004). In ICSI-derived embryos using sperm from infertile protein phosphatase 1cg knockout mice, there was developmental arrest preimplantation, but whether this was caused by aberrant gene function itself or by a chromosomal abnormality was unclear (Davies and Varmuza, 2003). ICSI has also been performed using sperm from BALB/c mice, a strain with a high percentage of malformed sperm but without a known gene mutation. Despite the malformed sperm, some viable offspring were produced, but there was also a high incidence of embryos with chromosomal abnormalities (Burruel *et al.*, 1996; Kishikawa *et al.*, 1999). Another ICSI experiment showed that the infertility of mice carrying two *t* complementary haplotypes (t^x/t^y) could be rescued by direct injection of spermatozoa into oocytes (Johnson *et al.*, 1995), indicating that the inherent defect involved interactions with oocytes—that is, zona pellucida penetration and sperm-egg fusion (Kuretake *et al.*, 1996b).

In addition to factors involved in spermiogenesis and sperm-fertilizing ability, microinsemination has been used to investigate oocyte factors indispensable for fertilization. For example, if the CD9 protein is absent on the oocyte membrane, there is no fusion with the sperm membrane, resulting in fertilization failure (Miyado *et al.*, 2000). However, if microinsemination is performed using oocytes with a CD9 deletion, offspring can be produced with normal efficiency. These findings demonstrate that CD9 plays a role in membrane fusion but is not involved in subsequent fertilization steps.

C. Genetic Preservation

Cryopreservation of embryos and sperm is an effective technique for preserving valuable animal species and laboratory strains. Cryopreservation of sperm is more efficient in terms of cost and space than is cryopreservation of embryos, and it is useful for banking TG and knockout mouse lines when inbreeding is not necessary (Nakagata, 2000). However, one drawback is decreased efficiency of IVF after sperm freeze–thawing, particularly in C57BL/6-strain mice (Nakagata, 2000), so a PZD method was developed (Nakagata *et al.*, 1997). However, ICSI can be performed even using sperm with motility disorders, and, with advanced technical procedures, the production rate of viable offspring is superior to that obtained with IVF (Szczygiel *et al.*, 2002). Live offspring have been produced by microinsemination even with sperm frozen without cryoprotectants (Wakayama *et al.*, 1998b). Viable offspring (mouse, rabbit, and rat) and normal-looking blastocysts (pig) have also been produced from spermatozoa that were freeze-dried and then stored at room temperature or under refrigeration (Hirabayashi *et al.*, 2005b; Kwon *et al.*, 2004; Liu *et al.*, 2004; Wakayama

and Yanagimachi, 1998). In addition, cryopreservation of spermatogenic cells at an earlier stage of development can reduce the time and effort required to collect such cells for experimental studies, and it is useful for preserving valuable genetic resources. Spermatogenic cells from several animal species can be cryopreserved in relatively simple solutions (e.g., PBS + 7.5% glycerol + 7.5% serum) with survival rates of 80–90% (Ogura *et al.*, 1997b). In mice, normal offspring have been produced with high efficiency using round spermatids after freeze–thawing (Ogura *et al.*, 1996a). This technology is useful for storing samples from animals with systemic disease (Ogura *et al.*, 1996b) or aging (Tanemura *et al.*, 1997), and for the transport of spermatogenic cells both domestically and internationally (Ikawa *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2003a). Live births in humans have also been reported using spermatids that had been freeze–thawed (Antinori *et al.*, 1997a).

Microinsemination techniques may enhance reproduction of endangered species because, in principle, fertilized oocytes can readily be obtained without optimization of several complex factors affecting fertilization (e.g., sperm capacitation). Application of the ICSI embryo-transfer techniques to the rescue of Felidae species may be promising, because the domestic cat can serve as a successful recipient of embryos from closely related nondomestic cats (Gomez *et al.*, 2004; Pope *et al.*, 1993). In domestic cats, the feasibility of producing viable offspring by ICSI has been demonstrated using both *in vivo*–matured (Pope *et al.*, 1998) and *in vitro*–matured oocytes (Gomez *et al.*, 2000).

D. Spermatogonial Stem-Cell Transplantation

Interest has also focused on spermatogonial stem-cell transplantation for use in genetic preservation and genetic engineering. This technology, which was originally developed by Brinster *et al.* (1994), involves allogeneic (intraspecies) or interspecies transplantation of spermatogonial stem cells, either purified or from testicular cell suspensions, for development to spermatozoa. When transplanted spermatogonial stem cells efficiently develop into mature spermatozoa, the normality of the stem cells and their spermatogenetic process can be confirmed by natural breeding, even after genetic modification *in vitro* (Nagano *et al.*, 2001a). However, in many other animal models with inefficient spermatogenesis, technical assistance with microinsemination is necessary. Transplantation between different strains (allogeneic transplantation) of mice is an example. As the seminiferous tubules are relatively immunologically privileged sites, better long-term survival of allogeneic donor stem cells than of skin transplants is expected. During this time (about 2 months), donor spermatogonial stem cells develop to round

spermatids, so microinsemination is required. This method has been used to produce viable offspring from C57BL/6 mice spermatids developed in the seminiferous tubules of C3H mice (Kanatsu-Shinohara *et al.*, 2003b). In other experiments, transplanted male germ cells (primordial germ cells or gonocytes) from fetuses have also been developed into spermatids and used to produce viable offspring (Chuma *et al.*, 2005; Ohta *et al.*, 2004).

Most research on spermatogonial stem cell transplantation has been conducted using primary cells from the testes. However, a spermatogonial germline stem-cell line (GS cells) established by Kanatsu-Shinohara *et al.* (2003c) retains its stem-cell characteristics after long-term passage in culture, or it can be genetically modified. This technique was further improved by adding some critical growth factors in the medium so that it can now be generally applied to major mouse strains including C57BL/6 (Kubota *et al.*, 2004). This GS cell technology is promising, in combination with microinsemination, for the highly efficient production of genetically modified animals including those that have been the subject of gene targeting.

Spermatogonial stem-cell transplantation is affected not only by immunological rejection but also by interspecies differences in the seminiferous tubule environment. For example, when the host is a mouse, rat and hamster sperm will develop with relatively high efficiency (Clouthier *et al.*, 1996; Ogawa *et al.*, 1999), but sperm development of spermatogonial stem cells from rabbits, dogs, and domestic species is difficult (Dobrinski *et al.*, 1999, 2000). With spermatogonial stem cells from monkeys, colonies will form only near the basement membrane (Nagano *et al.*, 2001b).

To overcome these obstacles, intact seminiferous tubules have been transplanted into different species to promote sperm development. If seminiferous tubules from pigs, goats, Dzungarian hamsters, or rabbits were transplanted into the back or testes of nude mice, spermatogonial cells developed into mature spermatozoa (Honaramooz *et al.*, 2002; Schlatt *et al.*, 2002; Shinohara *et al.*, 2002). A viable rabbit was born after microinsemination with rabbit spermatozoa, which had been developed in the mouse testis (Shinohara *et al.*, 2002).

E. *In Vitro* Spermatogenesis

It is very inefficient to produce mature spermatozoa *in vitro* from spermatogenic cells, probably because of the difficulty of mimicking the environment of the seminiferous epithelium *in vitro*. There are several reports of successful *in vitro* spermatogenesis from ES cells (Geijsen *et al.*, 2004; Toyooka *et al.*, 2003) or from premeiotic spermatogenic cells (Feng *et al.*, 2002; Gotoet *al.*, 1997; Rassoulzadegan *et al.*, 1993; Weiss *et al.*, 1997). However, in these reports, there was no conclusive evidence of the completion of

spermatogenesis, that is, birth of offspring from these *in vitro*–produced male germ cells. Marh *et al.* (2003) cultured mouse primary spermatocytes from the first spermatogenic wave in neonatal testes for 5B13 d, and they obtained round spermatids with a motile flagellum. By injecting these round spermatids into oocytes, they produced normal-looking offspring. Although preexisting spermatocytes were used, this is the only report of birth of offspring following microinsemination using *in vitro*–developing spermatogenic cells.

F. Gene Therapy for Spermatogenesis Failure

The causes of male infertility are not always genetic factors associated with the male germ line itself. At least some must come from dysfunction of Sertoli cells, multifunctional somatic support cells in the testis that make the scaffold of the seminiferous epithelium and produce several proteins to support development of male germ cells. Male mice with the Steel (Sl/Sl^d) mutation are azoospermic because they lack the membrane-bound form of this stem-cell factor on Sertoli cells. That the Sl/Sl^d mice have functional spermatogonial stem cells has been demonstrated clearly by the restored fertility of *W* mutant mice following Sl/Sl^d spermatogonial cell transplantation into the seminiferous tubules (Ogawa *et al.*, 2000).

Two groups (Ikawa *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2002), working independently and using different viral vectors (adenovirus and lentivirus vectors, respectively) found that the mouse *Sl* gene could be introduced into Sertoli cells in Sl/Sl^d mice, which then restored partial spermatogenesis. In both studies, they could not collect a sufficient number of spermatozoa from the epididymis for conventional IVF. Therefore, microinsemination procedures using spermatogenic cells, round spermatids, and elongated spermatids were performed, and normal pups were obtained at high rates after embryo transfer (19/87 and 12/43 per transfer, respectively). Thus, the microinsemination technique confirmed that spermatogenesis occurring in Sl/Sl^d mice after gene therapy was genetically normal, including germ-specific events such as meiotic division and paternal genomic imprinting.

G. Models for Treatment of Human Infertility

Microinsemination is also important for the treatment of human infertility in clinical practice. Such techniques for humans have advanced dramatically in recent years, as human oocytes can withstand injection and fertilization rates are high. From 1995 to 1997, some live births were reported using ROSI (Antinori *et al.*, 1997b; Fishel *et al.*, 1995; Tesarik *et al.*, 1995). However,

other studies in humans found low blastocyst and implantation rates of ROSI-derived embryos (Levran *et al.*, 2000; Urman *et al.*, 2002; Vicdan *et al.*, 2001). Further investigation of ROSI for human patients with late spermatogenic failure has been advocated (Aslam *et al.*, 1998; Urman *et al.*, 2002; Vanderzwalmen *et al.*, 1998). The Japanese Society of Fertility and Sterility currently does not support the use of ROSI. Some concern remains about the indiscriminate use of microinsemination in childless couples when the underlying genetics or pathologies associated with infertility have not been diagnosed, especially if transmission is likely to harm the offspring (Kuczynski *et al.*, 2002; Oehninger and Gosden, 2002).

It is generally accepted that nonhuman primates may provide the best experimental model for the human clinical practice. However, the first successful production of live monkey offspring by ICSI was first reported in rhesus monkeys in 1999 (Hewitson *et al.*, 1999), 7 years after the success in humans. Subsequent studies have reported live offspring in rhesus and cynomolgus monkeys using ejaculated sperm (Torii *et al.*, 2001), epididymal sperm (Hewitson *et al.*, 2002; Ng *et al.*, 2002), and spermatids (up to the fetal stage in cynomolgus monkeys) (Hewitson *et al.*, 2002; Ogonuki *et al.*, 2003b). In chimpanzees, blastocyst development using ICSI with ejaculated sperm has been reported (Suzuki *et al.*, 2004). These studies were also preceded by corresponding human cases (Schoysman *et al.*, 1993; Silber *et al.*, 1995; Tesarik *et al.*, 1995).

One of the issues to be resolved in the use of microinsemination in humans is "normality" of the fertilized oocytes and live offspring. This includes evaluation of the risk of the ICSI procedure itself on normal development. ICSI is performed with an injection pipette to immobilize (stun) a motile spermatozoon and then inject it into the oocyte. Injection of a motile spermatozoon can lead to a delay in pronuclear formation and a decreased fertilization rate (Catt *et al.*, 1995). In rhesus monkeys, the process of sperm swelling in ICSI embryos has been examined using electron microscopy (Ramalho-Santos *et al.*, 2000). The results show persistence of the acrosome and perinuclear theca (a cytoskeletal structure behind the acrosome), which normally disappear before entry into the ooplasm at fertilization. This may cause a delay in male nuclear decondensation. In other words, in normal fertilization, only the sperm nucleus enters the oocyte, whereas in ICSI the sperm-cell membrane is present during injection, which may cause some delay or inhibition of the interaction between the spermatozoon and oocyte. However, the process of immobilization (stunning) may "kill" the sperm. In microinsemination experiments in mice, immobilization of sperm and round spermatids and increased nuclear exposure time have both been associated with chromosome fragmentation and decreases in embryo development and live birth rates (Kuretake *et al.*, 1996a; Suzuki *et al.*, 1998; Tateno

et al., 2000). These factors can lead to serious problems in microinsemination in human patients. Further research is needed to determine what degree of stunning will not cause any chromosomal abnormalities and will enable smooth interaction between the sperm and oocyte.

Nakamura *et al.* (2002) evaluated a case of male infertility caused by globozoospermia (large round-headed sperm that lack acrosomes, which cannot bind to the zona pellucida) with an analysis of centrosomal function by microinsemination into bovine oocytes. The rate of male pronuclear formation was lower than with fertile donor sperm. Antibody staining of the ICSI-derived hybrid embryos also showed a lower rate of sperm aster formation. These asters are important in processes such as pronuclear migration and spindle formation in first somatic cell division. This centrosomal dysfunction involving aster formation may be one additional reason for the low fertility rates in men with globozoospermia. Analysis of centrosomal function by microinsemination of human sperm into animal oocytes may thus become an important screening study in the diagnosis and treatment of infertility.

Treatment of human infertility is based on the presumption of an abnormality in human reproductive cells, but evaluation using animal models may sometimes yield some unexpected results. In patients with no motile (viable) sperm, with sperm that died at an unknown stage, or with congenital spermatogenic arrest, further careful evaluation is needed to determine whether the sperm are suitable for microinsemination or whether any improved techniques are available. The use of animal models with long life spans similar to those of humans will thus be important in evaluating the normalcy of postnatal development and any effects of the technology on subsequent generations.

Monkeys are important models for human disease because of their many similar characteristics to humans and their long life span. In the field of developmental engineering, however, very few basic research data have been collected in monkeys. This highlights the need for further expansion of research in monkeys to serve as models for human infertility treatment. Cynomolgus monkeys form a well-established model for human IVF (Balmaceda *et al.*, 1984), but fertility rates are highly dependent on sperm motility. The use of ICSI to reliably inject a single spermatozoon may be a more promising technique than IVF to achieve fertilization. We investigated SOAF expression by injection of male cynomolgus monkey reproductive cells into mouse oocytes, and found that it is expressed in round spermatids (Ogonuki, 2001). We then performed ROSI using frozen round. The results of embryo transfer with fertilized oocytes showed development up to the fetal stage (Ogonuki, 2003b). At present, development of ROSI-derived embryos to the fetal stage without assisted oocyte activation has been reported only in humans and cynomolgus monkeys (Ogonuki, 2003b; Tesarik, 1995). This early expression of SOAF may thus be a characteristic feature of primates.

V. Nuclear Transfer Using Male Germ Cells

The production of viable offspring from later-phase pachytene and diplotene primary spermatocytes shows that—in at least some spermatocytes—paternal genomic imprinting is completed prior to the start of meiosis. Thus, to investigate genomic imprinting status, primary spermatocytes at earlier stages must be used. However, as mentioned above, the use of spermatocytes before the pachytene stage for microinsemination is difficult because of the structure of recombining chromosomes. Nuclei from spermatogonia at an earlier stage are still diploid and cannot be used for microinsemination. Construction of diploid embryos with spermatogonia therefore requires the use of nuclear-transfer cloning techniques (Fig. 4). In nuclear-transfer clones using unfertilized M II oocytes as recipients, the genomic-imprinting memory of donor cells does not change (Inoue *et al.*, 2002b), so the reconstructed embryos and fetuses accurately reflect the genomic imprinting status of the germ cells. Analysis of the gene-expression and DNA-methylation patterns of imprinted genes in a cloned fetus shows the imprinted status of the donor germ cell. Therefore, nuclear transfer cloning is a very effective way to study the genomic imprinting system in early germ cells.

Both gene-expression analysis and DNA-methylation analysis of cloned embryos from mouse primordial germ cells (PGCs) and gonocytes has clearly shown that paternal and maternal genomic imprinting memory in PGCs is gradually erased around day 11.5 of gestation and completely erased by day 12.5 (Lee *et al.*, 2002). This erasure causes bi-allelic expression or loss of expression, according to the imprinted gene examined. Thus, genomic imprinting of embryos from PGCs at day 12.5 or later is thought to reflect its "default status." In later fetal stages, paternal genomic imprinting is reestablished before birth (Lee, unpublished).

Embryos cloned from day-12.5 PGCs had developmental arrest at about day 9 of gestation. Interestingly, however, day–11.5 PGC clones (just one day earlier) had markedly improved development, with fetal development up to day 11.5 (Lee *et al.*, 2002). In day–10.5 PGC clones, some fetuses developed to parturition (Miki *et al.*, 2005). These developmental limitations can be explained at least in part by donor-cell genomic imprinting status. Interestingly, all term placentas of conceptuses cloned from day-10.5 PGCs showed hypertrophic development, characteristic of somatic-cell clones in the mouse (Wakayama and Yanagimachi, 1999a). This placental hypertrophy, together with the frequent postimplantation loss of such embryos (Wakayama and Yanagimachi, 1999b), indicates that the genomes of day-10.5 PGCs retain a somatic-cell genome type in terms of their reprogrammability in the egg cytoplasm. It is reasonable to assume that the female (oocyte) and male (sperm) germ-cell genomes undergo some epigenetic modifications during

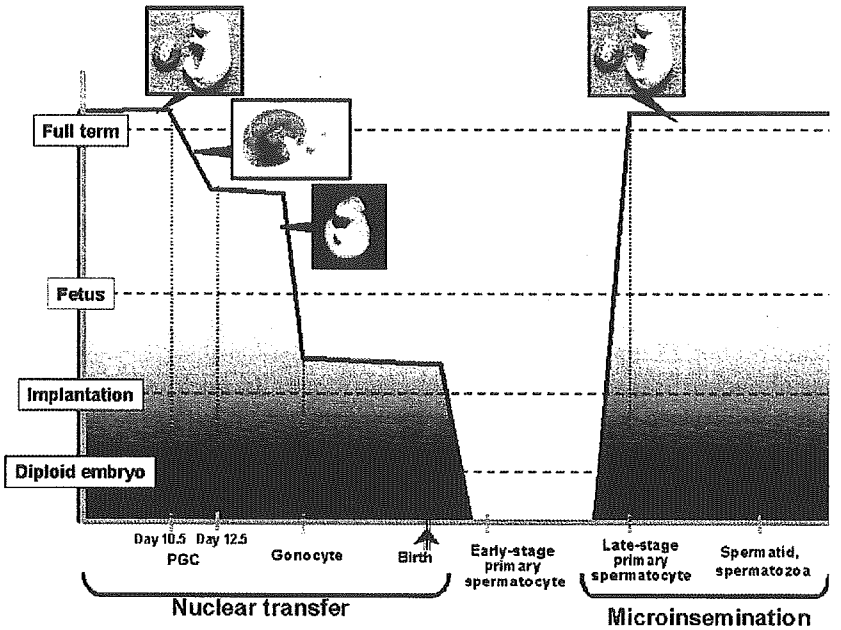

FIG. 9 The schematic relationship between the stages of male germ cells used for construction of diploid embryos and the developmental ability of resulting embryos in mice. Diploid embryos can be constructed by microinsemination using spermatogenic cells at stages ranging from pachytene spermatocytes to mature spermatozoa, and by using primordial germ cells (or gonocytes) by nuclear-transfer cloning. The complex wave of the graph for the latter represents the status of genomic imprinting. The gap for the early-stage primary spermatocytes is a result of the inability of the homologous chromosome pairs to segregate as haploid forms.

their early development, so that they can be correctly reprogrammed at fertilization to participate in the formation of a totipotent zygotic genome (Fulka *et al.*, 2004). Somatic-cell genomes presumably bypass this process and therefore are erroneously reprogrammed when transferred into the egg cytoplasm (Dean *et al.*, 2001). Our results indicate that this (unknown) epigenetic modification process, if present, must exist at day 11.5 or later in the mouse.

VI. Conclusions

Many types of male germ cells, from PGCs in fetal gonads to spermatozoa in adult testes, can participate in the formation of early embryos by microinsemination or nuclear transfer. Microinsemination with postmeiotic male germ