DNA analysis

The genomic DNA was isolated from tissue samples taken from the tail of each offspring using phenol/chloroform extraction, followed by ethanol precipitation. Ten micrograms of DNA were digested with *Eco*RI, and separated on 1.0% agarose gels. The DNA transfer and hybridization were performed as described previously (Kanatsu-Shinohara *et al.*, 2002). An *Nci*I-*Bgl*I fragment of the cDNA of the *Sl* gene (~430 bp, provided by Dr Y.Matsui) was used as a hybridization probe.

Results

Transplantation of W testis cells to Sl recipients

Owing to mutations in the *Sl* gene, spermatogenesis in Sl mice is impaired by dysfunction of the Sertoli cells (Flanagan *et al.*, 1991) (Figure 1A, B). The testes of Sl mice are significantly reduced in size (Figure 2A), and no differentiating germ cells can be detected by histological analysis (Figure 2B). However, there are a small number of undifferentiated spermatogonia that can differentiate into mature spermatozoa when they are exposed to normal Sertoli cells by germ cell transplantation (Ogawa *et al.*, 2000) (Figure 2C). To rescue this spermatogenic defect in Sl mice, testis cells were collected from neonatal W mice, and a dissociated single-cell suspension was microinjected into the seminiferous tubules of 4–6 week old Sl mice. W mice have normal Sertoli cells, but their germ cells cannot differentiate beyond the spermatogonia stage owing to mutations in the *c-kit* gene (Ohta *et al.*, 2003) (Figure 2D, E). Therefore, any spermatogenesis in the testes of Sl recipients must originate from the Sl spermatogonia, with support provided by the normal Sertoli cells transplanted from the W mice. At 20–30 days after transplantation, some of the recipients received an additional injection of Sl testis cells in order to increase the number of spermatogonial stem cells, because the total number of stem cells in the testes of Sl mice is only ~5% of that in the testes of wild-type mice (Shinohara *et al.*, 2000). The recipient mice were killed at 2–3 months after transplantation, and their testes were analysed histologically to determine the level of spermatogenesis. This time period corresponds to two or three spermatogenic cycles in mice, which would allow sufficient time to recover spermatogenesis from the spermatogonial stem cells (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). Although no evidence of spermatogenesis was found in Sl testis without transplantation (12 testes examined), the restoration of spermatogenesis was observed in 13 of 24 (54%) recipient testes. In testes with spermatogenesis, an average of 2.2% of seminiferous tubules contained germ cells (Figure 2F). The transplantation of additional Sl testis cells enhanced the recovery of spermatogenesis in the recipient Sl testis (Table I), suggesting that

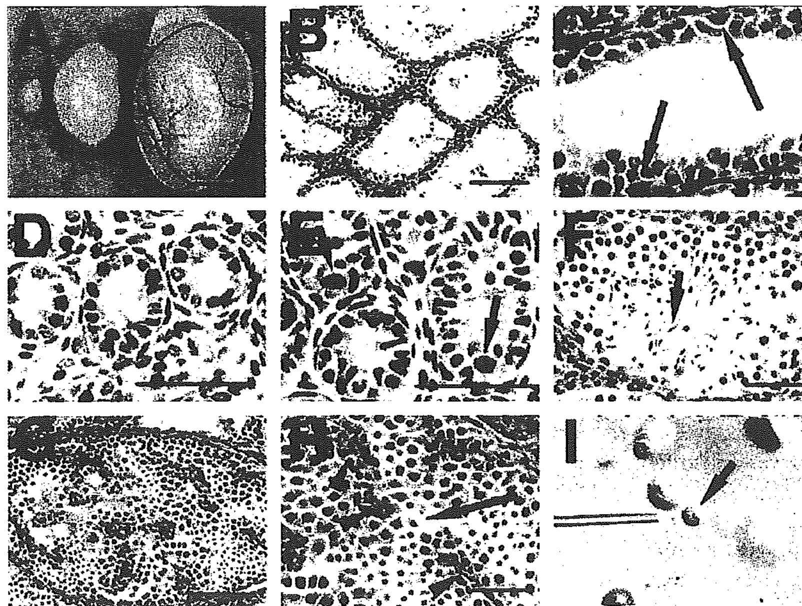

Figure 2. Transplantation of Sertoli cells into infertile Sl testis. (**A**) The macroscopic appearance of neonatal W (left), mature Sl (middle) and mature wild-type B6 (right) testes. Note the small size of the Sl testis. (**B** and **C**) The histological appearance of a mature Sl testis (recipient). Spermatogenesis is absent, but some spermatogonia are visible on the basement membrane (**C**, arrows). (**D** and **E**) The histological appearance of a neonatal W (**D**, donor) or wild-type (**E**) testis. Note the absence of germ cells in W testis. In contrast, germ cells are found in the neonatal wild-type testis (E, arrows). (**F**) A Sl recipient testis at 107 days after transplantation of W donor cells. Elongated spermatids were observed (arrow). (**G**) Spermatogenesis in the minitubules. Note the interdigitated basement membrane and the disorganized spermatogenesis. (**H**) Higher magnification of (**G**). Spermatogenesis occurs on both sides of the basement membrane (arrowheads), but some germ cells appear to migrate to the other side (arrow). (**I**) Spermatogenic cells released from a segment of seminiferous tubule. The arrow indicates a typical round spermatid used for microinsemination. Haematoxylin and eosin stain (**B–H**). Bar = 1 mm (**A**), 50 μm (**C**, **D**, **E**, **F**, **H**), 100 μm (**B**, **G**).

Table I. Spermatogenesis in infertile Steel (Sl) mice by infertile white spotting (W) mouse testis cell transplantation

Donor cells	No. of experiments	No. of injected testes	No. of testes with spermatogenesis (%)	No. of tubule cross-sections with spermatogenesis (%)[a]
Sl (control)	2	14	0 (0)	0 (0/1925)
Sl + W	1	6	5 (83.3)	2.5 ± 0.8 (24/979)*
W	3	18	8 (44.4)	0.6 ± 0.2 (16/2546)
Total (W donors)	4	24	13 (54.1)	1.1 ± 0.3 (40/3525)

Data are presented as means ± SEM. No evidence of spermatogenesis was found in untransplanted Sl testes (12 testes examined).
[a]In parentheses, total number of tubule cross-sections containing spermatogenesis in all recipient testes/total number of cross-sections examined in all recipient testes.
*Significantly more tubules showed spermatogenesis by additional transplantation of Sl testis cells verus W donor cells alone ($P < 0.01$ by t-test).

the number of spermatogonial stem cells in these mice is a limiting factor for restoration of fertility. Spermatogenesis in the Sl testis depended on the transplantation of testis cells from W mice, because no evidence of spermatogenesis was found after autologous transplantation of Sl testis cells. These results indicated that Sertoli cells from the W donor testes colonized the testes of the Sl recipients and provided membrane-bound wild-type Sl factor to the germ cells in the recipients, thus promoting differentiation.

The success of the restoration of spermatogenesis varied significantly among the samples; in some testes, up to eight tubule cross-sections (~5% of the tubules in the plane of the tissue section) contained various stages of spermatogenesis. These differentiated germ cells originate from spermatogonial stem cells, because other spermatogenic cells do not have the capacity for self-renewal and are lost by 35 days after transplantation (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). The spermatogonia, spermatocytes and round spermatids appeared morphologically normal. We also found tubules containing elongated spermatids that were apparently normal in several sections, but their incidence was significantly lower than that of the round spermatids. Spermatogenesis was found in apparently normal seminiferous tubules, but other areas with disorganized spermatogenesis were also detected (Figure 2G, H). In these 'minitubules', the seminiferous tubules had enlarged lumens, and irregular basement membranes subdivided the seminiferous tubules into several minitubules. Although these tubules often contained spermatogonia and spermatocyes undergoing meiosis, the formation of minitubules was not necessarily required for spermatogenesis, because apparently normal spermatogenesis was found in areas without minitubule formation. In addition, we occasionally found other areas in which minitubules were present without evidence of spermatogenesis, suggesting that the formation of minitubules does not always accompany successful spermatogenesis.

Generation of offspring by microinsemination

To confirm that the germ cells that developed in the host testes were fertile, we attempted to produce offspring from the Sl recipient mice using *in vitro* microinsemination, a technique commonly used to produce offspring from infertile animals and humans (Palermo *et al.*, 1992; Kimura and Yanagimachi, 1995). In two separate experiments, a total of 136 oocytes was constructed, using round spermatids (Figure 2I) and the 114 (83.8%) oocytes that developed to the 2-cell stage were transferred into the oviducts of Imperial Cancer Research (ICR) pseudopregnant recipients on the next day after microinsemination. The recipient females produced a total of four offspring, one male and three females (Figure 3A). These offspring had black coats with a white patch on the belly, which suggested that they were heterozygous Sl/+ or Sl^d/+ mice (Figure 3B). Southern blot analysis revealed that three of the live offspring were Sl^d/+ and one was Sl/+, indicating that the Sl and Sl^d haplotypes of the Sl/Sl^d germ cells had segregated during meiosis (Figure 3C). Breeding of the Sl/+ male and Sl^d/+ females produced completely white Sl/Sl^d offspring. These results indicated that the spermatogenic cells that developed in the testes of Sl mice after transplantation of W testis cells originated from endogenous Sl germ cells and that the offspring produced from these cells were fertile.

Discussion

In this study, we demonstrated that Sertoli cell transplantation rescues spermatogonial stem cells in the defective host microenvironment to allow spermatogenesis and the production of offspring from infertile animals. The result was unexpected, because previous transplantation studies in haematopoietic, melanogenic and spermatogenic systems have failed to demonstrate a beneficial effect in treating defective environment (McCulloch *et al.*, 1965; Mayer and Green, 1968; Shinohara *et al.*, 2003). While stem cell transplantation is a promising approach to the treatment of stem cell disorders (Weissman, 2000), difficulties in collecting and transplanting stromal cells limited the development of cell therapy for microenvironmental disorders (Dexter, 1982; Keating *et al.*, 1982; Simmons *et al.*, 1987). Our study now demonstrates that defects in the microenvironment of male germ cells can be corrected by stromal cell transplantation, which has important implications for the development of new techniques to treat disorders in other self-renewing tissues that are attributed to defective microenvironments.

Several methods have been used to manipulate the microenvironment of spermatogonial stem cells. We and others have shown that virus particles microinjected into the seminiferous

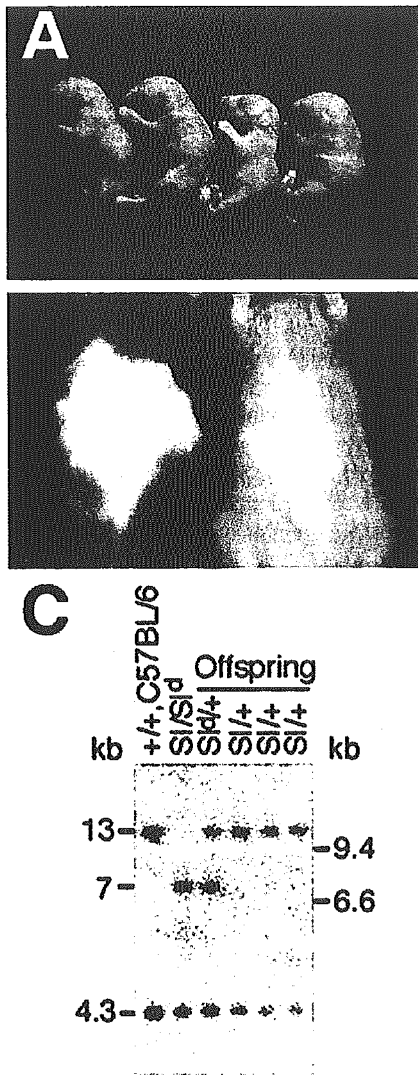

Figure 3. Offspring produced from Sl male mice that received transplanted testis cells from W mice donors. (**A**) The newborn offspring produced by microinsemination using round spermatids harvested from testes of Sl transplant recipients. (**B**) The mature offspring derived from the microinsemination. Note white patches on the belly, which suggest that the animals are either Sl/+ or $Sl^d/+$. (**C**) Southern blot analysis of *Eco*RI-digested genomic DNA from wild-type and Sl/Sl^d mice and from the offspring shown in **A**. The wild-type locus produced hybridization bands at 4.3 and 13 kb, the Sl locus produced no bands, and the Sl^d locus produced bands at 4.3 and 7 kb.

tubules can transduce Sertoli cells *in situ* (Ikawa *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2002). The transduction of Sl Sertoli cells in Sl mice with the wild-type *Sl* gene induced spermatogenesis from Sl stem cells, and normal offspring were produced. Sertoli cells can also be transduced by microinjection of DNA and subsequent electroporation of entire testis (Yomogida *et al.*, 2002). Although these approaches are useful in that they allow very efficient gene expression in Sertoli cells, there are at least two major drawbacks. First, the genetic rescue of defective Sertoli cell functions requires the identification of the responsible genes, which are currently not well characterized in humans. Second, the transduction of Sertoli cells is accompanied by the potential transduction of the germline cells. Recent studies have shown that virus vectors can be transmitted to offspring after the infection of stem cells (Nagano *et al.*, 2001; Kent Hamra *et al.*, 2002; Kanatsu-Shinohara *et al.*, 2004). These animal studies strongly suggest the potential risk of germline transduction, which precludes the use of virus vectors in clinical situations.

Sertoli cell transplantation provides an alternative method to overcome these problems. The success of the Sertoli transplantation technique probably depends on the unique ability of testicular cells to conserve their histogenic capacities. It was originally shown >25 years ago that dissociated newborn testis cells could reorganize *in vitro* into histotypic structures under conditions of slow rotation (Ohno *et al.*, 1978; Zenzes *et al.*, 1978). The reconstituted tubule-like structure contained germ cells, Sertoli cells and myoid cells, among others. The morphogenic activity is suppressed at the onset of puberty, but it can be induced if germ cells are removed (Zenzes and Engel, 1981). In our previous study, we extended this observation by demonstrating that dissociated testis cells reorganize into tubule-like structures *in vivo* after transplantation into seminiferous tubules (Shinohara *et al.*, 2003). The critical finding of this *in vivo* study, in contrast to the previous *in vitro* studies, was the induction of spermatogenesis in the tubule-like aggregates; whereas spermatogenesis could not be induced in the reaggregated tubules *in vitro*, spermatogenesis occurred efficiently *in vivo*. The normal Sertoli cells from the W mice mingled with the defective Sl Sertoli cells of the recipients to reform tubule structures and provide membrane-bound wild-type Sl factor to the germ cells of the Sl mice to permit spermatogenesis, indicating the considerable flexibility of the seminiferous tubule.

The most striking result of the present study was the production of offspring from the infertile Sl mice. The efficiency of offspring production was very low after microinsemination, which suggests that not all the germ cells may have undergone normal development. Another study also reported a lower development rate of embryos in microinsemination experiments using germ cells that developed after germ cell transplantation (Goossens *et al.*, 2003). Nevertheless, our result clearly demonstrates that at least some of the germ cells produced after Sertoli cell transplantation were functionally normal. Thus, the transplantation technique not only provides a method for basic studies on spermatogenesis but may also provide a new method for the treatment of male infertility. Currently, very little is known about the genes responsible for human male infertility, and no effective treatments are available for infertile men with potential Sertoli cell defects. The testes of Sl mice have histological features similar to those observed in the clinical condition known as Sertoli cell-only syndrome, which is found in 5–15% of infertile men (Del Castillo *et al.*, 1947). Although germ cell transplantation may be useful in some cases, the technique is inevitably accompanied by ethical problems and is only useful for infertile men with germ cell defects. Nevertheless,

given our results, it may be anticipated that healthy Sertoli cells from a donor testis could be transplanted into a heterologous recipient who has defective Sertoli cells to induce spermatogenesis from the normal stem cells of the patient. The present technique may also be used to achieve cross-species germ cell transplantation. When germ cells from phylogenetically distant donors, such as from primate or human, are transplanted into immunodeficient mouse recipients, their differentiation arrests at the stage of spermatogonial proliferation, possibly owing to the incompatibilities in the microenvironments (Nagano *et al.*, 2001, 2002). The transplantation of Sertoli cells may enable differentiation of these donors in mouse surrogates, thereby providing a biological assay system to characterize human spermatogenesis.

While our results show the remarkable flexibility of the spermatogenic system, improving the efficiency of spermatogenesis is the next important step required to facilitate a wide range of studies using Sertoli cell transplantation. Although premeiotic germ cells continued to proliferate for several months and spermatogenesis occurred up to the round spermatid stage, it was less efficient after the elongated spermatid stage. One possible explanation is that the transplanted Sertoli cells failed to reconstruct the spatially coordinated, cyclic gene expression pattern of normal seminiferous tubules. Alternatively, the limited area of colonization established by the donor Sertoli cells could also influence germ cell differentiation. A previous transplantation study showed that extensive proliferation of the spermatogonia population occurred in small colonies in the recipient tubules, but that meiotic differentiation occurred exclusively in colonies longer than 1 mm (Nagano *et al.*, 1999). However, in this study, Sertoli cell colonization occurs in a limited area without appropriate host conditioning. Endogenous Sertoli cells can be eliminated by treating the seminiferous tubules with cadmium; donor Sertoli cells can colonize extensive areas after such treatment (Shinohara *et al.*, 2003). Cadmium treatment, however, also removes the endogenous germ cells and is thus not appropriate in the present case (Shinohara *et al.*, 2003). It will be important to develop other methods to enhance Sertoli cell colonization without affecting the endogenous germ cell population. The resolution of these problems will not only improve the efficiency of Sertoli cell transplantation techniques but also increase our understanding of spermatogenesis.

Several new therapeutic approaches can be envisaged for human spermatogenic failure. A stem cell transplantation technique is available that allows offspring production from fresh or cryopreserved spermatogonial stem cells (Avarbock *et al.*, 1996; Ogawa *et al.*, 2000; Kanatsu-Shinohara *et al.*, 2003a). This technique will be particularly useful for restoring fertility to those who become infertile after malignancy therapy by chemicals or radiation. Although no method for fertility protection is currently available for prepubertal boys who do not have sperm, stem cell transplantation will provide a method to recover their fertility, since spermatogenesis occurs by transplantation of spermatogonial stem cells even from immature donors (Shinohara *et al.*, 2001). Recent development of spermatogonial stem cell culture techniques enables *in vitro* expansion of stem cells from a small biopsy sample for autologous transplantation (Kanatsu-Shinohara *et al.*, 2003b, 2004; Kubota *et al.*, 2004; Ogawa *et al.*, 2004). Future developments might even allow correction of defective genes at the spermatogonial stem cell level, and methods for genetic manipulations are being developed (Kanatsu-Shinohara *et al.*, 2005). In contrast, the Sertoli cell transplantation technique will be used to correct spermatogenic failure due to defects in Sertoli cells. Unlike stem cell transplantation, defective Sertoli cells can be replaced with healthy Sertoli cells from heterologous donors with less ethical restriction. As the method to culture Sertoli cells develops, it will be possible to correct the defect in Sertoli cells to be used for autologous transplantation *in vitro*. Our successful production of offspring from infertile Sl mice demonstrates the usefulness of the Sertoli cell transplantation technique, and indicates a promising opportunity to develop a new strategy for the treatment of human male infertility.

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Generation of Cloned Mice by Direct Nuclear Transfer from Natural Killer T Cells

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Summary

Cloning mammals by nuclear transfer (NT) remains inefficient. One fundamental question is whether clones have really been derived from differentiated cells rather than from rare stem cells present in donor-cell samples. To date, cells, such as mature lymphocytes, with genetic differentiation markers have been cloned to generate mice only via a two-step NT involving embryonic stem (ES) cell generation and tetraploid complementation [1–3]. Here, we show that the genome of a unique T-cell population, natural killer T (NKT) cells, can be fully reprogrammed by a single-step NT. The pups and their placentas possessed the rearranged *TCR* loci specific for NKT cells. The NKT-cell-cloned embryos had a high developmental potential in vitro: Most (71%) developed to the morula/blastocyst stage, in marked contrast to embryos from peripheral blood T cells (12%; $p < 1 \times 10^{-25}$). Furthermore, ES cell lines were efficiently established from these NKT-cell blastocysts. These findings clearly indicate a high level of plasticity in the NKT-cell genome. Thus, differentiation of the genome is not always a barrier to NT cloning for either reproductive or therapeutic purposes, so we can now postulate that at least some mammals cloned to date have indeed been derived from differentiated donor cells.

Results and Discussion

NKT-Cell Clones Have High Developmental Potency In Vitro

Previously, we analyzed the efficiency of somatic-cell cloning in mice by using two cell types and six genotypes for donors (2 × 6 factorial analysis of variance [ANOVA]) and found that immature Sertoli cells with the (B6 × 129) F1-mouse-strain genotype gave the best results in terms of birth rates of offspring after embryo transfer (about 10%) [4]. To extend this result, we sought cells that retain high genome reprogrammability among lymphocyte populations from such males. We eventually found that natural killer T lymphocytes (NKT cells) had such potency. NKT cells are small lymphoid cells that play regulatory roles, such as the inhibition of tumor development, protection against the development of autoimmune disease, and maintenance of transplantation tolerance [5], in the immune system. Furthermore, it has recently been demonstrated that NKT cells provide an innate-type immune response to certain glycosphingolipid-bearing microorganisms through recognition by their antigen receptor [6, 7]. We isolated NKT cells by fluorescence-activated cell sorting (FACS) of mononuclear cells from the livers of (B6 × 129) F1 male mice and used these for nuclear transfer (NT). NT was performed by direct injection into enucleated oocytes, as described previously [8]. Peripheral blood (helper) T cells isolated from the same strain of mice were used for NT experiments as controls. After 24 hr in culture, the majority of reconstructed oocytes developed into 2-cell embryos irrespectively of the cells used, as expected from their G0 cell-cycle state (Table 1 and Figure 1A). It is known that G0/G1-stage cell-cycle donors are readily synchronized with oocytes in this cloning technique [8]. Within the next 24 hr, whereas many of the T-cell clones arrested their development at the 2-cell stage, most NKT-cell clones reached the 4-cell stage (Table 1 and Figure 1A). Moreover, 71% of NKT-cell-clone embryos developed into the morulae/blastocyst stage, which was very significantly different from the rate of development of T-cell clones (12%, $p < 1 \times 10^{-25}$) (Table 1 and Figure 1A). This poor development of T-cell clones in vitro is consistent with that reported previously by Hochedlinger et al. [1]. However, the developmental potential of NKT-cell-clone embryos was unexpectedly high. In embryos constructed by NT, normal embryonic gene activation occurs only when the donor genome has been reprogrammed to the zygotic state [9]. For mice, most embryonic gene activation occurs in the early 2-cell stage [10], and, therefore, embryos that fail to activate their zygotic genes do not develop beyond this stage. Thus, the genome of NKT cells appears to be more readily reprogrammed than that of T cells, at least in terms of activation of the genes necessary for preimplantation development.

We further examined the plasticity of the NKT-cell genome by isolating nuclear-transfer ES cell (NTES cell) lines from NKT-cell embryos. When these embryos were harvested under conventional condition for NTES-cell establishment, six lines were successfully isolated. This efficiency (4% per 147 reconstructed embryos) was similar to that reported for clones from cumulus cells (2.3%–6.9%) or adult fibroblasts (1.1%–3.8%) [11] and much higher than the rates reported for clones from lymphocytes (0.2%–0.3%) [1].

The NKT-Cell Genome Supports Full-Term-Embryo Development by Single-Step Nuclear Transfer

When 272 embryos derived from NKT cells were transferred into pseudopregnant females, four (1.5%) developed to term offspring and 13 (4.8%) developed into

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Table 1. Development In Vitro and In Vivo of Embryos Cloned from NKT and T Cells

Cell Type	Time in Culture	No. Cultured	No. (%) Cleaved	No. (%) ≥ 4-Cell	No. (%) M & B*	No. Transferred	No. (%) Implanted	No. (%) Fetuses	No. (%) Placenta-Only
NKT cells									
	48 hr	280	260 (93)	241 (86)		185	112 (61)	3 (1.6)	5 (2.7)
	72 hr	292	274 (94)	241 (83)	207 (71)	87	49 (56)	1 (1.1)	8 (9.2)
T cells									
	48 hr	105	62 (59)	21 (20)		21	3 (14)	0 (0)	0 (0)
	72 hr	232	174 (75)	81 (35)	28 (12)	23	0 (0)	0 (0)	0 (0)

* indicates Morulae and blastocysts. See Figure 1A for statistical analysis.

placenta-only conceptuses (Table 1 and Figure 1A). All these 17 clone placentas showed mild or heavy hyperplasia (0.15–0.46 g) two to four times larger than the genotype-matched-control placentas produced by microinsemination (0.08–0.11 g) (Figure 1B); this is characteristic of the abnormal growth patterns of mouse somatic-cell clones [12, 13]. T-cell-clone embryos were also transferred into recipient females, as described above, but very few implanted and none survived through term (Table 1). The T-cell receptor (TCR) repertoire in NKT cells is highly restricted to the invariant Vα14 receptor, which in the mouse is encoded by the genes *Vα14–Jα281* and *Vβ8* [5]. Thus, it is feasible to trace the NKT donor genome in cloned animals. Southern-blot analysis of these clones with specific probes demonstrated that all clones and their placentas inherited the rearranged *TCRVα14* locus (Figure 1C). The *TCRVβ* locus was also rearranged for both alleles (Figure 1C). Polymerase chain reaction (PCR) amplification and DNA sequencing of the rearranged alleles also confirmed the clonality of the NKT-cell-cloned mice. Cloned mouse #1 inherited the rearranged allele of *Vα14–Jα281* derived from the C57BL/6 strain, whereas that of cloned mouse #2 was from the 129/Sv lineage (Figure 2A). Similarly, the *TCRVβ* sequences of clones #1 and #2 had the in-frame configurations *Vβ8S2-D1-Jβ2S5* and *Vβ8S3-D1-Jβ1S4*, respectively (Figure 2B). We then analyzed peripheral blood cells by FACS for the Vβ phenotypes of the two NKT clones. In both clones, unlike the control donor strain, the TCRVβ cells were all positive for TCRVβ8, clearly demonstrating the occurrence of allelic exclusion [14] in these cloned mice (Figure 2C). Clones #1 and #2 grew into normal-looking adults (Figure 1D). They are currently healthy in appearance at

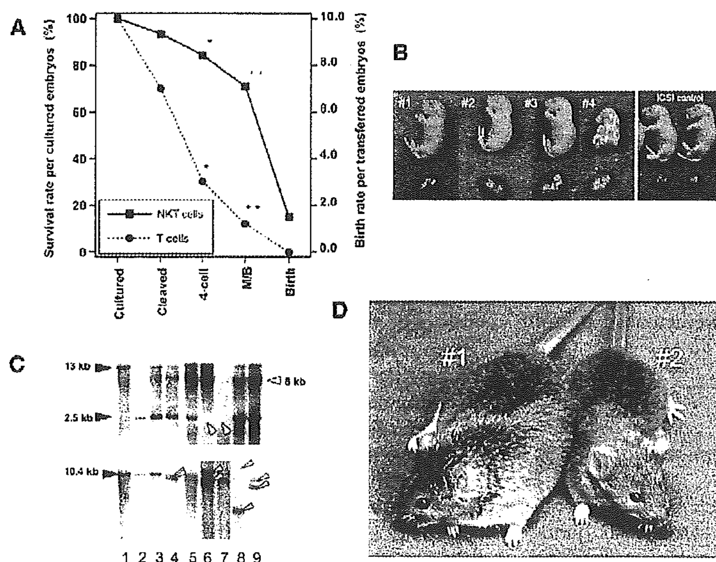

Figure 1. Development and Southern-Blot Analysis of NKT Clones

(A) Survival of embryos reconstructed from nuclei of NKT cells or T cells during in vitro culture. The data from 48-hr- and 72-hr-cultured groups in Table 1 have been combined. * indicates $p < 0.0001$; ** indicates $p < 1 \times 10^{-25}$.

(B) NKT clones and their placentas at birth. Clones #1 and #2 were alive and grew into normal adults (see [D]). Clone #3 was alive at birth but was cannibalized by its foster mother. Clone #4 was stillborn. The clones had placentas that were two to four times heavier than those of the genotype-matched intracytoplasmic sperm injection (ICSI) controls.

(C) Southern-blot analysis of the genomic DNA of NKT-cell-derived cloned mice for the detection of rearrangements of the *TCRVα14* (upper) and *TCRVβ* loci (lower). Black arrowheads indicate the nonrearranged bands, and white arrowheads indicate those that correspond to the rearranged loci. Genomic-DNA samples are from C57BL/6 (B6) mouse tail (lane 1); 129/Sv ES cells (lane 2); donor F1 (B6 × 129/Sv) mouse tail (lane 3); clone #1 tail (lane 4); clone #1 placenta (lane 5); clone #2 tail (lane 6); clone #2 placenta (lane 7); clone #3 placenta (lane 8); and clone #4 placenta (lane 9). At the *Vα14* locus (upper), rearrangement of the B6- and 129-strain alleles is indicated by the appearance of the 8 kb band and the disappearance of the 2.5 kb band, respectively. Clones #1, #3, and #4 carry a rearranged B6-strain allele (lanes 4, 5, 8, and 9), whereas clone #2 carries a rearranged 129-strain allele (lanes 6 and 7; note the absence of the 2.5 kb band; the 8 kb band probably represents an out-of-frame rearrangement of the B6 allele). The 13 kb band for the B6-strain allele persisted after rearrangement because of the presence of a pseudogene. At the *Vβ* locus (lower), both the B6- and 129-strain alleles have been rearranged, as indicated by the appearance of two new bands in clones #1, #2, #3, and #4 (lanes 4 and 5, 6 and 7, 8, and 9, respectively). For clones #1 and #2, rearrangement of the *TCR* loci was also confirmed by sequence analysis (see Figures 2A and 2B).

(D) NKT clones #1 and #2 that grew into adults. They showed a mild obese phenotype which is known to be associated with cumulus-cell clones [17].

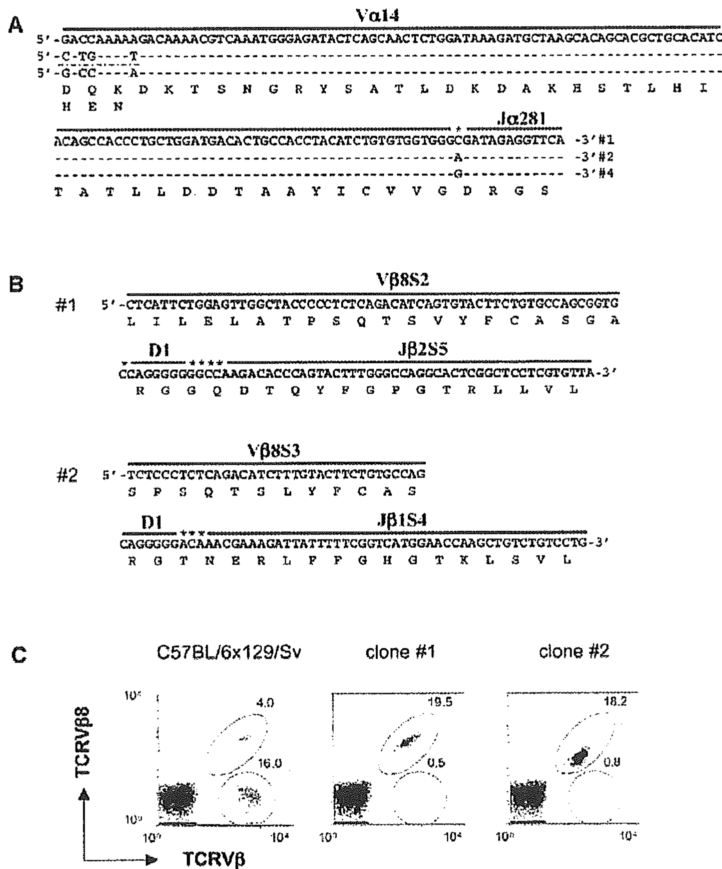

Figure 2. DNA-Sequence Analysis of the *TCRV* Loci and FACS Analysis of Peripheral Lymphocytes in NKT Clones

(A) The *TCRVα14* DNA sequences. The dot-dash line indicates polymorphism between the alleles of the C57BL/6 (B6) and 129 mouse strains. Clones #1 and #4 carry the rearranged B6-strain allele, whereas clone #2 carries the rearranged 129-strain allele. The asterisk indicates the *N*-position nucleotide that connects *Vα14* and *Jα281*. The derived DNA sequences show in-frame rearrangements. The bottom line indicates the amino acid sequence.

(B) The *TCRVβ* DNA sequences. That of clone #1 consists of the in-frame *Vβ8S2-D1-Jβ2S5* sequence, whereas that of clone #2 consists of the in-frame *Vβ8S3-D1-Jβ1S4* sequence.

(C) FACS analysis of peripheral lymphocytes in cloned mice. Expression of *Vβ8* on *TCRVβ*-positive T cells in the peripheral blood from the control (B6 × 129), clone #1, and clone #2 mice. Numbers in circles represent the percentage of gated lymphocytes. Disappearance of non-*Vβ8*-expressing T cells from the *TCRVβ*-carrying population in the two clones demonstrates exclusive expression (allelic exclusion) of the *Vβ8* allele at the *TCRVβ* locus.

the ages of 15 and 11 months, respectively, although they are relatively heavier than normal mice, as reported for mice cloned from cumulus cells [15] (Figure 1D). They proved to be fertile, producing litters of offspring that have normal numbers (nine to 17 pups per litter) and in which the rearranged alleles have been inherited through the germline. These F1 progeny are also fertile and have given birth to F2 mice, some of which are homozygous for the rearranged alleles, as expected (data not shown).

NKT-Cell Cloning as a Model for Nuclear-Transfer Studies

It has previously been reported that the nuclei of peripheral lymphocytes and olfactory sensory neurons, which both express stable markers of differentiated cells, can be cloned to generate mice [1–3]. These findings are very clear demonstrations of the reprogrammability of the differentiated nuclei. However, unlike most other clones, these were generated by a two-step NT, a technique involving ES-cell generation and tetraploid complementation to produce a viable placenta [1–3]. In this, the ES-cell stage allows for extra reprogramming time, and tetraploid-cell lines may contribute to most extra-embryonic tissues, which are commonly the more adversely affected components in cloned animals [16]. Furthermore, tetraploid cells rarely contribute to any part of the embryo proper through term [17]. Eggan et al. also attempted to produce clones directly from neuronal nuclei without tetraploid complementation, but again ES-cell intervention was necessary [2]. These facts leave open the possibility that the "successful" clones may have been produced by the accidental use of undifferentiated stem cells [18]. In this paper, we report the birth of offspring after single-step direct NT from NKT cells. The transferred genomes generated embryonic as well as extra-embryonic tissues, which play essential complementary roles in mammalian development. This provides the first direct evidence that fully differentiated cell nuclei carrying and expressing specific genetic markers can be reprogrammed within the oocyte cytoplasm to support full-term-embryo development. Thus, we can postulate that at least some of the mammals cloned to date are probably derived from differentiated donor cells rather than from stray stem cells. We observed a very high developmental ability of NKT-cell embryos in vitro (71% of the embryos reached the morula/blastocyst stage) and efficient generation of NKT-ES cells (4% per reconstructed embryos). Thus, certain fully differentiated cells may be a good source of donors for reproductive cloning in animals as well as therapeutic cloning in humans.

In mouse NT experiments, cloned offspring are most efficiently obtained when ES cells are used for donors

[19]. By contrast, cloning from T cells and B cells is extremely inefficient, and cloning fetal neural cells [20] is intermediate. Immature Sertoli cells from newborn males were reported to be better donor cells than cumulus cells, the germline support cells in female adults [6]. Taken together, these ideas show that it is conceivable that the efficiency (birth rate) of cloning depends on the state of differentiation of donor cells [21]. However, we demonstrate here that NKT cells with specific differentiated markers are suitable donors for the generation of cloned offspring and NTES-cell lines. Furthermore, although tissue-specific stem cells (e.g., hematopoietic cells and neural stem cells) are expected to be effective donor cells because of their innate differential plasticity [21], so far there has been no report of successful cloning by NT from these cells. Thus, we speculate that the capacity of the genome to be reprogrammed by NT is biologically distinct from the degree of genomic plasticity on the basis of its differentiation status (or its "stemness," in reverse). What determines this potential? Both NKT cells and T cells belong to the same hematopoietic-cell lineage and undergo similar DNA rearrangements to express TCR, but attempts to clone from these lineages show very different efficiency. Examination of the relationship between chromatin structure and the efficiency of cloning from different hematopoietic cells, including NKT cells, might help solve this question.

Experimental Procedures

Preparation of Donor Cells

Male (C57BL/6 x 129/Sv) F1 mice aged 2 to 5 months were used for the preparation of NKT cells and helper/inducer T cells. For the collection of NKT cells, we isolated dispersed liver mononuclear cells by using Percoll density gradients (Amersham Biosciences, Piscataway, New Jersey), and we sorted NKT cells via a Moflo flow cytometer (Dako Cytomation, Carpinteria, California) with phycoerythrin (PE)-α-galactosylceramide-loaded CD1-D tetramer (prepared in-house) and a fluorescein isothiocyanate (FITC)-labeled anti-TCRVβ (H57) monoclonal antibody (PharMingen, San Diego, California). The NKT cells were sorted twice and were more than 99% pure, as assessed by flow cytometry. For the collection of T cells, we isolated mononuclear cells from peripheral blood and stained them with PE-conjugated anti-CD3 and allophycocyanin (APC)-conjugated anti-CD4 monoclonal antibodies (eBioscience, San Diego, California). $CD3^{+}CD4^{+}$ helper/inducer T cells were sorted with a triple-laser flow cytometer, model FACS Vantage SE (BD Biosciences, San Jose, California). The purity of sorted cells was determined as more than 98% by flow cytometry. In our preliminary study, we confirmed 97%–99% viability after sorting for both NKT-cell and T-cell populations by propidium-iodide staining.

Nuclear Transfer

Nuclear transfer was performed as described previously, with minor modifications [4, 8]. Mature oocytes were collected from superovulated B6D2F1 females and enucleated and injected with NKT-cell nuclei via a Piezo-driven micromanipulator (Primetech Corporation, Ibaraki, Japan). The reconstructed oocytes were cultured in KSOM medium [22], and 4-cell embryos (48 hr in culture) or morulae/blastocysts (72 hr in culture) were transferred into the oviducts of ICR-strain pseudopregnant females at 0.5 days post coitus (dpc) with vasectomized males. At 19.5 dpc, the recipient females were killed and their uteri were examined for live or dead fetuses. Live fetuses were then reared by lactating ICR-strain foster mothers. Genotype-matched controls were produced by intracytoplasmic sperm injection as previously reported [23].

Southern-Blot Analysis

Genomic DNA (4 μg from the tail or 20 μg from the placenta) was digested with EcoRI (*TCRVα14* probe) or BamHI (*TCRVβ* probe). The bands were visualized with radiolabeled TCR *Vα14* and *Vβ* probes, which were derived from genomic regions 66569–67106 (AE008684) and 163526–164357 (AE000665.1), respectively.

Sequence Analysis

The *Vα14-Jα281* fragment was amplified with AmpliTaq-Gold (Roche, Nutley, New Jersey) in a PCR reaction mixture that contained RNase-treated genomic DNA and the primers 5′-GACCCAAGTGGAGCAGAGTC-3′ and 5′-AGGTATGACAATCAGCTGAGTCC-3′. The PCR products were purified with a PCR Purification Kit (Qiagen, Tokyo, Japan) and sequenced with a model 3100 Genetic Analyzer (Applied Biosystems/Hitachi, Tokyo, Japan). For *TCRVβ* sequencing, we used RNase-treated genomic DNA as the template in the PCR reaction to detect in-frame *Vβ-D-J* fragments. In brief, we used combinations of primers that corresponded to a given *TCRVβ* region and that encompassed either the 5′ end of the *TCRVβC1* gene or the 3′ end of the *J β2S7* gene. The PCR products were sequenced as described above.

Analysis of Peripheral Blood T cells

Lymphocytes were stained with allophycocyanin-labeled anti-TCRVβ (H57–597, PharMingen) and fluorescein-isothiocyanate-labeled anti-TCRVβ8 (F23.1, PharMingen) after the red blood cells were depleted with Red Blood Cell Lysis Buffer (Sigma, St. Louis, Missouri). FACS analyses were performed with a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences).

Animal Experimentation Procedures

All procedures described within were reviewed and approved by the Animal Experimental Committee at the RIKEN Institute and were performed in accordance with the RIKEN Guiding Principles for the Care and Use of Laboratory Animals.

Acknowledgments

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Microinsemination and Nuclear Transfer Using Male Germ Cells

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Microinsemination has been widely used in basic reproductive research and in human-assisted reproductive technology for treating infertility. Historically, microinsemination in mammals started with research on the golden hamster; since then, it has provided invaluable information on the mechanisms of mammalian fertilization. Thanks to advances in animal genetic engineering and germ-cell technologies, microinsemination techniques are now used extensively to identify the biological significance of genes of interest or to confirm the genetic normality of gametes produced by experimental manipulations *in vitro*. Fortunately, in mice, high rates of embryo development to offspring can be obtained so long as postmeiotic spermatogenic cells are used as male gametes—that is, round spermatids, elongated spermatids, and spermatozoa. For some other mammalian species, using immature spermatogenic cells significantly decreases the efficiency of microinsemination. Physically unstable chromatin and low oocyte-activating capacity are the major causes of fertilization failure. The youngest male germ cells, including primordial germ cells and gonocytes, can be used in the construction of diploid embryos by nuclear-transfer cloning. The cloned embryos obtained in this way provide invaluable information on the erasure and reestablishment of genomic imprinting in germ cells.
KEY WORDS: Microinsemination, Nuclear transfer, Spermatozoon, Spermatid, Spermatocyte, Primordial germ cell, Oocyte. © 2005 Elsevier Inc.

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I. Introduction

Sexual reproduction is characterized by the beginning of a new genetic identity through fertilization. In essence, the union of a haploid sperm and a haploid egg leads to a diploid zygote, but this is a process that involves complex biological and biochemical mechanisms (Yanagimachi *et al.*, 1994). Fertilization in mammals takes place within the body, and the subsequent embryo remains in the maternal body until it completes its normal course of development as a viable offspring. Therefore, research on fertilization in mammals differs from that in, for example, sea urchins and frogs, and it has necessitated the development of *in vitro* fertilization (IVF) technology to investigate the process of fertilization by microscopy.

IVF has helped elucidate the processes of capacitation and acrosome reaction of spermatozoa, and of sperm–egg membrane fusion. However, these reactions merely represent the conditions, or hurdles, for a spermatozoon to pass before it can deliver its nucleus into the oocyte cytoplasm (ooplasm). Therefore, one may attempt to bypass these sperm-associated processes to investigate what happens within the oocyte after sperm entry (e.g., resumption of meiosis [oocyte activation] and male pronuclear formation). For this reason, the technology that was developed following IVF was microinsemination.

Successful microinsemination in mammals was first reported during the 1970s (Uehara and Yanagimachi, 1976, 1977). The initial success of these studies was due mainly to the superior technology used, but golden hamster eggs, which they used in IVF experiments, were also well suited for intracytoplasmic injection. Mouse oocytes, which are much more readily available to the average laboratory than golden hamster eggs, are especially fragile during injection, and successful microinsemination in mice was not reported until 1995, by Kimura and Yanagimachi (1995a). Subsequent microinsemination experiments using golden hamster gametes, based on pronuclear formation and the DNA-synthesizing ability of sperm, led to further understanding of the chemical and physical nature of the sperm nucleus (Perreault, 1992; Yanagimachi *et al.*, 1992) and the nuclear-membrane integrity of sperm and spermatogenic cells (Usui *et al.*, 1997, 1999).

After initial successes, further advances in microinsemination were made using improved micromanipulation technologies and equipment. In particular, the use of inverted microscopes and micromanipulators made it possible to perform procedures under microscopy reliably and reproducibly, leading to dramatically improved oocyte viability and fertilization outcomes. With these technological advances came the new goal of producing viable offspring by microinsemination. Research was aimed at finding out whether fertilization processes bypassed by microinsemination (e.g., acrosome reaction

and sperm–oocyte membrane fusion) are required for individual development, and whether oocytes fertilized by microinsemination could really develop into viable offspring. Around 1990, normal offspring produced by microinsemination were reported in rabbits (Hosoi *et al.*, 1988), bovines (Goto *et al.*, 1990), and humans (Palermo *et al.*, 1992), thus demonstrating that the only necessary condition for normal fertilization and individual development was that the sperm nucleus be able to enter the ooplasm.

Starting in the mid-1990s, microinsemination experiments were performed using immature sperm (spermatids and spermatocytes). Live offspring were reported using round spermatids (Kimura and Yanagimachi, 1995b; Ogura *et al.*, 1994), secondary spermatocytes (Kimura and Yanagimachi, 1995c), and primary spermatocytes (Kimura *et al.*, 1998; Ogura *et al.*, 1998). These findings showed that the male gamete genome is already capable of supporting embryo development by the stage of meiotic prophase.

Microinsemination is currently used in human clinics to treat male infertility, as well as in basic biological research, which is the major topic of this review. For the former topic, there are many excellent reviews (e.g., Ludwig and Diedrich, 2002; Nyboe-Andersen *et al.*, 2004). When we speak about microinsemination, we generally refer to intracytoplasmic sperm injection (ICSI), but microinsemination is also performed with immature gametes, including elongated spermatid injection (ELSI) and round spermatid injection (ROSI). Although not discussed here, procedures that enable sperm to penetrate the zona pellucida, including partial zona dissection (PZD) and subzonal insemination (SUZI) are also, in a broad sense, microinsemination techniques (Iritani, 1991; Kawase *et al.*, 2002; Nakagata *et al.*, 1997). Diploid embryos can also be constructed from male germ cells before entering meiosis (e.g., primordial germ cells) using nuclear transfer techniques. This review outlines the current status of nuclear transfer using male germ cells from early stages of meiosis.

II. ICSI

As mentioned, ICSI started around 1990 and has been successful in producing live offspring or embryos in several animal species (Table I). Either mature ejaculated sperm or epididymal sperm have been used, depending on the animal species. This choice is based not on sperm fertilization ability but rather simply on the ease of sample collection; in general, ICSI is performed with epididymal sperm in small rodents and with ejaculated sperm in other animals, especially in farm animals (Catt *et al.*, 1996; Cochran *et al.*, 1998; Goto *et al.*, 1990; Lee *et al.*, 1998; Martin, 2000). To my knowledge, normal offspring have now been reported for 13 animal species (mastomys offspring were born from spermatids, but not from sperm).

TABLE I

Development of Embryos Following Microinsemination in Mammals

Species	Male germ cells used			
	Ejaculated sperm	Epididymal sperm	Testicular sperm	Spermatid
Mouse		Offspring (1)	Offspring (2)	Offspring (2)
Rat		Offspring (3)		Offspring (4)
Hamster		Offspring (5)	Pronuclear stage (6)	Offspring (7)
Mastomys		Two-cell (8)		Offspring (8)
Rabbit	Offspring (9)	Offspring (10)		Offspring (11)
Bovine	Offspring (12)			Blastocyst (13)
Horse	Offspring (14)			
Goat	Offspring (15)			
Sheep	Offspring (16)			
Pig	Offspring (17)			Pronuclear stage (18)
Cat	Offspring (19)			
Tammar wallaby		Eight-cell (20)		
Rhesus monkey	Offspring (21)	Offspring (22)		Offspring (22)
Cynomolgus monkey	Offspring (23)	Offspring (24)		Fetus (25)
Chimpanzee	Blastocyst (26)			
Human	Offspring (27)	Offspring (28)	Offspring (29)	Offspring (30)

(1) Kimura and Yanagimachi, 1995a; (2) Kimura and Yanagimachi, 1995b; (3) Hirabayashi *et al.*, 2002a; (4) Hirabayashi *et al.*, 2002b; (5) Yamauchi *et al.*, 2002; (6) Uehara and Yanagimachi, 1977; (7) Haigo *et al.*, 2004; (8) Ogonuki *et al.*, 2003a; (9) Hosoi *et al.*, 1988; (10) Ogonuki *et al.*, unpublished data; (11) Sofikitis *et al.*, 1994; (12) Goto *et al.*, 1990; (13) Goto *et al.*, 1996; (14) Cochran *et al.*, 1998; (15) Wang *et al.*, 2003; (16) Catt *et al.*, 1996; (17) Martin, 2000; (18) Lee *et al.*, 1998; (19) Pope *et al.*, 1998; (20) Richings *et al.*, 2004; (21) Hewitson *et al.*, 1999; (22) Hewitson *et al.*, 2002; (23) Torii *et al.*, 2001; (24) Ng *et al.*, 2002; (25) Ogonuki *et al.*, 2003b; (26) Suzuki *et al.*, 2004; (27) Palermo *et al.*, 1992; (28) Schoysman *et al.*, 1993; (29) Silber *et al.*, 1995; (30) Tesarik *et al.*, 1995.

Oocytes, spermatozoa, and fertilized oocytes have special biological and physical characteristics for each species. Therefore, microinsemination techniques, culture of fertilized oocytes, and embryo-transfer techniques have been developed specifically for each species. For example, the golden hamster was used in early microinsemination experiments, but because of an *in vitro* block of development in embryos (Bavister, 1989), successful production of viable offspring by microinsemination has only happened quite recently (Yamauchi *et al.*, 2002). The difficulty of culturing hamster embryos is clear: almost 30 years elapsed between the development of IVF for this species in the 1960s (Yanagimachi and Chang, 1963) and the birth of live pups (Barnett and Bavister, 1992). By contrast, such an *in vitro* developmental block does *not* usually occur in rabbit and human embryos. These two species were used in the earliest experiments that successfully produced live offspring by microinsemination (Hosoi *et al.*, 1988; Palermo *et al.*, 1992). In marsupials, the noneutherian mammals, the biological information on gametes and embryos is very limited and only four- to eight-cell embryos were obtained following ICSI in the tammar wallaby (Richings *et al.*, 2004). Technological points of ICSI are discussed below in relation to the particular biological characteristics of oocytes, sperm or spermatogenic cells, and embryos for each animal species.

A. Artificial Activation of Oocytes

The mechanism of oocyte activation (resumption of meiosis) by a mature spermatozoon involves diffusion of a sperm-borne oocyte-activating factor (SOAF) from the sperm cytosol into the ooplasm following sperm–oocyte membrane fusion (Swann, 1996). Swann *et al.* (2004) showed this factor to be phospholipase C (PLC) zeta. However, aside from SOAF, there are other oocyte activation factors (proteins) in the perinuclear matrix (Kimura *et al.*, 1998a; Perry *et al.*, 1999a). During ICSI with whole sperm, all cytosolic and perinuclear activation factors—as in normal fertilization by membrane fusion—are incorporated into the oocyte cytoplasm, so, in theory, assisted activation of oocytes during ICSI should not be required. However, assisted activation during ICSI is required in some animal species. For example, fertilized oocytes and viable offspring have been produced by sperm injection alone in bovines (Wei and Fukui, 2002) and pigs (Martin, 2000), but there is usually a low rate of embryo development in these species. It is likely that diffusion of the oocyte-activating factors into the ooplasm is delayed following ICSI, because at least a part of the sperm membrane is incorporated simultaneously. Artificially assisted activation significantly improves development to the blastocyst stage and thus leads to improved production of live offspring (Horiuchi *et al.*, 2002). Artificial

oocyte activation can be achieved using electrical pulses, alcohol, or calcium ionophores to facilitate calcium-ion influx into the cytoplasm (Yanagimachi, 1994). In addition, a protein synthesis inhibitor, cycloheximide (Prather, 2001), and agents that release Ca^{2+} from calcium stores (thimerosal, inositol 1,4,5-trisphosphate, and adenophostin) are sometimes used (Inoue *et al.*, 2002a; Sato *et al.*, 1998; Tao *et al.*, 2000).

One response of the oocyte to activation is the exocytosis of cortical granule enzymes. This leads to the zona reaction, resulting in blocking of polyspermic fertilization at the level of the zona pellucida by modifying the zona glycoproteins (Yanagimachi, 1994). Although often thought to be incomplete, the zona reaction may also occur following ICSI (Ghetler *et al.*, 1998). However, blocking polyspermic fertilization at the level of the oocyte plasma membrane requires fusion of the oocyte and sperm membranes. This has been shown experimentally *not* to occur following microinsemination (Maleszewski *et al.*, 1996).

B. Presence or Absence of Tail Structures: Centrioles and Midpiece Mitochondria

During fertilization, with the exception of certain mammals (e.g., Chinese hamster [Yanagimachi *et al.*, 1983]), the entire sperm (except the acrosome and most of the plasma membrane) is incorporated into the oocyte by fusion of the oocyte and sperm membranes. The sperm contents involved in subsequent development include the nucleus, oocyte-activating factors, and centrioles. In most animals, ranging from sea urchins and frogs to mammals, the sperm centrioles function as microtubule-organizing centers (MTOCs) in the fertilized oocyte. In mice, rats, and hamsters, however, asters present in the ooplasm function as the MTOCs (Navara *et al.*, 1995; Shin *et al.*, 1998) (Fig. 1). These asters are very active in anchoring the nucleus or chromosomes introduced into the ooplasm, and they therefore play a crucial role in the normal behavior of the donor chromosomes in somatic-cell nuclear transfer (Miki *et al.*, 2004a). Thus, normal fertilization occurs in these rodents (Family: Muridae) even in the absence of sperm centrioles. In addition, the sperm tails in these rodents are very long. Microinsemination of the head alone is likely to decrease oocyte damage and thus increase the rate of embryo development. Therefore, in ICSI of mice, rats, and hamsters, the sperm tail (containing the centrioles) is usually removed and only the sperm head is injected into oocytes (Fig. 2) (Hirabayashi *et al.*, 2002a; Kuretake *et al.*, 1996a; Yamauchi *et al.*, 2002).

In animals other than these rodents, ICSI routinely includes injection of the tail; however, at least in bovines and pigs, live births with injection of only the sperm head have been reported (Hamano *et al.*, 1999; Nakai *et al.*, 2003).

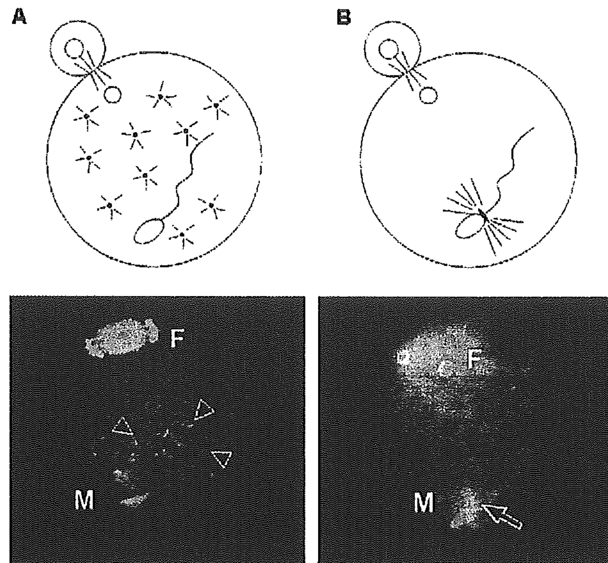

FIG. 1 Schematic drawings and representative photomicrographs of microtubule-organizing centers (MTOCs) during mammalian fertilization. (A) In mice, rats, and hamsters, microtubule asters preexisting in the ooplasm form the MTOCs. The image shows hamster oocytes at anaphase II. The sperm nucleus (M) is devoid of any microtubular structures, whereas cytoplasmic asters are present in the ooplasm (*arrowheads*). (B) In other species, MTOCs originate from sperm centrioles. The image shows a rabbit oocyte at anaphase II. A characteristic microtubule array (*arrow*), which will act as an MTOC, is being formed from the neck of the fertilizing sperm with the male chromosomes (M). F, female chromosomes. Image courtesy of Drs. Y. Terada and M. Tachibana.

As with oocytes in parthenogenesis and embryos in nuclear transfer, the oocytes in these animals have the potential to form MTOCs that function as necessary, indicating that the source of MTOCs is relatively flexible (see Section III.B). Although classified in the same order—Rodentia—guinea pigs differ from mice and rats (Muridae) in that the sperm centrioles function as MTOCs (A. Ogura, unpublished data). It is possible that guinea pigs should be classified apart from Rodentia, based on genome analysis (Graur *et al.*, 1991). On the other hand, Muridae (e.g., mice) may have followed a different course of evolution in this regard.

The midpiece of mammalian spermatozoa contains mitochondria, which supply ATP energy for sperm movement. Each mammalian spermatozoon contains about 10–1000 copies of mitochondrial DNA (mtDNA); this is markedly fewer than in oocytes, which possess about 10^5 copies of mtDNA (Diez-Sanchez *et al.*, 2003; Hecht *et al.*, 1984; Shitara *et al.*, 2000). Although all sperm mitochondria are introduced into oocyte cytoplasm after

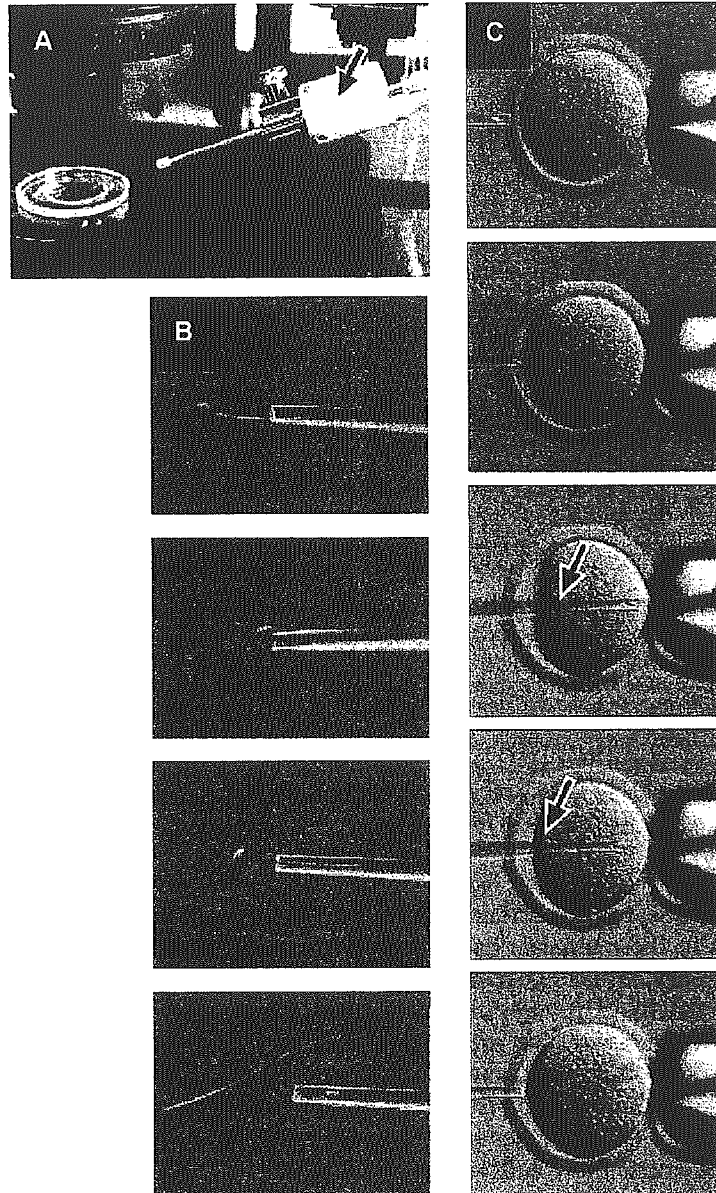

FIG. 2 The procedure of mouse intracytoplasmic sperm injection (ICSI). (A) For ICSI of mice and other laboratory rodents, use of a micromanipulator equipped with a piezo-impact unit (*arrow*) is essential because of the very fragile nature of the oolemma. (B) In mouse ICSI experiments, the sperm head is usually separated from the midpiece and tail at the opening of an injection pipette by application of a piezo-pulse. After the midpiece and tail are discarded, the

sperm–oocyte membrane fusion, they are eliminated in the early stages of embryogenesis (Kaneda *et al.*, 1995; Sutovsky *et al.*, 1996). Consequently, mtDNA is almost invariably strictly maternally inherited into the next generation (Birky, 1995; Giles *et al.*, 1980). This maintains a high level of homoplasmy (single type of mtDNA haplotype) in the individual.

Several studies have indicated that the mechanism by which the paternal mtDNA is eliminated from the ooplasm depends on a ubiquitin-mediated degradation system. One model proposes that the sperm mitochondrial membrane has a surface molecule, prohibitin, that is ubiquitinated during spermatogenesis (Thompson *et al.*, 2003) and is subsequently degraded by either lysosomal or proteosomal proteolysis in the embryo (Sutovsky *et al.*, 2000). This paternal mtDNA recognition mechanism seems to be species specific, because paternal sperm mtDNA persists in hybrid interspecies offspring in rodents and cattle (Kaneda *et al*, 1995; Sutovsky *et al.*, 1999).

We investigated whether the paternal mtDNA introduced into the ooplasm following ICSI is eliminated in a manner similar to that of normal fertilization (K. Inoue, unpublished data). Interestingly, as far as we examined, all the ICSI fetuses and offspring carried sperm-derived mtDNA in at least some, if not all, tissues, while the corresponding samples from IVF did not. Therefore, ICSI may increase the chance that sperm mtDNA will escape the embryo's recognition and degradation system and cause heteroplasmy (multiple forms of mtDNA haplotype) in the offspring.

Unlike normal fertilization involving sperm–oocyte membrane fusion, ICSI introduces a spermatozoon with its plasma membrane largely intact. Therefore, the timing of exposure of the sperm mitochondria to the ooplasm after ICSI might be slower than that of normal fertilization, and some sperm mitochondria may escape the surveillance mechanism. Whether ICSI increases the risk of heteroplasmy in human infertility treatment is still controversial, as there are conflicting results (St John *et al.*, 2000; Marchington *et al.*, 2002). We speculate that subtle differences in the status of the sperm plasma membrane and in the timing of fertilization events may have caused the discrepancy.

As round spermatids are already tagged by ubiquitin during spermatogenesis (Sutovsky *et al.*, 2000), paternal mtDNA can also be eliminated

head is aspirated back into the injection pipette. (C) The isolated sperm head is then injected into the ooplasm using the piezo-impact unit. After advancement through the zona pellucida with a few piezo pulses, the pipette is then inserted deep into the ooplasm. A sperm head is pushed forward to the tip of the pipette and a single piezo pulse of minimal intensity is applied. The plasma membrane is punctured at the pipette tip, as evidenced by a rapid relaxation and rebound of the membrane (compare arrows). This deep initial insertion of the pipette before rupture of the oolemma is important for success. The sperm head is then expelled into the ooplasm with a minimal amount of medium.

following ROSI. However, round spermatids contain about 10 times more mtDNA than mature spermatozoa, as much of the excess cytoplasm is discarded in the residual body and cytoplasmic droplet during spermiogenesis. This overload could cause spermatid mtDNA to remain in the ROSI embryos through the blastocyst stage (Cummins *et al.*, 1998; Shitara *et al.*, 2000). In mice, the paternal mitochondria are usually eliminated by the four-cell embryo stage (Kaneda *et al*, 1995; Sutovsky *et al.*, 1999). By contrast, mitochondria derived from somatic cells are not eliminated after injection into oocytes (Irwin *et al.*, 1999), presumably because they lack the ubiquitin-tagged marker. Therefore, small amounts of donor mtDNA can be detected in the adult tissues of most cloned bovines and mice after somatic-cell nuclear transfer (Inoue *et al.*, 2004; Steinborn *et al.*, 2000).

C. Presence or Absence of Acrosome

The sperm acrosome contains many enzymes that are not incorporated into the oocyte during normal fertilization, so the acrosome should preferably be removed before microinsemination. In most animals, however, injection of a spermatozoon with the acrosome is not a problem. This is probably because the oocyte is much larger than the acrosome. One exception, however, is the golden hamster, which has a large acrosome. Injection of its acrosome can damage the oocyte, so sperm pretreatment—for example by freeze–thawing—is necessary to remove the acrosome (Yamauchi *et al.*, 2002). In pigs, acrosome enzyme activity is high, so removal of the acrosome can also increase the effectiveness of microinjection (Katayama *et al.*, 2002). In humans, the increased fertilization efficiency associated with immobilizing the sperm before ICSI (breaking the cell membrane using an injection pipette) appears to be related to acrosome disruption before and after ICSI (Takeuchi *et al.*, 2004).

D. Use of Piezo-Driven Micromanipulators

Successful ICSI in mice, after it had been achieved in humans, livestock, and laboratory animals, was finally reported in 1995 (Kimura and Yanagimachi, 1995a). This success was delayed because mice oocytes are very sensitive to injection stimulation, and insertion of a microneedle immediately causes rupture of the oolemma. For this reason, the first viable offspring produced by microinsemination in mice were not with spermatozoa but rather with round spermatids, using electrofusion (Ogura *et al.*, 1994). It is apparently