lineage cells using B6 × 129F1 males as donors, and successfully obtained cloned pups derived from the nuclei of HSCs and natural killer T (NKT) lymphocytes [8,9]. Interestingly, HSCs were inherently inefficient donor cells and no HSC-derived pups were obtained when we used B6D2F1 females [8], the standard oocyte donors for mouse cloning experiments. In this study, therefore, we used B6 × 129F1 males as the source of NSCs and MSCs, and we now add the former to the clonable cell type list in mice. Table 3 shows the efficiencies of cloning mice using seven cell types with the male B6 × 129F1 genotype. These data show apparent cell type-specific differences in cloning efficiency ranging from 0% to 9.4% per embryo transferred. In view of the rates of four-cell embryos (per two-cells) and offspring (per transfer), NSCs seem to be moderately efficient sources of nuclei for transfer.

Following nuclear transfer into the ooplasm, the donor somatic cell genome should be reprogrammed to a state equivalent to that of a fertilized embryo for further development. During the first and second cell cycles in cloned mouse embryos, this reprogramming is manifested in the structural remodeling of the donor nucleus into pseudopronuclei and the initiation of embryo-specific transcription, termed zygotic gene activation (ZGA) [25]. Provided that nuclear transfer is performed a few hours before oocyte activation, nuclear structural remodeling, the timing of ZGA and of whole-genome ZGA activity in somatic cell-derived cloned embryos are indistinguishable from those in normally fertilized embryos [26, 27]. In contrast, analysis of the transcriptional levels of individual zygotic genes reveals that some are repressed in cloned embryos [8, 28], probably reflecting incomplete reprogramming of the donor cell genome.

Interestingly, the degree of this repression or the genes affected by nuclear transfer may vary with the donor cell type used [8]. Therefore, we hypothesize that the expression patterns of specific ZGA genes—especially those not expressed in unfertilized oocytes—may be good indicators of how effectively the donor cell genome is reprogrammed by nuclear transfer. In our current study, we analyzed six ZGA genes; of these, *Dppa4*, *ERV-L*, and *eIF-1A* appeared to give better information because their expression levels increased significantly from the basal levels at MII to significantly higher levels in the control two-cell IVF embryos (Fig. 4). The expression levels of these three genes did not differ significantly between NSC clones and IVF embryos. In contrast, the expression patterns for *eIF-1A* and *Dppa4* were significantly lower in fibroblast-derived clones than in IVF embryos ($P < 0.05$). These results along with the rates of four-cell embryos indicate that reprogramming was more efficient for the NSC genome than the fibroblast genome. This is consistent with the report of Blelloch *et al.*, who found more frequent generation of nuclear transfer ES cells from NSCs than from fibroblasts using the same B6 × 129F1 genetic background [29]. However, the birth rates in our study did not differ significantly between NSC and fibroblast clones, probably because of technical variations between the embryo transfer experiments and because of the low numbers of pups born in the cloning experiments. Our gene expression analysis using the two-cell embryos also suggests the importance of the donor genotype, because the overall gene expression patterns in our current study were better than those in our previous report using B6D2F1 female donors [8]. These findings show clear associations between the expression levels of certain genes and subsequent embryonic development. Taken

together, we expect that the reprogrammability of the different donor cells can be assessed as early as at the two-cell stage by analyzing the expression of appropriate genes as indicators.

In general, genetic factors as well as epigenetic factors may affect the development of cloned embryos considerably. The implantation failure found here for MSC-derived embryos is strongly suggestive of chromosomal abnormalities as documented by Bosch *et al.* [30]. Chromosomal abnormalities of cloned embryos may occur because of abnormal behavior of the donor chromosomes or chromosomal abnormalities preexisting in the donor cells. In the mouse, the chromosomal constitutions of cloned embryos are generally stable because of the presence of cytoplasmic asters that act as microtubule-organizing centers at fertilization [31]. During nuclear transfer, these asters gather together to form a spindle that anchors the donor chromosomes and contributes to the genetic stability of reconstructed embryos [32]. Therefore, chromosomal abnormalities in MSC-derived cloned embryos are likely to derive from those in the original donor cells. Our chromosomal analysis of the donor cells supports this assumption. The rates of MSCs with abnormal chromosomal numbers or morphology were extremely high. According to Miura *et al.*, repeated passages of mouse MSCs lead to spontaneous immortalization, which is very closely associated with chromosomal aberrations [33]. This should be considered when one clones mice from donor cells that have been passaged many times *in vitro*. By contrast, in bovines, MSCs were cloned successfully and normal offspring were born at the usual efficiency (7%, 1/13) probably due to more stable chromosomal constitutions of bovine MSC lines [34].

We conclude that tissue-specific stem cells in mice, namely NSCs, MSCs and HSCs, can show marked variations in their ability to produce cloned offspring, according to both the epigenetic and genetic status of their original genomes.

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Figure Legends

Figure 1. *In vitro* differentiation of NSCs and MSCs used as nuclear donors in this study. **(A):** Undifferentiated NSC neurospheres. **(B):** NSC-derived neurons (anti-MAP2 staining). **(C):** NSC-derived astrocytes (anti-GFAP staining). **(D):** NSC-derived oligodendrocytes (anti-O4 staining). **(E):** Undifferentiated MSC cells. **(F):** MSC-derived adipocytes (oil-red O staining). **(G):** MSC-derived osteoblasts (alkaline phosphatase staining). **(H):** MSC-derived chondrocytes (Alcian blue staining). Scale bar, 100 μm.

Figure 2. Cloned mouse pups born after nuclear transfer using NSCs as donors. Shortly after Caesarian section at full term, two pups recovered their movement and respiration.

Figure 3. Cytogenetic analysis of MSC and NSC lines. **(A, B):** The two chromosome types found in the MSC line used for nuclear transfer. Some MSCs had 41 chromosomes with monosomy 4, trisomy 6, and two Y chromosomes (arrowheads in **A**). Others had the normal number of chromosomes (2n = 40), but they also had the same monosomy 4 and trisomy 6 (arrowheads in **B**). Heteromorphism was observed on chromosome 16 in both types (arrows in **A** and **B**). The chromosomes of MSCs were especially prone to morphological and numerical abnormalities. **(C):** The distribution of the cells classified according to the chromosome numbers in different MSC lines. All MSC lines comprise cells with abnormal chromosome numbers. **(D):** The

distribution of the cells classified according to the chromosome numbers in two NSC lines. In contrast to the MSC lines, NCS lines comprise predominantly cells with the normal ploidy (2n = 40).

Fig. 4. Quantification by real-time RT–PCR of mRNA expression of various zygotic-activated genes in single oocytes and embryos. Genotype (B6 × 129F1)-matched two-cell IVF embryos, two-cell fibroblast-derived clone embryos (Fi) and two-cell NSC-derived clone embryos were analyzed. MII oocytes were derived from B6D2F1 females, as in the nuclear transfer experiments. Each dot represents a single embryo. Values are expressed relative to those in the IVF group (value = 1). Values with different letters differ significantly ($P < 0.05$, Scheffé's F test).

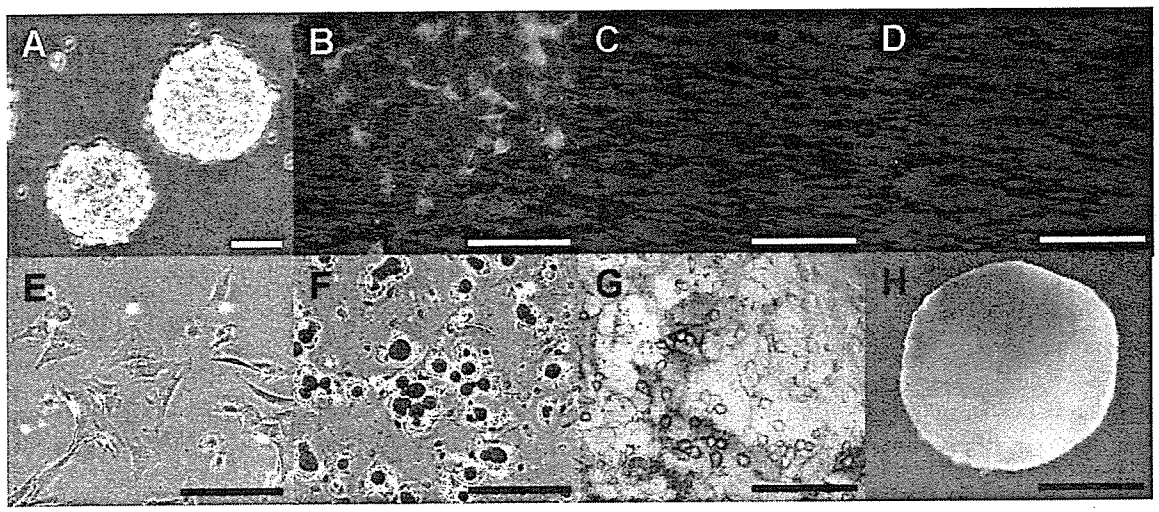
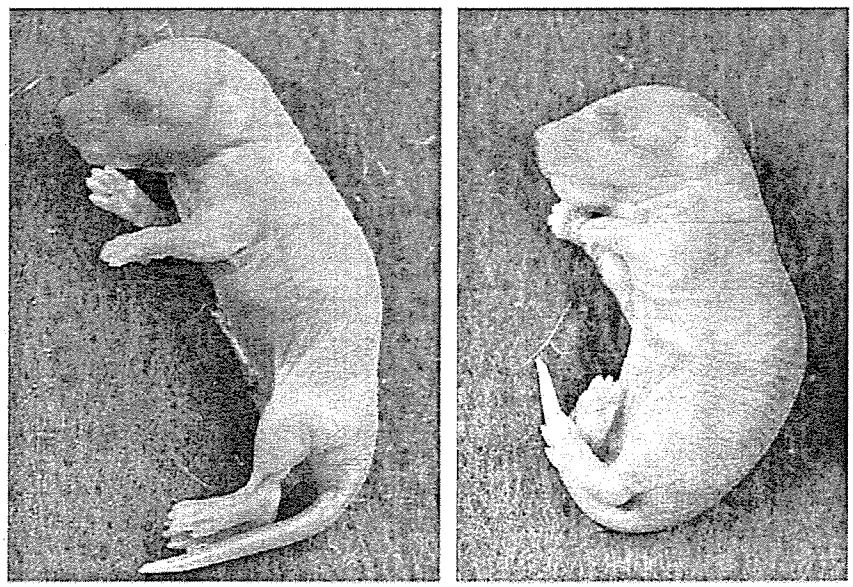


Fig. 1 (Ogura)



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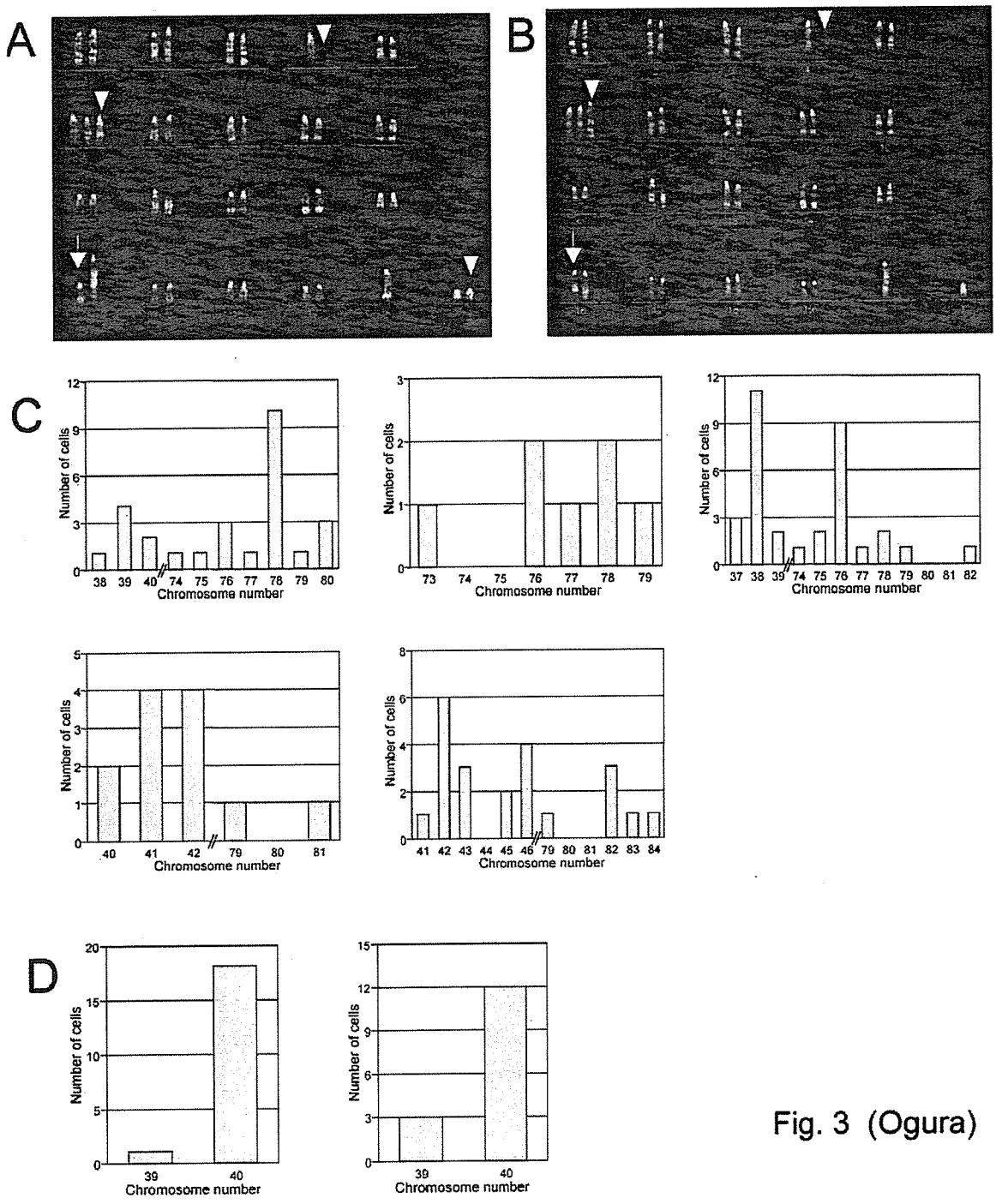

Fig. 3 (Ogura)

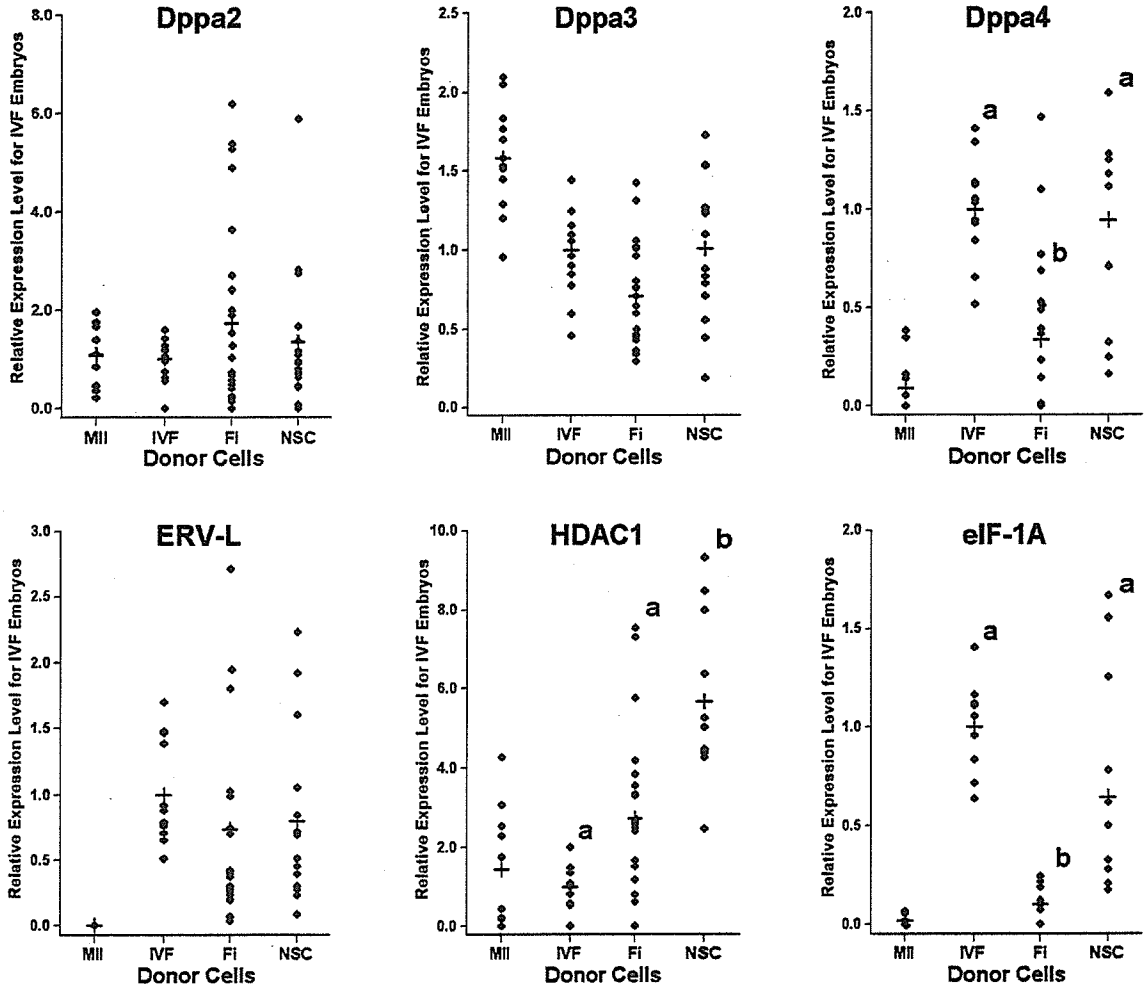

Fig. 4 (Ogura)

Table 1. Primer sequence for gene expression analysis

	Forward Primer	Reverse Primer	Product Size (bp)
Dppa2	5'-gcccagcctgcctccta-3'	5'-tgccatccgtactcaagttatgat-3'	83
Dppa3 (Stella/PGC7)	5'-atcgggaagaattaggagcttaca-3'	5'-caaaaaatagctttcacatctgctgt-3'	82
Dppa4	5'-aggaagtcagcaccaccgtagt-3'	5'-aagctggaagagggcaatggct-3'	263
ERV-L	5'-ggaatgaaggtatgggtcaatcc-3'	5'-ccttcaccttcagccagcac-3'	67
HDAC1	5'-gctgccctacaatgactactttga-3'	5'-ctcgttagtgttctggttggtcat-3'	91
eIF-1A	5'-caatgaaacggacacatttggt-3'	5'-agatgtcatcaatgtcttcatcatca-3'	89
Hprt	ABI Mm00446968_m1		

Table 2. *In vitro* and *in vivo* development of embryos cloned from NSCs, MSCs, and adult fibroblasts

Cell type	Embryos cultured	2-cell (%)	4-cell (% per 2-cells)	Embryos transferred (ET)	Embryos implanted (% per ET)	Term conceptuses (% per ET)	Offspring (% per ET)	Placenta-only conceptuses (% per ET)
NSC	488	328 (67.2)	249 (75.9)[a]	189	82 (43.4)[b]	10 (5.3)	3 (1.6)	7 (3.7)
MSC	232	172 (74.1)	79 (45.9)[a']	78	0 (0.0)[b']	0 (0.0)	0 (0.0)	0 (0.0)
Fibroblast	173	163 (94.2)	68 (41.7)[a']	33	19 (57.6)[b]	1 (3.0)	1 (3.0)	0 (0.0)

[a,a'; b,b'] $P < 0.05$ (Fisher's exact probability test).

Table 3. Efficiencies of cloning male mice from different cell types with the B6 × 129F1 genotype

Cell type	Embryos cultured	2-cell (%)	4-cell (% per 2-cells)	Embryos transferred (ET)	Embryos implanted (% per ET)	Term conceptuses (% per ET)	Offspring (% per ET)	References
Immature Sertoli	380	290 (76.3)	228 (78.6)	191	98 (51.3)	19 (9.9)	18 (9.4)	[4]
Adult fibroblast	355	334 (94.4)	194 (57.9)	126	82 (65.1)	4 (3.2)	3 (2.4)	[4,8]
Neural stem	488	328 (67.2)	249 (75.9)	189	82 (43.4)	10 (5.3)	3 (1.6)	This study
NKT lymphocyte	572	534 (93.4)	482 (90.3)	185	112 (60.5)	8 (4.3)	3 (1.6)	[9]
Hematopoietic stem	637	563 (88.4)	302 (53.6)	252	90 (35.7)	2 (0.8)	2 (0.8)	[8]
Primordial germ*	2018	1011 (55.4)	611 (60.4)	441	252 (57.1)	17 (3.8)	4 (0.9)	[35]
T lymphocyte	385	236 (61.3)	102 (43.2)	44	0 (0.0)	0 (0.0)	0 (0.0)	[9]
Mesenchymal stem	232	172 (74.1)	79 (45.9)	78	0 (0.0)	0 (0.0)	0 (0.0)	This study

Cell types are listed in the order of the offspring rate. *(B6D2F1 × 129)F1 females and males.

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Adenovirus-mediated gene delivery into mouse spermatogonial stem cells

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Spermatogonial stem cells represent a self-renewing population of spermatogonia, and continuous division of these cells supports spermatogenesis throughout the life of adult male animals. Previous attempts to introduce adenovirus vectors into spermatogenic cells, including spermatogonial stem cells, have failed to yield evidence of infection, suggesting that male germ cells may be resistant to adenovirus infection. In this study we show the feasibility of transducing spermatogonial stem cells by adenovirus vectors. When testis cells from ROSA26 Cre reporter mice were incubated *in vitro* with a Cre-expressing adenovirus vector, Cre-mediated recombination occurred at an efficiency of 49–76%, and the infected spermatogonial stem cells could reinitiate spermatogenesis after transplantation into seminiferous tubules of infertile recipient testes. No evidence of germ-line integration of adenovirus vector could be found in offspring from infected stem cells that underwent Cre-mediated recombination, which suggests that the adenovirus vector infected the cells but did not stably integrate into the germ line. Nevertheless, these results suggest that adenovirus may inadvertently integrate into the patient's germ line and indicate that there is no barrier to adenovirus infection in spermatogonial stem cells.

gene therapy | germ cell | spermatogenesis

Spermatogenesis depends on the continuous proliferation of spermatogonial stem cells. Although the number of spermatogonial stem cells is very small (comprising only 0.2–0.3% in the mouse testis) (1, 2), these cells undergo self-renewing division and produce differentiated cells while maintaining an undifferentiated state. Because spermatogonial stem cells transmit genetic information to the offspring, the introduction of genetic material into spermatogonial stem cells results in permanent modification of the germ line.

However, initial attempts to introduce genetic material into spermatogonial stem cells met with little success in part because of a lack of methods for transducing genetic material into stem cells. The first reported evidence of germ-line transduction used retrovirus vectors, which have relatively high infection efficiency and have been widely used in the transduction of stem cells in several self-renewing tissues (3). Spermatogonial stem cells were infected with retrovirus vectors *in vitro* and transplanted into the seminiferous tubules for offspring production. Transplanted stem cells colonized the empty seminiferous tubules of infertile recipient testes and reinitiated spermatogenesis, eventually leading to the production of transgenic animals (4, 5). Although these results opened up an opportunity for *in vitro* genetic manipulation of spermatogonial stem cells, they revealed a serious safety concern in human somatic gene therapy. Inadvertent infection of germ-line cells by gene therapy vectors could lead to vertical germ-line transmission of the virus and potential insertional mutagenesis.

Adenovirus is another important type of viral vector that is used in human gene therapy (6). Unlike retrovirus vectors, which can infect only dividing cells, adenovirus has relatively high transduction efficiency in target cells and infects both dividing and nondividing cells (6). Although adenovirus vectors can be prepared at higher titer than retrovirus vectors and infect a large range of host cells, including hematopoietic stem cells or embryonic stem cells (7, 8), many *in vitro* and *in vivo* attempts to transduce spermatogenic cells have not provided evidence of infection. In particular, the absence of a functional assay to directly measure stem cell activity has interfered with a conclusive determination of whether spermatogonial stem cells can be infected by adenoviruses. Although viruses that are injected intravenously or into nongonadal tissues can reach mouse or human testes, only Sertoli cells are preferentially infected by intratesticular injection, and there is no evidence of germ cell infection (9–14). Furthermore, there is no sign of infection after direct *in vitro* exposure of spermatogenic cells or mature sperm to adenovirus (15, 16). These studies suggest that male germ cells, including spermatogonial stem cells, cannot be infected by adenovirus vectors.

The purpose of this study is to examine the feasibility of adenovirus-mediated gene delivery to spermatogonial stem cells. We hypothesized that adenovirus infection did not occur in previous studies because of inefficient exposure of germ cells (or stem cells) to adenovirus and/or low sensitivity of the detection methods. To overcome these problems, we took advantage of the ROSA26 reporter mouse strain, which can sensitively monitor Cre-mediated deletion (17). By adding Cre recombinase into cells, it is possible to excise *loxP*-flanked DNA sequences in transfected cells (17). An adenovirus expressing the Cre recombinase gene was used to infect an enriched population of spermatogonial stem cells *in vitro*, and infected cells were transplanted into the seminiferous tubules of infertile animals. Recipient testes were analyzed for reporter gene expression, and offspring DNA was examined for virus gene integration.

Results

Infection of Spermatogonial Stem Cells *in Vivo*. In preliminary experiments we examined whether it is possible to infect spermatogonial stem cells *in vivo*. An adenovirus vector that expresses *LacZ* gene (AxCANLacZ) was introduced into the seminiferous tubules of immature and mature wild-type mice. In contrast to i.v. delivery, microinjection into seminiferous tubules allows more efficient direct exposure of germ cells to adenovirus. In particular, immature testes should provide better accessibility to spermatogonial stem cells because of the absence of a blood–testis barrier and multiple layers of germ cells (18). Previous studies using immature testes have shown efficient *in vivo* retrovirus infection of spermatogonial

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The authors declare no conflict of interest.

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Abbreviations: GS cell, germ-line stem cell; mGS cell, multipotent GS cell.

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