

# In Vitro-Differentiated Embryonic Stem Cells Give Rise to Male Gametes that Can Generate Offspring Mice

## Short Article

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### Summary

Male gametes originate from a small population of spermatogonial stem cells (SSCs). These cells are believed to divide infinitely and to support spermatogenesis throughout life in the male. Here, we developed a strategy for the establishment of SSC lines from embryonic stem (ES) cells. These cells are able to undergo meiosis, are able to generate haploid male gametes in vitro, and are functional, as shown by fertilization after intracytoplasmic injection into mouse oocytes. Resulting two-cell embryos were transferred into oviducts, and live mice were born. Six of seven animals developed to adult mice. This is a clear indication that male gametes derived in vitro from ES cells by this strategy are able to induce normal fertilization and development. Our approach provides an accessible in vitro model system for studies of mammalian gametogenesis, as well as for the development of new strategies for the generation of transgenic mice and treatment of infertility.

### Introduction

In sexually reproducing animals, all gametes of either sex arise from primordial germ cells (PGCs). In mam-

mals, PGCs arise from the proximal epiblast, a region of the early embryo that also contributes to the first blood lineages of the embryonic yolk sac (Lawson and Hage, 1994; Zhao and Garbers, 2002). In the mouse, PGCs migrate through the dorsal mesentery and enter the developing fetal gonad at the genital ridge; this occurs between E10.5 and E12.5. Once they arrive in the genital ridge, the PGCs in males are enclosed by Sertoli cells and become gonocytes (McLaren, 2000). The gonocytes proliferate for a few days and then arrest in G<sub>0</sub>/G<sub>1</sub> phase until birth. Within a few days after birth, the gonocytes resume proliferation to initiate spermatogenesis and to produce male gametes. Recent studies have demonstrated that embryonic stem (ES) cells differentiate into germ cells and more mature gametes (Hubner et al., 2003; Geijsen et al., 2004; Toyooka et al., 2003), although significant unanswered questions remain about the functionality of these cells. The derivation of germ cells from ES cells in vitro provides an invaluable assay both for the genetic dissection of germ cell development and for epigenetic reprogramming, and it may one day facilitate nuclear transfer technology and infertility treatments.

In the present study, we demonstrate that in vitro male gametes derived from ES cells are functional and are able to produce viable offspring.

### Results and Discussion

To examine the functionality of ES cell-derived male gametes, we developed a new strategy for the establishment of male germline stem cells, spermatogonial stem cells (SSCs), from ES cells. First, we produced two fusion genes, *Stra8-EGFP* and *Prm1-DsRed*, harboring coding regions of enhanced green fluorescent protein (EGFP) and red fluorescent protein (DsRed) under control of promoter regions of murine *Stra8* (Oulad-Abdelghani et al., 1996) and *protamine 1* (*Prm1*) genes (Zambrowicz et al., 1993), respectively (Figure 1A). Both reporter genes contain the *neomycin phosphotransferase II* gene (*NEO*), which is driven by the SV40 early promoter and enhancer for positive selection (Figure 1A). Previously, it was demonstrated that 1.4 kb of the 5' flanking region of the *Stra8* gene (Nayernia et al., 2004) and 0.5 kb of the 5' flanking region of the *Prm1* gene (Zambrowicz et al., 1993) are able to direct reporter gene expression specifically in premeiotic and haploid male germ cells, respectively. ES cells were transfected with the *Stra8-EGFP* fusion gene and were cultured for 2 months in the presence of G418 to select the cells that were positive for the *Stra8-EGFP* construct. The culture, transfection, and selection of cells were performed according to previously described protocols (Nayernia et al., 2004).

Previous studies have shown that a cocktail of soluble growth factors, including retinoic acid (RA), are able to sustain the survival and self-renewal of mouse germ cells in the absence of somatic cell support. After germ cell adhesion to an acellular substrate, such growth factors and compounds were able to prevent the occurrence

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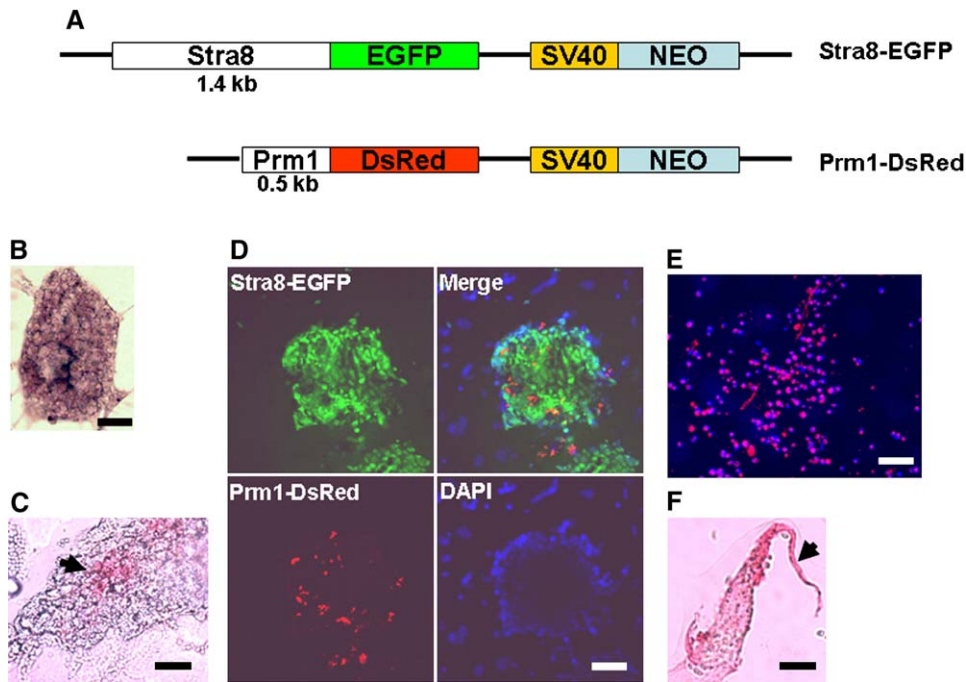


Figure 1. Strategy for the Establishment of ES Cell-Derived Male Germline Stem Cell Lines

(A) Schematic representation of the *Stra8-EGFP* and *Prm1-DsRed* reporter genes harboring a 1.4 kb and 0.5 kb promoter region of *Stra8* and the mouse *protamine 1 (Prm1)* gene, respectively. These regions are able to direct reporter gene expression specifically in premeiotic and haploid male germ cells, respectively. Both reporter genes contain *neomycin phosphotransferase II gene (NEO)*, which is driven by the SV40 early promoter and enhancer (SV40).

(B) HE staining of an embryoid-like cell colony.

(C) Immunostaining of an embryoid body-like cell colony with the anti-Mvh antibody shows differentiation of PGCs (arrow).

(D) Fluorescent images of *Stra8-EGFP* (green) and *Prm1-DsRed* (red) proteins in the ES-derived SSC12 cell line after 72 hr of RA induction. The merged image shows different cells with green and red fluorescence, indicating activation of *Stra8* and *Prm1* promoters in different cell types. DAPI: fluorescent image showing DNA localization.

(E) The merged fluorescent image of the supernatant from SSC12 cells after 72 hr of RA induction showing only red cells, which is an indication for release of *Prm1-DsRed*-positive cells into the medium.

(F) Magnification of a red cell from supernatant showing positive immunoreaction with a sperm tail-specific protein, PHGPx (arrow).

Bars in (B), (C), and (E) are 50  $\mu\text{m}$ , bars in (D) are 100  $\mu\text{m}$ , and bars in (F) are 10  $\mu\text{m}$ .

of significant levels of apoptosis in germ cells; stimulate their proliferation; and, when LIF was omitted from the cocktail, allow most of them to enter into and progress through meiotic prophase I (Farini et al., 2005; Koshimizu et al., 1995; Bowles et al., 2006). Furthermore, RA is known to promote the developmental progression of spermatocytes through early stages of meiosis (Leid et al., 1992; Akmal et al., 1997), though the molecular mechanisms underlying these effects remain to be elucidated. We therefore chose to use RA in our efforts to direct functional spermatogenesis in vitro.

After RA induction (final concentration of  $10^{-5}$  M), expression of *Stra8-EGFP* was detected in about 60% of all cells (data not shown). The positive cells were selected by using fluorescence-activated cell sorting (FACS) and were cultured for an additional 2 months under non-induced conditions. Since transgene silencing is a common problem in ES cells, it is important to note that these cells survived in medium containing G418 and remained GFP positive. It can therefore be concluded that the transgene is expressed. To examine whether the selected cells maintain the differentiation properties after RA induction, the cells were subjected to a second FACS sorting. Expression of *Stra8-EGFP* was detected in 90% of all cells (data not shown), indicating the main-

tenance of the differentiation potential of the sorted cells. The cells were transfected with the *Prm1-DsRed* fusion construct, and positive cell colonies were selected by PCR with primers specific for the *DsRed* coding region. Using this strategy, two cell lines, SSC7 and SSC12, were established. Both cell lines express EGFP without RA induction, which is an indication of the permanent activation of the *Stra8* promoter as well as differentiation toward male germ cells (data not shown). The cells formed embryoid body-like structures (Figure 1B) and expressed mouse vasa homolog (Mvh) protein (Figure 1C). Mvh is specific for differentiating germ cells from the late migration stage to the postmeiotic stage (Toyooka et al., 2000). After RA induction for 72 hr, the SSC7 and SSC12 cell lines are able to produce DsRed-positive (red) cells that arose from EGFP-positive cells (Figure 1D). Red cells are released into the medium and can be collected from the supernatant (Figure 1E). Using live phase contrast microscopy, the motility of cells in the supernatant of the SSC7 and SSC12 cell lines can be demonstrated after 72 hr of RA induction (Movie S1; see the Supplemental Data available with this article online). The formation of tail-like structures could be observed in some red cells (Figure 1F). Expression of DsRed protein is an indication that EGFP-positive cells

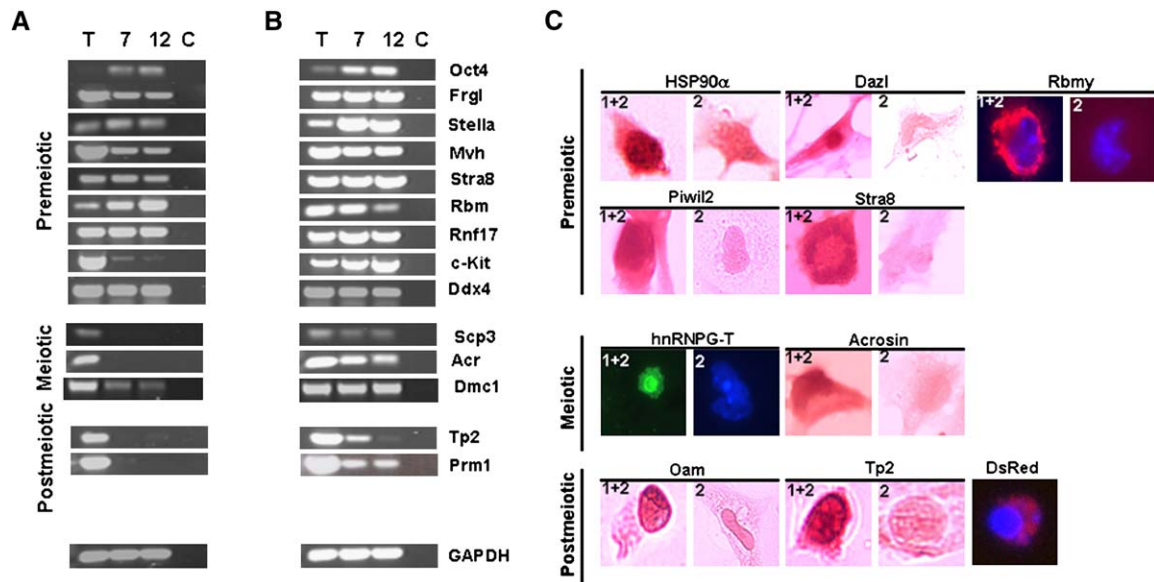


Figure 2. ES Cell-Derived Male Germ Cells Showing Premeiotic, Meiotic, and Postmeiotic Markers

(A) RT-PCR analysis of SSC7 and SSC12 cell lines under nondifferentiating conditions shows only expression of PGC (*Oct4*, *fragilis* [*Frgl*], *stella*, *mouse vasa homolog* [*Mvh*], and *Ddx4*) and premeiotic-specific genes (*Stra8*, *Rbm*, *Rnf17*, and *c-Kit* [Vincent et al., 1998]).

(B) RT-PCR analyses of SSC7 and SSC12 cells after treatment with RA (72 hr) show induction of meiotic (*synaptonemal complex protein 3* [*Scp3*], *acrosin* [*Acr*], and *Dmc1* [Tarsounas et al., 1999]) and postmeiotic (*transition protein 2* [*Tp2*] and *protamine 1* [*Prm1*]) gene expression. T, testis; C, nontemplate control.

(C) Immunohistochemical analysis and formation of sperm structures. In immunohistochemical analysis, the expression of premeiotic-specific proteins, Hsp-90 $\alpha$ , Dazl, Rbmy, Piwil2, and Stra8, is demonstrated (red). Meiosis was demonstrated by using immunostaining with an antibody against hnRNPG-T, which is expressed specifically in pachytene spermatocytes (green). The formation of an acrosome-like structure is shown by antibodies against outer acrosomal membrane (OAM) and Acr (red). Condensation of the nucleus is demonstrated by immunohistochemical analysis by using Tp2. DsRed is located in the cytoplasm of the cells with condensed nuclei. 1+2, primary and secondary antibodies; 2, only secondary antibody used as a negative control.

differentiated into haploid male germ cells and activated the *Prm1* promoter region.

#### Characteristics of ES Cell-Derived Male Gamete Differentiation

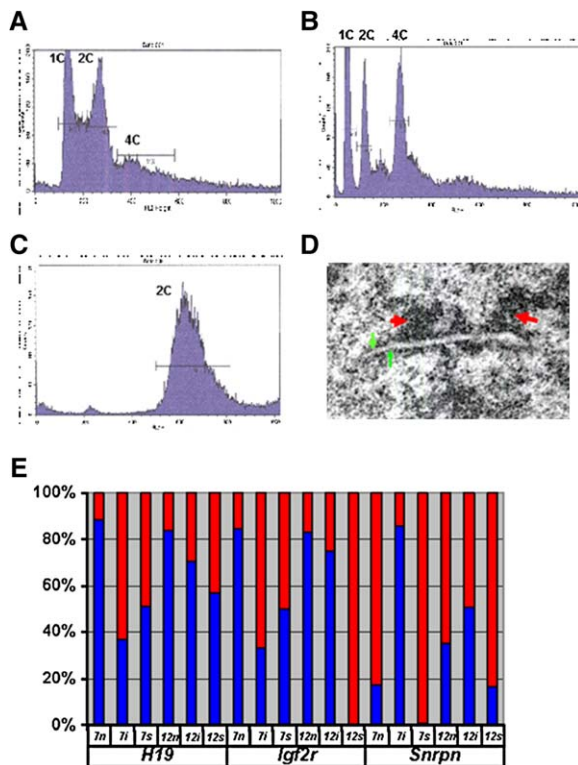
The cells were characterized by determining the expression of different markers for PGCs, premeiotic, meiotic, and postmeiotic male germ cells by RT-PCR analysis. RT-PCR showed expression of markers for the early stages of male germ cell development (*Oct4*, *fragilis*, *stella*, *Mvh*, and *Rnf17*) (Saitou et al., 2002; Scholer et al., 1990; Tanaka et al., 2000; Wang et al., 2001) in untreated and RA-treated SSC7 and SSC12 cells (Figures 2A and 2B). Furthermore, expression of *Ddx4*, which is a marker for late germ cells, was found (Figure 2). This gene is not expressed in ES cells (Zwaka and Thomson, 2005). Expression of PGC-specific genes in SSC7 and SSC12 cells indicates that a subpopulation of these cells can be characterized as PGCs. Immunohistochemical analysis with antibodies against Hsp-90 $\alpha$ , Dazl, Rbmy (Elliott et al., 1996), Piwil2, and Stra8 further confirmed this postulate (Figure 2C). It is worth noting that increased expression of *c-Kit* after RA treatment (Figures 2A and 2B) indicates that both cell lines are able to differentiate to spermatogonia.

To investigate whether SSC cell lines undergo meiosis in vitro, we determined the DNA content of the cells by flow cytometric analysis. As shown in Figure 3A, ~30% of SSC cells undergo meiosis and produce a haploid cell population (1C in Figure 3A) after 72 hr of

RA induction. Without RA induction, ~3% of SSC cells undergo meiosis (data not shown). Furthermore, we analyzed the formation of the synaptonemal complex in SSC cell lines after RA induction by using electron microscopy. The formation of the synaptonemal complex in SSC cell lines after RA induction is additional evidence for meiotic differentiation (Figure 3D). After 24 hr of induction, the cells are able only to differentiate to early spermatids, but not to late spermatids, which were shown by markers specific for these stages (Figure S1; see the Supplemental Data available with this article online).

To analyze postmeiotic differentiation on the molecular level, we examined expression of different stage-specific genes in RA-treated and untreated SSC7 and SSC12 cells. Using RT-PCR, we analyzed expression of *synaptonemal complex protein 3* (*SCP3*) and *acrosin* (*Acr*), two markers for meiotic differentiation (Nayernia et al., 1996; Yuan et al., 1998), and *transition protein 2* (*Tp2*) and *Prm1*, two postmeiotic markers. Expression of *SCP3* and *Acr* was observed after RA induction ( $10^{-6}$  M, 72 hr, Figure 2B). *Tp2* is a basic chromosomal protein that functions as an intermediate in the replacement of histones by protamines, and its mRNA is first detectable in step 7, round spermatids (Kistler et al., 1996). As shown by RT-PCR, both cell lines were positive for *Tp2* and *Prm1* after 72 hr of induction by RA (Figure 2B), and these data provide evidence for postmeiotic differentiation of these SSC cell lines. Furthermore, the formation of acrosome, the occurrence of nuclear





**Figure 3. Identification of Haploid Male Germ Cells by FACS**  
(A) The SSC12 cell line shows ~30% haploid cells (1C) after 72 hr of RA treatment.  
(B) Testis cell suspension shows populations of cells that are in the haploid stage (1C), G0/G1 phase (2C), and G2/M phase (4C).  
(C) No haploidization was detected in feeder layer cells.  
(D) The formation of synaptonemal complex (Page and Hawley, 2004) in SSC12 cells after RA treatment identified by electron microscopy. Chromatin (red arrows) and the lateral element (green arrows) are shown.  
(E) Profiles of H19, Igf2r, and Snrpn methylation in noninduced (n), induced (i), after FACS, and supernatant (s) red cells of SSC7 and SSC12 cell lines. The percentages of unmethylated (red bars) and methylated (blue bars) CpG sites are given on the y axis.

condensation, and the formation of a tail-like structure in the SSC12-derived postmeiotic cells was confirmed by immunocytochemical analysis with three specific antibodies to Acr and outer acrosomal membrane protein (OAM), to Tp2, and to midpiece-specific protein PHGPx (phospholipid hydroperoxide glutathione peroxidase), respectively (Figures 1F and 2C). DsRed protein was detected in the cells with condensed nuclei (Figure 2C).

During spermiogenesis, structural and biochemical modifications of chromatin are responsible for the final nuclear maturation of spermatozoa. Chromatin condensation is achieved through replacement of somatic-type histones first by transition proteins and finally by protamines. Several staining procedures have been used to analyze the structural and biochemical modifications of chromatin in spermatozoa. Aniline blue staining can discriminate between stages of spermatid differentiation. Early stages have nuclei containing lysine-rich histones that will react by taking up the aniline blue stain, whereas the protamine-rich nuclei of mature spermatozoa, with abundant arginine and cysteine, remain un-

stained. Using aniline blue staining, we observed an increase of unstained cells from 3% in untreated cells to 56% in cells released to the medium (Figure S2). This result is an indication of the modification of chromatin of SSC cells during in vitro differentiation.

DNA methylation is an epigenetic regulator of gene expression and acts as an important molecular mark underlying the parental-specific expression of genes subject to genomic imprinting. Several studies have now demonstrated that the erasure of at least methylation imprints occurs in the germline (Surani, 2001). To obtain conclusive evidence that the SSC7 and SSC12 cell lines were indeed germ cells, we analyzed whether these cells manifested erasure of epigenetic imprints, which is a unique property of germ cells. We analyzed the methylation status of the imprinting control region (ICR) of *H19*, the differentially methylated region 2 (DMR) of *Igf2r*, and DMR1 of the *small nuclear ribonucleoprotein N* (*Snrpn*) genes (Lucifero et al., 2002) in independent clones of the SSC7 and SSC12 cells. The analysis was carried out at different CpG sites by a bisulfite sequencing assay. The *H19* ICR and the *Igf2r* DMR2 showed the expected decrease of methylation after RA induction, whereas an unexpected increase of methylation was observed for the *Snrpn* DMR1 (Figure 3). These findings suggest that after RA induction the grandparental methylation imprints are efficiently erased at many, but not all, alleles of two of the three analyzed genes. In supernatant cells, the *Igf2r* DMR2 was completely unmethylated at all analyzed sites in SSC12, and the *Snrpn* DMR1 was more or less completely unmethylated at the analyzed sites in both SSC7 and SSC12 (Figure 3E). These methylation profiles are consistent with previously published methylation profiles in haploid male germ cells (Lucifero et al., 2002), indicating that at least some methylation imprints were correctly reprogrammed in all or the majority of cells in the supernatant. In contrast, the *Igf2r* DMR2 displayed hemizygous methylation patterns in the supernatant of SSC7. Similarly, the supernatants of both SSC7 and SSC12 showed hemimethylated patterns for the *H19* ICR (Figure 3E).

#### Functionality of ES Cell-Derived Male Gametes

The testis cell transplantation technique provides access to the mammalian germline and has been used in experimental animal models to study stem cell/niche biology and germline development (Dobrinski, 2005). To investigate SSC capacity and the further development of SSC7 and SSC12 cell lines in vivo, cells were transplanted into one of the testes of germ cell-depleted recipient mice. The other testis served as an internal control. Histological analysis of testes after 4 months showed the appearance of spermatogenesis-like structures (Figure S3A) and sperm in the lumen (Figure S3B) of two of ten transplanted mice. Colonization of SSC7 and SSC12 cells was evaluated by histological sectioning. Each slide was viewed at a magnification of 400× for the analysis. In five mice, teratoma were detected. No regeneration of spermatogenesis was observed in non-transplanted control testes (Figure S3C) controlled by serial histological analysis. However, all sperm were immotile or showed reduced motility (data not shown). To examine the origin of detected postmeiotic cells, the sections were evaluated by fluorescence microscopy,

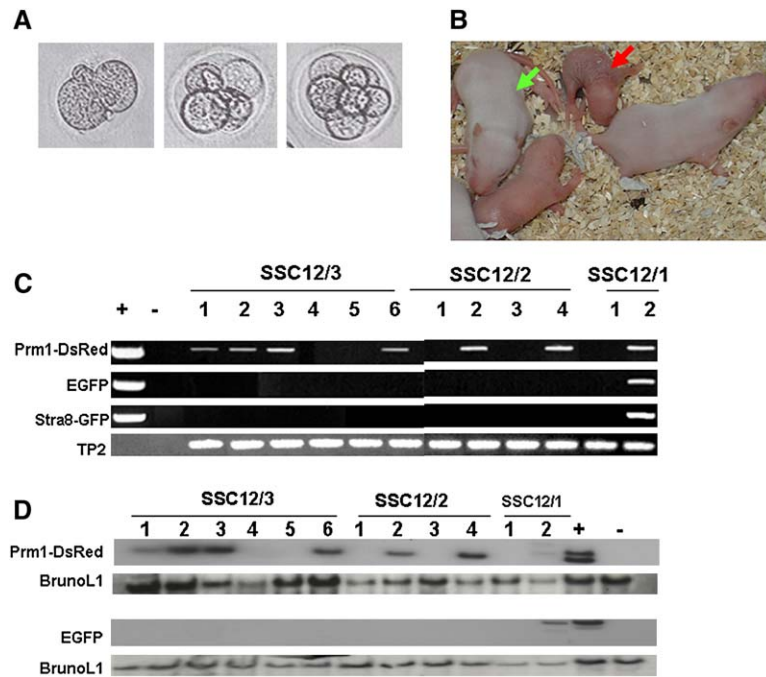


Figure 4. Functionality of ES Cell-Derived Male Gametes

(A) Development of preimplantation embryos derived from intracytoplasmic injection (ICSI) of Prm1-DsRed-positive haploid cells into the CD1 or NMRI oocytes.

(B) Full-term development of transferred two-cell embryos derived from ICSI with Prm1-DsRed-positive haploid cells. The red arrow shows an agouti mouse (5 days after birth) that carried both transgenic alleles, Stra8-EGFP and Prm1-DsRed; the green arrow demonstrates a white mouse (7 days after birth) that carried only the Prm1-DsRed reporter gene.

(C) Genomic PCR with DNA isolated from tail biopsies shows seven mice with the Prm1-DsRed transgene and a mouse with the double transgene, Stra8-EGFP and Prm1-DsRed. Genomic PCR with primers specific for the TP2 gene serves as a positive control for PCR. +, PCR with Stra8-EGFP and the Prm1-DsRed reporter gene. -, nontemplate negative control. SSC12/1, SSC12/2, and SSC12/3: ICSI experiments with haploid germ cells of the SSC12 cell line.

(D) Southern blot analysis with genomic DNA isolated from tail biopsies of the born mice. The blots were hybridized with DsRed- and EGFP-specific transgene probes, as well as with a loading control probe for the endogenous gene BrunoL1.

and red spermatids (Figure S3B) were detected in the lumen, which is evidence that sperm are derived from transplanted cells. Furthermore, using PCR analysis on genomic DNA isolated from sperm, it was shown that sperm contain the Prm1-DsRed transgene (Figure S3D). This finding indicates that SSC7 and SSC12 cells can be partially differentiated into postmeiotic germ cells and can develop into sperm in vivo.

Intracytoplasmic injection of in vitro-generated red cells (haploid cells) into unfertilized oocytes of wild-type females was carried out to examine the oocyte-activating competence of male gametes and the development of preimplantation embryos. Polar body extrusion, pronuclear formation, and normal features of preimplantation embryos (Figure 4A) indicate that in vitro ES-derived male gametes are able to activate the unfertilized oocytes and initiate early embryonic development. We succeeded in obtaining progeny by transferring the two-cell embryos into the oviducts of pseudopregnant females (Figure 4B). Out of 65 embryos transferred into oviducts, 12 animals were born. The results of the PCR analysis were confirmed by Southern blot analysis by using a DsRed-specific probe (Figure 4D). To examine expression of the *Prm1-DsRed* transgene, testis sections were evaluated by fluorescence microscopy. Red spermatids were detected in the testis of one of the transgenic progeny (Figure S4). One animal carried both transgene alleles (Stra8-EGFP and Prm1-DsRed), and six animals carried only the *Prm1-DsRed* transgene (Figure 4C). The offspring were either smaller or larger than controls and died prematurely (5 days to 5 months after birth). Some offspring were not transgenic. Presumably, this is related to the likelihood of transgene loss (subsequent to the relaxation of selection), recombination during meiosis, and/or allele segregation.

To support our conclusion that disturbed establishment of male germline-specific methylation imprints in ES-derived gametes leads to abnormal methylation patterns and phenotypic abnormalities in the offspring, bisulfite sequencing assays were applied to spleen tissues of three animals conceived by ICSI with ES-derived haploid cells. Interestingly, the *H19* ICR and the *Snrpn* DMR1 showed strong hypomethylation in spleen tissues of mice 3/3 (number/designation) and 3/6, which both displayed overgrowth, whereas the *Igf2r* DMR2 was hypermethylated in the spleen of mouse 3/3 (Figure S5). In contrast, the DMR1 of *Snrpn* was strongly hypermethylated in the spleen of the growth-retarded mouse, 2/2 (Figure S5). Thus, similar methylation abnormalities were observed in the two mice with overgrowth, and opposite abnormalities were seen in the mouse with growth retardation. The spleen of the wild-type mouse showed normal methylation patterns for all three analyzed regions. Although similar to somatic cell nuclear transfer technologies, ES-derived gamete differentiation may interfere with the essential reprogramming events in gametogenesis, and a certain percentage of ES-derived sperm could display correct methylation imprints required for normal embryonic development.

### Conclusions

These results indicate that our established ES cell-derived lines SSC7 and SSC12 can produce functional gametes in vitro that are able to fertilize oocytes and support full-term development to embryos and adult animals. Thus, male gametes produced by our strategy were able to initiate and to support embryonic and post-natal development after ICSI. Previously, different groups showed the establishment of spermatogonial cell lines from testis (Feng et al., 2002; Kubota et al.,

2004; Ogawa et al., 2004; Hofmann et al., 2005). However, only the cell lines established by Feng et al. (2002) showed differentiation to spermatocytes and spermatids in vitro (Feng et al., 2002). Recently, two groups demonstrated the derivation of male gametes from ES cells (Toyooka et al., 2003; Geijsen et al., 2004). However, full-term development of embryos produced by ES-derived gametes was not shown. Furthermore, in contrast to our strategy, haploid gametes were obtained after 3 weeks of induction with RA. We established SSC cell lines that undergo meiosis and produce male gametes after only 72 hr. This result indicates that our cell lines sustain differentiation characteristics of SSCs.

Although we succeeded in obtaining progeny from ES cell-derived male gametes, technical improvements are necessary. Improvement of oocyte microinjection is important to obtain more two-cell embryos. In this study, we used 210 oocytes for microinjection; 65 oocytes developed to 2-cell embryos, and 7 animals that carried transgene alleles were born. The fertilization rate might have been affected by damage during oocyte injection or by the heterogeneity of microinjected male gametes. To demonstrate the expression of *Prm1-DsRed* in transgenic mice, we prepared testes of positive animals and monitored them for DsRed-positive cells. In one transgenic line, we observed DsRed-positive spermatids (Figures S4A and S4B). This is an indication for specific expression of the *Prm1-DsRed* fusion transgene in testis.

Our findings suggest that development of male germ cells from ES cells is a cell-autonomous process that is controlled by the microenvironment generated in different developmental stages. Our report, together with the recent demonstration of oocyte and sperm generation from ES cells (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004; Clark et al., 2004), provides new possibilities for investigating germ cell development, epigenetic reprogramming, and germline gene modification.

## Experimental Procedures

### Fusion Gene Constructs

Construction of the *Stra8-EGFP* construct is previously described (Nayernia et al., 2004). For construction of the *Prm1-DsRed* fusion gene, the 5' flanking region of the mouse *Prm1* gene (Zambrowicz et al., 1993), the +652/+2 fragment (+1 indicates the start of translation), was amplified by genomic PCR with the 5'-GTCTAGTAATGTC-CAACACCT-3' and 5'-GGTGCTGGCTTGGCCGGGAGC-3' primers and was cloned into the pDsRed plasmid (BD Bioscience).

### Isolation of Recombinant ES Cell-Derived Germ Cells

Mouse embryonic stem cell line R1 (XY) (provided by Dr. A. Nagy, University of Toronto, Canada) was cultured in an undifferentiated state on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts with Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 15% FCS, 2 mM L-glutamine (GIBCO-BRL), 50  $\mu$ M  $\beta$ -mercaptoethanol ( $\beta$ -ME; Promega), 1  $\times$  non-essential amino acids (NEAA; GIBCO-BRL), and 103 U/ml LIF as described previously (Joyner, 2000). Linearized plasmid DNA (30  $\mu$ g) was electroporated into ES cells. Colonies resistant to G418 (400  $\mu$ g/ml) were selected. Resistant colonies were tested by PCR, and colonies that contain the *Stra8-EGFP* construct were selected and cultured in an undifferentiated state. Cultures were proliferated in the above-described medium for an additional 2 months (four passages) and were then frozen. Thereafter, cells were cultured on

a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts with basic ES cell medium. To induce differentiation, medium was changed to medium containing retinoic acid (RA) (Sigma) at a final concentration of  $10^{-5}$  M, and the cells were cultured for 10 days. Positive cells (60%) were sorted by FACS. Briefly, cells were dissociated with 0.25% trypsin/EDTA, neutralized with DMEM with 10% FCS, washed twice with PBS, and then resuspended in PBS containing 0.5% BSA. Approximately  $2 \times 10^6$  cells/ml in PBS/BSA were used for sorting. The flow cytometry was performed on a FAC-Star Plus (Becton Dickinson) equipped with dual 488 nm argon and 633 nm helium neon lasers. Sorted cells were cultured in RA-free medium. After 8–10 weeks (4 passages), medium was changed with medium supplemented with RA ( $10^{-6}$  M), and after 12 hr, GFP-positive cells (90%) were sorted by FACS. Thereafter, the cells were cultured in basic medium supplemented with LIF on fibroblast feeder layers and transfected with the *Prm1-DsRed* construct. Positive cell colonies were selected after PCR analysis. Two cell lines were established and designated as SSC7 and SSC12. For differentiation, the cells were cultured on gelatine-coated dishes, without LIF.

### RNA Isolation and RT-PCR

Total RNA was isolated, and cDNA synthesis was carried out with oligo-dT primers as described (Nayernia et al., 2004). RT-PCR amplification was performed by using specific primers for *EGFP*, *Stra8*, *Tex18*, *Dazl*, *Rbm*, *Oct4*, *c-kit*, *Scp3*, *Pgk2*, and *Tp2*. RT-PCR was achieved after 35–40 cycles of 94°C, 30 s; 50°C–62°C, 30 s; 72°C, 45 s, depending on the primer sets. Primer sequences are available as Supplemental Data.

### Aniline Blue Staining

A total of 10  $\mu$ l of the cell sample was spread onto glass slides and allowed to dry at room temperature. Smears were fixed in 3% glutaraldehyde (12.5 ml glutaraldehyde 25% in 87.5 ml PBS) for 30 min at room temperature. Slides were then stained with 5% w/v aniline blue in PBS (pH 3.5). Each slide was then washed with PBS and left to dry in the air. Stained and unstained cells were counted and examined under the microscope at 600 $\times$  magnification.

### Immunocytochemistry and Fluorescence Microscopy

Adherent cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 1 hr at ambient temperature. Fixed cells were rinsed in PBS (pH 7.4) and subsequently incubated overnight with primary antibodies specific to integrin  $\alpha$ 6, integrin  $\beta$ 1, heat shock protein 90 $\alpha$  (Hsp90 $\alpha$ ), *c-kit*, *Oct4*, *Tp2*, or outer acrosomal membrane (OAM) protein. All primary antibodies, except those for *Tp2*, *Acr*, *Rbm*, *hnRNPG-T*, *Dazl*, *Piwil2*, *Stra8*, and *OAM*, were purchased from Santa Cruz. Other antibodies were provided in collaboration with other research groups (see Supplemental Data). Cells were rinsed three times and incubated in the appropriate Cy3-, FITC-, or AP-conjugated secondary antibody (Sigma). All incubations were performed in PBS (pH 7.4), 5% BSA, and 0.1% Triton X-100. For nuclear stainings, fixed cells were incubated for 5 min with DAPI (4', 6'-diamidino-2-phenylindole) (Vector) dye. Microscopy was performed on a Zeiss fluorescence microscope.

### Transplantation into Seminiferous Tubules of Testes

Recipients used were 129/Sv and FVB mice. At 6–8 weeks of age, the recipient mice were injected i.p. with busulfan (40 mg/kg body weight), which destroys endogenous spermatogenesis. Recipients were used for transplantation  $\geq$  4 weeks after injection. Cultured SSC7 and SSC12 cells were harvested by using 0.25% trypsin plus 1 mM EDTA, and the cells were suspended in DMEM culture medium. The cell concentration for transplantation was  $10$ – $30 \times 10^6$  cells/ml. Approximately 10  $\mu$ l of the cell suspension was transplanted via rete testis into seminiferous tubules of one testis. The other testis served as an internal control. Ten animals were transplanted, and ten animals were used as control. As an external control, busulfan-treated, nontransplanted males were used. After 4 months, recipient mice were killed, and testes were examined by immunocytochemistry, image cytometry, and RT-PCR analysis. All of the experimental procedures complied with national regulations for the Care and Use of Laboratory Animals (similar to the U.S. National Research Council guidelines).



### Intracytoplasmic Microinjection

Microinjection of red cells was performed according to the published procedures (Suzuki and Yanagimachi, 1997) with the following modifications. Female CD-1 or NMRI wild-type mice were superovulated, and oocytes were collected 10–12 hr after hCG administration. The cumulus cells were removed by hyaluronidase treatment, and the oocytes were washed and subsequently cultured in fertilization medium (MediCult, Jyllinge, Denmark). In vitro-generated red cells were collected from the supernatant of cells in the differentiated state and were injected into the oocytes by using a micromanipulator. After injection, oocytes were cultured in M16 (Sigma) at 37°C and 5% CO<sub>2</sub> and were monitored for embryonic development or retransferred to pseudopregnant females. Born mice were analyzed by PCR and Southern blot analysis by using genomic DNA isolated from tail biopsies.

### Electron Microscopy

Cells were fixed with 5% glutaraldehyde in 0.2 M phosphate buffer, postfixed with 2% osmium tetroxide, and embedded in epoxy (Epon) resin. Different cell colonies were examined by electron microscopy.

### Methylation Experiments

Bisulphite treatment was performed by using the MethylEasy DNA Bisulphite Modification Kit (Human Genetic Signatures, Macquarie Park, Australia). PCR amplification for the *H19* ICR, the *Igf2r* DMR2, and the *Snrpn* DMR1 was carried out as previously described (Lucifero et al., 2002). The PCR products were cloned by using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany). Positive clones were sequenced by using the CEQ DTCS Quick Start Kit (Beckman Coulter, Krefeld, Germany) and were analyzed on a Beckman CEQ 8000 Genetic Analysis System. The following numbers of CpG sites and clones were analyzed for in vitro studies (cell culture): H19 7n, 139 CpG sites/10 clones; H19 7i, 138/9; H19 7s, 320/20; H19 12n, 215/15; H19 12i, 233/15; H19 12s, 198/14; *Igf2r* 7n, 156/23; *Igf2r* 7i, 119/17; *Igf2r* 7s, 110/16; *Igf2r* 12n, 167/24; *Igf2r* 12i, 95/14; *Igf2r* 12s, 105/15; *Snrpn* 7n, 276/20; *Snrpn* 7i, 471/32; *Snrpn* 7s, 156/11; *Snrpn* 12n, 245/20; *Snrpn* 12i, 186/13; *Snrpn* 12s, 256/16. The following numbers of CpG sites and clones were analyzed for in vivo studies (tissues): H19 24, 208 CpG sites/13 clones; H19 9, 231/15; H19 27, 231/17; H19 19, 156/11; *Igf2r* 24, 82/13; *Igf2r* 9, 92/14; *Igf2r* 27, 125/18; *Igf2r* 19, 112/16; *Snrpn* 24, 268/17; *Snrpn* 9, 207/13; *Snrpn* 27, 205/13; *Snrpn* 19, 207/13.

### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, primer sequences, and antibodies used in this study and supplemental figures depicting the time course of differentiation, assessment of chromatin condensation, transplantation, and histological sections of transgenic mice and are available at <http://www.developmentalcell.com/cgi/content/full/11/1/125/DC1/>.

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