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Methylation Status and Expression of Human Telomerase Reverse Transcriptase mRNA in Relation to Hypermethylation of the *p16* gene in Colorectal Cancers as Analyzed by Bisulfite PCR-SSCP

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Original Articles

Methylation Status and Expression of Human Telomerase Reverse Transcriptase mRNA in Relation to Hypermethylation of the *p16* gene in Colorectal Cancers as Analyzed by Bisulfite PCR-SSCP

Kiyoaki Nomoto[1], Masato Maekawa[1], Kokichi Sugano[1], Mineko Ushiama[1], Noriko Fukayama[1], Shin Fujita[2] and Tadao Kakizoe[3]

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Background: The expression level of human telomerase reverse transcriptase (hTERT) is correlated with telomerase activity and is expressed at high levels in malignant tumors. It is of interest whether expression of *hTERT* is regulated by methylation of the CpG island in the promoter of the *hTERT* gene. We examined *hTERT* expression and methylation status of the *hTERT* and other genes including *p16*.

Methods: We analyzed methylation status by bisulfite treatment and polymerase chain reaction with single-strand conformation polymorphism analysis (PCR-SSCP) and expression of the *hTERT* by RT-PCR, in 13 cancer cell lines, eight white blood cell samples and 24 colorectal cancer tissues.

Results: In the cancer cell lines, hTERT was expressed and the CpG island of the *hTERT* promoter was methylated. Most colorectal cancer tissues showed similar results. The promoter of *hTERT* was methylated in six cases, partially methylated in 17 cases and unmethylated in one case. All cases with methylation of *hMLH1* or *p16* also showed methylation of *hTERT*; however, some of the cases lacking *p16* methylation also had *hTERT* methylation.

Conclusion: Increased expression of *hTERT* is related to hypermethylation of *hTERT* in colorectal cancerous tissues as well as some cancer cell lines and disconcordant with hypermethylation of *p16*.

Key words: methylation – human telomerase reverse transcriptase – p16 – colorectal cancer – expression

INTRODUCTION

Regulation of expression of the telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT), is considered the major determinant of enzyme activity (1,2). hTERT is expressed at high levels in malignant tumors and cancer cell lines but not in normal tissues or telomerase-negative cell lines. A close correlation has been found between *hTERT* expression and telomerase activity in a variety of tumors (3–6). The 5′-regulatory region of the *hTERT* gene was recently cloned and characterized (7–10). The *hTERT* gene promoter lacks a TATA or CCAAT box, but a number of potential transcription factor binding sites, including potential binding sites for SP1, MAZ (Myc-associated zinc finger protein), a bHLHZ class of transcription factors (E-boxes), c-Ets-2 and AP-2 (7–10) are present. Luciferase assays assessing promoter activity revealed that a 59-bp region (–208 to –150) is required for maximal promoter activity (7). The 5′ end of the *hTERT* cDNA contains GC-rich sequences, indicating the presence of a CpG island. *hTERT* expression may be regulated by methylation.

Promoter methylation plays an important role in the regulation of gene transcription, X chromosome inactivation, genomic imprinting and carcinogenesis (11–13). CpG islands are found within the promoter regions of ~60% of human genes (14) and these CpG islands normally are not methylated regardless of the expression status of the genes. Aberrant DNA methylation is an important alternative mechanism for muta-

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tions in coding regions that lead to inactivation of tumor suppressor genes and mismatch repair genes during neoplasia (12,13,15). It is of interest whether expression of *hTERT* is also regulated by methylation of the CpG island in the promoter of the *hTERT* gene. We hypothesized that the *hTERT* CpG island would be unmethylated to permit expression of *hTERT* in most telomerase-positive cells. Data from two previous reports, however, showed that the *hTERT* promoter region was mostly methylated and that *hTERT* was expressed in some cancer cell lines (16,17). Also, only a few clinical cancer specimens were examined (17). Here we tried to investigate the methylation status of a portion of the promoter of the *hTERT* gene in some colorectal cancerous tissues as well as some cancer cell lines, using bisulfite treatment and polymerase chain reaction with single-strand conformation polymorphism analysis (PCR-SSCP) (BiPS) (18). We also examined the association between expression of *hTERT* mRNA and methylation status of genes including *p16, hMLH1* and *HIC1* in colorectal cancer tissues.

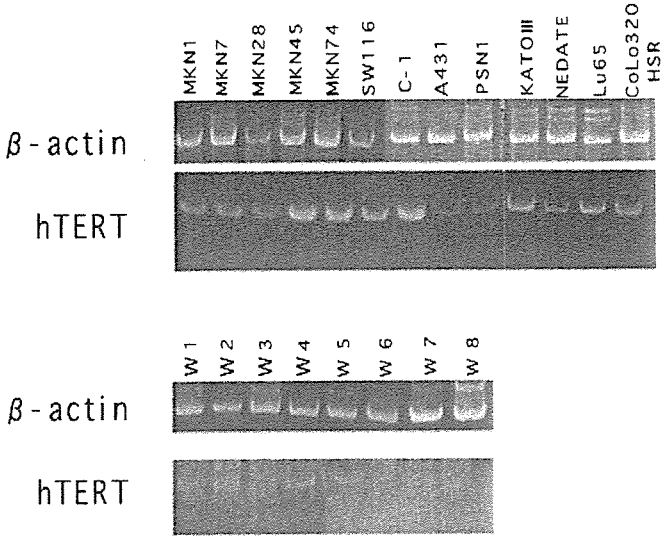

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the expression of β-actin (inner control) and *hTERT* in cancer cell lines and peripheral white blood cells (PWB) (W1–W8). The origins of the cancer cell lines are described in Materials and Methods. The amplified products of hTERT and β-actin are 145 and 621 bp, respectively.

MATERIALS AND METHODS

Subjects

Thirteen tumor cell lines were provided as subjects. MKN1, MKN7, MKN28, MKN45, MKN74, KATO III (Japanese Cell Resource Bank) and NEDATE (National Cancer Center Hospital) cell lines were derived from stomach cancers. SW1116 (American Type Culture Collection), C-1 and Colo320HSR cell lines (National Cancer Center Research Institute) were derived from colorectal cancers. PSN1, Lu65 and A431 cell lines (National Cancer Center Research Institute) derived from pancreas, lung and vulva cancers, respectively, were also used. Colorectal cancer tissues, normal accompanying mucosa or tissue and metastatic lesions of liver and normal liver tissue resected at surgery from 24 patients were obtained from the National Cancer Center Hospital, Japan. Experimental use of specimens in addition to pathological examination was consented to by each patient. Peripheral white blood cells (PWB) were obtained from eight normal volunteers.

DNA Extraction, RNA Preparation and cDNA Synthesis

DNA was extracted from the tissue specimens according to a method described previously (19). Total RNA was prepared from the various cancer cell lines, and the PWB, the colorectal cancer tissues and corresponding normal mucosa by a standard guanidium thiocyanate–phenol–chloroform–isoamyl alcohol extraction method (20). Approximately 0.5–1.0 μg of total RNA was reverse transcribed with random hexamers as primers and SuperScript RNase H reverse transcriptase (GIBCO BRL, Gaithersburg, MD).

BiPS Analysis

The BiPS procedure was performed as described previously (18). Briefly, bisulfite treatment was done with reagents provided in the Oncor CpGenome DNA Modification Kit (Intergen, Purchase, NY). Control methylated DNA was prepared with SssI methylase (New England Biolabs, Tokyo, Japan) from genomic DNA of PWB. PCR primers were designed to be complementary to the chemically modified DNA with no CpG sites in the corresponding region of the genomic DNA. The primer sequences for amplification of *hTERT* were 5′-GGGTTTTTAGTGGATT-3′ (sense) and 5′-AAACTAAAA-AATAAAAAAACAAAAC-3′ (antisense). The primer sequences corresponded to nucleotide positions 1432–1447 and 1536–1512, respectively, of the *hTERT* gene (GenBank AF098956). The region selected for amplification has been related to the promoter activity (7–10). The expected size of the RT-PCR product was 105 basepairs containing nine CpG sites. PCR was performed with AmpliTaq Gold (PE Applied Biosystems, Branchburg, NJ) and the hot start procedure. SSCP analysis was performed using 15% non-denaturing polyacrylamide gel and silver staining detection (Daiichi Pure Chemicals, Tokyo, Japan).

Sequencing Reaction of the Methylated DNAs

Extra bands (possibly methylated) detected by BiPS analysis were excised from the gels and reamplified. The PCR products were treated with shrimp alkaline phosphatase and exonuclease III (Amersham Pharmacia Biotech, Amersham, Bucks, UK) to remove excess PCR primers and nucleotides and then sequenced directly by the dideoxy sequencing procedure with a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

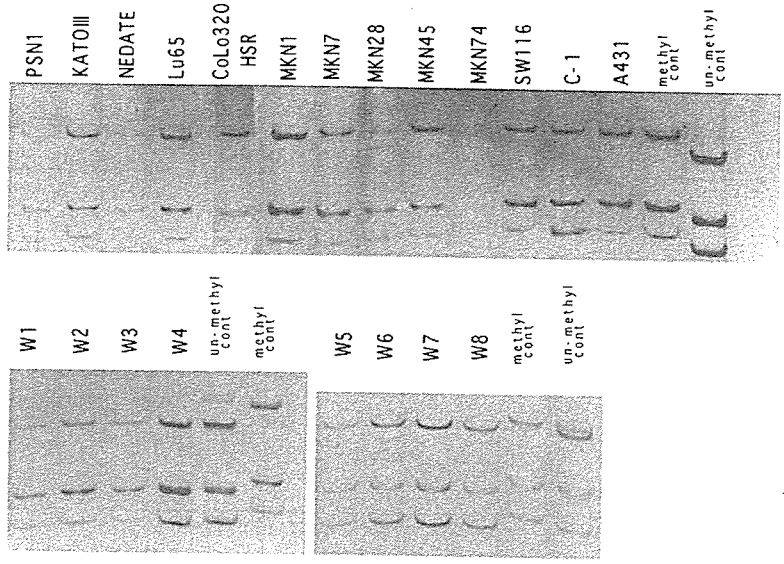

Figure 2. BiPS analysis of the *hTERT* gene promoter in cancer cell lines and PWB (W1–W8).

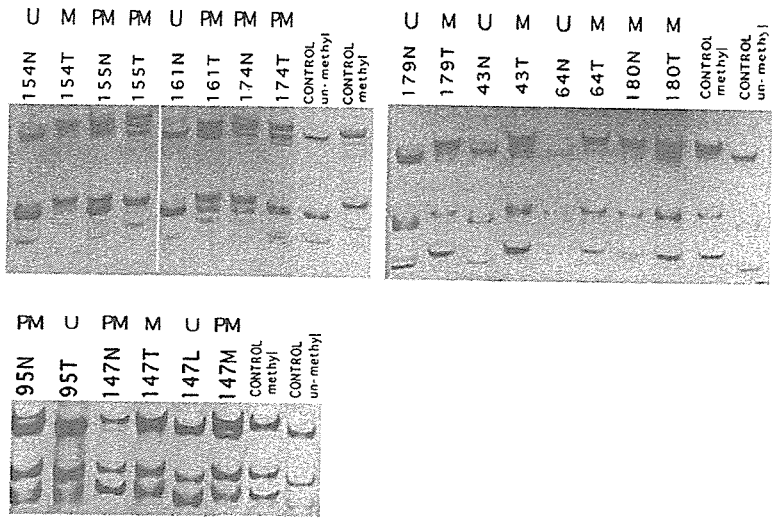

Figure 3. BiPS analysis of the *hTERT* gene promoter in colorectal cancer specimens. The methylation status of *hTERT* gene was analyzed and classified visually as U (unmethylated), M (methylated) or PM (partially methylated). Cancer tissue (T), corresponding normal mucosa (N), normal liver (L) and metastatic lesion of liver (M) were analyzed.

Assessment of *hTERT* mRNA Levels by RT-PCR

Expression of *hTERT* was analyzed by RT-PCR with the primers described by Nakamura et al. (2). β-Actin mRNA was amplified as described (21) for internal control. PCR products were separated by electrophoresis on 8% polyacrylamide gels and visualized by ethidium bromide staining and UV illumination.

RESULTS

Cancer Cell Lines and Peripheral White Blood Cells

According to RT-PCR analysis, β-actin and *hTERT* were expressed in all 13 cell lines examined (Fig. 1). In PWB, β-actin was expressed in all eight samples, whereas no or only trace amounts of the expected length of *hTERT* were observed. With BiPS analysis, 11 cell lines showed three bands that migrated to the same positions as those of fully methylated control DNAs (Fig. 2). One cell line, MKN28, yielded four bands. Although one of the bands migrated to a similar position as the unmethylated control DNA, it was identified unexpectedly as methylated. MKN74 was not informative owing to unsuccessful PCR amplification. All PWB samples showed bands that migrated to the same position as the unmethylated control DNA bands (Fig. 2).

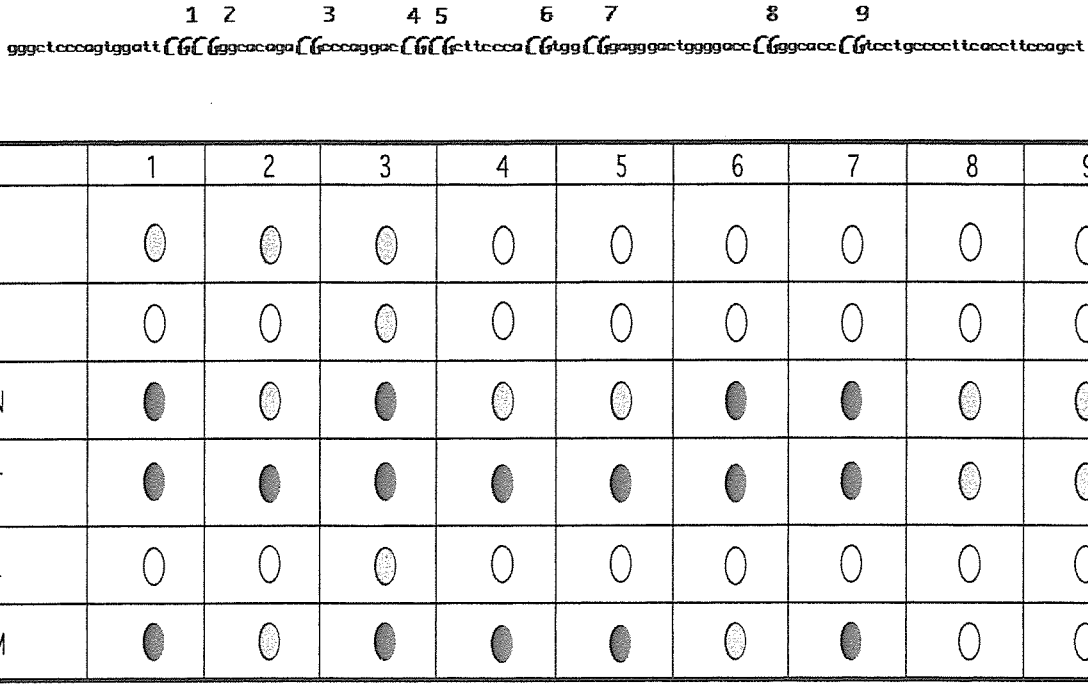

Figure 4. Methylation within the CpG island in the *hTERT* gene promoter. PCR products amplified after bisulfite treatment were sequenced and partial sequences were schematically represented. Open ovals indicate unmethylated CpG sites and closed ovals indicate methylated sites. Shaded ovals indicate a mixed pattern of methylated and unmethylated DNA.

Colorectal Cancer Tissues

In normal mucosa, *hTERT* expression was negative in 18 cases, weakly positive in three cases and positive in three cases (data not shown). In cancerous tissues, *hTERT* expression was negative in three cases and positive in 21 cases.

We classified the BiPS patterns of cancerous tissues. The *hTERT* gene promoter was methylated in six cases, partially methylated in 17 cases and mostly unmethylated in one case (Fig. 3). Sequence analysis of the amplified products revealed complex methylation patterns. Methylation patterns for *hTERT* consisted of mixtures of unmethylated and methylated DNA at each CpG site (Fig. 4). In Case No. 147, we were able to analyze metastatic foci as well as the primary tumor and found that normal mucosa and metastatic liver tissue had mixed patterns of methylated and unmethylated bands; cancerous tissue showed only methylated bands and normal liver tissue showed only unmethylated bands. This may be due to differences in the methylation status between organs or the cell population analyzed.

Relation Between Methylation Status or Expression of *Htert* Gene and Other Genetic Properties

Nearly all colorectal cancerous tissues analyzed showed aberrant methylation of *hTERT* gene (Table 1). Of the 24 cases, seven, five and 20 cases showed methylation of *hMLH1*, *p16* and *HIC1*, respectively (15). All cases with methylation of *hMLH1* or *p16* also showed *hTERT* methylation. All cases with microsatellite instability also showed *hTERT* methylation. The one case without *hTERT* methylation was one of four cases lacking methylation of *HIC1* gene.

DISCUSSION

We had hypothesized that the *hTERT* CpG island would be unmethylated to permit expression of *hTERT* in telomerase-positive cells. Our present data and data from two previous reports (16,17), however, showed that the *hTERT* promoter is methylated and that *hTERT* is expressed in some cancer cell lines and cancer specimens. Methylation of the CpG island in the *hTERT* gene promoter appears not to be responsible for direct repression of *hTERT* expression.

5-Aza-2′-deoxycytidine (5-aza-CR), a demethylating agent, caused down-regulation of *hTERT* expression in several cancer cell lines (16,22). This alteration in expression levels may be influenced by the cell cycle regulator protein p16 and the *p16* gene is silenced by hypermethylation in some cancer tissues and cancer cell lines. Kitagawa et al. (22) indicated that up-regulation of *p16* and subsequent down-regulation of c-*myc* are major pathways for *hTERT* repression by 5-aza-CR. It has been reported that myc proteins activate *hTERT* transcription directly through the E-box located within the core promoter (7,9,23). It is thought that hypermethylation of *p16* silences the gene and increases c-*myc* expression, resulting in up-regulation of *hTERT* expression. If this is so, the elevation of telomerase activity observed in most cancer tissues and cancer cell lines is due to *p16* methylation. In the present study, however, we investigated methylation not only of *hTERT* but also of

Table 1. Genetic properties and DNA methylation status in colorectal cancer

Patient	Age (years)	Gender	*hTERT* methylation*	*hTERT* expression	*hMLH1* (%)	*p16* No. (%)	*HIC1* (%)
43	51	M	M	+	91	0	75
64	38	F	M	+	62	0	32
71	87	M	M	+	38	0	43
154	33	F	M	+	0	0	62
180	78	M	M	+	0	0	0
179	53	M	M	–	0	0	60
88	60	F	PM	+	0	66	59
102	79	F	PM	+	95	65	80
16	73	M	PM	+	32	57	43
21	73	F	PM	+	64	48	65
76	51	M	PM	+	0	40	39
7	53	M	PM	+	0	0	55
17	53	M	PM	+	0	0	0
18	69	F	PM	+	0	0	80
22	61	F	PM	+	0	0	67
23	54	M	PM	+	0	0	0
27	67	M	PM	+	0	0	43
86	60	M	PM	+	0	0	45
94	51	F	PM	+	24	0	52
147	65	M	PM	+	0	0	45
161	34	M	PM	+	0	0	76
174	65	M	PM	+	0	0	19
155	48	M	PM	–	0	0	91
95	21	M	U	–	0	0	0

Percentages are calculated % of methylated bands. The percentages are from our previously reported results (15). *M, methylated; PM, partially methylated; U, unmethylated.

p16 and we showed that up-regulation of *hTERT* expression does not originate from hypermethylation of *p16* because the frequency of *p16* hypermethylation was lower than that of up-regulation of *hTERT*. The *Myc* oncogene activates *hTERT* expression and c-myc works as a transcription factor in conjunction with Max protein and the Mad family proteins (23–25). Therefore, the Myc/Max/Mad network affects *hTERT* expression and methylation of the genes encoding these proteins might affect *hTERT* expression. Recently, it was reported that progesterone regulates *hTERT* expression via activation of the mitogen-activated protein kinase signaling pathway (26). Methylation status of the genes in this signaling pathway may also affect *hTERT* expression. Additional genes that are silenced by hypermethylation may cause down-regulation of *hTERT* expression. To clarify this point, further studies are needed.

In normal mucosa of six cases, *hTERT* expression was weakly observed. BiPS analysis revealed partial methylation of *hTERT* gene. This may be due to the presence of cancer cells present in normal mucosa or to mononuclear cells or immature cells expressing *hTERT*. Some telomerase activity is in peripheral blood cells (27) that originate from a subset of somatic cells such as lymphocytes, intestinal mucosal cells and skin basal cells.

We compared the methylation status of the promoter region of the *hTERT* gene with expression of other methylated genes (*HIC1*, *p16*, *hMLH1*) (15). The hypermethylation of *hTERT* gene promoter was found most frequently among the examined genes and did not correlate well with the methylation status of other genes. It suggests that silence of the *hTERT* expression is more critical in carcinogenesis than that of the other genes.

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HST-1/FGF-4 gene activation induces spermatogenesis and prevents adriamycin-induced testicular toxicity

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We previously demonstrated expression of the *HST-1/FGF-4* gene in the testis of normal adult animals, which suggests its possible role in spermatogenesis. For an understanding of its functional significance in the testis, conditional transgene expression was used. Precise genetic switches can be efficiently generated in a straightforward manner using adenovirus-carrying Cre recombinase, which means our new strategies promise to contribute substantially to a better and prompt understanding of the functions of genes *in vivo* by controlling the expression of any gene to any organ at any desired time. Our new method demonstrated for the first time that the specific gain of function of the *HST-1/FGF-4* gene in the testis resulted in markedly enhanced spermatogenesis. To further investigate the function and therapeutic potency of HST-1/FGF-4, transgenic mice with enhanced HST-1/FGF-4 expression in the testis were exposed to adriamycin (ADR), an anticancer drug causing severe testicular toxicity. Degree of damage to spermatogenesis was assessed by sperm count, testicular weight, histology, and DNA ploidy. Induced expression of HST-1/FGF-4 markedly enhanced the recovery of ADR-induced testicular damage. Furthermore, adenoviruses carrying the *HST-1/FGF-4* gene ameliorated testicular toxicity of ADR. These results with new adenovirus-mediated Cre/lox conditional mice indicated that HST-1/FGF-4 could be an important factor for spermatogenesis, presenting a new paradigm to treat impaired fertility.
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Introduction

The *HST-1* gene was first cloned in our laboratory as a transforming gene in the NIH3T3 assay system and identified as a gene belonging to the fibroblast growth factor (FGF) gene family, FGF-4 (Sakamoto *et al.*, 1986; Yoshida *et al.*, 1991). The product, HST-1/FGF-4 is especially involved in embryogenesis (Suzuki *et al.*, 1992; Niswander and Martin, 1992, 1993; Niswander *et al.*, 1994; Ochiya *et al.*, 1995). Up-regulation of *HST-1/FGF-4* gene expression is associated with human testicular tumors (Yoshida *et al.*, 1988). As to its biological activities, we have previously shown that it is a factor with a potent angiogenic activity (Yoshida *et al.*, 1994) as well as a potent inducer of platelet production from megakaryocytes (Sakamoto *et al.*, 1994; Konishi *et al.*, 1995, 1996). Most of the other FGF family genes have their expression not only in the embryonic stage but also in several adult tissues. However, it was noted that the *HST-1/FGF-4* gene is normally dormant in adult tissues, and the physiological functions of its production of adult bodies were still unknown. We have designed highly sensitive RT–PCR analyses and found that adult murine and human *HST-1/FGF-4* gene expression were detected predominantly in the testis, nervous system, and intestines, being weakly recognized in other tissues. By *in situ* hybridization, we also showed cell type-specific *HST-1/FGF-4* gene expression in Sertoli cells in the testis and in Purkinje cells in the cerebellum (Yamamoto *et al.*, 2000). These results suggest that the *HST-1/FGF-4* gene may also play some specific roles in adult tissues as well as in embryos.

The presence of *HST-1/FGF-4* gene expression in Sertoli cells within the testis was of great interest with regard to spermatogenesis, since the whole process of differentiation of male germ cells depends on a complex network of endocrine and paracrine communication involving a variety of supporting cells (Sharpe, 1994). In the seminiferous tubule, spermatogenic cells

are in continuous contact with Sertoli cells, which indicates that Sertoli cells play a critical role in germinal-cell differentiation (Griswold *et al*., 1988). A number of endocrine agents and locally produced paracrine factors were shown to control and maintain Sertoli cell function and differentiation (Griswold, 1988). Among them, FGF-2 was found to significantly increase the number of Sertoli cells and also stimulate the proliferative activity of the gonocytes *in vitro* (Van Dissel-Emiliani *et al*., 1996). FGFR-1 and FGFR-4 are known to be localized in Sertoli cells in rat testis (Van Dissel-Emiliani *et al*., 1996; Cancilla and Risbridger, 1998; Le Magueresse-Battistoni *et al*., 1994) and normal human germ cells (Steger *et al*., 1998). All these reports suggest possible significance of *FGF* gene family and their receptors in spermatogenesis.

The studies we present here were designed to show that the *HST-1/FGF-4* gene promotes spermatogenesis *in vivo* and that it may participate in the regulation of spermatogenesis. For this purpose, as an efficient and accurate method for controlled *in vivo* transgene modulation by site-directed Cre recombinase, we established a transgenic animal carrying the dormant *HST-1/FGF-4* gene. This is a powerful tool for both designing such genetic switches and speeding the creation of gene-activated animals by the use of Cre site-specific DNA recombinase (Sauer, 1998). Precise genetic switches can be efficiently generated in a straightforward manner using an adenovirus that carries Cre recombinase (Kanegae *et al*., 1995). Our recombination-based conditional gene activation strategies *in vivo* can be used to design induced expression of any gene to any tissue at any desired time, and will have a profound impact on fundamental biology and the design of better therapeutic models of human diseases.

By administration of Cre-expressing adenoviruses *in vivo*, we accomplished conditional high expression of the *HST-1/FGF-4* transgene and, more importantly, found that high expression in these mice resulted in an increased sperm count and resistance to age-dependent and ADR-induced impaired spermatogenesis. Moreover, ADR-induced testicular toxicity was ameliorated by injection of adenoviruses carrying the *HST-1/FGF-4* gene into testis. Thus, these data suggest that HST-1/FGF-4 is an important factor in spermatogenesis and indicate the possibility that this gene may present a new approach for male infertility.

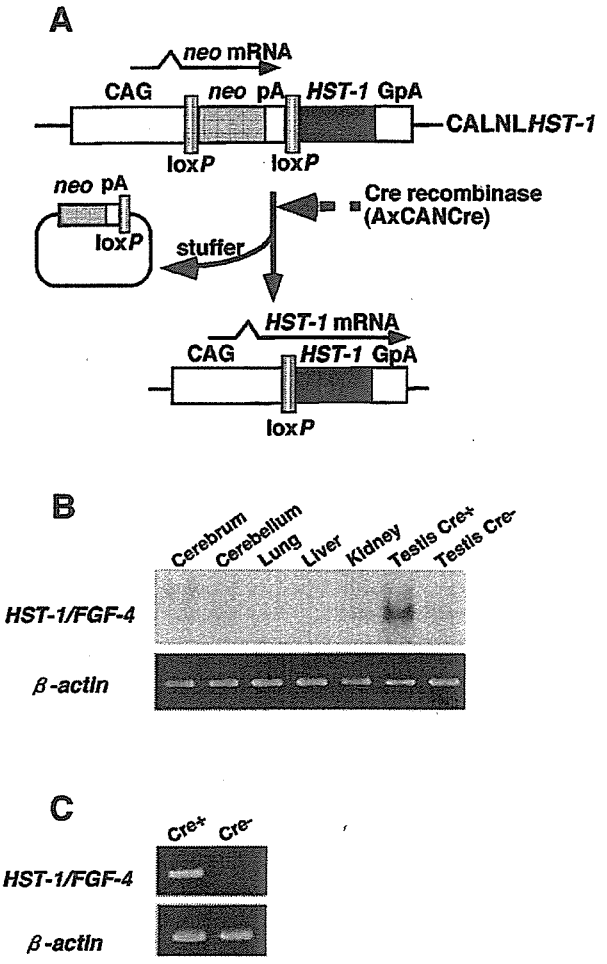

Figure 1 Cre-mediated activation of *HST-1/FGF-4* gene in mice. (**a**) Activation of a human *HST-1/FGF-4* gene in the CALNL*HST-1* switching unit by Cre recombinase. The Cre-mediated excisional deletion removes both a neo coding region and a poly(A) sequence, consequently generating a functional *HST-1/FGF-4* gene expression unit. SpA, SV40 early poly(A) site; GpA, rabbit-β-globin poly(A) site. (**b**) Northern blot analysis of activation of the dormant *HST-1/FGF-4* gene in the testes of the transgenic mice. Each lane contained 10 μg of total RNA. (**c**) RT–PCR analysis of purified Sertoli cells from Cre-mediated gene activated testes. Two days after the AxCANCre or control AxCAwt administration, testes were removed. After the purification of Sertoli cells, poly(A)$^+$ RNA was extracted and subjected to RT–PCR analysis to detect *HST-1/FGF-4* gene activation. The same RNA samples were subjected to RT–PCR analysis of *β-actin* transcripts to verify their integrity

Results

Inducible HST-1/FGF-4 *gene activation in mice using the Cre/lox system*

To assess the functional role of *HST-1/FGF-4* gene products in the testes for spermatogenesis *in vivo*, Cre recombinase-regulated conditional transgenic mice were generated (Figure 1a). In this system, the recombinase is provided by the adenovirus-carrying *Cre* gene (AxCANCre) (Kanegae *et al*., 1995). When a sufficient amount of Cre is supplied, the stuffer sequence is excised as circular DNA, and then the CAG promoter and the *HST-1/FGF-4* gene are joined via a single loxP site, thereby initiating target gene expression in the target tissue. Eight independent transgenic-mouse founder lines harboring the switching unit were obtained. No abnormalities among founders and/or their progenies were observed. To see whether conditional gene expression was attained in transgenic mice, 1.0×10^8 p.f.u of AxCANCre per testis was administered. As a control, the other testis of the pair

was left untreated. One day after the administration, the testes and other tissues were removed and total mRNAs were obtained. The activation of the dormant transgenic *HST-1/FGF-4* gene by Cre was assessed by Northern blot analysis of transgene sequences in transgenic mice. The expected HST-1/FGF-4 signal was indeed detected in testis from the Cre-injected sample, but not with mRNA from the Cre-uninjected samples (Figure 1b). It was also shown that mRNAs from other tissues such as brain, lung, liver, and kidney revealed no mRNA products. Testes-specific Cre-mediated gene activation was confirmed in three independent transgenic-mouse founders.

Our previous reports suggest that the Cre-mediated switching of gene activation was determined nearly 100% in cultured cells (Kanegae *et al.*, 1995). To determine the frequency of gene activation and cell type in testes, *in situ* hybridization analyses were performed. The results showed that human *HST-1/FGF-4* mRNA was primarily detected in spermatocytes, and Sertoli cells and some spermatogonia in Cre injected mice (Figure 2a), while the expression of the transgene cannot be demonstrated in mice that were not injected with Cre (Figure 2b). In addition, RT–PCR analysis showed that human *HST-1/FGF-4* gene activation had actually occurred in purified Sertoli cells from Cre-injected testes (Figure 1c). These results confirm that *HST-1/FGF-4* transgene activation had occurred only in the testes, as predicted, via precise site-directed deletion of the stuffer sequences in the conditional transgenic mice carrying the dormant transgene.

Enhanced spermatogenesis in mice from HST-1/FGF-4 expression

To study the effects of HST-1/FGF-4 in spermatogenesis, three independent transgenic mice lines were administered 1.0×10^8 p.f.u. of AxCANCre per testis, and then testis weights and sperm counts were examined. An increase in sperm count was first observed at 2 weeks after the administration in all mice lines with AxCANCre as compared to the control animals. The most evident increase in the sperm count was observed 4 weeks after the administration (Figure 3): $17.2 \pm 2.2 \times 10^6$/ml of AxCANCre and $12.9 \pm 0.5 \times 10^6$/ml of AxCAwt ($P < 0.001$). No gross abnormalities in sperm parameters including morphology and motility were observed. Increased testis size was seen 4 weeks after the AxCANCre administration. Testes from mice administered AxCANCre exhibited a 15% increase in testes weight as compared to the control animals. A cross-section of the seminiferous tubules of these testes was comparable with that of the control mice; the number of spermatogonia had increased, while that of Sertoli cells had not (data not shown). The diameter of the seminiferous tubules was not significantly changed. BrdU incorporation analysis showed that AxCANCre-administered testes contained an increased number of BrdU-positive spermatogonia relative to the control group (Figure 4). The labeling indices were $28.9 \pm 3.7\%$ in mice with AxCANCre and $5.7 \pm 1.1\%$ in mice with control AxCAwt ($P < 0.001$). These results suggest that forced *HST-1/FGF-4* gene

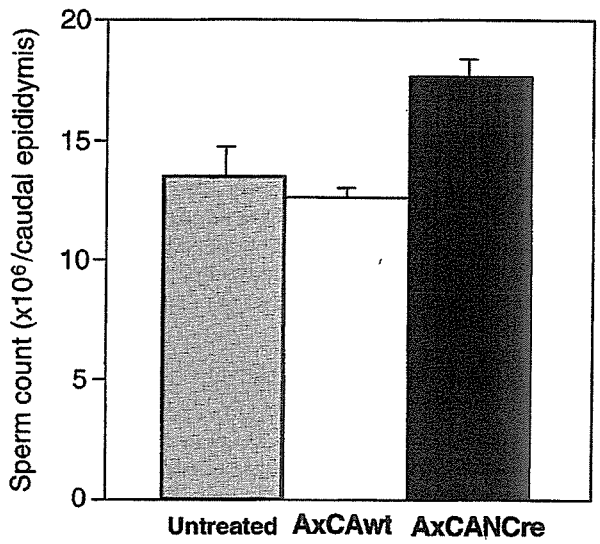

Figure 3 Sperm production of Cre-injected transgenic mice. The epididymis sperm count was estimated 4 weeks after the AxCANCre (■) or AxCAwt (□) administration. As a control, sperm count from untreated (■) mice were performed. The number of testis was 10

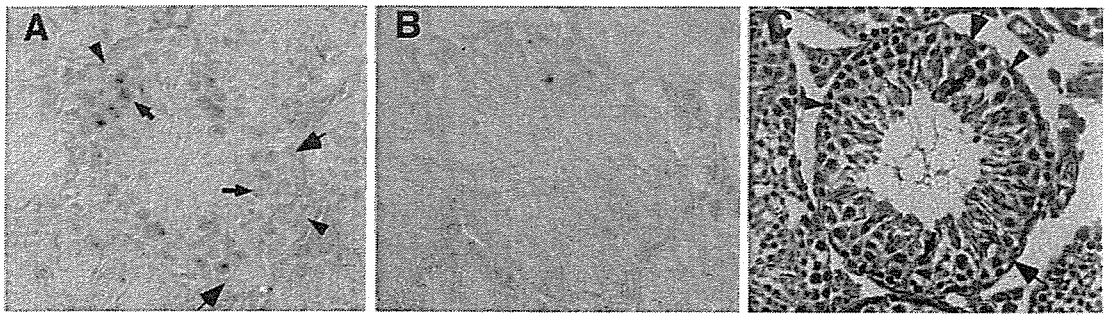

Figure 2 Profiles of testes after HST-1/FGF-4 switches on. *In situ* hybridization of antisense human *HST-1/FGF-4* RNA probe to sections of testes from transgenic mice 7 days after the intratesticular administration of AxCANCre (**a**) or control AxCAwt (**b**). Hematoxylin-eosin staining was performed in the same testes section (**c**). Large arrow: Sertoli cells; small arrow; spermatocytes; arrowhead: spermatogonia

expression in testes may affect gonocyte proliferation, resulting in enhanced spermatogenesis.

HST-1/FGF-4 blocks age-dependent sperm decrease

The effect of HST-1/FGF-4 on age-dependent change of sperm count was analysed. Increased sperm count continued to be observed at least 45 weeks after the AxCANCre administration and these mice with a high number of sperm were normally fertile. The sperm count of control animals that received AxCAwt decreased in an age-dependent manner, and at 75 weeks after the adenovirus treatment, the sperm count shows $2.4 \pm 0.3 \times 10^6$/caudal epididymis. In contrast, at the same age, mice with AxCANCre showed a sperm count of $11.2 \pm 2.4 \times 10^6$/caudal epididymis ($P < 0.001$), which is equivalent to that of control young adult at 10-weeks-old (Figure 5). Histological analysis showed that the number of spermatogonia and spermatocytes was not diminished when testes were injected with AxCANCre (Figure 6b). However, testes from control mice administered with AxCAwt showed a diminished number of both types of cells, indicating age-dependent changes (Figure 6a). Furthermore, mice with AxCANCre revealed normal fertility at 86-weeks-old ($n=4$, 100% fertile) whereas control mice were infertile ($n=4$, 0% fertile). The results obtained indicated that HST-1/FGF-4 worked as a protective agent against age-dependent changes in male fertility.

Effects of HST-1/FGF-4 on drug-induced testicular toxicity

Experiments were performed to investigate the effects of HST-1/FGF-4 on drug-induced toxicity against testis. One week prior to the administration of ADR to induce testicular toxicity, one group of male transgenic mice was injected with 1.0×10^8 p.f.u. of AxCANCre into testis. As a control, an adenovirus vector without *HST-1/FGF-4* gene was administered into testis. These mice received one intraperitoneal injection of ADR every week for 3 weeks at a dose of 2.0 mg/kg. Thereafter, they were periodically sacrificed for the examination of number, motility and the morphology of the sperm together with weight of the testis. ADR treatment caused a decrease in body weight, testis weight, sperm counts and sperm motility and an increase in the percentage of morphologically abnormal sperm in the control mice. The sperm counts of the control mice reached to the lowest levels in week 7 after the ADR treatment of $2.0 \pm 1.1 \times 10^6$/caudal epididymis, corresponding to an 86% decrease (Figure 7a). In contrast, the AxCANCre-injected group showed the lowest levels in week 5 of $6.6 \pm 1.2 \times 10^6$/caudal epididymis ($P < 0.003$), corresponding to only a 59% decrease, with their sperm counts returning to the pretreatment level by week 13. Changes in testis weight showed that AxCANCre-injected mice revealed enhanced recovery from ADR damage while the testis weight of the control mice significantly decreased 7 weeks after the ADR treatment and remained reduced for an additional 8 weeks (Figure 7b). Sperm motility was significantly decreased in the control mice 4 weeks after the ADR treatment ($12 \pm 2\%$), while the AxCANCre-administered mice showed $42 \pm 5\%$ sperm motility ($P < 0.01$) and then rapidly recovered to a normal level by week 10 ($64 \pm 7\%$). Although the data are not shown here, a comparison of the DNA histogram by flow cytometry revealed that AxCANCre-administered mice showed significant recovery of haploid cells (spermatid, 1C) at week 13, in agreement with the recovered point of sperm count.

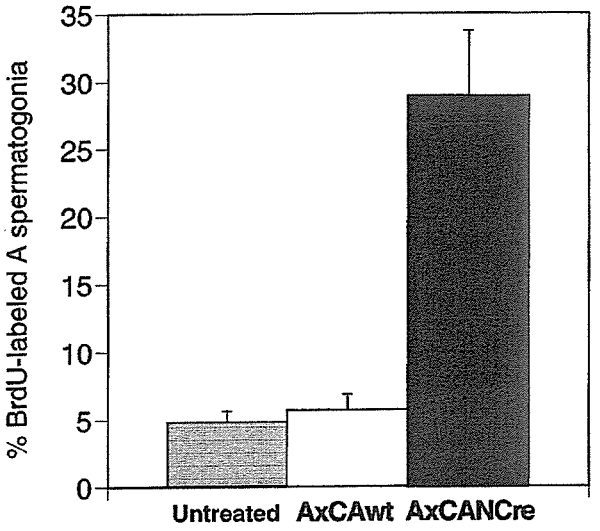

Figure 4 Animals treated with AxCANCre or control AxCAwt received intraperitoneal injection of 5-BrdU 2 h before being sacrificed. The labeling index in A spermatogonia was determined and compared between AxCANCre (■), AxCAwt (□), and untreated (■). The number of animals in each experiment was six

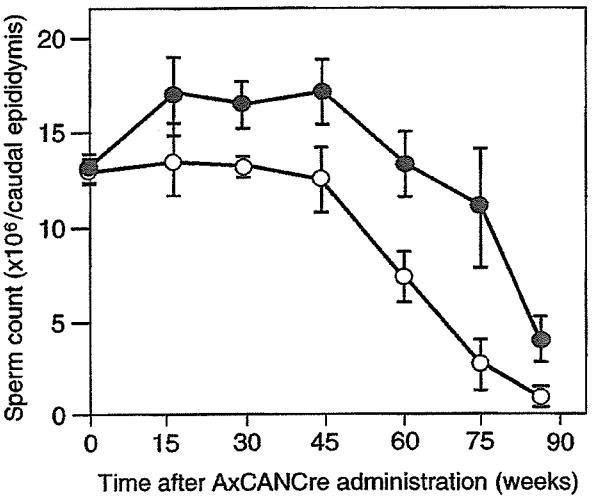

Figure 5 Age-dependent changes in sperm production. The epididymis sperm count was estimated every 15 weeks after the AxCANCre (●) or AxCAwt (○) administration. The number of animals was eight

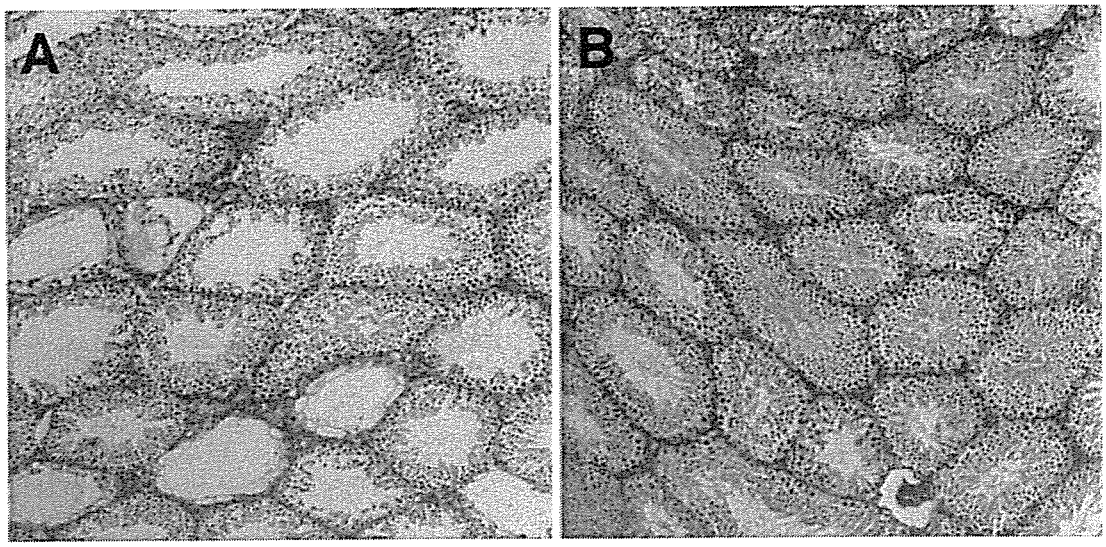

Figure 6 Histological findings of effect of HST-1/FGF-4 against age-dependent testicular changes 75 weeks after the treatment. (**a**) Control testes with AxCAwt. (**b**) Appearance of testes from AxCANCre-injected mice. The number of testis examined in each group was six

Furthermore, testicular histology revealed that the number of spermatogonia and spermatocytes recovered in the AxCANCre-injected mice 10 weeks after the ADR treatment (Figure 8c). However, testes from control mice administered with AxCAwt showed a diminished number of both types of cells 10 weeks after the ADR treatment, indicating that severe testicular toxicity was present (Figure 8b) as compared to normal testis (Figure 8a). The results obtained indicated that HST-1/FGF-4 worked as a protective agent against ADR-induced testicular toxicity.

Effects of HST-1/FGF-4 on fertility in ADR-treated mice

We assessed the effect of HST-1/FGF-4 on ADR-treated male fertility 10 weeks after the ADR treatment. Transgenic male and normal female mice were housed together overnight on a 1 : 1 basis in the home cage of the male. No pregnant animals were obtained in the control mice with ADR-treated alone. In contrast, successful pregnancies were observed in three of five male mice with AxCANCre. The mean litter size was six to seven. Consequently, these results indicate that HST-1/FGF-4 contributes to the recovery of ADR-induced male infertility.

Adenovirus-mediated HST-1/FGF-4 *gene transfer effectively recovered sperm count in ADR-treated mice*

To explore the therapeutic usefulness of the *HST-1/FGF-4* gene on impaired spermatogenesis, 1.0×10^8 p.f.u. of adenovirus carrying the *HST-1/FGF-4* gene (Ax*HST-1*) per testis was administered into testis of mice 4 weeks after the 2 mg/kg ADR treatment. As a control, adenovirus without *HST-1/FGF-4* gene (AxCAwt) was injected. The sperm count of the Ax*HST-1*-injected group showed a dramatic recovery 10 days after the adenovirus injection ($P<0.05$), while the sperm count of the control groups administered with AxCAwt severely decreased (Figure 9). In the Ax*HST-1*-treated mice, a significant decrease in the diameter of the seminiferous tubules was also observed (data not shown). These results suggest that the gene transfer of *HST-1/FGF-4* has a protective effect on experimentally induced testicular toxicity in mice.

Discussion

A method for activation or inactivation of a transgene in animals has recently been accomplished by using site-specific recombinases Cre and FLP (Werdien *et al*., 2001). However, this current system of transfer recombinase *in vivo* using either gene transfer or crossing Cre-expressing animals was low in efficiency and time-consuming. Here we report for the first time that a simple inoculation of Cre-expressing adenovirus into a target organ allows genetic switching-on in transgenic animals. Our adenovirus-mediated recombinase-based conditional gene activation strategies *in vivo* can be used to design induced expression of any gene to any tissue at any desired time, and will have a profound impact on fundamental biology and the design of better therapeutic models of human diseases. By establishing Cre/lox conditional transgenic animals in which testis-specific gain of function of the *HST-1/FGF-4* gene resulted in enhanced spermatogenesis, we have demonstrated that this gene has a protective effect against testicular toxicity induced by an anticancer drug. In those animals, impaired fertilization ability also recovered. In addition, we have successfully shown the therapeutic effectiveness of the adenovirus carrying *HST-1/FGF-4* gene against oligozoospermia in mice.

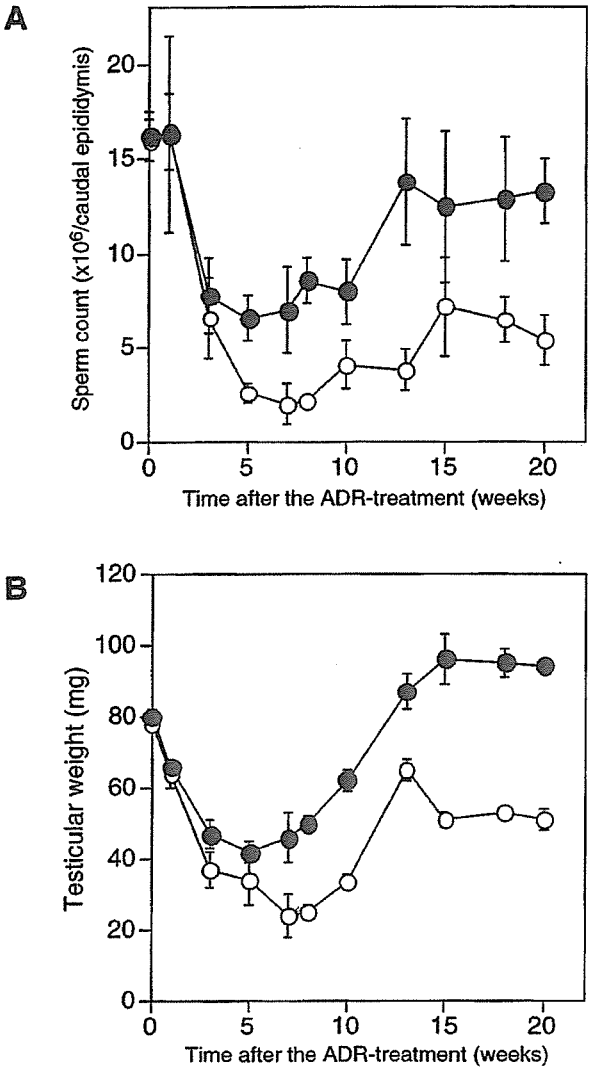

Figure 7 (a) Effect of HST-1/FGF-4 on ADR-induced testicular toxicity. After the ADR treatment of mice pretreated with AxCANCre (●) or control AxCAwt (○), epididymal sperm count was periodically examined. Sperm count remarkably recovered in mice with AxCANCre. (b) Effect of HST-1/FGF-4 on testicular weight. After the ADR treatment of mice pretreated with AxCANCre (●) or control AxCAwt (○), testicular weight was periodically evaluated

To our knowledge, this is the first demonstration of gene therapy against drug-induced male infertility in animal models.

The present results that the *HST-1/FGF-4* gene can correct anticancer drug-induced male infertility provides us with the possibility of treatment of human drug- or radiation-induced oligozoospermia. There are controversial disputes over decreases in sperm counts in young, seemingly healthy people worldwide, and some people attribute the decrease to the presence of endocrine disrupters. Whether there is in fact a decrease in sperm counts worldwide is yet to be determined, but male infertility accompanied by decreased sperm counts is a serious problem for some people (Ford, 2001). Therapeutic approaches for male infertility have been developed including spermatogonial stem cell transplantation (Avarbock *et al.*, 1996; Ogawa *et al.*, 2000). These methods, however, are highly invasive and expensive treatments. Our results suggest that *HST-1/FGF-4* gene therapy can be used to treat some people with oligozoospermia unrelated to drug toxicity, indicating hope of development of better treatments for male infertility.

The mechanism by which HST-1/FGF-4 stimulates spermatogenesis *in vivo* has not been established. FGF-2 produced from Sertoli cells was found to significantly increase the number of Sertoli cells and also stimulate the proliferative activity of the gonocytes *in vitro* (Van Dissel-Emiliani *et al.*, 1996). The presence of continuous expression of the *Hst-1/Fgf-4* gene in Sertoli cells was a novel finding, and this observation has led us to speculate that HST-1/FGF-4 could be involved in specific signals during sperm maturation. For example, *Hst-1/Fgf-4* gene expression in testis was found during the period that the animals retained their fertility (Yamamoto *et al.*, 2000) and the expression was decreased thereafter. In addition, inhibition of *HST-1/FGF-4* gene expression by antisense ODNs against this gene decreased the sperm count *in vivo* (unpublished observations). Furthermore, the significance of HST-1/FGF-4 is supported by the reports that FGFR-1 and FGFR-4 are known to be localized in Sertoli cells in rat testis and that FGFR-1, -2, -3 and -4 are present in adult germ cells (Cancilla and Risbridger, 1998; Le Magueresse-Battistoni *et al.*, 1994). We have confirmed that FGFR-1 and FGFR-2, which are known as high-affinity receptors for HST-1/FGF-4, are expressed in murine spermatogonia (data not shown). Considering that overexpression of HST-1/FGF-4 does not lead to apparent growth of Sertoli cells in testis, enhanced spermatogenesis in our system may result from HST-1/FGF-4 acting as a paracrine growth and/or differentiating factor on spermatogonia.

ADR, an anticancer drug well known for its testicular toxicity, was used in this study. It is known that ADR inhibits RNA and DNA synthesis through interaction into the double-helix, thereby interfering with cell division (Simpkins *et al.*, 1984; Simpkins and Pearlman, 1984) and induces chromosomal aberrations (Russo and Levis, 1992). ADR tended to kill type A spermatogonia at all stages of the spermatogenic cycle (Matsui *et al.*, 1993) and induces apoptotic death (Sjoblom *et al.*, 1998; Shinoda *et al.*, 1999). There is a possibility that HST-1/FGF-4 protects against ADR-induced apoptotic death of spermatogonia. FGFs has been shown to protect tissue damage in animal models of drug-induced apoptosis (Wagle and Singh, 2000) and *in vitro* cultured cells (Miho *et al.*, 1999; Haimovitz-Friedman *et al.*, 1994). The cellular and molecular mechanisms of FGFs effects have not been fully defined; thus, further investigation of the protective effect of HST-1/FGF-4 on ADR-mediated germ cell death is required.

Male germ cell tumors often overexpressed the *HST-1/FGF-4* gene (Strohmeyer *et al.*, 1991). Therefore,

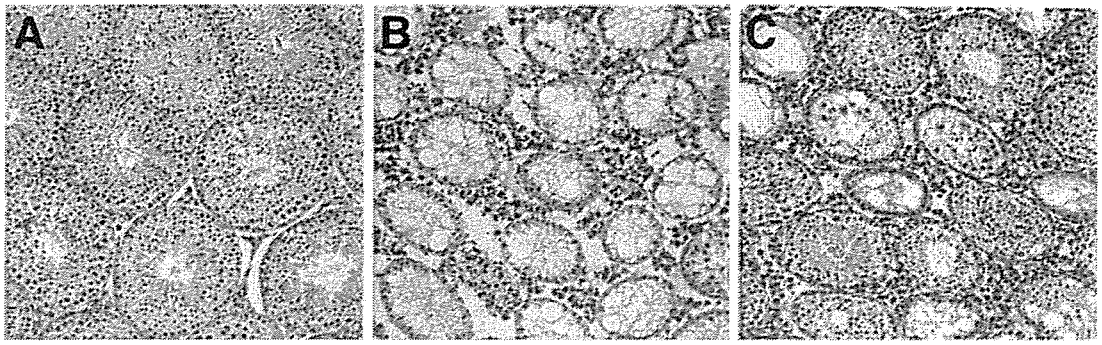

Figure 8 Histological findings of effect of HST-1/FGF-4 against ADR-induced testicular toxicity. (a) Untreated normal testis. (b) Appearance of testes from AxCAwt-injected mice: tubules were severely atrophied 4 weeks after the ADR treatment. (c) Spermatogenesis was observed in some seminiferous tubules in testes from AxCANCre-treated mice

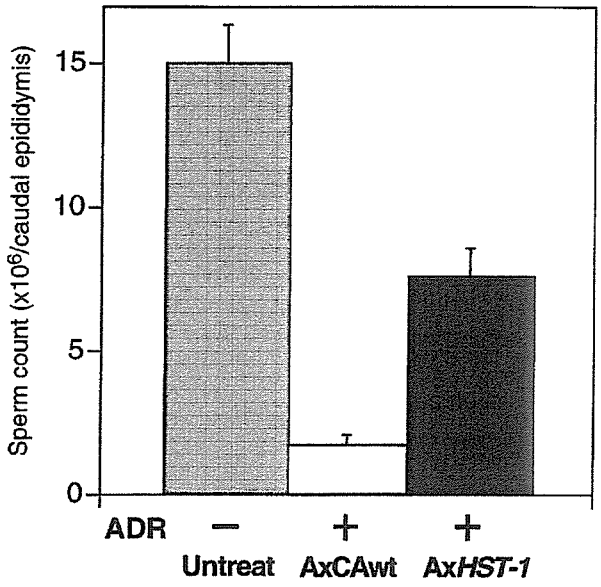

Figure 9 Effective recovery of the sperm count in mice with adenovirus-mediated gene transfer. Ax*HST-1* was injected into mice with ADR-induced testicular toxicity. As a control, AxCAwt was injected. The sperm count was estimated 10 days after the adenovirus treatment. The number of animals was five. (■) Ax*HST-1*, (□) control AxCAwt, and (■) untreated normal

there is potential for HST-1/FGF-4 to stimulate tumor growth *in vivo* when this gene is used in a clinical setting for human gene therapy. Although our conditional transgenic mice with continuous *HST-1/FGF-4* gene expression in testes produced no tumor growth through more than a period of 1 year and 10 months (unpublished observations), further careful analyses are required before our adenovirus-carrying *HST-1/FGF-4* gene can become an ideal gene therapy for male infertility.

Adventitious germ-line integration of transferred gene in the course of somatic gene therapy is an ethical concern that must be addressed. In our study, adenovirus injection was performed by intratesticular injections, thereby germ cells and sperm were directly exposed to a high titer of adenovirus. However, in our experiments, we have not observed germ-line integration of the transferred genes. In addition, it has been reported that mouse germ-line integration event is minimal in adenovirus injection into testes (Hall *et al.*, 2000). However, further careful studies are required to see whether germ-line integration of exogenous gene is not occurred to the damaged testes before our method is applied for human gene therapy.

Finally, our results have important implications for clinical applications of the *HST-1/FGF-4* gene for oligozoospermia and male infertility, in addition to furthering an understanding of the mechanisms involved in the effects of this gene on spermatogenesis.

Materials and methods

Recombinant DNA constructs and conditional transgenic mice

The CALNL*HST-1* switching unit was constructed according to the original method (Kanegae *et al.*, 1995). In brief, 1901 bp of a full-length of human *HST-1/FGF-4* cDNA fragment was cloned into the *Swa*I site of plasmid pCALNLw consisting of the CAG promoter, a loxP sequence, a neo-resistant gene (1.0 kb of the *Bgl*II–*Sma*I fragment from pSV2neo), a second loxP site, and the poly(A) signal from pCAGGS, resulting in a transgene named CALNL*HST-1*. The transgene was linearized and injected into C57BL/6J mouse (CLEA Japan Inc., Japan) zygotes at a concentration of 2 μg/ml to generate transgenic mice according to an established procedure (Hogan *et al.*, 1986). Transgenic founder mice were mated to C57BL/6J mice, and offspring were screened for the presence of the transgene by Southern blot analysis of genomic DNA isolated from tail biopsies at the age of 3 weeks.

Switching of the transgene by AxCANCre

A recombinant adenovirus expressing Cre recombinase (AxCANCre) was prepared as described (Kanegae *et al.*, 1995). As a control, an empty adenovirus vector was used (AxCAwt). The virus stock was concentrated and purified at

1.0×10^9 p.f.u./ml as described previously (Kanegae *et al.*, 1994). Transgenic mice were administered 1.0×10^8 p.f.u. of AxCANCre or control AxCAwt per testis. Mice were sacrificed at various times, and testis weights, caudal epididymal sperm counts and sperm motility were investigated.

Testes specific HST-1/FGF-4 *gene activation by Northern blot hybridization*

In order to evaluate the testes specific gene expression in Cre-switching mice, Northern blot hybridization analysis was performed. One day after the intratesticular administration of AxCANCre into right testis, total RNA was harvested from testes and other organs. Left testis was remained untreated as a control. Ten μg of each total RNA sample was separated on a denatured 1% agarose gel, transferred to a NitroPlus nitrocellulose membrane (Micron Separations Inc., MA, USA), and the blot was hybridized with the α^{32}P-dCTP-labeled full-length of human *HST-1/FGF-4* cDNA as a probe in the presence of 50% formamide for about 18 h. As an internal reference, the same filter was later hybridized to a *β-actin* probe. The images were incorporated into Macintosh computer through a STORM image scanner (Molecular Dynamics). Intensity of each band on the image was measured and the data were processed by an image processing software ImageQuant.

Localization of HST-1/FGF-4 *gene expression by* in situ *hybridization*

Digoxygenin-labeled sense or antisense riboprobes were synthesized by T3 or T7 RNA polymerase from cDNA plasmids containing the entire coding sequences of human *HST-1/FGF-4* cDNA in the presence of digoxygenin-dUTP (Roche Diagnostics, GmbH, Germany). *In situ* hybridization experiments using fresh-frozen testis sections and digoxygen-in-labeled riboprobes were carried out essentially as described previously (Ishii *et al.*, 1997; Wilkinson, 1992).

Cell isolation

Sertoli cells were isolated from AxCANCre injected testes. In brief, testes were decapsulated in ice-cold Minimum essential medium (MEM, Life Technologies Inc., MD, USA) and subjected to enzymatic digestion in accordance with the previous report (Orth and Boehm, 1990). Seminiferous cord fragments free from interstitial cells were harvested and were treated with 0.05% trypsin 5.3×10^{-4} M EDTA (Life Technologies Inc., MD, USA) for 7 min at 37°C. The final single cell suspension in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc., MD, USA) containing 2% FBS was plated on gelatin-coated 100-mm plate at 5×10^5 cells density and incubated for 6 h at 37°C. The adherent cells were washed with serum-free MEM three times to remove gonocytes and then subjected to total RNA purification. At that moment, adherent cells consist of 98% < Sertoli cells.

RNA preparation and Northern blot hybridization

AxCANCre were injected into a right testis of the conditional transgenic mouse. Left testis of the transgenic mouse remained untreated. Twenty-four hours after the injection, total RNAs were prepared from both Cre-injected and -uninjected testis, and other tissues such as brain, lung, liver, and kidney by using Isogen solution (Nippon Gene, Tokyo) according to the manufacturer's protocol. Expression of mRNA for *HST-1/FGF-4* gene was examined by Northern blot hybridization under high stringency condition with α^{32}P-labeled human *Fgf-4* cDNA fragment as a probe. Native mouse *Hst-1/Fgf-4* gene expression has not been detected in adult tissues by human *FGF-4* cDNA probe because of its low expression level. The same RNA samples were subjected to RT–PCR analysis of *β-actin* transcripts.

RT–PCR analysis

Cellular and tissue total RNA samples were prepared using Isogen solution (Nippon Gene, Tokyo) according to the manufacturer's protocol. An aliquot of total RNA isolated was treated with DNase (DNase I, amplification grade; Life Technologies Inc., MD, USA), and poly(A)$^+$ RNAs were purified in Sertoli cell samples. RT–PCR reaction was performed using QIAGEN OneStep RT–PCR kit (QIAGEN K.K., Japan) with the following primers: 5′-TTCTTCGTGGCCATGAGCA-3′ and 5′-TGACCTTCATG-GTGGGCGACA-3′ for human *HST-1/FGF-4*. After 30 min at 50°C for reverse transcription reaction, the reaction was terminated at 94°C for 15 min, and PCR was then performed with 35 cycles in a Perkin-Elmer Thermal Cycler using a cycle profile of 1 min at 97°C, 1 min at 60°C and 40 s at 72°C. After RT–PCR, aliquots were run on 3.0% agarose gels, stained with ethidium bromide, and then photographed under UV illumination. This primer set does not recognize native mouse *Hst-1/Fgf-4* gene expression under our RT–PCR conditions. *β-actin* cDNA was amplified as an internal control using primers 5′-GACATCAAAGAGAAGCTGTGC-3′ and 5′-TAGGAGC-CAGAGCAGTAATC-3′.

Labeling index and immunohistochemistry

Animals received an intraperitoneal injection of 5-BrdU (100 mg/kg body weight, Sigma-Aldrich, Japan) 2 h before being sacrificed. To determine the BrdU labeling index, the testes of each animal were fixed in Carnoy's fixative and embedded in paraffin. Sections were made and processed immunohistochemically using the BrdU staining kit (Roche Diagnostics, GmbH, Germany) according to the recommended method. Using a microscope, the percentage of BrdU-labeled cells was determined by counting at least 200 seminiferous tubules cross-sections per testis.

ADR treatment and assessment of spermatogenesis

Transgenic mice (male, 10 weeks old) were divided into two groups. One group received AxCANCre, and the other group received control AxCAwt. The number of mice in each group was 30. Seven days after the adenovirus injection, adriamycin (ADR), an anticancer drug, was intraperitoneally administered to 10- to 11-week-old C57BL/6J or transgenic male mice at a dose of 2 mg/kg once a week for 3 weeks, and then body weight, testis weight, sperm count, sperm motility and DNA ploidy were observed periodically. The experiment was repeated three times. Sperm were counted by the following method. Spermatozoa were collected by mincing caudal epididymis and incubated in modified Krebs-Ringer bicarbonate solution (Ivanova *et al.*, 1999) with 4 mg/ml of bovine serum albumin (Sigma-Aldrich, Japan) at 37°C. The sperm suspension was incubated for 30 min at 37°C, and sperm count was determined by an added 1/10 volume of 5 M NaCl. Sperm motility was assessed by light microscopy after incubating for 1 h at 37°C as described, and the data were represented as a percentage of motile sperm per more than

1000 counts. The DNA histograms were analysed according to the described method by using propidium iodide (PI) staining (Hellstrom *et al.*, 1990). DNA histograms were collected by automated flow cytometry. For the examination of testis histology, standard periodic acid Schiff and hematoxylin and eosin staining were performed after the fixation with Bouin solution and counted 200 seminiferous tubules cross-sections per testis (Grell *et al.*, 1980). The germ cells were recognized and counted by using morphological criteria according to the described methods (Oakberg and Huckins, 1976). Mice were housed with normal C57BL/6J female mice overnight on a 1:1 basis to examine male fertility.

AxHST-1 administration into testis

C57BL/6J mice received ADR at a dose of 2 mg/kg once a week for 3 weeks. Four weeks later, mice were divided into two groups and received either an intratesticular injection of Ax*HST-1*, which was previously used for significant expression of HST-1/FGF-4 *in vivo* (Takahama *et al.*, 1999), or control AxCAwt at a dose of 1.0×10^8 p.f.u./testis. Ten days after the treatment, they were sacrificed for the examination of cauda epididymal sperm profiles and the morphology and weight of the testes.

Statistical analysis

The results are represented as means$\pm$s.d. Student's *t*-test was performed for statistical evaluation, with $P<0.05$ considered as significant.

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Original Article

Medically and economically appropriate follow-up schedule for prostate cancer patients after radical prostatectomy

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Abstract

Background: Our goal was to determine the optimal frequency and method of follow-up after radical prostatectomy to minimize medical cost without adversely affecting patients.

Methods: Two hundred and twenty-one patients who underwent a radical prostatectomy with or without adjuvant androgen deprivation from 1989 to 1999 were selected for the study. Eighty percent of the patients received postoperative androgen deprivation. Tumor recurrence was strictly defined as detectable serum prostate specific antigen (PSA) and/or clinical findings such as local tumor detection or bone metastasis. Thirty of 221 patients experienced tumor recurrence. Risk of tumor recurrence, procedures for detection of recurrence, and PSA doubling time after biochemical failure were analyzed.

Results: None of the 30 patients who were examined showed definitive local recurrence or metastatic sites on the imaging study at the time of initial PSA detection, and there were no observed recurrences in the absence of detectable serum PSA. In patients who showed elevated PSA within 12 months after radical prostatectomy, PSA levels rapidly increased with doubling times ranging from 1.2 to 13.7 months. Excluding those patients, the doubling time of PSA levels ranged from 2.8 to 31.5 months.

Conclusions: Prostate specific antigen screening is sufficient to detect treatment failure after radical prostatectomy, irrespective of adjuvant hormone therapy. Based on the calculated doubling time, the longest advisable interval between checks of PSA levels is estimated to be four months within the first year after radical prostatectomy, and biannually or annually thereafter. Continuously elevated PSA levels or clinical symptoms indicate surveys for local recurrences and distant metastases.

Key words adjuvant hormone therapy, follow-up, medical cost, radical prostatectomy.

Introduction

The number of men in the USA and Japan undergoing radical prostatectomies dramatically increased after the introduction of testing for serum prostate specific antigen (PSA).[1] Unfortunately prostate cancer recurs in some patients after radical prostatectomy. Pound *et al.* reported that local recurrence developed in 2.97% and distant metastasis developed in 6.2% of their 1916 patients after radical prostatectomy was conducted. Few of their patients were treated with adjuvant therapy.[2] Partin *et al.* reported recurrence rates of four percent for local recurrence, eight percent for distant metastases, and 23% for an isolated elevation of PSA only in their stage T1a to T3a prostatic cancer patients after radical prostatectomy.[3] However, the appropriate postoperative follow-up schedule and examination methods, and their cost-effectiveness and clinical benefits are controversial issues.[4]

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