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Pluripotent Stem Cells Induced from Testicular Tissue of a Man with Klinefelter Syndrome (47, XXY) by Four Transcription Factors (*OCT4, SOX2, KLF4,* and *C-MYC*)

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1. Introduction

Klinefelter syndrome (KS) is the most common chromosomal aberration in men, with an estimated frequency of 1:500 to 1:1000 among live deliveries (Lanfranco et al., 2004). KS is characterized by an X-chromosome polysomy, with X disomy being the most common variant (47, XXY). Although the classic description of men with KS has been that they are tall with eunuchoid body proportions, low testosterone, sparse facial and pubic hair, small, hard testicles, micropenis, sterility, and mild to moderate cognitive deficits, it is now well known that this description is not accurate. Rather, men with KS exhibit a broad spectrum of phenotypes, and they are represented in a variety of professions and can be found at all socioeconomic levels (Lanfranco et al., 2004).

The cardinal problems in men with KS—progressive testicular failure (and thus azoospermia or cryptozoospermia), small testes (5 to 7 cm^3), and low testosterone—are found in most men with KS, and men with these symptoms should undergo prompt cytogenic evaluation. Most men with KS are diagnosed as adults, when they present with infertility or hypogonadism (Graham et al., 1988). It is strongly recommended that men with KS undergo genetic counseling, because they have an increased medical risk for various diseases, including diabetes, cardiovascular disease, and cancer, and their offspring have an increased risk for chromosomal abnormalities (Lanfranco et al., 2004).

It is predicted that the aberrant expression of X chromosome-linked genes plays a role in the failure of spermatogenesis seen in men with 47, XXY. However, the mechanisms underlying the infertility of men with KS is still poorly understood, and its treatment is both difficult and rarely successful. The elucidation of the molecular mechanisms of X chromosome inactivation might therefore allow us to better predict the extent of reproductive failure in KS patients and to design novel therapies.

It was recently reported that introducing a set of transcription factors related to pluripotency can directly reprogram human somatic cells to produce induced pluripotent stem (iPS) cells (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007). We reasoned that iPS cells derived from KS patients could be useful as a tool for studying the mechanism of X chromosome inactivation. In addition, iPS cells derived from testicular tissue might be a potential source for cell-based therapies to treat some cases of male infertility.

In this study, we successfully obtained iPS cells derived from the testicular tissue of men with KS, by introducing four transcription factors (*OCT4, SOX2, KLF4*, and *C-MYC*), using the lentiviral vector system. In the future, such iPS cells might be useful for uncovering the mechanisms of X-chromosome inactivation, and could lead to novel treatments for infertility.

2. Materials and methods

2.1 Human subjects

In accordance with the regulations set forth by the Human Investigations Committee of Toho University School of Medicine, written informed consent was obtained from male patients being treated for infertility, who visited the Reproduction Center of Toho Medical Center Omori Hospital. Testicular tissues were obtained from one patient with KS by testicular sperm extraction.

2.2 Adult human testis tissues with KS

First, we confirmed that there were no mature sperm in the testicular tissues from KS patients. These tissues included the rete testis, Leydig cells, Sertoli cells, and fibroblasts. The tissues were used fresh. Cell suspensions from the testis tissues were prepared by enzymatic digestion (Ogawa et al., 1997). The dissociated testis cell suspension was plated onto 10-cm culture dishes coated with 0.1% gelatin (Sigma) in a standard culture medium (Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 7% fetal bovine serum (FBS), 2 mM glutamine (Sigma), and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma)). These cells were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere for 17 days.

2.3 Cell culture

293FT cells were purchased (Invitrogen). The 293FT cells were maintained in the standard culture medium. Mitomycin C-treated MEF feeder cells were purchased (ReproCELL). The MEF feeder cells were plated in 0.1% gelatin-coated 10-cm culture dishes in the standard culture medium. iPS cells were generated and maintained in Primate ES medium (ReproCELL) supplemented with 4 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems). To grow the iPS cells, the Primate ES cell medium was supplemented with 4 ng/ml bFGF and 10 μM Y27632 (Wako) and changed every second day. For passaging, the iPS cells were rinsed with Hank's balanced salt solution (HBSS) (Invitrogen) once and incubated in dissociation medium (ReproCELL) at 37°C. All cultures were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere.

2.4 Plasmid construction

The open reading frames of human *OCT4, SOX2, KLF4*, and *C-MYC* were amplified by RT-PCR and subcloned into pLenti 6.3/V5-TOPO (Invitrogen). A stop codon was inserted to avoid expressing the V5 protein. All the plasmids were constructed by Invitrogen.

2.5 Lentivirus production and infection

The ViraPower™ HiPerfome™ Lentiviral Expression Kit (Invitrogen) was used to produce the lentiviruses. 293FT cells were plated at 6 X 10⁶ cells per 10-cm dish and incubated overnight. We prepared four dishes of 293FT cells to produce four lentiviruses (encoding of

OCT4, SOX2, KLF4, and *C-MYC*). The next day, the 293FT cells were transfected with 3 μg pLenti 6.3/V5-TOPO and 9 μg ViraPower packaging mix with the Fugene 6 transfection reagent (Roche), according to the manufacturer's instructions. The medium was collected 24 and 48 hours after the transduction and replaced with new medium. The collected supernatants were designated as the first and second virus-containing supernatants. Medium was also collected 72 hours after transduction, as the third (final) virus-containing supernatant. The virus-producing cells were then discarded. The virus-containing supernatants were filtered through a 0.45-μm pore filter (Millipore). An equal amount of the supernatants containing each of the four lentiviruses was mixed, transferred to the culture dish containing testis cells, and incubated at 37°C.

2.6 iPS cell generation

The virus-containing medium was used to replace the standard medium in the testis cell cultures, 24 hours after the third virus-mediated transduction of medium from the virus-producing cells. The medium was changed every second day. Six days later, the cells were harvested by trypsinization, and 5 X 10^4 cells were placed on MEF feeder cells (10-cm dish) in Primate ES cell medium supplemented with 4 ng/ml bFGF. The medium was changed every second day. We monitored these cells daily for morphological changes.

2.7 Immunofluorescence microscopy and immunostaining

The Human Embryonic Stem Cell Marker Antibody Panel was used (R&D Systems). Cells were washed twice with phosphate-buffered saline, fixed with 4% (w/v) paraformaldehyde for 20 min, permeabilized for 60 min with phosphate-buffered saline containing 0.1% (v/v) Triton X-100, and then blocked for 3 h with phosphate-buffered saline containing 20% donkey serum. For immunostaining, the fixed samples were incubated with anti-human alkaline phosphatase monoclonal, anti-human NANOG polyclonal, anti-human OCT4 polyclonal, anti-human SSEA-1 monoclonal, and anti-human SSEA-4 monoclonal antibodies (all from R&D Systems) as indicated, washed three times with PBS containing 0.1% (v/v) Triton X-100, and probed with the appropriate secondary antibodies (anti-goat IgG antibody conjugated with Alexa 488 or anti-mouse IgG antibody conjugated with Alexa 488) (Molecular Probes). Nucleic acid was detected using SYTOX[R] Orange Nucleic Acid Stain (Molecular Probes).

2.8 Reverse-Transcription (RT)-PCR

Total RNA was prepared using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen). RT-PCR was performed using the SuperScript™III One-Step RT-PCR System with Platinum[R] Taq DNA Polymerase (Invitrogen) for human SSC markers, with the following conditions: 55°C for 30 min for reverse transcription; 94°C for 2 min to inactivate the reverse transcriptase and activate the polymerase; 40 cycles of 94°C for 15 s, 55°C for 30 s, 68°C for 1 min; and 68°C for 5 min, for the final extension. The products were as follows: for *OCT4*, 315 bp; *NANOG*, 285 bp; *GAPDH*, 513 bp; *STELLAR*, 174 bp; *GDF3*, 150 bp; *DAZL*, 178 bp; and *VASA*, 199 bp, (Klimanskaya et al., 2006) (Ezeh et al., 2005).

2.9 Short tandem repeat analysis and karyotyping

DNA fingerprinting analyses with short tandem repeat (STR) markers were performed at SRL Laboratory, Japan. Chromosomal G-band analyses were performed at Nihon Gene Research Laboratories, Japan.

2.10 In vitro assay (embryoid body formation)

Confluent iPS cells in a 10-cm dish were harvested by trypsinization. The iPS cells were then transferred to a Poly (hydroxyethyl methacrylate-co-methyl methacrylate; HEMA-MMA)-coated dish in Primate ES cell medium. The medium was changed every other day, and the cells were maintained in floating culture for 8 days.

2.11 In vivo assay (teratoma formation)

Confluent iPS cells in a 6-well dish were harvested by trypsin treatment, collected into tubes, and spun, and the pellets were suspended in ES medium. The cells were then injected into the testes of SCID mice (8-weeks old) (Charles River). Twelve weeks after injection, the tumors were dissected and fixed with 10% formalin. Pathological analyses were performed at the Tokyo Central Pathology Laboratory, Japan.

3. Results

3.1 Generation of ES-like cell colonies from the testicular tissue of an infertile man with KS

Testicular tissue was obtained from an infertile man with KS (Fig. 1a). Colonies with a human ES cell-like morphology first became visible 15-16 days after their transduction with transcription-factor-expressing viruses under human ES cell-supporting conditions. The morphology of these colonies was similar to that of human ES cells. We obtained about 20 ES cell-like colonies from 5×10^4 testicular cells in this experiment (data not shown). The colonies were collected on days 20-25, and transferred into 24-well plates on MEF feeder cells. Colonies that continued to expand and maintained an ES cell-like cell morphology were further passaged, while those that failed to expand or produced aberrant colonies were discarded. Ultimately, only one ES cell-like cell line was obtained (Fig. 1b).
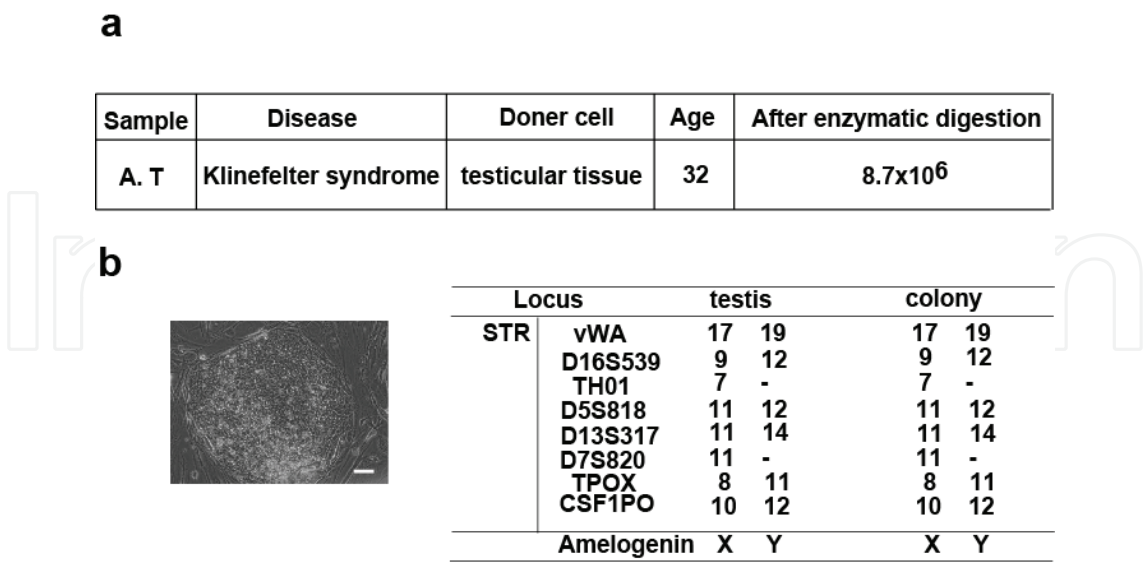
a

Sample	Disease	Doner cell	Age	After enzymatic digestion
A. T	Klinefelter syndrome	testicular tissue	32	8.7×10^6

b

Locus		testis		colony	
STR	vWA	17	19	17	19
	D16S539	9	12	9	12
	TH01	7	-	7	-
	D5S818	11	12	11	12
	D13S317	11	14	11	14
	D7S820	11	-	11	-
	TPOX	8	11	8	11
	CSF1PO	10	12	10	12
Amelogenin		X	Y	X	Y

Fig. 1. Generation of ES-like cells from human testicular tissue from an adult with KS by four transcription factors (*OCT4, SOX2, KLF4,* and *C-MYC*). a, Overview of the human testicular sample. b, Morphology and STR analysis of the ES-like cells. Bar = 60 μm

We then performed evaluations to confirm that this ES-like cell line was derived from testicular tissue. The pattern of 15 short tandem repeat (STR) loci and the Amelogenin locus was a perfect match between the ES cell-like colonies and one testicular tissue sample, as assessed using the PowerPlex^R 16 System (Promega). The results for 8 STRs and Amelogenin are shown in Figure 1b. In this analysis, Amelogenin only indicated the presence of the X or Y chromosome, and could not distinguish polysomies of the X or Y chromosome. These findings indicated that the ES cell-like colonies were generated from the testicular tissue and were not a result of cross-contamination.

3.2 Human testicular cells and ES-like cells derived from a KS patient expressed *OCT4, NANOG, STELLAR*, and *GDF3*, but not *DAZL* or *VASA*

RT-PCR showed that the ES-like cells derived from the KS patient expressed many marker genes for undifferentiated ES cells, including *OCT4, NANOG, STELLAR*, and *GDF3* (Fig. 2). We also confirmed that the testicular tissues from the same KS patient expressed *OCT4, NANOG, STELLAR*, and *GDF3* (Fig. 2). Neither the ES-like cells nor the original testes tissues expressed the germ-cell marker *DAZL* or *VASA* (Fig. 2). We could not find any differences between the ES cells and the testicular tissue derived from the KS patient by RT-PCR.
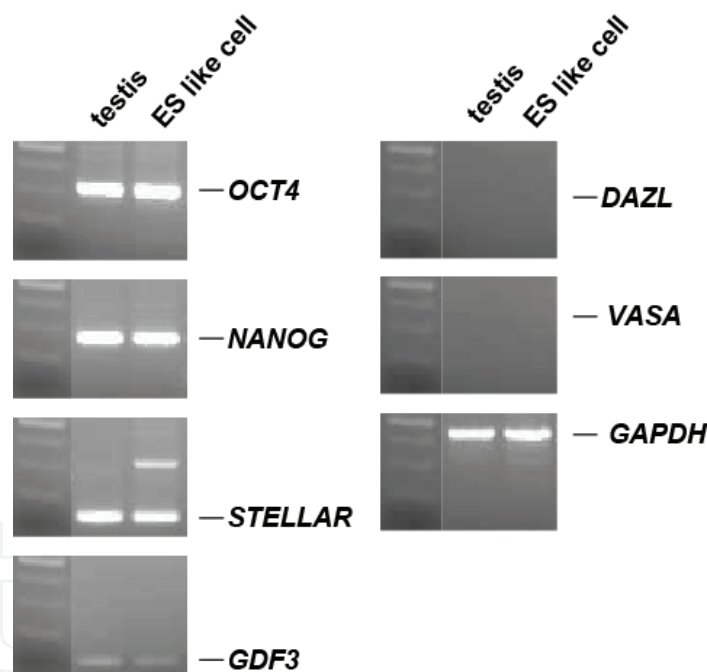

Fig. 2. Total gene expression in the ES-like cells and testis cells derived from a KS patient by RT-PCR. RT-PCR analysis for the expression of *OCT4, NANOG, STELLAR, GDF3, DAZL, VASA,* and *GAPDH* in ES-like cells and testis cells. The upper band in the RT-PCR results for *STELLAR* is nonspecific. *GAPDH* was used as a loading control.

3.3 Confirmation by chromosomal analysis that the iPS cells derived from the KS patient had a karyotype of 47, XXY

Karyotyping is the gold standard for diagnosing KS. To examine the karyotype of the iPS cells, we subjected twenty cells to chromosomal G-band analysis. The results confirmed that the iPS cells had the karyotype 47, XXY, which the same as that of the donor (Fig. 3).
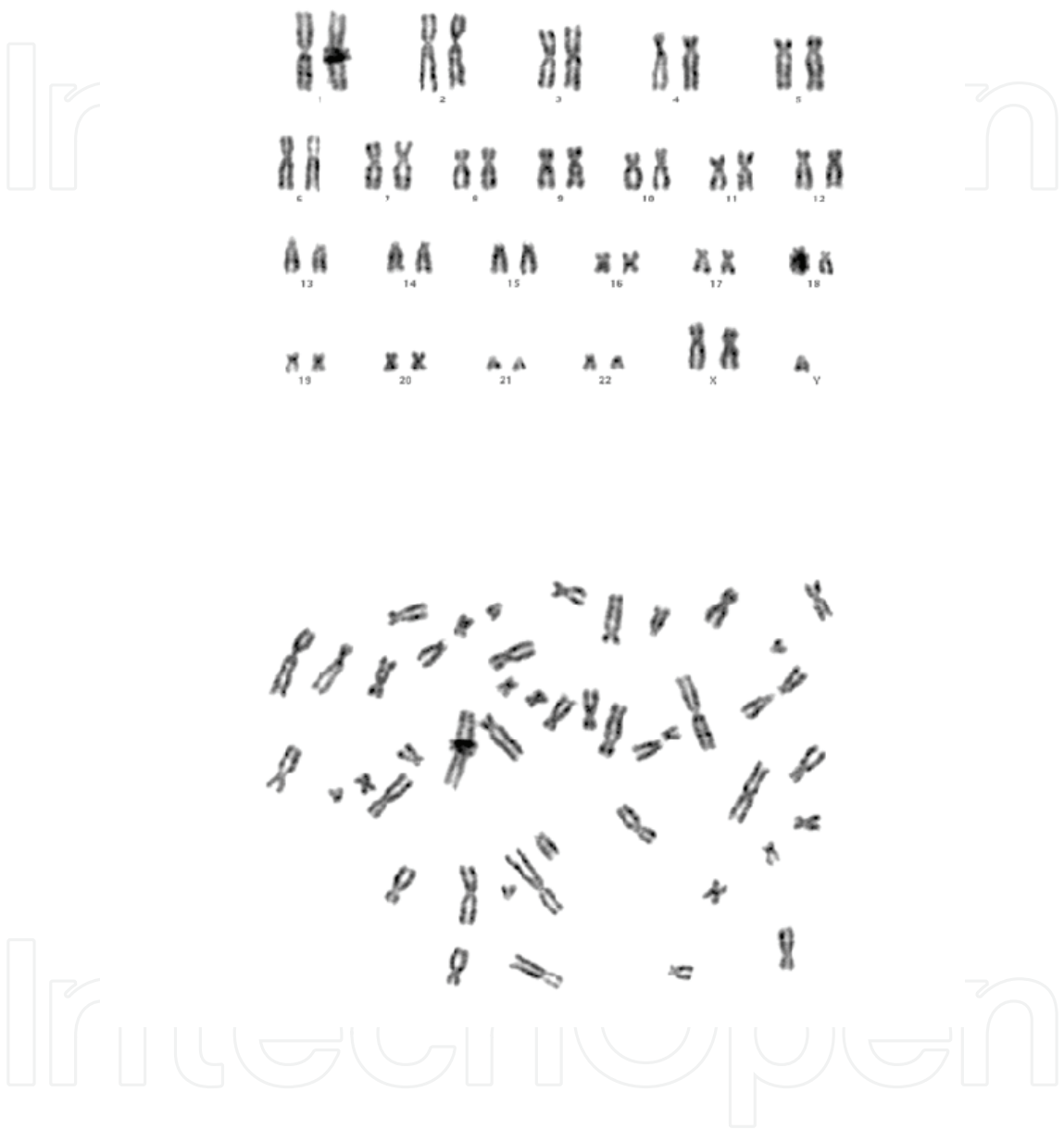
300

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Fig. 3. G-banding chromosome analysis of the ES-like cells. A karyotype analysis of the ES-like cells derived from a patient with KS

3.4 iPS cells derived from a KS patient expressed human ES-cell markers by immunostaining

We characterized the iPS cells derived from the KS patient by immunostaining, and found that they expressed human ES-cell-specific surface antigens, including alkaline phosphatase (AP), NANOG, OCT4, and SSEA-4, but not SSEA-1 (Fig. 4a-e).
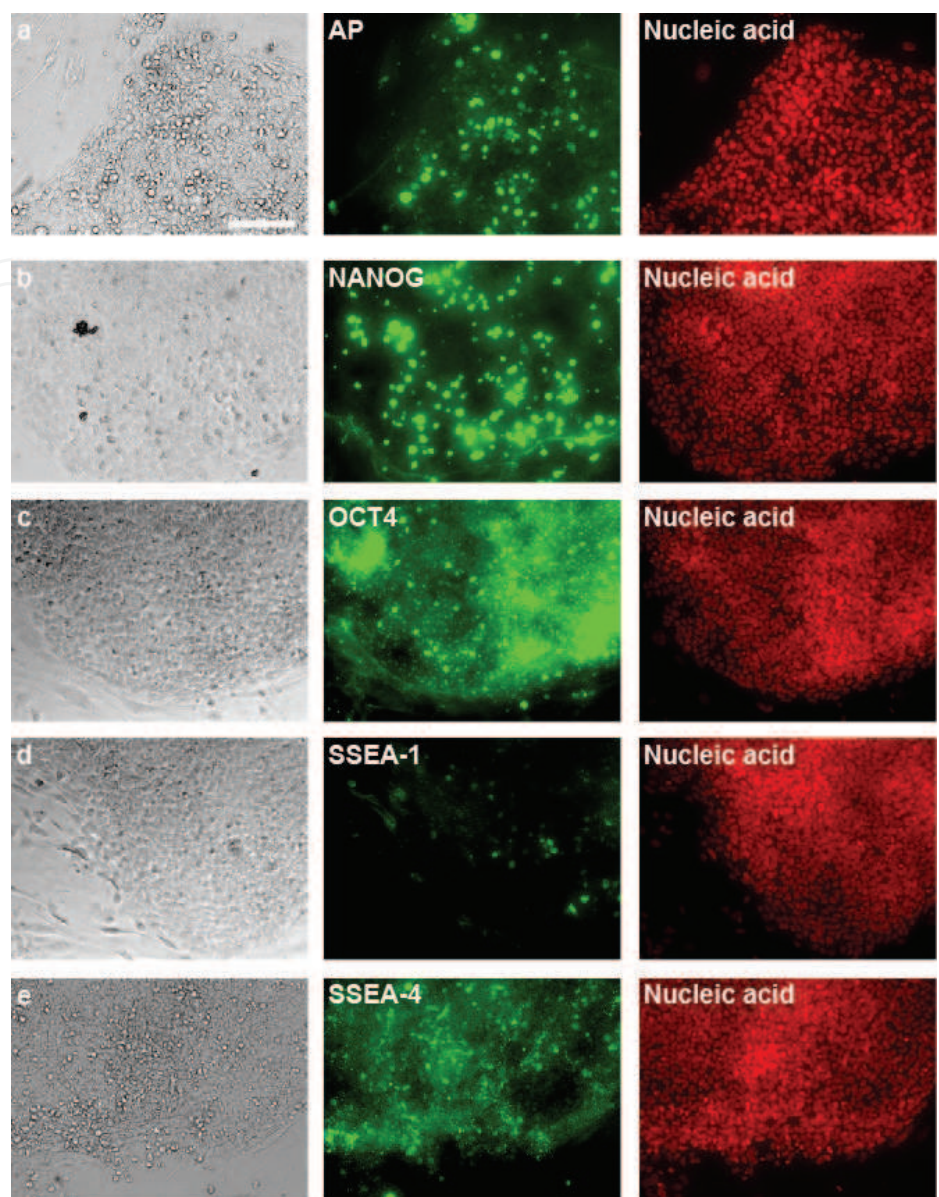
Fig. 4. Characterization of ES-like cells derived from a KS patient by immunostaining. a-e,
Immunostaining of ES-like cells for alkaline phosphatase (AP) (a), NANOG (b), OCT4 (c),
SSEA-1 (d), and SSEA-4 (e). Nuclei were stained with SYTOX^R Orange. Bar=100 μm.

3.5 iPS cells derived from a KS patient have the potential for multipotency in vitro and in vivo

To assess the pluripotency of the iPS cells in vitro, we examined their ability to form
embryoid bodies. After 8 days in floating culture, embryoid bodies derived from the iPS cell
were identified (Fig. 5a).

We next investigated whether the iPS cells derived from a KS patient were pluripotent in
vivo, by transplanting them into the testis of SCID mice. We observed tumor formation in
the testis 12 weeks after the injection. Histological inspection showed that the cells had
differentiated into representatives of all three germ layers in vivo (Fig. 5b).
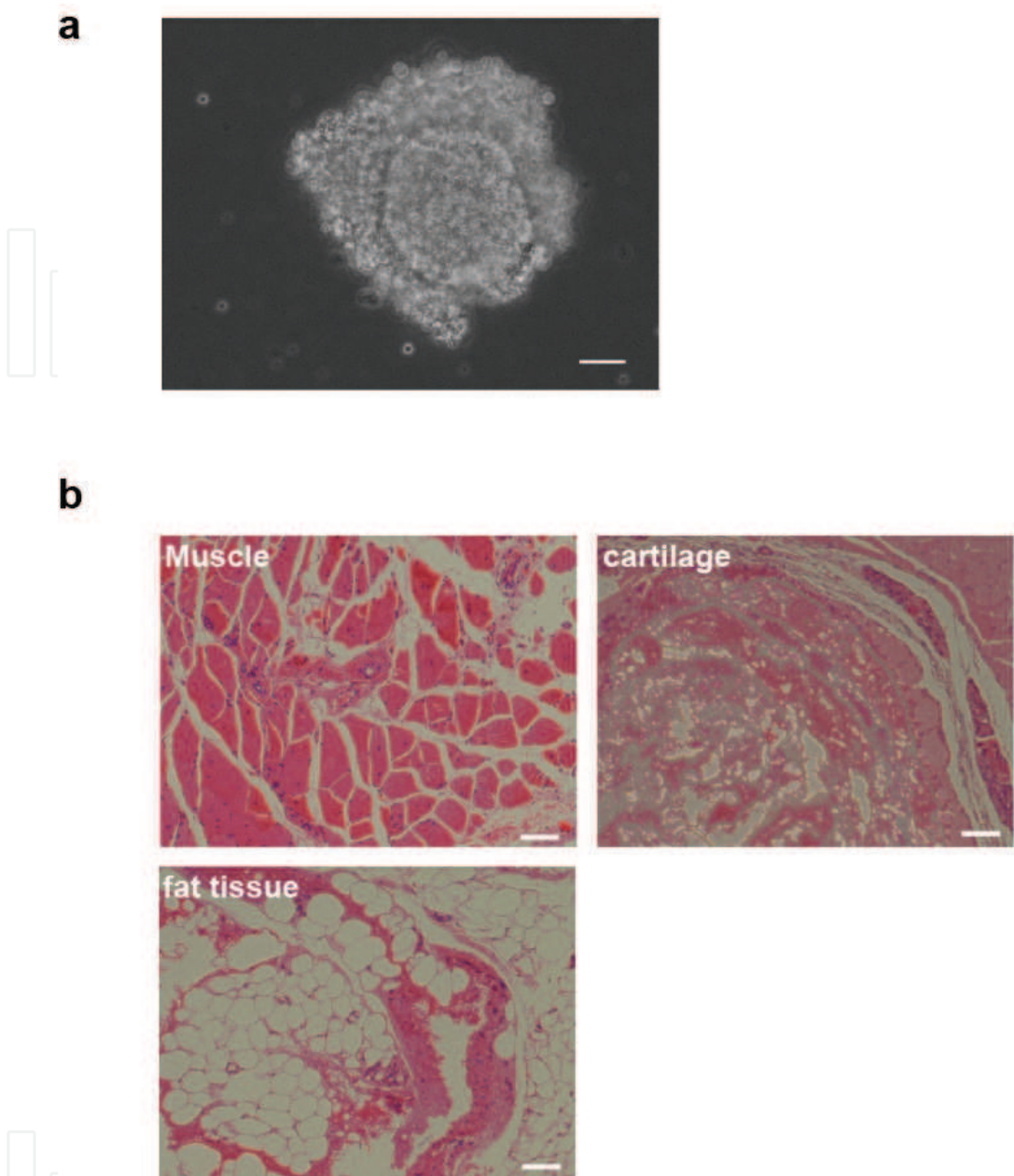
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Fig. 5. Embryoid body formation and teratoma formation from the ES-like cells derived
from a KS patient. a, Embryoid body derived from the iPS cells in vitro. Bars = 30 μm.
b, Hematoxylin and eosin staining of sections of tumors generated from the ES-like cells
derived from a KS patient. Bars = 60 μm.

4. Discussion

Male infertility has increasingly become a major health and social concern worldwide. At
the same time, the development of molecular genetics and stem cell biology methods has led
to major advances in genomic medicine. However, the genetic abnormalities that cause male
infertility are still largely unknown.

Many of the most severe cases of nonobstructive azoospermia are in men with KS, which is
the most common known genetic cause of azoospermia. In 90% of these cases, KS results

from non-mosaic X chromosomal aneuploidy, in which the men carry an extra X chromosome (47, XXY). The other 10% of patients are mosaics who carry a combination of XXY/XY chromosomes (Graham et al., 1988). Approximately half of the Klinefelter's cases are thought to be paternally derived, and recent evidence suggests that KS may be related to advancing paternal age, although this finding is controversial (Jacobs et al., 1988; Lowe et al., 2001).

It is predicted that the aberrant expression of X chromosome-linked genes plays a role in the spermatogenic failure seen in men with 47, XXY (Vawter et al., 2007). However, the abnormal inactivation of the X chromosome in KS patients has not been demonstrated. Therefore, it is important to investigate the molecular mechanisms of X chromosome inactivation in KS, both to predict the extent of reproductive failure and to offer some treatment in the future.

The condition of nonobstructive azoospermia is typically characterized by small-volume testes and elevated follicle-stimulating hormone (FSH). Men with nonobstructive azoospermia have severely deficient spermatogenesis, resulting in so little sperm being produced in the testis that the sperm reach the ejaculate. Typically, a man with nonobstructive azoospermia cannot biologically father a child. Recently, however, microdissection testicular sperm extraction (micro-TESE) has shown promise for enabling nonobstructive azoospermia patients, including those with KS, to father a child (Schlegel, 1999; Schlegel, 2009). However, very little sperm is retrieved from the testis of nonobstructive azoospermia patients using this technique. We are therefore looking for new approaches to treat male infertility, including that due to nonobstructive azoospermia.

The reprogramming of human somatic cell nuclei towards pluripotency was recently achieved by the introduction of four defined factors, generating iPS cells (Lowry et al., 2008; Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007). This remarkable technique enables the generation of patient-specific cells that can be used to model human diseases in tissue culture. We hypothesized that the generation of iPS cells derived from the testicular tissues of infertile men might provide a cell source for individual cell-based therapy that also avoids the ethical concerns associated with the use of stem cells. In support of this possibility, the establishment of pluripotent stem cells from the adult human testis under specific culture conditions was reported (Conrad et al., 2008) (Kossack et al., 2009). However, a later report indicated that the pluripotent stem cells of Conrad et al have a gene-expression profile that is similar to fibroblasts, and not similar to human ES cells (Ko et al., 2010). Therefore, the pluripotency of Conrad and colleagues' stem cells has been called into question. In addition, fibroblasts can be easily established from human testicular cultures (Chen et al., 1975).

Here we successfully generated iPS cells from the testicular tissue of KS patients by expressing four transcription factors in them (*OCT4*, *SOX2*, *KLF4*, and *C-MYC*). The morphology, proliferation, surface markers, and gene expression of these cells were similar to those of human ES cells. In the future, the investigation of iPS cells derived from KS patients may elucidate the mechanisms of infertility in KS patients and lead to a breakthrough in its treatment.

In particular, the X chromosome is the only chromosome in humans where one sex (female) has double the amount of genetic material as the other sex. In animals and hybridoma models, the presence of two active X chromosomes is lethal; in females, one of the X

chromosomes undergoes random X chromosome inactivation (XIC) during embryogenesis, which is executed by a noncoding RNA called X chromosome inactivating transcript (XIST) (Hong et al., 2000). Therefore, since KS is not lethal, it is unlikely that both X chromosomes are active in men with KS, and XIC must occur. Such an abnormal inactivation of the X chromosome, which ordinarily would never happen in men, might be associated with some of the reproductive and cognitive sequelae associated with KS.

Data from several groups led to the assumption that most men with KS are born with spermatogonia (Lin et al., 2004) (Yamamoto et al., 2002) (Wikstrom et al., 2004). However, during early puberty, most likely after the initiation of spermatogenesis, the spermatogonia undergo massive apoptosis due to increased FSH levels in boys with KS (Wikstrom et al., 2004). It was later found that boys with KS have an adequate number of sperm in the ejaculate during early puberty (Paduch et al., 2009). The mechanism by which spermatogenesis is lost from puberty to adulthood is unknown. Three possible mechanisms have been suggested. The first is an intratesticular hormonal imbalance that results in hypersensitivity to the increasing intratesticular testosterone and estradiol concentrations (Paduch et al., 2009). The second is Sertoli cell dysfunction (Paduch et al., 2009). The third suggests that there are defects in spermatogonial stem cell renewal (Paduch et al., 2009). In particular, the abnormal pairing of X and Y chromosomes may lead to the loss of spermatocytes during meiosis (Paduch et al., 2009).

In the future, an understanding of the molecular mechanism governing X chromosome inactivation should help us resolve how male infertility develops and how its treatment might be improved.

5. Acknowledgements

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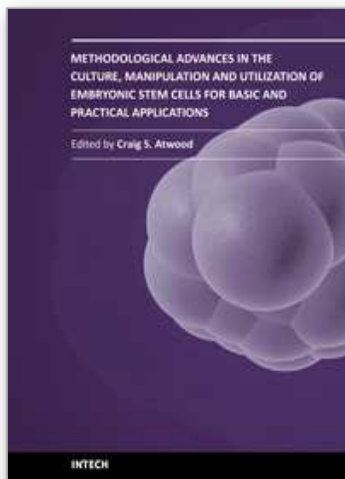
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