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Generation of Haploid Spermatids with Fertilization and Development Capacity from Human Spermatogonial Stem Cells of Cryptorchid Patients

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SUMMARY

Generation of functional spermatids from azoospermia patients is of unusual significance in the treatment of male infertility. Here, we report an efficient approach to obtain human functional spermatids from cryptorchid patients. Spermatogonia remained whereas meiotic germ cells were rare in cryptorchid patients. Expression of numerous markers for meiotic and postmeiotic male germ cells was enhanced in human spermatogonial stem cells (SSCs) of cryptorchidism patients by retinoic acid (RA) and stem cell factor (SCF) treatment. Meiotic spreads and DNA content assays revealed that RA and SCF induced a remarkable increase of SCP3-, MLH1-, and CREST-positive cells and haploid cells. Single-cell RNA sequencing analysis reflected distinct global gene profiles in embryos derived from round spermatids and nuclei of somatic cells. Significantly, haploid spermatids generated from human SSCs of cryptorchid patients possessed fertilization and development capacity. This study thus provides an invaluable source of autologous male gametes for treating male infertility in azoospermia patients.

INTRODUCTION

Male gametogenesis is a process by which spermatogonial stem cells (SSCs) divide and differentiate into haploid spermatids. Any error during male gametogenesis can result in male infertility, which is a major health problem around the world (De Kretser and Baker, 1999). Infertility affects around 15% of couples, and male factors account for 50% (Schlegel, 2009). Azoospermia has been observed in 1% of the general populations and accounts for 10%–15% of male infertility (Jarow et al., 1989; Willott, 1982). Nonobstructive azoospermia (NOA) affects 10% of infertile men, and notably it has been diagnosed in 60% of azoospermic men (Jarow et al., 1989; Matsumiya et al., 1994). Cryptorchidism is one of the most common causes that result in NOA (Sinnar et al., 2011). Severe cryptorchidism could lead to male infertility, since male germ cells (especially haploid spermatids) are significantly reduced or completely lost in cryptorchid testes (Zivkovic et al., 2009). It has been reported that the transition of gonocytes into A_{dark} spermatogonia in cryptorchid testes is impaired (Kamisawa et al., 2012). Therefore, it is of great significance to establish an effective method to induce differentiation of human spermatogonia from cryptorchid testes into haploid spermatids for the treatment of male infertility. Previous studies have been focused on the in vitro models of male germ cell maturation (Tesarik, 2004). However, there is currently no efficient approach for generating haploid spermatids in vitro from spermatogonia of human testes.

Complete spermatogenesis in vitro to obtain male gametes has not yet been achieved in humans, although certain progress has been made in the derivation of male germ cells from mouse or human embryonic stem cells (ESCs) (Aflatoonian et al., 2009; Chen et al., 2007; Clark et al., 2004; Hübner et al., 2003; Kee et al., 2006; Mikkola et al., 2006; Nayernia et al., 2006; Tilgner et al., 2008; West et al., 2008). There are ethical issues obtaining human ESCs, which is a major obstacle for their potential use in the clinic. It has recently been demonstrated that the induced pluripotent stem cells (iPSCs) could generate primordial germ cells and finally haploid spermatids (Easley et al., 2012; Hayashi et al., 2011; Imamura et al., 2010; Park et al., 2009). Of great concern, male germ cells derived from human iPSCs may not be used for treating male infertility due to tumor-forming risks, which result from the reprogramming of somatic cells by gene transfer using viral vectors and their genetic instability. Therefore, more attention has been paid to generating male gametes from human spermatogonia of patients.

It has been suggested that several growth factors, such as bone morphogenetic proteins (BMPs), glia cell line-derived neurotrophic factor (GDNF), stem cell factor (SCF), and retinoic acid (RA), were crucial for the maintenance of normal spermatogenesis in rodents. The SCF/KIT system plays an

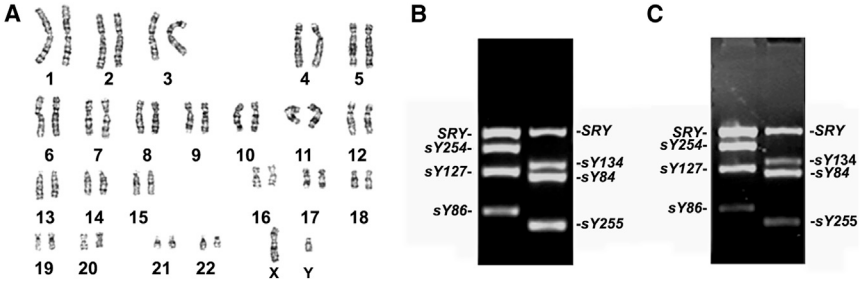

Figure 1. Karyotype and the Completeness of Genomic DNA Sequence of Numerous Y Chromosome Genes in Cryptorchid Patients

(A) Karyotype analysis displaying chromosome karyotype in cryptorchid patients.

(B and C) Multiplex PCR showing the expression of numerous Y chromosome genes, including *SRY*, *sY254*, *sY127*, *sY86*, *sY134*, *sY84*, and *sY255*, in a normal man (B) and in a cryptorchid patient (C).

See also Table S1.

essential role in spermatogonial proliferation, differentiation, survival, and subsequent entry into meiosis (Mithrapabhu and Loveland, 2009), and SCF has been shown to induce mouse spermatogonia to differentiate into round spermatids in vitro (Feng et al., 2000). Furthermore, SCF is required for the proliferation of mouse differentiating spermatogonia, specifically type A_1 to A_4 spermatogonia (Hasthorpe, 2003; Tajima et al., 1994). RA, the active derivative of vitamin A, controls the entry of germ cells into meiosis in both mice and humans (Childs et al., 2011; Ohta et al., 2010). Interestingly, RA could induce the transition of undifferentiated spermatogonia to differentiating spermatogonia and mediates the timing of meiosis by the activation of the SCF/KIT pathway (Pellegrini et al., 2008; Zhou et al., 2008). Therefore, RA and SCF were chosen in this study to induce the differentiation of human spermatogonia from cryptorchid testes. It has been recently reported by our peers and us that human SSCs can be clearly identified and cultured for a short- and long-term period (He et al., 2010; Sadri-Ardekani et al., 2011; Sadri-Ardekani et al., 2009). Round spermatids with unknown function can be derived from mouse spermatogonia (Feng et al., 2002). Nevertheless, the generation of functional haploid spermatids from SSCs in vitro has not yet been achieved in humans. Here, we present molecular and cellular evidence demonstrating the differentiation of human SSCs from cryptorchid patient into cells with phenotypic characteristics, DNA content, and fertilization and development capacity of haploid spermatids. Of unusual significance, our ability to generate human functional haploid spermatids from cryptorchid testes could offer an important source of functional and autologous male gametes for treating male infertility in azoospermia patients.

RESULTS

Cryptorchid Patients Had a Normal Karyotype and Excluded Y Chromosome Microdeletion or Gene Mutation

We first checked the chromosome karyotype and the expression of numerous Y chromosome genes of cryptorchid patients. Karyotype analysis revealed that cryptorchid patients possessed a normal chromosome karyotype (Figure 1A). Multiplex PCR was used to check whether cryptorchid patients had Y chromosome microdeletion. As shown in Figure 1C, numerous Y chromosome genes, including *SRY*, *sY254*, *sY127*, *sY86*, *sY134*, *sY84*, and *sY255*, were detected in cryptorchid patients, which was comparable to the expression of these genes in normal men (Figure 1B), suggesting that cryptorchid patients did not have Y chromosome microdeletion. Mutation analyses using gene sequencing were performed to screen the mutation of *INSL3* (insulin-like 3), *RXFP2* (relaxin/insulin-like family peptide receptor 2), and *AR* (androgen receptor) genes in cryptorchid patients and normal men, and no mutation of those genes was observed (data not shown). Therefore, testicular tissues of these cryptorchid patients were used to induce differentiation.

The clinic data of cryptorchid patients are shown in Table S1 (available online). The levels of testosterone (T) and estradiol (E2) of cryptorchid patients were within the normal ranges. However, both left and right testicular volumes of cryptorchid patients were significantly smaller than those of normal men. The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL) in cryptorchid patients were statistically higher than those of normal men.

Human SSCs Remained whereas Meiotic Male Germ Cells Were Very Rare or Lost in the Testes of Cryptorchid Patients

Histological and immunohistochemical analyses of cryptorchid patients were performed to evaluate the spermatogenesis status of testicular tissues. The testes from obstructive azoospermia (OA) patients due to inflammation but with normal spermatogenesis in vivo served as the controls. Histological examination showed that seminiferous tubules of cryptorchid testes had a reduced tubular diameter and a thickened basement membrane (Figure 2B) compared to the control testes (Figure 2A). There were human spermatogonia along the basement membrane in cryptorchid testes (Figure 2B); however, differentiated