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A method for rapid generation of transgenic animals to evaluate testis genes during sexual maturation

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ABSTRACT

In certain forms of idiopathic infertility, there is failure of follicle stimulating hormone (FSH) and testosterone (T) to initiate spermatogenesis despite the presence of Sertoli cells and germ cells in the testis. In postnatal rats (up to 11 days of age) and infant monkeys (3–4 months old), robust division and differentiation of spermatogonial stem cells is not discerned, even though serum levels of FSH and T are similar to those found during adulthood. Lack of spermatogenesis together with normal hormone levels is a situation similar to that found in certain categories of male infertility. To investigate this intriguing situation, Sertoli cells were cultured from infant and pubertal rats and monkeys and differential gene expression by testicular Sertoli cells was evaluated by DNA microarray using the Agilent microarray system. To determine the role of candidate genes in regulation of spermatogenesis, transgenic animals over-expressing these genes must be generated. However, present techniques for generation of transgenic animals have limited utility for production of several transgenic animals within a short period of time. Therefore, we have developed a technique for making transgenic animals by the testicular route which is less labor intensive and less time consuming. This technique is also ethically superior since fewer mice are required than in existing alternative methods of transgenesis.

1. Introduction

Testicular Sertoli cells have a crucial role in the initiation and maintenance of spermatogenesis (Sharpe, 1994; Griswold, 1998). The tight junctions made by adjacent Sertoli cells generate a barrier which does not allow the blood borne materials to enter into the lumen of seminiferous tubules. This prevents immunological events which may harm the advanced germ cells since several of their proteins appear for the first time in an individual at the time of puberty and hence are antigenic in nature (Turek et al., 2003). Functions of the testicular Sertoli cells are known to be regulated by follicle stimulating hormone (FSH) and testosterone (T), mainly via their receptors expressed in these cells (Baccetti et al., 1998). Expression of several Sertoli cells genes and their products are modulated by these hormones (Sutton et al., 1998; Benbrahim-Tallaa et al., 2002; McLean et al., 2002; Sadate-Ngatchou et al., 2004). Lack of sufficient knowledge regarding Sertoli cells mediated regulation of spermatogenesis has severely hampered the diagnosis and treatment of infertility due to seminiferous tubule dysgenesis.

Approximately 10–15% of couples are affected by infertility. A male factor can be diagnosed in approximately 50% of these and about 30–40% of male infertility has unknown origins (Bhasin et al., 1994). Failure of FSH and T to initiate spermatogenesis in spite of the presence of Sertoli cells and germ cells in the testis of some men is intriguing (Schaison et al., 1993; Nieschlag and Leifke, 1997). To address this issue, it is important to have an appropriate testis model of

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active and inactive spermatogenesis, so that a comparative study of differential gene expression may be undertaken. In developing a workable model, a major challenge is to determine the role of differentially expressed genes through production of transgenic animals for undertaking functional genomic studies (Houdebine, 1997). Present techniques for making transgenic animals are cumbersome and also technically demanding. Additionally, a large number of zygotes must be harvested from mice, which must be killed to produce founders for creating the desired transgenic lines. In this review, an attempt is made to address these two issues of determining differential gene expression in establishing a new user-friendly method for generation of transgenic mice to evaluate important testis genes.

2. The infant testis model

The endocrine activity of the hypothalamic–pituitary–testicular axis, as reflected by the circulating levels of leutinizing hormone (LH), FSH and T in infant rats (0–9 days old) and monkeys (0–4 months old) is similar to that found in pubertal and adult animals. Despite adult-like concentrations of LH, FSH and T in infant male monkeys and rats, the activity of the germinal epithelium is limited to proliferation of undifferentiated Type A spermatogonia; a noticeable number of primary spermatocytes cannot be detected until puberty in monkeys (at 3–3.5 years) and day 15 of age in rats (Plant, 1994; Dym et al., 1995). The lack of initiation of spermatogenesis in the presence of adequate hormones in infants (for example, rats younger than 9 days and monkeys younger than 4 months) is a situation similar to that found in certain categories of male infertility, where hormone treatments fail to induce spermatogenesis in spite of the presence of Sertoli cells and germ cells in the testis. Thus, the infant testis can be used as a surrogate model for research related to infertility primarily due to testicular failure.

One hypothesis to explain this testicular quiescence, despite a robust hormonal drive, is that the infant Sertoli cells fail to transduce the hormonal (FSH and T) signals necessary for the occurrence of spermatogenesis. In response to FSH and T stimulation, pubertal Sertoli cells are presumed to produce factors required by spermatogonia for initiation of their development and differentiation, thereby creating a favorable environment inside the seminiferous tubule. Hence, comparative evaluation of FSH and androgen-mediated gene expression by Sertoli cells from pubertal testis (containing advanced germ cells) and infant testis (containing only spermatogonia) might reveal developmental deficits in FSH and androgen-mediated gene expression in infant Sertoli cells, which might be responsible for the spermatogenic quiescence during infancy.

3. Differential gene expression studies

All animals for this study were kept according to the Guidelines provided by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA) and protocols were approved by the Institutional Animal Ethics Committee. Wistar rats (*Rattus rattus*, 5 and 12-days-old) and rhesus monkeys (*Macaca Mulatta*, 3 months and 1.5–2 year old) were obtained from the Animal Facility of the National Institute of Immunology (New Delhi, India). Since it is difficult to determine the exact age of puberty in primates, juvenile rhesus monkeys (1.5–2 year old) were surgically implanted with catheters via the femoral or internal jugular vein under sterile conditions and treated with pulses of gonadotropin releasing hormone (GnRH, one pulse/3 h) for about a month to induce puberty as described previously (Majumdar et al., 1995a). Within 4–5 weeks of GnRH treatment, circulating levels of T increased and testis size increased by 4-fold or more, and this was associated with the presence of spermatocytes indicating induction of puberty (Devi et al., 2006). From the testis of infant and pubertal rats and monkeys, Sertoli cells were isolated and cultured according to previously described procedures (Majumdar et al., 1995b, 1998). On day 3 of rat as well as monkey Sertoli cells culture, residual germ cells were removed by hypotonic shock. Rat Sertoli cells were treated with recombinant ovine FSH (oFSH, 50 ng/ml) and T (10^{-7} M) for about 12 h. At the end of treatment, when 5-day-old rat Sertoli cells were of 9 days postnatal age on day 4 of culture, and 12-day-old rat Sertoli cells cultures were of 16 days postnatal age on day 4 of culture, the cells were added to RNAlater (Qiagen, USA) to prevent degradation of RNA, before extraction of RNA. After similar experimentation using Sertoli cells from pubertal and 3-month-old monkeys, cells were treated with RNAlater for the extraction of RNA. For monkey studies, recombinant monkey FSH (5 ng/ml) was used instead of oFSH.

Age-specific genes expressed by the Sertoli cells from rats and monkeys (infant and pubertal) were determined by DNA microarray analysis using Agilent rat arrays and human arrays (for monkeys) respectively. About 4700 genes were up-regulated and 3500 genes were down-regulated in Sertoli cells cultured from 12-day-old rats as compared to that of those cultured from 5-day-old rats. Similarly about 1300 genes were up-regulated and more than 1000 genes were down-regulated in pubertal monkey Sertoli cells as compared to those in infant Sertoli cells. Several important genes including the FSH receptor, glial derived neurotropic factor (GDNF), octamer transcription factor-1 (OCT-1) and wingless type 4 (Wnt4) were up-regulated in spermatogenically active Sertoli cells.

Genes of interest which show more than a 2–3-fold up- or down-regulation may either be recovered from amplification of genomic DNA or procured commercially (as cDNA clones) for the purpose of generating transgenic animals. A gene which is up-regulated in pubertal Sertoli cells can be ligated to the promoter of a gene which is active in infant Sertoli cells, so that pubertal genes can be over-expressed in infants and vice versa. This would help in evaluating the functions of candidate genes by determining if a gene specifically expressed during puberty can induce spermatogenesis in infants or a gene specifically found during infancy can limit spermatogenesis in the pubertal animals. Although this strategy is very attractive, it is possible only when one can efficiently generate several transgenic animals within a short period of time.