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Murine Germ Cells Do Not Require Functional Androgen Receptors to Complete Spermatogenesis Following Spermatogonial Stem Cell Transplantation

DANIEL S. JOHNSTON, LONNIE D. RUSSELL, PATRICK J. FRIEL AND MICHAEL D. GRISWOLD[1]

School of Molecular Biosciences and Center for Reproductive Biology, Washington State University (DSJ, PJF, MDG), Pullman, WA 99164 and Department of Physiology, School of Medicine, Southern Illinois University (LDR), Carbondale, IL 62901

ABSTRACT The spermatogonial stem cell transplantation technique was employed to determine if murine germ cells require functional androgen receptors to complete qualitatively normal spermatogenesis. Testicular cells from testicular feminized mice were injected into the seminiferous tubules of azoospermic mice expressing functional androgen receptors. Recipient testes were analyzed between 110 and 200 days following transplantation. Multiple colonies of complete and qualitatively normal donor-derived spermatogenesis were seen within the seminiferous tubules of each recipient testis, demonstrating that murine germ cells do not require functional androgen receptors to complete spermatogenesis.

Introduction

Androgens are absolutely required for normal spermatogenesis in mammals. The initiation and maintenance of normal spermatogenesis are dependent on androgen action (1,2). While the effects of androgens on spermatogenesis are well documented, the precise cellular distribution of the androgen receptors (ARs) within the testis, and thus the sites of direct androgen action, remain unclear.

There is general agreement that ARs are present in Leydig cells, Sertoli cells and peritubular cells of mammalian testes (3). However, the expression of ARs within germ cells remains controversial. There are numerous reports supporting the presence (4-8) and absence of ARs (3, 9, 10) within the germ cells of mammalian testes. At present, there are mixed results as to whether ARs are expressed within mammalian germ cells and thus, the question remains as to whether the direct action of androgens on germ cells is required for spermatogenesis.

The goal of this study was to determine whether murine germ cells require ARs for normal spermatogenesis. The spermatogonial stem cell transplantation technique was employed to investigate if testicular germ cells from testicular feminized mice (*Tfm*), which do not express functional ARs, could complete spermatogenesis within the seminiferous tubules of azoospermic mice expressing functional ARs.

Tfm mice are androgen insensitive due to a point deletion within the coding region of the AR that leads to premature translation termination and an inactive receptor (11). The AR is located on the X chromosome in mice, and thus males receiving an X-chromosome containing the mutated AR gene are affected with the *Tfm* condition while females with one copy are carriers for the condition (12). The sterility associated with the *Tfm* condition in males prevents the production of androgen insensitive females animals carrying two mutated ARs. *Tfm* animals fail to undergo masculinization *in utero* and genetic males carrying the *Tfm* mutation have female genitalia, lack Wolffian duct structures and do not respond to pharmacological doses of testosterone (13,14). *Tfm* males have small abnormal testes that contain a few germ cells (15). A small percentage of germ cells make it to the first meiotic division (16) and the most advanced germ cells are arrested at the first meiotic division (15).

Correspondence: Griswold@mail.wsu.edu
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Materials and Methods

Animals. Protocols for the use of animals in these experiments were approved by the Washington State University Animal Care and Use Committee and in accord with the National Institutes of Health standards established by the Guidelines for the Care and Use of Experimental Animals. W/W^v mice were purchased from The Jackson Laboratory. C57BL/6 *Tfm* mice were obtained from a breeding colony raised at Washington State University from progenitors purchased from The Jackson Laboratory. *Ta*+/+*Tfm* heterozygous females were used in all matings. The hair-structure marker Tabby (*Ta*) facilitates detection of mutant and nonmutant phenotypes. The *Tfm* condition was verified *post mortem* by the absence of all male and female reproductive organs except the testes and vagina (12).

Tfm mice expressing *the Eschericia coli lacZ* structural gene were produced by mating *Ta*+/+*Tfm* heterozygous females with homozygous B6,129 Ros*a* 26 male mice. Rosa 26 mice express the *lacZ* structural gene in nearly all cell types, including cells at all stages of

spermatogenesis. Male offspring from these matings expressing the *Tfm* condition were designated *Tfm*/Rosa, and expressed the *lacZ* structural gene in a manner similar to homozygous Rosa 26 animals. In these studies, identification of spermatogenesis derived from *Tfm*/Rosa germ cells was authenticated by expression of the reporter gene and subsequent staining with X-Gal.

Preparation of donor germ cells. Donor testicular cells were isolated from approximately 2-5 month old *Tfm* or *Tfm/Rosa* males. Preparation of germ cells was essentially as previously described (17), with the exception that more vigorous pipetting was required. When a single cell suspension was obtained the cells were counted using a hemacytometer, pelleted by centrifugation at 600 X g for 5 minutes and suspended in injection media (18) at a concentration of 6.8 million cells/ml. Prior to injection, the viability of the cells was determined by trypan blue exclusion. In all cases, viability of the cells was greater than 90%.

Recipients. Two to five month old homozygous mutant W/W^v animals were used as recipients for the *Tfm* germ cells. B6,129 male mice were treated with 40 mg/kg of the chemotherapeutic agent busulfan at 4-6 weeks of age (19) and used as recipients of *Tfm*/Rosa testicular cells 4-6 weeks thereafter. At this dose busulfan destroys nearly all endogenous spermatogonial stem cells, thereby abolishing spermatogenesis and creating space on the basal surface of the tubules for transplanted stem cells to seed and develop.

Spermatogonial stem cell transplant technique. The transplantation technique used in these studies has been previously reported by Boettger-Tong et al. (17). During these studies, between 55-80% of the surface tubules were filled during each injection. The volume injected into each recipient W/W^v testis was approximately 3 µl. Approximately 7 µl was injected into each B6,129 recipient testis. In most cases the uninjected contralateral testis served as the negative control for these experiments.

Histological analysis of recipient testes. Recipient males were sacrificed 110-200 days following transplantation. For recipient W/W^v testes, the tunica was removed from the testis and fixed in 4% paraformaldehyde in PBS, pH 7.4 for 12 hours at 4°C. Subsequently, tissues were equilibrated in 70% ethanol at 4°C before imbedding in paraffin. Five µm sections were prepared, stained with hematoxylin and eosin and observed under Olympus SZX12 and Nikon Microphot-FX microscopes under brightfield illumination. For B6,129 recipient animals, the tunica was removed and the testis was immersed in 4% paraformaldehyde in PBS, pH 7.4 for 2 hr at 4°C. The testes were washed 3X for 30 min in *lacZ* rinse buffer [0.2M sodium phosphate, Ph 7.3, 2 mM magnesium chloride, 0.02% NP-40, 0.01% sodium deoxycholate] at 4°C with gentle agitation. The testes were stained in *lacZ* rinse buffer supplemented with 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide and 1 mg/ml X-Gal for 18 hours at 34°C. The tissues were equilibrated in 70% ethanol.

Results

Analysis of resulting Tfm derived spermatogenesis in W/W^v recipients. Donor cells isolated from the testis of *Tfm* mice were injected into 7 recipient W/W^v testes. Recipient testes were analyzed between 110 and 200 days following transplantation. In each case, donor derived spermatogenesis was observed in the seminiferous tubules of the recipient. Approximately 6% of the sectioned recipient tubules showed transplanted spermatogenesis. The characteristic arrangement of the successive stages of sperm cell differentiation was normal. Numerous released spermatozoa were observed in the lumen of some seminiferous tubule sections (Fig. 1). Sperm morphology, including nuclear positioning, flagellum morphology and acrosomal formation appeared normal on all developing and released sperm. Furthermore, appropriate cycle and wave patterns were observed.

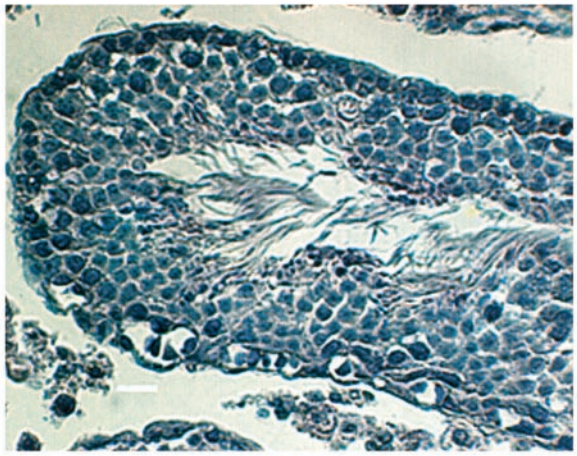

Fig 1. Spermatogenesis derived from transplanted *Tfm* germ cells. Testicular germ cells from a C57BL/6 *Tfm* animal were transplanted into the testis of a 3-month-old W/W^v recipient. The recipient testis was collected 200 days after injection and processed as described in Materials and Methods. Scale bar represents 500µm.

Although W/W^v animals are sterile due to a defect in the *c-kit* receptor, a few endogenous germ cells are typically found in the testes of these animals (20, 21). To verify that observed spermatogenesis in the W/W^v recipients was *Tfm* derived, seminiferous tubules containing complete spermatogenesis were collected from slides and subjected to genomic PCR analysis using a primer pair specific for

murine androgen receptor. The primer pair was designed so that the resulting PCR product spanned the region containing the point mutation at bp 1162 (11). When the PCR products were subcloned and sequenced, six of the ten clones contained insert derived from *Tfm* genomic DNA (data not shown). Clones derived from wild-type AR DNA are believed to be derived from the recipient's Sertoli cells, Leydig cells, macrophages and peritubular myoid cells.

Analysis of resulting Tfm/Rosa derived colonization in B6,129 recipients. Donor cells isolated from *Tfm*/Rosa mice were injected into approximately 15 recipient B6,129 testes. Recipient testes were analyzed between 110 and 200 days following transplantation. X-gal staining of recipient testes showed numerous colonies of *Tfm*/Rosa derived cells in the seminiferous tubules on the surface of the testes. In general, the number of donor-derived colonies was greater than typically observed following analysis of transplantation of wild-type Rosa 26 testicular cells. A representative B6,129 recipient testis is shown in Figure 2.

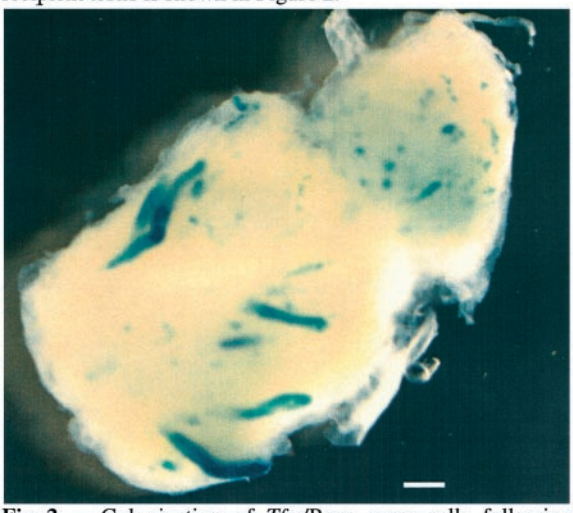

Fig 2. Colonization of *Tfm*/Rosa germ cells following spermatogonial stem cell transplantation. Testicular germ cells from a three-month-old *Tfm*/Rosa animal were transplanted into the testis of a busulfan-treated B6,129 animal. The recipient testis was collected approximately 150 days after injection, fixed and stained with X-Gal as described in Materials and Methods. Blue regions of tubules indicate areas of spermatogenesis derived from transplanted *Tfm*/Rosa germ cells. Scale bar represents 20 μm.

Discussion

The results in this paper demonstrate that androgens do not directly regulate the genes within murine germ cells that are required for proliferation, differentiation, meiotic competence or spermiation. It can be assumed that any effects of testosterone and its derivatives on germ cells are mediated via communication from the testicular somatic cells. Given the nature of the intimate relationship between Sertoli cells and developing germ cells, it is most widely assumed that Sertoli cells are responsible for mediating the effect of androgens in the spermatogenic process, although a peritubular influence can not be discounted.

Often when seminiferous tubules (21,22) or germ cells (19) have been transplanted from mutant to normal animals, the reciprocal transplant has also been performed. Typically, transplantation in one direction will permit normal spermatogenesis while spermatogenesis in the reciprocal transplant will fail, verifying the point of failure. In this case, however, performing the reciprocal transplant was not possible, as the highly abnormal *Tfm* testes are not amenable to injection. Since all Wolffian duct derived structures are absent, no gubernaculum is present and the testes are restricted to the abdomen. Also, *Tfm* testes do not have a rete and the seminiferous tubules contain no lumen, making injection of donor germ cells into *Tfm* seminiferous tubules impossible.

One or two areas of complete spermatogenesis were noted in each W/W^v recipient testis. Due to the small size of the W/W^v testes and the limited volume that they can hold, we estimate that approximately 23,000 *Tfm* testicular cells were injected into each recipient testis. Therefore, the degree of colonization observed was greater than that observed when normal adult animals were used as donors (24). This finding was supported by the observation that an unusually large number of *Tfm*/Rosa colonies were observed on the surface of the recipient B6,129 testes as compared to analyses of recipient testes transplanted with equal numbers of wild-type Rosa 26 cells for equal periods of time. This is likely due to the fact that advanced germ cells are not present in the *Tfm* testis and a high percentage of the injected cells were actually stem cells. This hypothesis is consistent with the report of Shinohara and Brinster showing that when germ cells were isolated from the testes of cryptorchid animals and used as donors, the number of donor derived colonies increased dramatically (25).

In 1975 Lyon et al. (15) proposed the hypothesis that germ cells do not require the direct action of androgens for proper development. Male chimeric mice were produced from *Tfm* and normal mice by embryo aggregation and demonstrated to be fertile. More importantly, some of the offspring were derived from sperm carrying the *Tfm* mutation, showing that in the context of the chimeric animal male germ cells do not require the direct action of androgen to complete spermatogenesis or for fertility. However, due to the close contact between *Tfm* and wild-type germ cells, the possibility remained that that the normal germ cells supported the mutant germ cells in some manner. The studies reported here demonstrate unequivocally that in the complete absence of wild-type germ cells, germ cells lacking functional ARs can repopulate an azoospermic testis and complete qualitatively normal spermatogenesis.

A key assumption in our strategy was that the donor *Tfm* testes would contain stem cells. The results of this study show conclusively that stem cells are present within *Tfm* testes and that following transplantation into a normal androgen responsive environment, the stem cells proliferate

and develop qualitatively normal spermatogenesis in a manner analogous to wild-type stem cells.

While these data do not prove that germ cells cannot respond directly to androgen in normal mice, they clearly demonstrate that there is no requirement for the direct action of testosterone on germ cells during the process of murine spermatogenesis.

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