Increased male reproductive success in Ts65Dn “Down syndrome” mice

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Abstract The Ts65Dn mouse is trisomic for orthologs of about half the genes on Hsa21. A number of phenotypes in these trisomic mice parallel those in humans with trisomy 21 (Down syndrome), including cognitive deficits due to hippocampal malfunction that are sufficiently similar to human that “therapies” developed in Ts65Dn mice are making their way to human clinical trials. However, the impact of the model is limited by availability. Ts65Dn cannot be completely inbred and males are generally considered to be sterile. Females have few, small litters and they exhibit poor care of offspring, frequently abandoning entire litters. Here we report identification and selective breeding of rare fertile males from two working colonies of Ts65Dn mice. Trisomic offspring can be propagated by natural matings or by in vitro fertilization (IVF) to produce large cohorts of closely related siblings. The use of a robust euploid strain as recipients of fertilized embryos in IVF or as the female in natural matings greatly improves husbandry. Extra zygotes cultured to the blastocyst stage were used to create trisomic and euploid embryonic stem (ES) cells from littermates. We developed parameters for cryopreserving sperm from Ts65Dn males and used it to produce trisomic offspring by IVF. Use of cryopreserved sperm provides additional flexibility in the choice of oocyte donors from different genetic backgrounds, facilitating rapid production of complex crosses. This approach greatly increases the power of this important trisomic model to interrogate modifying effects of trisomic or disomic genes that contribute to trisomic phenotypes.

Introduction

The discovery by LeJeune et al. (1959) that individuals with Down syndrome (DS) inherit three copies of the G group chromosome now known as chromosome 21 (Hsa21) established the basis for subsequent research in this area. The advent of molecular biology and of human gene mapping provided information about the genes on Hsa21 at the time when developments in mouse embryology (i.e., transgenic mice) provided the opportunity to examine gene dosage effects on all aspects of mammalian function. By localizing the positions of genes across different mammalian genomes, comparative mapping identified genes from Hsa21 on mouse chromosomes (Mmu) 16, 17, and 10 (Reeves et al. 2001).

Gene mapping led to the production of transgenic mice with extra copies of individual genes mapped to Hsa21 or homologous regions of the mouse genome (Groner 1995). Early on, however, it was not clear that gene dosage would have effects in mice analogous to those in human beings. Early work in mice with trisomy for all of Mmu16 initiated thinking in this direction (Gropp et al. 1975); but while this model inspired further developments in the field, the Ts16 mouse does not represent a genetic model of trisomy 21 and is no longer utilized as such.

However, the fundamental question of the existence of “mouse Down syndrome” became addressable by the transformative experiments of Muriel Davison beginning...
in the late 1980s. Using a bold experimental approach, she produced aneuploid mice carrying additional large segments of chromosomes resulting from induced translocations (Davisson et al. 1990). The chromosomes involved were defined by G-banding and those that involved distal Mmu16 were assessed using somatic cell genetic approaches to map breakpoints precisely (Davisson et al. 1993). Hundreds of interesting translocations were produced, but the “crown jewel” of these was unquestionably the Ts65Dn mouse, in which a reciprocal translocation between the distal end of Mmu16 and the pericentromeric region of Mmu17 resulted in a small marker chromosome, T65Dn. This marker can segregate onto an otherwise normal karyotype as an extra chromosome, producing Ts65Dn mice which have 41 chromosomes (and 41 centromeres) resulting in trisomy for more than 100 genes that are found on Hsa21. This mouse made it possible to ask whether a trisomic mouse has phenotypes corresponding to those in DS, the answer to which was a resounding affirmative (Reeves et al. 1995).

This initial characterization of Ts65Dn included the first demonstration of hippocampal-based deficits in this model, a finding quickly confirmed by other prominent investigators around the world (Coussons-Read and Crnic 1996; Escorihuela et al. 1995; Holtzman et al. 1996). Indeed, this specific finding inspired the development of tests for hippocampal function in children with DS (Pennington et al. 2003). Ts65Dn has become the standard model for DS research, providing insights into diverse aspects of DS phenotypes, including brain development and function, development of neural crest-derived structures including the skeleton of the face and mandible, and resistance to cancer in people with DS to name just a few (Moore and Roper 2007). The model is sufficiently robust that it has recently been used to discover potential therapeutic approaches to amelioration of cognitive deficits specific to DS (Reeves and Garner 2007; Salehi et al. 2007).

Like all models, Ts65Dn does not perfectly reflect the human condition. First, orthologs of only about half of the genes from Hsa21 are trisomic in these mice and a number of genes from Mmu17 that are not orthologous to Hsa21 are present in the T65Dn marker chromosome (Gardiner et al. 2003). These trisomic mice cannot be fully inbred and so Ts65Dn is maintained as an advanced intercross between the C57BL/6 (B6) and C3H strains. From a practical standpoint, the model is limited by very difficult husbandry. Males are generally sterile so that the line has been propagated only through females since its development. As with all mice, litters are variable in size, but Ts65Dn have smaller litters than euploid, and while the frequency of the extra chromosome is Mendelian at midgestation, perinatal attrition occurs so that litters include only about 25–35% trisomic pups at weaning (Roper et al. 2006; Williams et al. 2008). Ts65Dn females are poor mothers and approximately 40% of litters are abandoned or otherwise produce no survivors (Moore 2006; Williams et al. 2008). Thus, it is often not possible to generate sufficient siblings to carry out experiments in the most closely related, age-matched mice. These issues make it necessary to maintain large colonies to produce sufficient mice, make it difficult to breed multiple mutations (or even one) onto the trisomic background, limit procedures requiring induced ovulation, e.g., for the production of embryos from which to develop embryonic stem (ES) cells, and pose an overall practical limit to applications for the model.

Methods

Mice

Female B6EiC3Sn-a/a-Ts(1716)65Dn mice (herein Ts65Dn) were obtained from the Jackson Laboratory (JAX) (http://jaxmice.jax.org/strain/001924.html) and maintained by crossing females to male (C57BL/6J x C3H/HeJ)F1 mice (B6C3). In some cases, male B6EiC3Sn-a/a were used as fathers. Trisomic mice and ES cell lines were identified by qualitative PCR and fluorescence in situ hybridization (FISH) (Lorentzi et al. 2010; Moore et al. 1999). Institute of Cancer Research (ICR) strain mice were obtained from Taconic Farms, Inc. Mice for these experiments were generated and housed at Franklin and Marshall College (F&M) or at Johns Hopkins School of Medicine (JHU). All procedures were approved by Institutional Animal Care and Use Committees.

Male offspring were singly housed with females upon weaning and fertile males were identified by production of litters. Male Ts65Dn mice produced from trisomic females, from trisomic males by IVF, or from trisomic males by natural mating were screened for fertility for 3 or more months. Fertile trisomic mice of both sexes were incorporated into our breeding colonies.

Cryopreservation of sperm

Several protocols were attempted and the highest-efficiency preservation of Ts65Dn sperm was accomplished using the JAX cryopreservation service protocol which is based on that of Ostermeier et al. (2008). Sperm concentration was increased with trisomic males compared to the amounts for euploid mice as described below. Briefly, sperm was recovered from one or two males, as described below, in cryoprotective medium, 18% raffinose (w/v) (Sigma R7630), 3% skim milk (w/v) (BD Diagnostics 232100), and 477 mM monothioglycerol (Sigma M6145)
in culture-grade water. After 10 min, the dish was swirled gently to equilibrate distribution. Sperm was stored in French straws (IMV AAA201) and placed in liquid nitrogen vapor phase for 10 min in a cooling device sufficient to achieve a cooling rate of approximately 37°C/min. After 10 min, straws were plunged into liquid nitrogen for storage.

In vitro fertilization

Superovulation of female donor mice was accomplished by giving 5 IU Pregnant Mare Serum Gonadotropin (PMS, CalBiochem #367222) via intraperitoneal (IP) injection about 60 h prior to the IVF. This was followed about 48 h later by IP injection of 5 IU Human Chorionic Gonadotropin (HCG, CalBiochem #230734). Sperm preparation was modified from standard protocols to optimize conditions for Ts65Dn donors. Fertilization dishes were prepared with four to five drops (250 μl) of Research Vitro Fert (RVF, K-RVFE-50 Cook Medical) on 60-mm culture dishes (per dish—1 drop per 4 females). Drops were covered with embryo-tested mineral oil and placed in a 5% CO2 incubator to equilibrate (≥20 min). Culture dishes were prepared by placing 50-μl drops of RVF on 60-mm culture dishes and covered with embryo-tested mineral oil. Plates were placed in a 5% CO2 incubator until needed. Fresh sperm was collected 12 h after females were primed by HCG injection. The donor male was euthanized and the caudal epididymides and vas deferens were removed and placed in a 35-mm culture dish containing 1 ml RVF per male. Using a 28-g insulin syringe and a pair of fine forceps, six cuts were made in the epididymides. The vas deferens was progressively squeezed with syringe and forceps to express the sperm. The dish with sperm was placed on a 37°C slide warmer for 10 min to allow the sperm to swim out. Ten-microliter aliquots of sperm were transferred to each drop of the fertilization dish and cultured with embryo-tested mineral oil. Plates were placed in a 5% CO2 incubator until needed. Fresh sperm was collected 12 h after females were primed by HCG injection. The donor male was euthanized and the caudal epididymides and vas deferens were removed and placed in a 35-mm culture dish containing 1 ml RVF per male. Using a 28-g insulin syringe and a pair of fine forceps, six cuts were made in the epididymides. The vas deferens was progressively squeezed with syringe and forceps to express the sperm. The dish with sperm was placed on a 37°C slide warmer for 10 min to allow the sperm to swim out. Ten-microliter aliquots of sperm were transferred to each drop of the fertilization dish and cultured in a 5% CO2 incubator. After fertilization, dishes were returned to the incubator and two-cell embryos were transferred to recipient mothers the following day. Introduction of two-cell embryos to prepared recipients was carried out using standard procedures as described (Hogan et al. 1994).

Development of ES cells

Following IVF and progression to the two-cell stage, embryos that were not transferred to pseudopregnant mothers were cultured in a 5% CO2 incubator for 3 or more days in 50-μl drops of M16 embryo culture medium (Millipore MR-010-D) under embryo-tested mineral oil. Blastocysts were removed and cultured on gelatin-coated 4-well dishes without feeder cells for 4–5 days in ES media which is high-glucose DMEM, 15% fetal calf serum, 1× MEM nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 100 mM β-mercaptoethanol (Sigma) containing 0.5 unit/ml ESGRO (Millipore) and 36 μM PD98059 (Sigma). PD98059 was used only during the initial 4–5 days of culture. Outgrowths of each inner cell mass were isolated and trypsinized in 20-μl drops of 0.25% trypsin under oil at 37°C for 10 min and then dispersed with a finely drawn glass pipette and transferred to a well of a 96-well plate containing mitomycin C-treated mouse embryonic fibroblast feeder cells and ES media. ES cell colonies appeared in 2–5 days. They were isolated based on morphology, plated on fibroblast feeder cell layers, and expanded in ES media containing 0.5 unit/ml ESGRO.

Results

Rare Ts65Dn males are fertile

When first characterized, Ts65Dn males were believed to be infertile (Davison et al. 1993) as are males who have Down syndrome. While there are anecdotal reports of fertile Ts65Dn males, the frequency is low and has not been established precisely. It is not unexpected that males with an extra centromere, as occurs due to the presence of the T65Dn marker chromosome, would be infertile; however, at least some of the reduced fertility is apparently due to the trisomic genes involved. A version of Ts65Dn exists in which the small marker chromosome is involved in a Robertsonian translocation to Mmu12. This mouse, called Ts[Rb(12.1716)]2Cje, has the same gene dosage imbalance as Ts65Dn but a normal number of centromeres (Villar et al. 2005). The original report indicated that males were fertile but that only 1–5% of their progeny were trisomic. Different frequencies for transmission of both Ts2Cje and Ts65Dn have been observed in different colonies, but greatly reduced male transmission of extra genetic material is a characteristic of both strains (RR and CM, unpublished observations).

In the course of aging mice for various studies, young males were housed with females from about the time of weaning, with occasional litters resulting (in CM’s colony). Several males were identified that fathered multiple litters, transmitting the T65Dn marker chromosome to a subset of progeny. Over the course of 4 years, we produced a total of 453 progeny from natural matings of fertile Ts65Dn males, including similar numbers of males and females (Table 1). Litter sizes at weaning were similar, with Ts65Dn males producing 5.4 pups per litter (54 litters total) and Ts65Dn females producing on average 4.9 pups per litter (140 litters) (p = 0.136). However, about two of every five litters born to Ts65Dn females is abandoned or killed, while we
saw little or no loss of litters among the euploid mothers. Of the litters from trisomic males, 28% of offspring were trisomic at weaning, as expected for Ts65Dn progeny (Moore et al. 2006; Roper et al. 2006).

Sperm from fertile Ts65Dn males is effective for IVF In addition to male infertility, generation of Ts65Dn mice is hampered by husbandry concerns. IVF offers the possibility of using euploid surrogate mothers with good husbandry characteristics. Standard procedures were used to prepare sperm from Ts65Dn males and, in general, fewer motile sperm were evident. Several different dilutions were used for the initial IVF procedure. Optimal results were obtained when sperm were recovered from two epididymides/vas deferens placed in 1 ml of human tubal fluid (HTF) medium, which was then diluted further to a concentration two to three times higher than is standard for euploid strains for fertilization, i.e., 10–15 μl per 250 μl of HTF medium instead of the 5 μl that would be used from euploid males. The yield is variable based on concentration and volume of sperm obtained, but in general six to ten straws are obtained. Each can be used for IVF in which the number of progeny is limited primarily by the number of oocytes obtained and the number of surrogate mothers into which they are transferred.

Oocytes were prepared from superovulated B6C3 females and incubated with prepared sperm for 4 h, washed, and incubated overnight using standard procedures (see Methods). Two-cell embryos were transferred to pseudopregnant ICR females, which bear large litters and provide excellent husbandry. In three separate rounds of IVF using fresh sperm and 30–40 B6C3F1 oocyte donors per round, a total of 53 embryo transfers were performed. Thirty-eight litters were identified resulting in 271 pups (7.1 pups per litter on average) (Table 1). There were no recorded losses of whole litters. Although we have not sought to recover the maximum possible number of pups (by maximizing the number of superovulated females), one round of IVF resulted in 153 pups. There was no significant sex bias (55% male) and 31% were trisomic at weaning as expected from natural matings of Ts65Dn (Roper et al. 2006; Williams et al. 2008).

Sperm freezing allows further flexibility for IVF Cryopreservation of sperm is an important colony management tool and also provides experimental flexibility, e.g., for rapid expansion of colonies. We first used a conventional cryopreservation procedure that has proven effective for a number of mixed genetic backgrounds to freeze Ts65Dn sperm (Sztein et al. 1997). However, few motile sperm were recovered after thawing and no two-cell embryos were produced. It has long been known that the success rates for recovery of frozen sperm are strain dependent. Historically, B6 has provided poor recovery of frozen sperm, but several procedures have recently reported success with this strain (Ostermeier et al. 2008). JAX has developed a robust procedure for cryopreservation of sperm from a number of strain backgrounds and this technique proved to be efficacious to cryopreserve and recover Ts65Dn sperm.

As we recovered fewer motile sperm from Ts65Dn, we modified the protocol to provide a threefold increase in concentration for cryopreservation. In the two-cell test, 80% of B6C3 oocytes fertilized with sperm that had been frozen progressed to the two-cell stage. We performed a small test IVF with an aliquot of cryopreserved sperm to produce three litters, including three male and three female trisomic Ts65Dn mice. This was a single small proof-of-concept experiment, but scale-up is readily accomplished simply by superovulating more females to produce additional oocytes and transferring to more recipient mothers. The ability to cryopreserve sperm further expands flexibility for genetic experiments.

Embryonic stem cells from Ts65Dn mice Availability of ES cells for Ts65Dn has been a community goal for several years. Attempts to create Ts65Dn ES cells by a conventional route, i.e., timed pregnancy of superovulated Ts65Dn females with recovery of blastocysts that can then be propagated in culture, have proven to be problematic. Superovulation works poorly in this strain so there is a very low production of embryos. Sacrifice of a large number of Ts65Dn females is problematic as these represent the breeding stock for the colony. With the production of hundreds of embryos by IVF, a large number of blastocysts could be recovered for production of ES cells. Ten ES lines were recovered and karyotyped. All had a normal chromosome complement plus or minus the T65Dn marker chromosome. Two of the ten lines that we

Table 1 Characteristics of offspring from natural mating and IVF with male Ts65Dn

|          | No. of Ts65Dn | No. of euploid | % male | % trisomic |
|----------|---------------|----------------|--------|------------|
| Natural  |               |                |        |            |
| Female   | 59            | 175            | 48.3   | 28.3       |
| Male     | 69            | 150            |        |            |
| Total    | 128           | 325            |        |            |
| IVF      |               |                |        |            |
| Female   | 35            | 87             | 55.0   | 31.0       |
| Male     | 49            | 100            |        |            |
| Total    | 84            | 187            |        |            |

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characterized (16.7%) were trisomic derivatives of Ts65Dn (Fig. 1).

Ts65Dn mice generated from males are similar to those from females

Since Ts65Dn mice are typically produced from a trisomic mother, both maternal and embryonic components of the placenta are trisomic in trisomic pups, while their euploid siblings have a trisomic maternal component but a euploid embryonic component. This may contribute to the fact that Ts65Dn mice are smaller than their euploid littermates at birth and throughout life (Moore 2006; Roper et al. 2006). When the T65Dn chromosome is transmitted by the father, the mother’s contribution is always euploid.

All animals produced here were used in experiments examining various effects of ploidy as opposed to providing a comprehensive set of growth data for IVF versus natural mating. However, we selected a cohort of animals produced by IVF that were sacrificed between 3 and 4 months of age and compared their weights to those of trisomic mice from natural mating of trisomic mothers. Weights for Ts65Dn mice 3–4 months old that were born to trisomic mothers compared to the weights of those produced by IVF were lower, but not significantly so (28.4 vs. 30.4 g, \( p = 0.2, \ n = 31 \) male Ts65Dn born from mothers, 10 males from IVF). Some Ts65Dn mice generated from IVF were used in experiments with behavioral endpoints. Their impairment in performance in the Y-maze and the Morris water maze was the same as we and others have previously reported for Ts65Dn mice from trisomic mothers (data not shown).

Heritability of male fertility in trisomic mice?

Exact frequencies of the incidence of Ts65Dn male fertility from founder stocks at JAX are not available, although this frequency was long held to be 5% or less. The initial finding of fertile Ts65Dn males was serendipitous and the time of exposure to females was variable depending on the use of these mice in other experimental procedures. Furthermore, the percentage of males in our working colonies that were produced by IVF or who have a relative produced by IVF has increased as more procedures are successful and trisomic progeny, male and female, are incorporated into breeding procedures. We now identify fertile males with much higher frequency than when the process started at F&M (Table 2), but the possibility that improved screening contributes to this cannot be ruled out.

All but one of the fertile trisomic males that we have observed can be traced by euploid or trisomic lineage to a single founder Ts65Dn female introduced into the F&M colony from JAX in 2005. However, the T65Dn chromosome in a given fertile trisomic male can be traced to one of five different Ts65Dn females (crossed to euploid males who had a fertile trisomic male relative). We do not know the relationships of these Ts65Dn females, but they were shipped to F&M or to JHU at different times over 5 years and are presumably only distantly related. At any rate, it appears unlikely that a variant of the T65Dn marker chromosome is primarily responsible for male fertility.

Discussion

Ts65Dn has become the standard model for Down syndrome work but remains limiting for practical reasons related to cost, husbandry, and the expertise required to grow and type these mice. We established a useful approach to overcome the most important logistical limitations of Ts65Dn. Using fresh or cryopreserved sperm from fertile males, we generated large cohorts of siblings.
from mothers that carry large litters and provide good husbandry, assuring that more mice survive to weaning. While we used B6C3 F1 females as oocyte donors here, inbred lines, such as B6, can also provide large numbers of oocytes from superovulation. Progeny from such IVF procedures with Ts65Dn would be on average 75% B6 and 25% C3H; since they are full sibs, half of the remaining variable portion of the genome (25%) would be identical by descent (ibd), i.e., any pair of mice from this scenario would share on average 87.5% of their genetic information, without even accounting for the portions of the B6 and C3H genomes that are already ibd. It can be argued that many phenotypes that are detectable only with less than this small amount of genetic variability are of minimal relevance to natural outbred populations, i.e., people with trisomy 21.

Large cohorts of closely related siblings are useful for a variety of experiments, especially behavioral testing or outcomes with seasonal variation where it would be very difficult to generate sufficient offspring by conventional matings with Ts65Dn females in a short period of time. In another example, establishment of ES cells for Ts65Dn has been problematic due to low levels of superovulation and hence small numbers of blastocysts recovered from trisomic females. We obtained multiple trisomic ES lines using excess blastocysts from IVF. Finally, the ability to do complex crosses with multiple mutant alleles carried in the superovulated females is greatly enhanced by this approach.

Increasingly, contributions of individual genes to Ts65Dn phenotypes are being assessed by breeding null alleles or segmental deletions onto the trisomic background (e.g., Olson et al. 2004, 2007; Salehi et al. 2006; Sussan et al. 2008). Matings produce the desired genetic combinations 25% of the time for one gene; this frequency is further reduced to about 15–20% by the sub-Mendelian recovery of Ts65Dn at weaning. In a conventional breeding colony, only males are used for experiments since all females need to be put into production to generate experimental animals; thus, 10% of weanlings might be suitable for this experiment. A three-way cross to combine alleles of two genes on the Ts65Dn background would be expected to produce the desired genotype in 5% of weaned progeny. These frequencies assume no deleterious interaction of trisomy with the modified genes, which is frequently not the expected outcome.

Thus, months can be required to produce sufficient animals to support analysis. Doing IVF with fresh sperm requires that a large number of female mice between 3 and 4 weeks of age be available near the time when the male is identified as fertile, since fertile trisomic males become sterile much younger than their euploid counterparts. To do this would require an enormous standing breeding colony. While production of 30 doubly mutant females 3–4 weeks of age remains a considerable task, this step is no longer limited to a short window of male fertility since frozen sperm can be used. In our largest IVF, we recovered more than 450 oocytes from superovulated females, resulting in more than 300 two-cell embryos the next day.

We observed a far higher than expected frequency of male fertility from male Ts65Dn with a fertile male ancestor. These observations raise the possibility that QTL associated with this trait could be mapped in mice from these pedigrees. In practical terms, the use of fertile Ts65Dn males or cryopreserved sperm for generation of trisomic mice allows us to rapidly produce large cohorts of closely related trisomic mice for complex genetic analysis of the effects of trisomy on development and function.

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