Identification of Trisomy 16 Murine Embryos by Fluorescence In Situ Hybridization

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Trisomy 21 (Ts 21, Down syndrome) is the most commonly observed human autosomal trisomy in newborns (13). Individuals with Down syndrome exhibit a number of characteristic phenotypic manifestations, including mental retardation and abnormalities of the face, heart, gastrointestinal tract, and nervous system (2,3).

Animal model systems have been employed to study the effects of chromosomal trisomies in embryonic development. Murine breeding strategies that allow for the generation of trisomies of any desired chromosome employ mice carrying Robertsonian chromosomal translocations involving the chromosomes of interest (11). Mating of males carrying two pseudometacentric Robertsonian chromosomes, with a common arm, with females of normal karyotype results in the generation of embryos with balanced, trisomic, and monosomic chromosomal complements. Examination of the various phenotypes produced by different murine trisomies reveals distinct similarities between Down syndrome and murine trisomy 16 (Ts 16) (9). Ts 16 mice display defects in cardiac, craniofacial, nervous system, and endocrinological development similar to those observed in Down syndrome (8,9,13). In addition, murine chromosome 16 shares a large syntenic region with a portion of human chromosome 21 (19). Therefore, the Ts 16 mouse has come to be accepted as a model system for studying the molecular etiology of Down syndrome (4,6,8,12,13,19).

Developmental abnormalities associated with Down syndrome and with murine Ts 16 are thought to be caused by a gene dosage effect: crucial genes on the trisomic chromosome are present in greater copy number than normal and are, therefore, overexpressed, resulting in the characteristic phenotypic features of the trisomy (3). Molecular understanding of the Down syndrome pheno-

type will require more than just determination of the expression of genes present on critical regions of chromosome 21 because many genes, some even present on euploid chromosomes (7,17), exhibit expression levels that differ considerably from the expected 1.5-fold increase (12,20). Thus, development of the abnormal phenotype is probably the result of abnormal regulation of gene expression affecting euploid chromosomes as well as the trisomic chromosome. Identification of those genes whose regulation is affected by trisomy 21 (or, in the mouse, Ts 16), whether or not they are present on chromosome 21, will be of crucial importance in the understanding of the molecular basis of Down syndrome.

As a first step in identifying differential gene expression in tissue of Ts 16 compared with euploid embryos, we have developed a procedure for the identification, by means of fluorescence in situ hybridization (FISH), of embryos trisomic for chromosome 16. Ts 16 mice and their normal littermate control animals were generated by mating doubly heterozygous Robertsonian translocation male mice (Rb(6:16)24LubxRb(16:17)7BnrF₁) (The Jackson Laboratory, Bar Harbor, ME, USA) with normal CD-1 female mice (Harlan Sprague Dawley, Indianapolis, IN, USA). Matings were accomplished by placing one male with two females overnight; the presence of a vaginal plug the following morning [gestation day (gd) 0] was considered evidence of mating. Pregnant females were euthanized on gd 12 by carbon dioxide asphyxiation, and the embryos were rapidly dissected from the uteri. Individual embryos were minced with a scalpel blade, and the tissue was trypsinized in 0.5 mL 0.05% trypsin/EDTA solution (Life Technologies, Rockville, MD, USA). The tissue was incubated at 37°C for 30 min with intermittent shaking, and the digestion was terminated by the addition of 5 mL Complete Medium [OPTI-MEM™ (Life Technologies) containing Earle's salts, 25 mM HEPES buffer, and supplemented with 2 mM glutamine, 5% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA)], 100 µg/mL streptomycin, and 100 U/mL penicillin. The tissue was centrifuged at 1000× g for 10 min

at 4°C, and the pellet was resuspended in 10 mL Complete Medium. The resuspended cells from each embryo were plated in 60-mm tissue culture dishes and cultured for 2–3 days in Complete Medium until approximately 90% confluent. The cells were then treated with 0.15 mL 10 µg/mL KARYO-MAX® Colcemid® Solution (Life Technologies) in 2.5 mL Complete Medium for 3 h. After removal of the Colcemid, the cell monolayer was washed with calcium magnesium-free PBS (CMF-PBS) and trypsinized by addition of 2 mL 0.05% trypsin/EDTA solution for 5–10 min. The cells were resuspended by addition of 3 mL Complete Medium, and the suspension was centrifuged at 1000× g for 10 min at 4°C. The cell pellet was washed in CMF-PBS, centrifuged at 1000× g for 10 min, and the final pellet was resuspended in 10 mL 0.56% KCl. After incubation for 20 min at room temperature, 1 mL fixative (methanol/acetic acid, 3:1) was added. The suspension was centrifuged as above, and the pellet was resuspended in 10 mL fixative. The suspension was centrifuged at 1000× g for 10 min, and the pellet was resuspended in 1 mL fresh fixative and stored at 4°C until used.

For preparation of chromosome spreads, cell suspensions were centrifuged at 1000× g for 10 min, the pellet was resuspended in 1 mL freshly prepared fixative, and samples were delivered dropwise from a Pasteur pipet to clean glass slides from a height of 8–10 in. The chromosome spreads were allowed to dry overnight at room temperature.

Chromosome 16 was detected with a commercially available mouse chromosome 16-specific fluoroscein isothiocyanate (FITC)-labeled DNA probe (Cambio, Cambridge, UK). The probe was prepared from PCR-amplified DNA obtained from fluorescence-activated cell sorting (FACS)-sorted chromosomes (18) (details provided by Cambio). The signal was visualized with an FITC Amplification kit (Cambio). Chromosome spreads were serially dehydrated in ethanol, washing twice in 70% and 90% and once in 100% ethanol, and allowed to air-dry. Chromosomes were denatured by incubation in 70% formamide, 30% 2× SSC (0.03

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M sodium citrate, pH 7.0, 0.3 M sodium chloride) at 65°C for 10 min. Slides were then plunged immediately into ice-cold 70% ethanol, dehydrated in serial ethanol solutions as described above, and air-dried. Solution containing the chromosome 16 probe (as supplied by the manufacturer) was warmed to 37°C, mixed well, denatured at 68°C for 10 min, and incubated for 60–90 min at 37°C. Fifteen microliters of probe were then added to each slide, overlaid with a cover slip, sealed with rubber cement, and incubated overnight at 42°C. After removal of the dried rubber cement, the cover slips were removed with 2× SSC warmed to 45°C, and the slides were washed sequentially in 50% formamide in 2× SSC at 45°C for 5 min (twice) and 0.1× SSC at 45°C for 5 min (twice). Chromosome spreads were then exposed to detection reagents and counterstains exactly as recommended in manufacturer's instructions (FITC Amplification Kit; Cambio). The detection reagents were diluted with a protein solution provided in the kit to block nonspecific hybridization of antibodies used in the detection of signal. The spreads were first treated with 100 µL rabbit anti-FITC antibody, overlaid with a Parafilm® cover slip, and incubated in a humidified box for 20 min at 42°C. After removal of the cover slip, the spreads were washed three times with a Wash Solution containing 0.05% Tween® 20 in 4× SSC at 42°C, and then treated with 100 µL FITC-labeled goat anti-rabbit IgG antibody, overlaid with a Parafilm cover slip, and incubated in a humidified box for 20 min at 42°C. The spreads were again washed three times with Wash Solution at 42°C and counterstained with DAPI and propidium iodide in mountant. The slides were viewed with a 100× objective (Olympus BX60® microscope; Olympus America, Melville, NY, USA) with oil immersion, and images were captured using CytoVision System™ software (Applied Imaging, Santa Clara, CA, USA).

A chromosome spread from an embryo with a normal complement of chromosomes (20 pairs) is shown in Figure 1A. Two labeled chromosomes (chromosome 16) are readily visible, one single-armed or acrocentric (16 a) and the other bi-armed or metacentric

(16 m). The FISH pattern obtained with a chromosomal spread from a Ts 16 embryo is shown in Figure 1B, with three labeled chromosomes (chromosome 16) evident. The quality of the metaphase spread varied both between, and within, preparations. However, even when the individual chromosomes were not clearly visible, three labeled chromosomes could be discerned (Figure 1C) in chromosomal spreads from Ts 16 embryos. Thus, even with chromosome spreads that are not of the highest quality, our protocol, which combines standard FISH procedures and the convenience of a commercially available probe, allows for the identification of a trisomic karyotype. This may be particularly important when processing samples in which repeated fixation is not an option. This protocol is most useful with metaphase spreads and is not recommended with interphase nuclei. No chromosomal spreads were found that contained signals representing nonspecific binding of probe to DNA from other chromosomes, or to other nuclear components. Furthermore, omission of the chromosome 16 probe during the hybridization procedure did not result in detection of a signal associated with a chromosome. Five trisomic signals were scored out of 30 spreads examined. It is likely that this low number of trisomic-positive spreads is due to factors that influence the frequency of aneuploids in the progeny of matings involving Robertsonian chromosomes, including the genetic background and sex of the Robertsonian parent (6).

We have developed a procedure for the identification, by means of FISH, of embryos trisomic for chromosome 16 in a litter comprising euploid and triploid individuals. The identification is based on the hybridization of a murine chromosome 16-specific probe to a metaphase spread of chromosomes derived from primary culture of cells from individual embryos at day 12 of gestation. Dissociated cells from each embryo were plated in 60-mm tissue culture dishes. Based on the rapid growth characteristics of embryonic cells, a smaller piece of embryonic tissue may be used for generation of the chromosome spreads, leaving more tissue for gene expression analysis.

Published methods for identification of trisomic embryos are based on morphological features of the embryo (15,16) or traditional karyotyping (1,5). The former is useful only for embryos

Figure 1. FISH detection of chromosome 16 in chromosome spreads from mouse embryonic cells. (A) Normal complement of chromosomes in a chromosomal spread of a wild-type embryo cell, with two labeled chromosomes, one single-armed or acrocentric (16 a), the other bi-armed or metacentric (16 m). (B) Three labeled chromosomes (Ts 16) from Ts 16 individual. (C) Three labeled chromosomes [16] in a chromosomal spread of a Ts 16 embryo cell in which all chromosomes are poorly distinguished.

Benchmarks

at gd 14 or later (15), while the latter requires some expertise in cytogenetics. A technique for the identification of segmental trisomy progeny in the Ts65DN mouse (an additional mouse model of Down syndrome) using interphase FISH of tail fibroblasts has also been reported (21). Our procedure, though similar, uses a commercially available probe generated from PCR-amplified DNA from FACS-sorted chromosomes (Certificate of Analysis Information for Mouse Chromosome 16-specific Paint; Cambio). Whole chromosome paints for murine chromosomes 1–19, X and Y are also commercially available for use in detection of chromosomal abnormalities in mice (<http://www.cambio.co.uk>).

The recent sequencing of human chromosome 21 provides great impetus for research on the molecular basis of Down syndrome (10). Due to the partial synteny between mouse chromosome 16 and human chromosome 21, the Ts 16 mouse has been used as a model for the study of aspects of this syndrome (4,6,8,12,13,19). Although the Ts 16 mouse used in the present studies exhibits perinatal mortality, it provides a valuable animal model to examine aspects of the Down syndrome phenotype such as aberrant craniofacial development. Mouse chromosome 16 has partial synteny with human 22q11, a region implicated in DiGeorge syndrome (14). Cardiac defects in the Ts 16 mouse also make it useful in the study of the formation of the atrioventricular junctions and atrial and ventricular septation (22). Analysis of aberrant gene expression in the tissues affected in these syndromes and defects will likely contribute to our understanding of the molecular and cellular basis of the associated malformations.

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**P. Nugent, M.J. Barch¹,
W.M. Weston², R.M. Greene,
and M.M. Pisano**

*University of Louisville
Birth Defects Center
and School of Dentistry*

*¹University of Louisville
School of Medicine
Louisville, KY, USA*

*²GlaxoSmithKline
Pharmaceuticals
Collegeville, PA, USA*

De-Chorionation of Fixed Zebrafish Embryos

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The zebrafish has become an important model for investigating vertebrate development because of a number of attractive characteristics, including high reproductive potential and the optical clarity and accessibility of the embryos. The embryo is surrounded by a protec-