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Embryonic stem cell rescue of tremor and ataxia in myelin-deficient *shiverer* mice

Hoi Pang Low [a,*,1], Béatrice Gréco [a,1], Yusuke Tanahashi [a,2], Judith Gallant [b], Stephen N. Jones [b], Susan Billings-Gagliardi [a,b], Lawrence D. Recht [a,3], William J. Schwartz [a]

[a] Department of Neurology, 55 Lake Avenue North, University of Massachusetts Medical School, Worcester, MA 01655, USA
[b] Department of Cell Biology, 55 Lake Avenue North, University of Massachusetts Medical School, Worcester, MA 01655, USA

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ABSTRACT

Transplantation of neural precursor cells has been proposed as a possible approach for replacing missing or damaged central nervous system myelin. Neonatal and adult myelin-deficient *shiverer* (*shi*) mice, bearing a mutation of the myelin basic protein (*MBP*) gene, have been used extensively as hosts for testing cell engraftment, migration, and myelination, but relatively little progress has been made in reversing *shi* motor deficits. Here we describe a prenatal cell replacement strategy, showing that embryonic stem cells injected into *shi* blastocyst embryos can generate chimeric mice with strong and widespread immunoreactive MBP expression throughout the brain and a behavioral (motor) phenotype that appears essentially rescued.

1. Introduction

The prospect that repair of the damaged central nervous system (CNS) might be achieved by exogenous cell replacement has generated much recent excitement. Therapeutic efficacy will surely depend on the identity of both the transplanted cell type and the local host environment, and animal models are proving valuable for assessing the possible influences of these factors.

One powerful model has been the mutant mouse *shiverer* (*shi*), bearing a large deletion in the myelin basic protein (*MBP*) gene [1]. Homozygous *shi* mice fail to produce the four "classic" isoforms of MBP, and their CNS shows extensive dysmyelination [2]. *Shi* mice exhibit severe tremor beginning at about postnatal day 10, later develop hindlimb ataxia, and survive for only a few months. Both the morphological and motor phenotype of *shi* mice can be genetically rescued by expression of the wild-type *MBP* transgene [3].

Shi mice are a useful transplant host because any myelin produced by engrafted wide-type donor cells will be MBP-positive and can be unequivocally identified by light microscopic immunocytochemistry. Neonatal and adult *shi* mice have been transplanted with oligodendrocytes; oligodendrocyte progenitors; neural "stem" cells, either freshly-dissected, immortalized, or growth-factor expanded *in vitro*; non-neural precursor cells; and embryonic stem (ES) cell-derived progenitors by injection into CNS parenchyma or ventricles (for reviews, references, and some recent examples, see [4–9]). The cells engraft, migrate, and myelinate, but – with the exception of two reports describing diminished tremor [10] and somewhat improved rotarod performance [11] – fail to rescue the mutant's motor deficits. Most recently, Windrem et al. [12] have reported some success by first generating immunodeficient *shi* mice, using sorted fetal human glial progenitor cells, and implanting the cells into five cerebral and cerebellar sites in pups within a day of birth. There was prolonged survival in 23% of the transplanted mice, with two-thirds of these survivors apparently rescued.

To our knowledge, a prenatal cell replacement strategy has been reported only once, in which aggregation chimeras were created using wild-type and *shi* embryos at the 8-cell stage; although few in number, the resulting animals showed patches of MBP-positive and -negative axons intermixed within white matter [13]. Here we

* Corresponding author. Tel.: +1 508 856 1128; fax: +1 508 856 6778.
E-mail address: hoipang.low@umassmed.edu (H.P. Low).
[1] These authors contributed equally to the study.
[2] Current address: Department of Physiology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan.
[3] Current address: Departments of Neurology and Neurosurgery, Stanford University School of Medicine, 300 Pasteur Drive, Palo Alto, CA, United States.

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show that ES cells injected into *shi* blastocyst embryos can generate chimeric mice with strong and widespread immunoreactive MBP expression throughout the brain and a motor phenotype that appears essentially rescued.

2. Materials and methods

2.1. Animals and blastocyst injections

Shi mice are maintained in our colony on a B6C3F1 hybrid-based stock, greater than 99.9% congenic at other loci. Twice per year, homozygous *shi* males are outcrossed to B6C3F1 wild-type females to produce unaffected heterozygous *shi* breeders for the next generation. For the current study, homozygous *shi* offspring were identified by their pronounced tremor and ataxia. Blastocysts from homozygous intercrosses were flushed from the uteri of *shi* females 3.5 days postcoitum. Each *shi* blastocyst was injected with 10 to 12 ES cells (Omnibank no. OST32815) bearing a retroviral promoter trap insertion that functionally inactivates one allele of the *Ini1* gene; expression of a reporter β-galactosidase (β-gal)-neomycin gene fusion cassette within the retroviral insertion is regulated by the *Ini1* promoter [14]. Ten ES cell-injected blastocysts were surgically implanted into the uterine horn of a pseudo-pregnant Swiss Webster dam, and pups were delivered about 17 days later. All animal procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Homozygous *shi* mice genetically rescued with the wild-type *MBP* transgene (*shi*/*shi; MBP*/*MBP*) were initially a gift of Carol Readhead [3], and they have been bred onto the same hybrid background on which the *shi* mice are maintained. *Ini1*-heterozygous(*Ini1*+/-) mice were created and maintained in our colony as previously described [14].

2.2. Phenotype assessment

Pups were assessed at least every other week from 3 to 4 weeks of age until 9 weeks at time of sacrifice by one of us (S.B.-G.) experienced in the handling and observation of developing *shi* and normal mice. Animals were scored on the presence of tremor and ataxia, with 0 as "absent," 1 as "barely detectable," 2 as "mild-moderate," and 3 as "severe." For reporting purposes, we categorized the motor phenotype of each mouse at sacrifice as: "rescued" if it was scored 0 — tremor and 0 — ataxia (abbreviated 0,0), "essentially rescued" (0,1) or (1,0), "not rescued" (3,3), and "intermediate" (all other combinations).

2.3. Animals sacrifice and brain assessment

Mice were deeply anesthetized with ketamine (25 mg/kg IP) and xylazine (5 mg/kg IP) and perfused with ice-cold heparinized phosphate-buffered saline (PBS), followed by ice-cold 4% buffered paraformaldehyde fixative. Brains were removed, post-fixed for 4 h, and 50 μm thick coronal sections were cut on a vibratome, or saturated with 20% sucrose and cut on a freezing microtome, and stored at −20 °C in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.25 mM polyvinylpyrrolidone in PBS).

For X-gal histochemistry to detect β-gal activity, free-floating sections were rinsed in PBS and incubated in a solution of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.01% deoxycholic acid, 0.02% Nonidet P-40, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal; Gold Biotechnology, St. Louis, MO) overnight at 37 °C. After rinsing in PBS and water, the sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA), rinsed in water, mounted onto slides, dried, and coverslipped in Permount. For MBP immunofluorescence, free-floating sections were rinsed in Tris-buffered saline (TBS), blocked in 10% normal goat serum and 0.4% Triton X-100 in TBS for 30 min to 1 h, followed by incubation with anti-MBP antibody (mouse; 1:1000; Covance, Berkeley, CA) diluted in 2% normal goat serum and 0.4% Triton X-100 in TBS overnight at 4 °C. After washing, sections were incubated with Alexa Fluor secondary antibody (1:200; Invitrogen/Molecular Probes, Eugene, OR) for 2 h at room temperature in the dark, washed, and coverslipped in Prolong anti-fade reagent (Invitrogen/Molecular Probes).

For cellular double labeling, free-floating sections were first treated with 1% hydrogen peroxide for 20 min, blocked with 10% normal goat serum and 0.4% Triton X-100 in TBS for 1 h, and incubated with antibodies directed against either Olig-1 (rabbit; 1:200; Chemicon International, Temecula, CA), glial fibrillary acidic protein

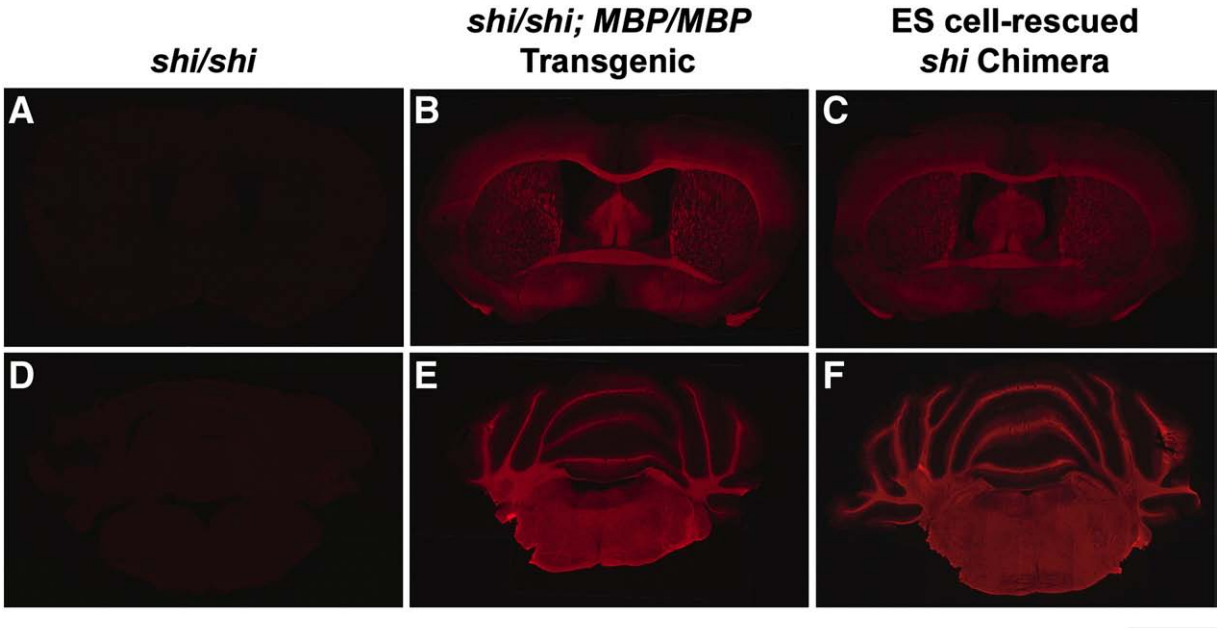

Fig. 1. Coronal brain sections at two rostrocaudal levels labeled for immunoreactive MBP from homozygous *shi* mice (*shi*/*shi*) (A,D), a homozygous *shi* mouse genetically rescued with the wild-type *MBP* transgene (*shi*/*shi*; *MBP*/*MBP*) (B,E), and chimeras derived from ES cell-injected *shi* blastocysts (ES cell-rescued *shi* Chimera) (C,F). Scale bar represents 2 mm.