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Therapeutic cloning in individual parkinsonian mice

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Abstract

Cell transplantation with embryonic stem (ES) cell progeny requires immunological compatibility with host tissue. ‘Therapeutic cloning’ is a strategy to overcome this limitation by generating nuclear transfer (nt)ES cells that are genetically matched to an individual. Here we establish the feasibility of treating individual mice via therapeutic cloning. Derivation of 187 ntES cell lines from 24 parkinsonian mice, dopaminergic differentiation, and transplantation into individually matched host mice showed therapeutic efficacy and lack of immunological response.

Previous work has shown generation of pluripotent mouse ntES cells via somatic cell nuclear transfer with adult fibroblasts or cumulus cells as nucleus donors^{1,2}. Such ntES cell lines have been shown to be equivalent in developmental potency compared with fertilization-derived ES cell lines^{3,4} and can be derived via nuclear transfer into normal adult, aged or failed-to-fertilize mouse oocytes⁵. Since these first reports on mouse ntES cell derivation, there have been only two *in vivo* attempts at therapeutic cloning, in Rag2-deficient⁶ and in parkinsonian⁷ mice, each using a single ntES cell line derived from a genetically related donor and not from the affected mouse to be treated. Although transfer of such nonautologous nuclei can bypass immunological incompatibility in inbred mice, the use of therapeutic cloning in humans will require transfer of autologous nuclei. Here we show the feasibility of treating multiple parkinsonian mice with dopamine neurons derived from individually matched ntES cell lines.

Z/EG26, wild-type 129/Sv or BALB/c mice at 3.5–4 weeks of age were rendered parkinsonian via intrastriatal 6-hydroxydopamine (6-OHDA) injections⁸. Tail-tip biopsies ($n = 24$) were obtained from Z/EG26 and 129/Sv mice at 5–6 weeks of age and shipped to Japan, where the

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AUTHOR CONTRIBUTIONS V.T. designed the experiments, performed some of the *in vivo* experiments and analysis, supervised the entire *in vivo* section of the work and contributed to the manuscript; M.T. performed all *in vitro* ES cell culture experiments and analyses; G.P. performed most of the *in vivo* experiments and analysis and contributed to the Supplementary Methods section; J.M. assisted with the immunohistochemistry and analysis of *in vivo* experiments; B.C. assisted with animal care and *in vivo* tests; G.A.-S. assisted with the *in vivo* experiments; S.W., E.M., H.O. and T.W. performed the fibroblast isolation and culture and the nuclear transfer and ES cell line derivation; T.W. also contributed to experimental design; L.S. designed the experiments and contributed to the manuscript.

Note: Supplementary information is available on the Nature Medicine website.

tail tissue was dissociated and fibroblast cultures were established. Nuclei were microinjected into B6D2F1 mouse oocytes and ntES cells derived as described previously^{2,9} (Supplementary Fig. 1 online). A total of 187 ntES cell lines were generated from 24 parkinsonian mice (Z/EG and 129/Sv), with a minimum of one line per mouse (Supplementary Table 1 online).

In order to genetically identify autologous ntES cell progeny in host mice, we used a Cre-lox reporter strain. Z/EG26 mice express *lacZ* ubiquitously, but Cre-mediated excision removes *lacZ* and thus activates eGFP¹⁰. ntES cell lines established from Z/EG26 nucleus donors were transfected with a Cre recombinase expression plasmid¹¹. Cells were expanded and subjected to neural induction and differentiation into midbrain dopamine neurons⁷. Identification of midbrain-like dopamine neuron progeny was confirmed by co-expression of tyrosine hydroxylase with midbrain-specific transcription factors such as engrailed and Pitx3 (Supplementary Fig. 1d). A minimum yield of 20% midbrain dopamine neurons (cells positive for tyrosine hydroxylase and engrailed) out of total treated cells was required before progression to *in vivo* study.

Lesioned mice were monitored long-term by a battery of behavioral tests starting at 3 weeks after lesioning^{12,13} (Supplementary Methods online). Out of the 24 parkinsonian mice used for ntES cell derivation, only those maintaining stable motor asymmetry over 6 months after lesioning were used for transplantation ($n = 6$). A total of 100,000 ntES cell-derived cells containing >20% midbrain dopamine neurons were grafted as a cell suspension into the host striatum ipsilateral to the lesion. Each mouse (129/Sv and Z/EG26) received dopamine neuron progeny obtained from a ntES cell line derived from its own tail fibroblast donor nuclei. Mice were allowed to survive for 11 weeks, during which behavioral tests were repeated at 2–4-week intervals. The average age of the mice at the time of killing was 12 months. Control mice consisted of a group of age-matched 6-OHDA-lesioned BALB/c mice ($n = 7$) that received an allograft of 100,000 dopamine neuron progeny derived from a single Z/EG26 ntES cell line.

Mice grafted with matched ntES cell-derived dopamine neurons showed a significant amelioration of parkinsonian phenotype in all behavioral tests (Fig. 1a–d). Stereological analysis revealed an average of $19,091 \pm 14,497$ tyrosine hydroxylase-positive cells per graft in five out of six mice (Fig. 1e). The remaining mouse showed signs of neural overgrowth with a graft composed of >100,000 tyrosine hydroxylase-positive cells and large numbers of tyrosine hydroxylase-negative neural precursors and neurons, but it was devoid of non-neural cells or of Oct4-expressing undifferentiated ES cells (Supplementary Fig. 2 online). An average of 40% of the tyrosine hydroxylase-positive cells within the autologous grafting group co-expressed Girk2, a marker widely used for the identification of A9 midbrain dopamine neurons (Fig. 1f).

Mice that received allografts of ntES cell-derived dopamine neurons showed poor graft survival, with three mice having no surviving cells and an overall average of 166 ± 83 tyrosine hydroxylase-positive cells ($n = 7$). Potential causes of low survival include the advanced age of the mice at transplantation (9–12 months)¹⁴ and the complete mismatch of both major and minor histocompatibility genes¹⁵ between the two strains. Alternatively, the results could be due to cell line-specific differences in tyrosine hydroxylase yield *in vitro* or *in vivo*.

We therefore compared tyrosine hydroxylase-positive cell yield among six ntES cell lines derived from six independent mice (Fig. 2a). Data obtained in triplicate show that all ntES cell lines were capable of generating tyrosine hydroxylase-positive neurons, with yields ranging from 12% to 45% (mean $31\% \pm 11\%$). We next tested variations in tyrosine hydroxylase-positive cell yield among four independent ntES cell lines derived from a single mouse (Fig. 2b); all four lines derived from mouse 242 yielded tyrosine hydroxylase-positive neurons, with similar variability to that observed across different mice (compare to Fig. 2a).

Karyotypic analysis was performed in a random subset of ntES cell lines, testing for potential inter-mouse and intra-mouse karyotypic variability ($n = 11$; Fig. 2c and Supplementary Table 2 online). The majority of the tested lines maintained a high proportion of karyotypically normal cells, with loss of a sex chromosome as the most frequent abnormality observed.

Another potential source for the variability in the outcome observed between autologous and allogenic grafts is differential *in vivo* dopamine neuron survival among ntES cell lines. To address this issue experimentally and to control for batch-to-batch variability, we tested the *in vivo* performance of additional ntES cell lines. Cells used for grafting into allogenic (BALB/c, $n = 6$) and congenic (129/Sv, $n = 6$) hosts were obtained from the same ntES cell differentiation batch. Cells derived from two additional ntES cell lines were tested, one 129/Sv and one Z/EG26. BALB/c and 129/Sv (congenic with Z/EG) hosts received unilateral 6-OHDA lesions at 9 months of age. Dopamine neuron derivation was carried out as above. Each mouse received 100,000 cells, and all mice survived grafting and behavioral testing. In the BALB/c group (allografts) the average number of tyrosine hydroxylase-positive cells at 10 weeks was $1,020 \pm 968$ cells. Two BALB/c mice—one deriving dopamine neurons from the 129/Sv cell line and the other from the Z/EG26 ntES cell line—had no surviving cells. In the 129/Sv group, all mice had surviving grafts, with the tyrosine hydroxylase-positive cell number ranging from 302 to 19,308 cells (average $8,784 \pm 4,293$ cells). Two mice in this group showed graft overgrowth. These grafts contained large numbers of precursor cells arranged in rosette-like structures, often surrounded by immature tyrosine hydroxylase-positive cells (Supplementary Fig. 2 online). This suggests ongoing neurogenesis of tyrosine hydroxylase-positive cells *in vivo*, thus explaining the exceedingly high tyrosine hydroxylase-positive cell numbers observed in some mice. However, most of the cells in these grafts were positive for markers of neural precursors and young neurons and negative for non-neural lineage markers, compatible with neural overgrowth rather than teratoma formation. It should be noted that the graft overgrowth seen here originated from a different cell line (289G) than that observed in the autologous group (NY1). 289G is a Z/EG26 line that had a normal karyotype and did not result in overgrowth when transplanted autologously. NY1 had loss of the X chromosome (Supplementary Table 2) as a major karyotypic abnormality. Future studies must incorporate selection strategies for enrichment of dopamine neurons and elimination of immature cells.

Mice that received congenic grafts showed significant improvements in behavioral scores compared with allogenic grafts (Fig. 2d–g). These results confirm and extend our data from the autologous grafting group, demonstrating superior graft survival and behavioral results associated with decreased immunogenicity.

Histological analysis in the allograft (BALB/c) group showed evidence of a strong chronic inflammatory host response characterized by large numbers of activated CD11b⁺ and CD68⁺ microglial cells, as well as CD45⁺ infiltrates (Supplementary Fig. 3 online). Autologous grafts were devoid of inflammatory cells, whereas congenic grafts showed a mild degree of inflammation, with CD68⁺ and CD11b⁺ cells located focally around graft cores. Occasional clusters of CD45⁺ cells with lymphocytic morphologies were seen at the graft periphery (Supplementary Fig. 3).

Although technically complex, with an average interval of >10 months from lesioning to transplantation endpoint, these data demonstrate the feasibility of treating individual parkinsonian mice via therapeutic cloning and suggest considerable therapeutic potential for the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Behavioral and histological analyses of autologous grafts

(a) Amphetamine-induced rotations in mice receiving matched dopamine neuron grafts. (b) Spontaneous rotation behavior in the same mice. (c, d) Cylinder test (c) and adhesive tape-removal test (d) were additional spontaneous behavior measures used to assess graft function and restoration of behavioral asymmetry. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to pretransplantation values (Dunnett test). (e) Representative graft showing large numbers of eGFP⁺ (left) and tyrosine hydroxylase-positive cells (right). (f) Girk2 immunohistochemistry confirmed the presence of substantia nigra (A9) type midbrain dopamine neurons located primarily at the graft periphery. Right panel shows a higher power image of cells in left panel. Scale bar in e, 100 μm; scale bars in f, 50 μm in left panel and 10 μm in right panel. All mouse experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the Sloan Kettering Institute for Cancer Research.

Figure 2. *In vitro* and *in vivo* studies addressing variability of dopamine neuron yield and *in vivo* survival among multiple ntES cell lines

(a, b) Percentage tyrosine hydroxylase-positive cells out of total cells as shown by a representative sample of six ntES lines derived from different individual mice (a) or among lines derived from a single mouse (b). Whereas all ntES lines were capable of generating tyrosine hydroxylase-positive neurons, they did show significant variability. Data were derived from three independent sets of differentiations. TH/Tuj1, tyrosine hydroxylase-positive cells over β -tubulin-positive cells. (c) Representative spectral karyotype analysis of an ntES line, showing euploidy. The majority of the lines maintained a high proportion of karyotypically normal cells, with loss of a sex chromosome representing the most common abnormality. (d–g) ntES cell line performance was also tested *in vivo* by grafting dopamine neuron progeny from 2 different lines into a group of allogenic (BALB/c) and congenic (129/Sv) mice. Behavioral analysis shows statistically significant improvement in amphetamine rotation scores in the congenic hosts compared to the allogenic hosts (d). A similar pattern was noted in a battery of spontaneous (non-drug-induced) behavioral assays, the cylinder test (e), tape removal test (f) and beam traversal test (g). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ in a and b (Scheffe test, compared against each other) and in (d–g) (Dunnett test, compared to pre-transplantation scores).