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Properties of secondary and tertiary human enteric nervous system neurospheres

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Abstract Advances in enteric nervous system (ENS) stem cell biology have raised the possibility of treating Hirschsprung's disease with ENS stem/progenitor cell (ENSPC) transplantation. This study aimed to expand ENSPC numbers by the growth and redissociation of neurospheres and assess their differential potential.

Methods: Human ENS neurospheres were cultured as previously described and redissociated to generate secondary and tertiary neurospheres. Neurospheres were assessed for the presence of neuronal (PGP9.5), glial (S100), and stem cell (p75, nestin markers). The degree of immunofluorescence was quantified using the ImageJ program. Secondary/tertiary neurospheres were transplanted into mouse distal colon grown in tissue culture.

Results: Secondary/tertiary neurospheres could be generated with exponentially increasing numbers. Tertiary neurospheres showed a significant increase in the proportion of p75 staining but a significant decrease in the proportion of S100 staining. After transplantation, secondary/tertiary neurosphere–derived cells positive for PGP9.5 and S100 could be identified.

Conclusions: It is possible to exponentially expand neurosphere and therefore ENSPC numbers by repeated dissociation and culture. There is a loss of S100-positive cells in secondary/tertiary neurospheres, but the ENSPCs remain capable of differentiating into neurons and glia when transplanted into an embryonic gut environment.

Despite advances in the understanding of the molecular and genetic defects that result in Hirschsprung's disease [1], and advances in surgical technique, Hirschsprung's disease still results in significant long-term problems, with a recent study reporting 1 in 5 teenagers experiencing fecal incontinence [2]. Developments in enteric nervous system (ENS) developmental biology have raised the possibility that stem

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cell transplantation may be a viable therapeutic option [3]. Currently, successful embryonic neural crest stem cell transplantation leading to an improvement in function in an animal model of Hirschsprung's disease has been described [4]. However, the use of embryonic allografts poses not only ethical problems but also potential issues of transplant rejection and immunosuppression. We have therefore hypothesized that transplantation of postnatally derived autologous ENS stem cells into the residual aganglionic bowel after surgery for Hirschsprung's disease will avoid such difficulties and improve long-term functional outcome.

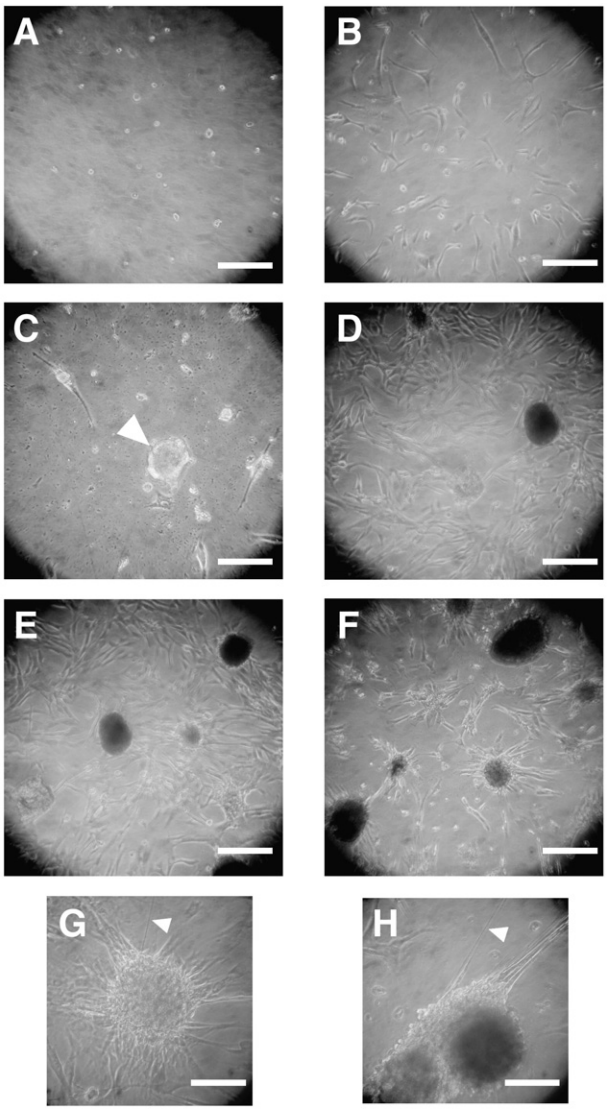

Fig. 1 Generation of secondary and tertiary neurospheres from primary neonatal human neurospheres. A-E, Secondary neurospheres formed after dissociation of primary neurospheres. A, Day 0 of culture. Initial single cell suspension. B, Day 7. Cells are seen to adhere to the bottom of the Petri dish with some assuming neuronal phenotypes. C, Day 14. A neurosphere has begun to form (arrowhead) and other, nonneurosphere-derived cells have begun to reduce in number. D, Day 21. Neurosphere colonies are well established and continue to increase in number. E, Day 28. Neurosphere colonies reach stable numbers and sizes. F, Tertiary neurospheres formed after dissociation of secondary neurospheres and 28 days in culture. Neurosphere size, number, and morphology are comparable to primary and secondary neurospheres. G and H, Tertiary neurospheres after 21 days in culture demonstrating neurite outgrowth (arrowheads) and therefore the capacity for neuronal differentiation. Scale bars: A-F, 200 μm; G-H, 100 μm.

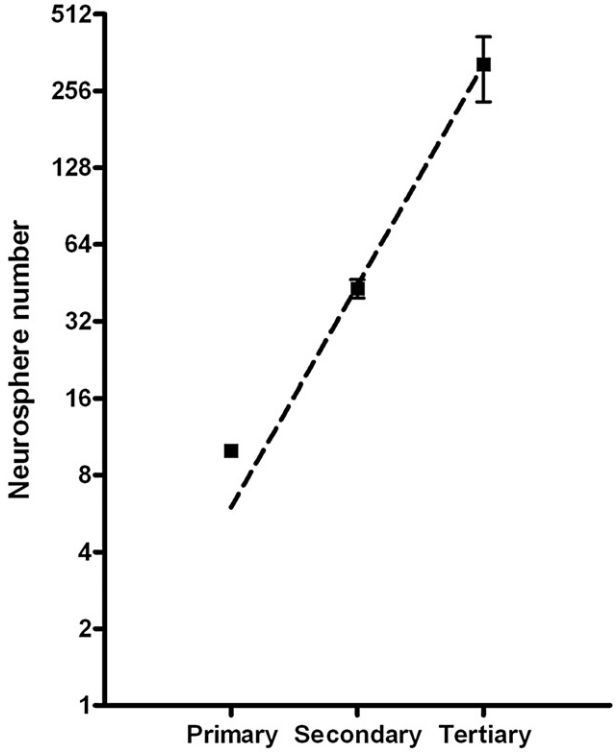

Fig. 2 Graph showing neurosphere number after dissociation of 10 primary neurospheres and subsequent numbers of secondary and tertiary neurospheres produced. Growth is exponential with the dotted line representing the exponential function $y = e^{2+x}$. Error bars represent standard error of the mean.

Studies by ourselves [5] and others [6] have demonstrated that it is possible to generate ENS stem cells (ENSC) and progenitor (ENSPC) cells from postnatal human bowel, including the ganglionic colon of children with Hirschsprung's disease. Furthermore, we have recently demonstrated that the transplantation of such cells in the form of neurospheres into an aganglionic tissue culture model of embryonic mouse hindgut results in the restoration of coordinated contraction and function [7]. However, the initial numbers of neurospheres generated using such techniques are unlikely to be sufficient for a successful clinical treatment. Neurospheres are cellular aggregates containing neural stem cells and their progeny (including neurons and glia), and one of their properties is that they can be dissociated to a single cell suspension and then reform into secondary and tertiary neurospheres with an exponential increase in cell number [5,8].

This study therefore aimed to generate secondary and tertiary neurospheres from postnatal human bowel samples and characterize them in terms of their capacity to generate further ENSC, neurons, and glia, and also to assess the