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Research Report

PA6-induced human embryonic stem cell-derived neurospheres: a new source of human peripheral sensory neurons and neural crest cells

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ABSTRACT

Human embryonic stem cells (hESC) have been directed to differentiate into CNS cells with clinical importance. However, for study of development and regeneration of the human PNS, and peripheral neuropathies, it would be useful to have a source of human PNS derivatives. We have demonstrated that peripheral sensory neuron-like cells (PSN) can also be derived from hESC via neural crest-like (NC) intermediates, and from neural progenitors induced from hESC using noggin. Here we report the generation of higher purity PSN from passagable neurospheres (NSP) induced by murine PA6 stromal cells. hESC were cultured with PA6, and colonies that developed a specific morphology were cut from the plates. Culture of these colonies under non-adhesive conditions yielded NSPs. Several NC marker genes were expressed in the NSP, and these were also detected in 3–5week gestation human embryos containing migrating NC. These NSPs passaged for 2–8weeks and re-plated on PA6 gave rise to many Brn3a+/peripherin+ cells, characteristic of early sensory-like neurons. Re-culturing PA6-induced NSP cells with PA6 resulted in about 25% of the human cells in the co-cultures differentiating to PSN after 1week, compared to only about 10% PSN obtained after 3 weeks when noggin-induced NSP were used. Two month adherent cultures of PA6-induced NSP cells contained neurons expressing several PSN neuropeptides, and voltage-dependent currents and action potentials were obtained from a molecularly identified PSN. hESC-derived PA6-induced NSP cells are therefore an excellent potential source of human PSN for study of differentiation and modeling of PNS disease.

1. Introduction

Most research on the generation of neurons from hESC has focused on CNS phenotypes with clinical importance, such as spinal motoneurons and mesencephalic dopaminergic neurons (i.e. (Singh et al., 2005; Roy et al., 2006)). However, many pathologies of PNS neurons exist, such as Familial Dysautonomia, infections of PSN by viruses, and damage to PSN and/or their processes resulting from diseases such as diabetes. It is therefore important to establish methods for generating PNS neurons for pre-clinical studies. We initially succeeded in eliciting PSN differentiation from hESC (Pomp et al., 2005) using the SDIA method of Sasai and colleagues (Kawasaki et al., 2000; Mizuseki et al., 2003). hESC cells co-cultured with PA6 cells

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murine stromal cells for 4 weeks induced neurons that co-express: 1) peripherin (peri) and Brn3a, and 2) peripherin and tyrosine hydroxylase, combinations characteristic of PSN and sympathetic neurons respectively (Mizuseki et al., 2003). Peripheral sensory and sympathetic neurons develop in amniote embryos from the NC. Very little is known about human NC at the cellular or molecular level, because it is present only in very young embryos of 3–5 weeks gestation that are difficult to obtain from pregnancy terminations. Analysis of expression of mRNAs identified in other species as NC markers revealed presence of many of these markers in the co-cultures before PSN appeared. Expression of NC marker genes including *SNAI1* (*SNAIL*), *dHAND*, and *SOX9* were increased at 1 week of co-culture relative to naive hESC, and several genes known to be down-regulated upon in-vivo differentiation of NC derivatives, were downregulated with extended PA6-hESC co-culture. This expression pattern is consistent with the initial formation of NC, and their subsequent differentiation into NC derivatives, such as neurons. The SDIA protocol we used has the drawback, however, of only generating less than 1% PSN.

In order to improve upon this method, we recently used neural precursors propagated as neurospheres (NSP) as the starting material for PSN induction (Brokhman et al., 2008). NSP were generated using the BMP antagonist noggin (Itsykson et al., 2005), and co-cultured with PA6 cells. This protocol increased by 10-fold the percentage of PSN from the 1% obtained in the SDIA approach by Pomp et al. The temporal expression of markers in the cultures was again consistent with the PSN having differentiated from NC-like precursors in the co-cultures and NSP themselves.

We here examine whether NSP generated by another method could further increase the yield of PSN from hESC. PA6 cells have been used to generate NSP from murine ESC (Kitajima et al., 2005), and CNS-like neural precursors from hESC (i.e. Ko et al., 2007; Song et al., 2007; Freed et al., 2008),. We generated NSP from hESC co-cultured with PA6, by identifying neurogenic colonies with a distinct morphology. These colonies were manually cut from the feeders, grown in a neurogenic medium, and rapidly yielded NSP. The NSP prepared by induction with PA6 feeders contained markers of neural precursors, neurons and glia. In contrast to the NSP generated with noggin, PA6-NSP contained AP2+/NCAM+ putative NC cells. We found that PA6-NSP when dissociated and re-seeded on PA6 yielded a further increase in PSN to more than 25% of cells in cultures. Long term cultures (2 months) of PA6-derived NSP cells yielded preliminary data on the electrophysiological properties and neuropeptide-expression by hESC-derived putative PSN. This method, with minimal further refinement, shows promise for the generation of populations of PSN for modeling human pathologies and drug development.

2. Results

2.1. *Generation of neural precursor containing spheres from co-cultures of hESC and PA6*

Changes are observed in hESC colonies within 7 days of seeding on PA6 feeder layers (Pomp et al., 2005). On culture day 12, the large majority of hESC colonies display one of two types of morphology: heterogeneous (Het) and homogeneous (Hom) (Fig. 1A–B). Instead of allowing the hESC to differentiate for 3–4 weeks as in Pomp et al, the colonies were mechanically removed from the feeder layer on days 12 to 14 of co-culture, and cultured as floating aggregates. About two days later, profound differences between aggregates derived from the HOM and HET colonies began to appear. The aggregates derived from the HOM colonies became spherical, appeared homogeneous in terms of cell density, did not contain cysts and resembled "neurospheres" (NSP) derived from CNS or ESC (i.e. Itsykson et al., 2005, Figs. 1C, E). In contrast, floating HET developed into cystic, irregular structures with areas of different cell density, with an appearance similar to early embryoid bodies (EBs) (Fig. 1D). RT-PCR of RNA from PA6-induced NSPs (from HOM colonies) for the definitive endodermal marker (*FOXA2*) and the extraembryonic endodermal

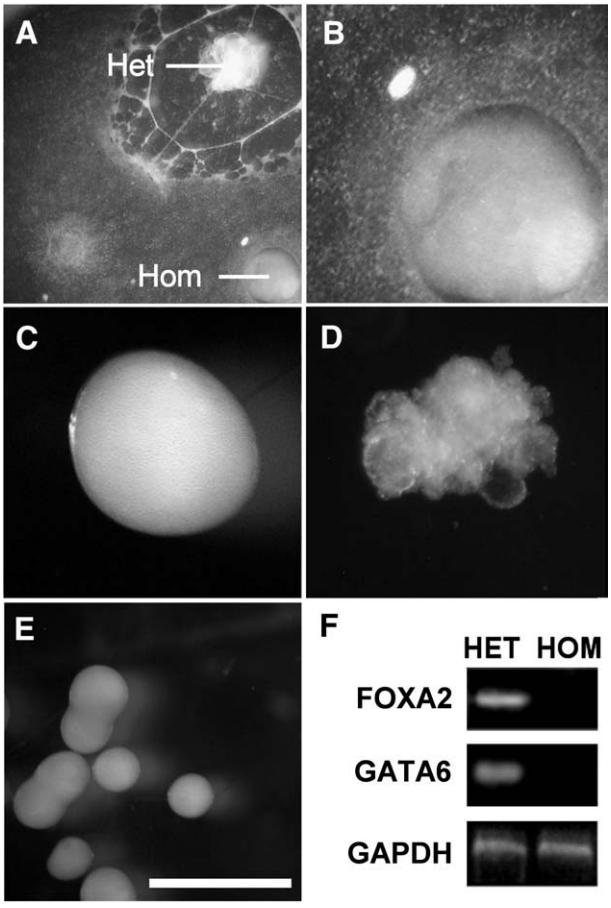

Fig. 1 – Generation of neurospheres from hESC using PA6 co-culture. (A) Two types of hESC colonies predominate after 12–14 days of co-culture of hESC and PA6 cells: heterogeneous (Het) and homogeneous (Hom) colonies. (B) shows a homogenous colony from panel (A) at higher magnification. The Hom colonies gave rise to neurospheres (C), while the heterogeneous colonies developed into cystic structures similar to embryoid bodies (D) when grown in suspension in serum-free medium. A number of NSP propagated for about 1 month, some of which have fused together, are seen in E. Bar = 1 mm. (F) Expression shows that spheres made from HET colonies, but not those from Hom colonies express non-ectodermal transcripts.