REVIEW ARTICLE

Generation of Neural Crest Progenitors From Human Embryonic Stem Cells

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ABSTRACT The neural crest (NC) is a transient population of multipotent progenitors arising at the lateral edge of the neural plate in vertebrate embryos, which then migrate throughout the body to generate diverse array of tissues such as the peripheral nervous system, skin melanocytes, and craniofacial cartilage, bone and teeth. The transient nature of neural crest stem cells make extremely challenging to study the biology of these important cells. In humans induction and differentiation of embryonic NC occurs very early, within a few weeks of fertilization giving rise to technical and ethical issues surrounding isolation of early embryonic tissues and therefore severely limiting the study of human NC cells. For that reason our current knowledge of the biology of NC mostly derives from the studies of lower organisms. Recent progress in human embryonic stem cell research provides a unique opportunity for generation of a useful source of cells for basic developmental studies. The development of cost-effective, time and labor efficient improved differentiation protocols for the production of human NC cells is a critical step toward a better understanding of NC biology. *J. Exp. Zool. (Mol. Dev. Evol.) 314B:95–103, 2010.* © 2009 Wiley-Liss, Inc.

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The neural crest (NC) gives rise to progenitor cells that migrate throughout the body to generate diverse array of tissues and later differentiate into neurons and glial cells of the peripheral nervous system (PNS), skin melanocytes, craniofacial cartilage, and the dentin, dental pulp and alveolar bone (Dupin et al., 2007; Chung et al., 2009). A multimodal gene regulatory network mediates the complex process of NC formation and involves the early induction and maintenance of the precursor pool, emigration of the progenitors from the neural tube via an epithelial–mesenchymal transition, migration of progenitor cells along distinct pathways and differentiation into diverse cell types (Sauka-Spingler and Bronner-Fraser, 2008; Nikitina et al., 2009). Many complex pathologies such as DiGeorge, Treacher Collins, Waardenburg and Down syndromes, neurofibromatosis and Hirschsprung's disease are associated with the disruptions in NC development (Taneyhill and Bronner-Fraser, 2005; Wurdak et al., 2006; Dixon et al., 2007; Tobin et al., 2008; Roper et al., 2009). Thus, elucidation of human NC development and the mechanisms by which it is disrupted in human disease will improve our understanding of molecular basis of disease pathogenesis and, ultimately, provide opportunities for novel therapeutic interventions.

Our knowledge of human NC biology will be profoundly enhanced by the study of in vitro differentiated human embryonic stem cells (hESCs). Moreover breakthrough studies in somatic cell reprogramming to generate induced pluripotent stem (iPS) cells have opened the door to the exciting opportunity of generating patient-specific cells for regenerative medicine (Okita et al., 2007; Takahashi et al., 2007).

Significant effort has been devoted to characterizing the neural potential of hESCs and protocols have been developed for neural induction and terminal differentiations (Schwartz et al., 2008). Although several dozen publications describe the methods of neural cell differentiation from embryonic stem cells and similar conditions are routinely used for the generation of central nervous system (CNS) and PNS, most effort has been implemented toward specification of CNS while there are only limited studies of the ability of embryonic stem cells to differentiate into

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neural crest stem cells (NCSCs). Here we present a mini-review of recent efforts aimed at derivation of NCSCs and their terminally differentiated derivatives from hESCs. To date several methodologies have been developed that describe the derivation of NCSCs from hESCs. These protocols involve stromal-derived inducing activity (SDIA), embryoid body (EB) and neural rosettes-derived methods as well as modulation of signaling pathways.

SDIA-DERIVED METHODS

Sasai and colleagues have shown that neurons are efficiently induced from murine and primate ESCs when co-cultured with the mouse stromal line PA6 (Kawasaki et al., 2000; Mizuseki et al., 2003). Although early exposure of SDIA-treated cells to BMP4 suppresses neural differentiation, late BMP4 exposure (during days 5–9) of cells co-cultured with PA6 stromal cells induced cells to differentiate into NC cells and dorsalmost CNS cells, with autonomic system and sensory lineages induced preferentially by high and low BMP4 concentrations, respectively (Mizuseki et al., 2003). The ability of SDIA-treated cells to differentiate into ectoderm-derived sensory tissues such as retinal pigment epithelium (Kawasaki et al., 2002) and lens cells (Ooto et al., 2003) has also been demonstrated. The stromal cell feeder layer has been widely used subsequently to enrich both mouse embryonic stem cell (mESC) and hESC for neuronal precursors (Buytaert-Hoefen et al., 2004; Takagi et al., 2005; Ko et al., 2007; Song et al., 2007; Elkabetz et al., 2008; Freed et al., 2008; Pomp et al., 2008)

hESCs co-cultured using PA6 stromal cells have been induced to express NC markers and generate peripheral sensory neurons (PSN) (Pomp et al., 2005; Brokhman et al., 2008; Jiang et al., 2008; Pomp et al., 2008). The weakness of the earlier study was the low yield (less than 1%) of desired PSNs (Pomp et al., 2005). To improve efficiency, the authors switched to combining generation of neurospheres (NSPs) and co-culturing with PA6 cells. A ten-fold increase in the yield of PSNs was achieved when NSPs generated using the BMP antagonist, Noggin were dissociated and used as starting material to co-culture with PA6 (Brokhman et al., 2008). NSP formation in the presence of Noggin in a defined culture system reduced the heterogeneous nature of EB differentiation and allowed hESC to develop mainly into NPs, while differentiation into other lineages was suppressed. Further improvement in the method was achieved by using PA6 cells for NSP induction. Using this approach NSPs were generated by co-culture of hESCs with PA6 cells for 2 weeks and further differentiation into PSN (Pomp et al., 2008). Compared with previous methods (Pomp et al., 2005; Brokhman et al., 2008) the use of PA6 generated NSPs significantly reduced the time required for differentiation and increased not only the yield (more than 25%) but also the purity of PSNs. In addition this study was the first to report the expression of known NC markers *SNAIL, SOX9, MSX1* and *FOXD3* in a 4-week gestation human embryo. Earlier reports showed the expression of p75 (Josephson et al., 2001) and *SOX10* (Bondurand et al., '98) in human embryos at this stage.

The above studies demonstrated that differentiated embryonic stem cells selectively induced terminal differentiation of NC derivatives; however, actual NC cells themselves have not been identified in these studies. The first demonstration of the isolation and directed differentiation of NCSC-like cells was performed using mESCs by means of combining ST2 stromal cell co-culture and FACS sorting (Motohashi et al., 2007). It was shown earlier by the same group that co-culturing mESCs with ST2 stromal cells showed efficient derivation of melanocytes after 21 days (Kunisada et al., 2003). Based on this observation the mESC culture conditions were improved to induce exclusively melanocytes and NCSC-like cell population obtained after the c-KIT sorting. These cells were able to differentiate into melanocytes, neurons and glial cells in vitro. Interestingly studies that have demonstrated the emergence of NC cells from both mESC (Rathjen et al., 2002; Mizuseki et al., 2003) and hESC (Lee et al., 2007; Jiang et al., 2008; Pomp et al., 2008) have shown the potential of these NCSCs to differentiate into sensory neurons, autonomic neurons, smooth muscle cells and glial cells, but not to melanocytes, at least without sorting.

Recently Jiang et al. (2008) showed that the efficiency of NCSC differentiation from hESCs can be dramatically improved by combining SDIA co-culture with FACS-based enrichment. After only 1 week of co-culture of H9 hESCs with PA6 cells about half of colonies were positive for p75 and the vast majority of these cells were also positively stained for HNK1, a general NCSC marker, which was used for characterization of avian embryo (Bronner-Fraser, '86) and human NCSC (Lee et al., 2007). As prolonged incubation and sorting times are required for dual staining the authors used single marker (p75) FACS sorting, which proved sufficient for successful enrichment of multipotent cells with genetic, phenotypic and functional characteristics of NCSCs. These cells not only gave rise to peripheral nerves, glial and myofibroblastic cells under differentiation conditions in vitro but importantly, also cells that differentiated into NC derivatives when transplanted into developing chick embryos in vivo. However, sorting for a single marker raises purity concerns. As a result optimizing conditions for using multiple markers for cell isolation is important. It remains unclear whether combining c-KIT sorting with p75 and/or HNK-1 will permit selective isolation of SDIA-induced human NCSC that have maintained melanocytic differentiation capacity.

In another study cranial NC-like cells were derived from hESCs through the formation of EBs and subsequent FACS sorting for $FZD3^+$/$CAD11^+$ without the use of stromal cells. More than 1% of cells were double positive for selected markers and were able to self-renew and maintain multipotent differentiation potential (Zhou and Snead, 2008). More detailed information on procedures for generating NCSCs from ESCs is depicted in Table 1.

Table 1. Characterization of ESC-derived NCSC-like cells.

NCSC derivation method	References	Cells	Comments	Markers for FACS sorting	Markers used for assessing	Terminal differentiation						
						PNS	Glia	Myofib-roblasts	Chond-rocytes	Osteo-cytes	Melano-cytes	Adipo-cytes
SDIA-derived protocols	Mizuseki et al., 2003	mESCs (EB5), primate ESCs	PA6/BMP4	–	*Ncx, Slug, Snail, Msx1, Hand1, Ap2/Ncam, Brna3/Peri*	Y		Y				
	Motohashi et al., 2007	D3 mESC, GFP-D3 ESC	9–12 days on ST2	c-Kit$^+$/ CD45$^-$	*Snai1, Slug, Pax3, Sox10, Mitf-M*	YY	Y	Y			YY	
	Pomp et al., 2005	HES1, HUES1, HUES7	PA6 <1% yield	–	*SNAIL, HAND1, SOX9, MSX1, FOXD3, AP2, E-CAD*	Y						
	Brokhman et al., 2008	HES1, HUES7	Nog-NSP-PA6 ~10% yield	–	*SNAIL, HAND1, SOX9, P75,, NCX,SOX10, FOXD3*	Y	Y					
	Pomp et al., 2008	HUES7, HUES9	PA6-NSP-PA6 25% yield	–	*AP2, NCAM, SNAI1, SOX9, SOX1, MSX1, PAX3, FOXD3*	Y	Y					
	Jiang et al., 2008	H9 (WA-09), H1 (WA-01)	Only 7 days on PA6, ~50% of cells p75$^+$	p75$^+$	*p75, HNK1, SNAI1, AP2, SLUG, SOX10*	YY	YN	YY				
EB-based protocols	Rathjen et al., 2002	mESCs, E14, D3	EBM for 9 days	–	*Sox10*	Y	Y	Y				
	Zhou and Snead, 2008	H9, HUES1, HUES3	10 days EB	FZD3$^+$/ CAD11$^+$	*SOX10, p75*	Y	Y	Y	Y	Y		
Neural Rosette-derived protocols	Lazzari et al., 2006	ICM from Bovine embryo	ON STO feeder		*Slug, Msx1, Sox10, FoxD3*	Y	Y	Y	Y		Y	
	Lee et al., 2007	WA-09, I-8 RUES1-eGFP	MS5 co-culture for ~28 days to get NR	p75$^+$/ HNK1$^+$	*SOX10, PAX3,, SNAIL, AP2, BRN3A, ERBB3*	YY	YN	YY	YY	Y		Y
	Elkabetz et al., 2008	H9 (WA-09), H1 (WA-01), RU-01eGFP	MS5 co-culture and EB derived NR	FORSE1$^-$/ N-CAD$^+$	*PAX3, AP2, NGFR, MSX1, SOX10, TWIST1, ERBB3, P75*							
Signaling pathway change	Chambers et al., 2009	WA-09, iPS14, iPS27	11-day treatment Noggin and SB	PAX6$^-$	*AP2, HNK1, PAX7, p75, HMB45*							

In vivo differentiation results are depicted in bold.

Despite improvements, the major drawback of SDIA-based methods is the use of undefined culture conditions such as stromal cells (PA6, ST2 or MS5). Stromal cell contamination also may cause difficulties for focused studies of NC biology. ST2 stromal cells have been effectively excluded by flow cytometry using GFP-mESCs for initial differentiation (Motohashi et al., 2007). Alternatively, fluorescent stromal cells can be generated for SDIA protocols enabling negative sorting of desired cell populations.

NEURAL ROSETTE-DERIVED NCSCS

The neural rosette is the developmental signature of neuroprogenitors in cultures of differentiating embryonic stem cells; rosettes are radial arrangements of columnar cells that express many of the proteins expressed in neuroepithelial cells in the neural tube. Although differing in some details, the development of neural rosettes proceeds under alternative conditions in which pluripotent hESCs either differentiated into EBs (Zhang et al., 2001; Li et al., 2005) or proliferated in the presence of stromal cell feeders (Perrier et al., 2004). Whatever the mechanism, selective recovery of neural rosettes was shown to generate enriched populations of neuroprogenitors expressing early neuroectodermal markers such as PAX6 and SOX1 (Zhang et al., 2001; Perrier et al., 2004; Wilson and Stice, 2006).

NCSCs were derived from hESCs at the neural rosette stage by FACS sorting for p75 and HNK1 (Lee et al., 2007). Here the neural rosettes were generated by the methods of Perrier et al. (2004) through co-culturing hESCs with MS5 stromal cells. Neural differentiation was induced by withdrawal of bFGF/EGF and exposure to BDNF, GDNF, NGF and dbcAMP. This treatment regimen yielded both peripheral sympathetic neurons (TH^+/$Peripherin^+$) and sensory neurons ($Brn3a^+$/$Peripherin^+$). Schwann cell differentiation was also induced from these expanded $p75^+$/$HNK1^+$ cultures by treatment with CNTF, neuregulin1 beta, and dbcAMP. However these early NCSCs could not form Schwann cells in vivo. Similar results were obtained in a recent study, when despite successful glial differentiation in vitro NCSCs did not differentiate into Schwann cells in either developing or adult hosts in vivo (Jiang et al., 2008). These facts support the view that cell-intrinsic temporal changes in epigenetic state, including direct methylation of the GFAP promoter, rather than an artifact of in vitro culture is an explanation of results on Schwann cell differentiation. The general drawback of the rosette-intermediate process is that it is time-consuming. It also requires undefined culture conditions due to the use of stromal cells or generation of heterogeneous EB formation.

NC precursor cells were also derived from neural rosettes generated from bovine embryos (Lazzari et al., 2006). In this study neural rosettes were directly derived from the inner cell mass of both cloned (nuclear transfer) and fertilized (in vitro fertilization) bovine embryos. After in vitro expansion, neural rosettes gave rise to p75-positive NC precursor cell lines ($>90\%$) capable of long-term proliferation and differentiation into autonomic and sensory peripheral neurons, glial cells, melanocytes, smooth muscle cells, and chondrocytes, recapitulating in vitro the unique plasticity of the NC lineage (Lazzari et al., 2006). This study provides a model of neural induction directly from pre-implantation embryos of a large mammalian species and gives unprecedented in vitro access to early steps of nervous system development. The characterization of highly proliferative cell lines of NC identity demonstrates the possibility of obtaining them directly from the embryo, PNS and ectomesenchymal derivatives for potential cell therapy and tissue engineering applications.

Recent advances revealed that hESC-derived neural rosettes (termed as R-NSCs) represent a novel stem cell state that can be prospectively isolated, regionally specified, and expanded in vitro in the presence of SHH/Dll4/Jag without losing rosette properties (Elkabetz et al., 2008). Expression profiling analysis identified a unique set of genes specific to only R-NSCs (*PLZF, EVI-1, DACH1*). It has been demonstrated that R-NSCs derived from hESCs (using both stromal cell-based and EB-based protocols) as well as from mESCs and mouse E8.25 anterior neural plate tissue share the same characteristics suggesting that R-NSCs might represent the neural stem cell stage in vivo at the neural plate stage (Elkabetz et al., 2008). Isolation of $Forse1^+$/$N\text{-}cad^+$ cells from R-NSCs was used as a marker for prospective isolation of anterior neural rosette cells (Elkabetz et al., 2008). On the other hand, $Forse1^-$/$N\text{-}cad^+$ R-NSCs were shown to have the capacity to generate NC lineages providing a novel strategy for studying early human NC development in vitro. The potential of R-NSCs to recreate the full neuronal diversity would ultimately improve access to therapeutically relevant cell types.

TRANSGENESIS

Embryonic stem cell differentiation may be redirected through the expression of transcription factors involved in cell fate decision either through over-expression or using inducible gene expression systems. The limitation of such approaches is that most transcription factors should be present only during defined developmental windows. The use of promoters active only at a particular stage of differentiation (for example *Sox1*) allows the physiological expression pattern of transcription factors to be mimicked more closely. The onset of *Sox1* expression correlates with the formation of neural plate, and its expression is downregulated when neural cells differentiate (Wood and Episkopou, '99) making it an ideal candidate for the enrichment of neuroprogenitors. Generating cells that express GFP under the control of *Sox1* promoter allowed sorting for early neuroectodermal cells (Aubert et al., 2003; Ying et al., 2003). Genetic selection of *Sox1*-GFP expressing neural precursors by FACS sorting was shown to effectively remove unwanted proliferating

cell types and avoid tumor formation after transplantation (Chung et al., 2006). An alternative approach might be to use a tissue-specic promoter to express antibiotic resistance allowing the selective killing of undesired cells. Although most differentiation studies implementing *Sox1*-GFP cells involved generation of CNS derivatives, attempts to derive NCSCs are also in progress (Rodgers et al., 2009). A major drawback of this method is the poor survival of some cell types after cell sorting and low yields of the desired cells.

A conditional transgenesis approach was used to establish a NC cell line from mouse trunk NC (Maurer et al., 2007). Using a transgenic mouse line, allowing for spatio-temporal control of the transforming *c-myc* oncogene, the JoMa1 cell line was derived. These cells express NCSC markers in a transgene-activity-dependent manner. A conditional 4-OHT inducible *c-MycER* when "switched on", keeps the cells in a multipotent proliferative state and when "switched off" allowed directed differentiation into neurons, glia, smooth muscle cells, melanocytes and chondrocytes. This unique approach would allow the generation of NCSC lines by crossing c-MycER^T mice with mice harboring mutations affecting NC development, thus enabling further insight into genetic pathways controlling NC differentiation. Overall this system would provide a powerful tool for studying NCSC biology.

MODIFICATION OF SIGNALING PATHWAYS

Optimization of cell differentiation through targeting different intracellular pathways is becoming a promising research avenue. Growing evidence suggests that self-renewal and differentiation of hESCs can be regulated using small molecules that modulate intracellular signaling pathways (Ding et al., 2003; Hao et al., 2008; Ying et al., 2008). Inhibition of Activin/Nodal signaling was shown to promote specification of hESCs into neuroectoderm indicating a role for this pathway in controlling early neural development of pluripotent cells (Smith et al., 2008). Therefore, in their quest to improve the efficiency of the generation of neuroprogenitors from hESCs, Chambers et al. (2009) used inhibitors of SMAD signaling to promote neural conversion of human ES and iPS cells. The synergistic action of Noggin and SB431542 was sufficient to induce rapid and complete neural conversion of $>80\%$ of hESCs under adherent culture conditions. After the comparison of early neural precursor cells obtained by dual SMAD inhibition with previously described R-NSC (Elkabetz et al., 2008) the authors suggested that these neuroprogenitors may represent the most primitive hESC-derived neural precursor state that is then capable of rosette formation. Interestingly, FACS sorting revealed that PAX6$^+$ cells exhibited an anterior CNS character, whereas PAX6$^-$ cells under the same conditions expressed NC markers *AP2α*, *HNK1*, *PAX7* and *p75*. Moreover it was shown that the ratio of PAX6$^+$/PAX6$^-$ could be regulated by manipulating initial cell seeding densities; when higher cell density was applied more cells became Pax6$^+$, whereas lower density favored the formation of Pax6$^-$ NC progeny (Chambers et al., 2009). The schematic representation of various protocols implemented in derivation of NCSCs from hESCs is shown in Fig. 1.

ADULT TISSUE-DERIVED NCSC

Multipotent NC cells with a differentiation capability similar to that of NCSCs have been identied in several embryonic and adult locations and recently reviewed by Delfino-Machin et al. (2007).

Several laboratories have reported that cells isolated from the adult skin of mice and humans have self-renewal features and differentiate into neurons, glial cells, smooth muscle cells, melanocytes, chondrocytes, and adipocytes (Fernandes et al., 2004; Toma et al., 2005; Wong et al., 2006). Other studies have shown that cells in adult hair follicles are also capable of differentiating into derivatives of NCCs and these cells were thought to be derived from NCCs according to the cell lineage analysis (Sieber-Blum et al., 2004; Amoh et al., 2005a,b; Yu et al., 2006). Pluripotent stem cells identified in the adult mouse hair follicle and termed EPI-NCSCs (Sieber-Blum et al., 2004) currently have the best claim to be truly multipotent NCSCs, being able to generate cells expressing markers appropriate for neurons, glia, myobroblasts, chondrocytes and melanocytes. The transcriptional prole of EPI-NCSCs in culture identified a 19 gene "molecular signature" that distinguished them from epidermal stem cells (which generate keratinocytes), despite sharing a niche in the bulge of hair follicles (Hu et al., 2006). In addition, EPI-NCSCs share a portion of the stem cell gene expression profile of iPS cells (Sieber-Blum and Hu, 2008). Owing to their ability to migrate, EPI-NCSCs can be isolated as a highly pure population of multipotent progenitors and they share some characteristics with pluripotent stem cells without being tumorigenic. As the patient's own EPI-NCSCs could be used for transplantation, this would avoid graft rejection. Recent studies demonstrated the use of adult tissue-derived NCSCs in an effort to repair disorders such as the lesioned spinal cord and EPI-NCSCs grafted into the adult spinal cord do not show evidence of tumorigenesis (Sieber-Blum et al., 2006; Song et al., 2008).

NCSC-derived cells, such as melanocytes or glial cells, showed unstable NC cell phenotypes and could dedifferentiate and then redifferentiate into other NC derivatives. Pigmented cells isolated from the skin of quail embryos were able to generate glial cells and myobroblastic cells, which are the derivatives of NC cells, when exposed to EDN3 act as a mitotic signal (Dupin et al., 2000, 2003; Trentin et al., 2004; Real et al., 2006). Schwann cells isolated from quail embryonic nerves generated myobroblastic cells (Real et al., 2005). The melanoblasts, which are thought to be restricted in their fate to melanocytes, were shown to have a multipotential cell fate being able to generate neurons, glial cells, and smooth muscle cells in addition to melanocytes, even after they have already migrated toward the target sites in the skin (Motohashi et al., 2009).

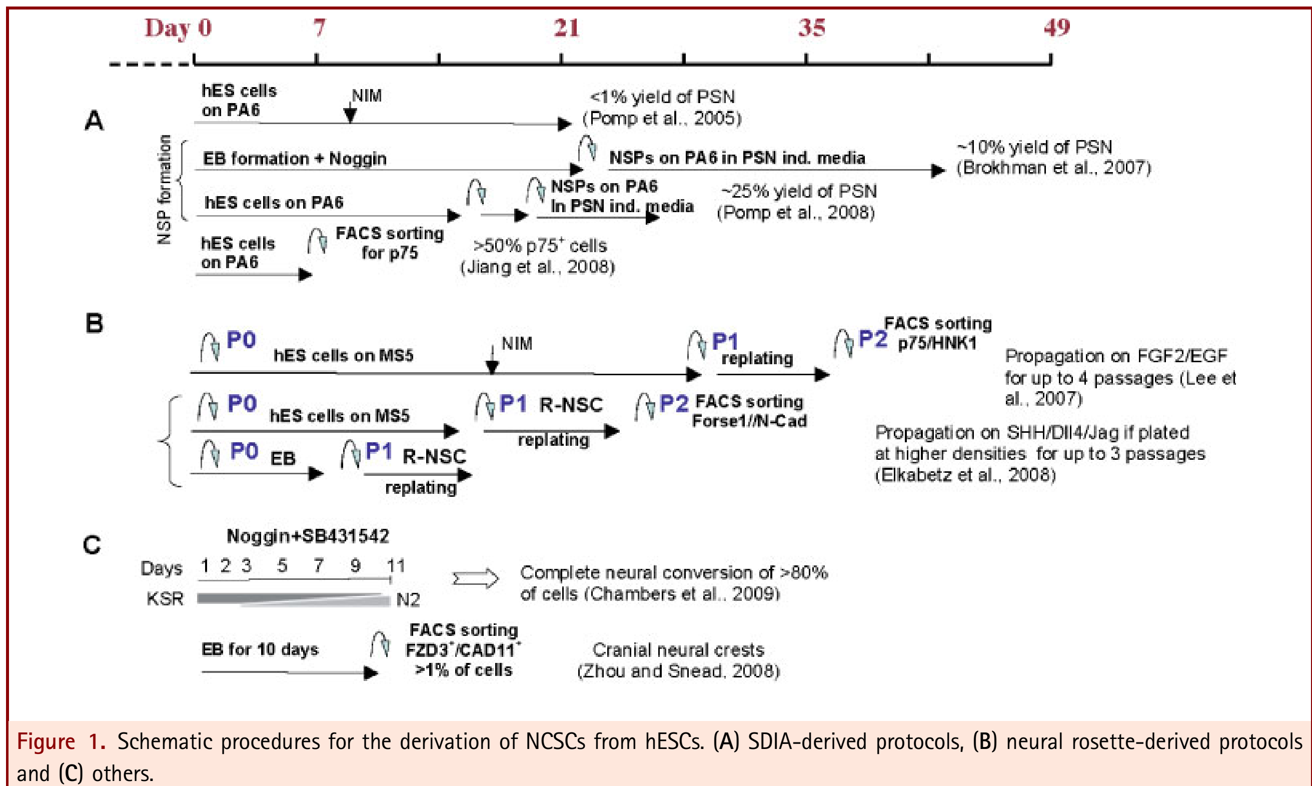

Figure 1. Schematic procedures for the derivation of NCSCs from hESCs. (A) SDIA-derived protocols, (B) neural rosette-derived protocols and (C) others.

CONCLUDING REMARKS

There has been considerable effort in recent years to identify and isolate NCSCs derived from ESCs. The ability to rapidly and reproducibly generate large numbers of NCSCs for biological studies will be invaluable for studies of disrupted NC development and to elucidate the pathogenesis of both benign and malignant human NC disorders. There are several issues that are important to address for cells to be suitable for use in human therapy including establishing a system free of any potential harmful substances. Many protocols involve materials of animal origin, which could be a source for potential pathogens. In addition feeder cells can transfer xenogenous glycans to hESC and induce an immune response. Moreover maintaining naïve embryonic stem cells on fibroblast feeder layers make these protocols very labor intensive. Although recent studies reported fully feeder-free method of culturing hESCs (Ludwig et al., 2006), the use of feeder-free hESC for SDIA protocols or derivation of neural rosettes has not yet been reported. In contrast to hESCs, the use of feeder-free mESCs such as the *Sox1*-GFP cell line has been implemented. Co-culture systems with stromal cells for neural induction also cause the same problems as the most of these are of animal origin (murine PA6, ST2 MS5 cell lines). One possibility is to replace them with human stromal cells but generation of such lines has proved to be technically challenging and thus such lines have not been described yet. The use of other cell types of animal origin such as mouse Sertoli cells (Yue et al., 2006), or chicken somites (Sagha et al., 2009) for co-culturing have been demonstrated to induce neuronal differentiation. On the other hand using selective survival approaches involving transgenes for the generation of neurons raises similar concerns. It is not clear at this point whether vector-mediated transgenesis of hESCs will be suitable for clinical applications. The use of lentiviral vector-mediated integration of the perspective transgenes into the host cell genome might present the risk of malignant transformation. Therefore, it is important to develop other strategies such as nonintegrating transgenesis or cell-permeant proteins, to introduce these transcription factors into the host cells.

The majority of neural induction protocols for hESCs rely on EB formation, stromal feeder co-culture or selective survival conditions. Each strategy has considerable drawbacks, such as poorly defined culture conditions, protracted differentiation and low yield. Neural induction by the dual SMAD inhibition helped to overcome some disadvantages of NCSCs from hESCs derivation protocols; it is simple, requires less time, less work, and provide better yields and purity, and defined culture conditions (Chambers et al., 2009).

Another challenge in current differentiation protocols is a lack of synchronicity of the generated cell populations. Transplantation of cells after FACS sorting for NCSC markers effectively eliminated in vivo tumor formation indicating effective removal of predifferentiated cells via sorting (Lee et al., 2007). Given the

disparities in the protocols it is conceivable that the SDIA and R-NSC approaches and signaling pathway modulation result in cells that correspond to in vivo-derived cells of different developmental stages. Direct comparison of NCSCs derived by different techniques would be important to determine whether or not these NCSCs possess equivalent differentiation and self-renewal capacities in vitro and in vivo. In addition, comparison of hESC-derived NCSCs with adult tissue-derived cells will provide insights into the presumed ontogeny of NC cells and clarify the relationship between these different stem cells.

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