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Analysis of the neurogenic potential of multipotent skin-derived precursors

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Abstract

Multipotent precursors similar to stem cells of the embryonic neural crest (NC) have been identified in several postnatal tissues, and are potentially useful for research and therapeutic purposes. However, their neurogenic potential, including their ability to produce electrophysiologically active neurons, is largely unexplored. We investigated this issue with regard to skin-derived precursors (SKPs), multipotent NC-related precursors isolated from the dermis of skin. SKP cultures follow an appropriate pattern and time-course of neuronal differentiation, with proliferating nestin-expressing SKPs generating post-mitotic neuronal cells that co-express pan-neuronal and peripheral autonomic lineage markers. These SKP-derived neuron-like cells survive and maintain their peripheral phenotype for at least 5 weeks when transplanted into the CNS environment of normal or kainate-injured hippocampal slices. Undifferentiated SKPs retain key neural precursor properties after multi-passage expansion, including growth factor dependence, nestin expression, neurogenic potential, and responsiveness to embryonic neural crest fate determinants. Despite undergoing an apparently appropriate neurogenic process, however, SKP-derived neuron-like cells possess an immature electrophysiological profile. These findings indicate that SKPs retain latent neurogenic properties after residing in a non-neural tissue, but that additional measures will be necessary to promote their differentiation into electrophysiologically active neurons.

Keywords: Stem cells; Neurogenesis; Neural crest; Transplantation; Hippocampal slice; Growth factors

Introduction

The embryonic neural crest (NC) is a population of ectodermally-derived precursors that has unique migratory properties and differentiation characteristics. These multipotent stem cells originate near the boundary between the primitive neural plate and adjacent epidermis along most of the vertebrate rostro-caudal axis. They subsequently migrate throughout the body to produce diverse neural and mesodermal cell types. Among the known derivatives of the neural crest are the neurons, glia, fibroblasts, and endocrine cells of the autonomic, sensory, and enteric nervous systems, the bone, cartilage, meninges, connective tissue, pericytes, and dermis of the head, smooth muscle of the outflow tract of the heart, and melanocytes and sensory receptors of the skin (Le Douarin and Dupin, 2003; Le Douarin and Kalcheim, 1999).

Multipotent cells that have characteristics reminiscent of embryonic NC stem cells have been isolated from several postnatal tissues, including skin, gut, dental pulp, and the heart (Fernandes et al., 2004; Kruger et al., 2002; Miura et al., 2003;

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Tomita et al., 2005). We recently reported that multipotent NC-related cells can be isolated from skin, a particularly abundant and accessible tissue (Fernandes et al., 2004). These skin-derived precursors, or SKPs, can be cultured from the dermis of rodent (Fernandes et al., 2004; Toma et al., 2001) and human (Toma et al., 2005) skin. Recent studies have revealed two important features of SKPs. First, SKPs are multipotential. When colonies were generated from single rodent (Toma et al., 2001; Fernandes et al., 2004) or human (Toma et al., 2005) SKPs, using both limiting dilution and methylcellulose-based methods of clonal analysis, they contained distinct sub-populations of cells with the properties of neurons, glial cells, smooth muscle cells, and adipocytes. Second, SKPs are a type of NC-related precursor. Cultured SKPs expressed a variety of markers of primitive embryonic NC precursors, including characteristic NC transcription factors, and they behaved similarly to host NC precursors when transplanted into the embryonic chick neural crest migratory stream. Fate mapping studies with Wnt1-cre;R26R compound transgenic mice, which express β-galactosidase in neural crest-derived cells, confirmed that at least the SKPs derived from facial skin were neural crest-derived (Fernandes et al., 2004). Moreover, SKPs from dorsal back skin can differentiate into functional myelinating Schwann cells (McKenzie and Miller, 2002), a cell type only derived from the neural crest.

The persistence of NC-related precursors within accessible postnatal tissues raises the possibility of their use for a variety of research and therapeutic purposes. For example, NC-related precursors have been useful for modeling how mutations in genetic diseases, such as Hirschprung's Disease (Iwashita et al., 2003), or in NC cancers, such as melanomas (Fang et al., 2005), affect the fate decisions, properties, and functions of embryonic NC cells. NC-related precursors could also potentially be expanded in vitro to produce larger numbers of NC cells for high throughput screening or for cell replacement transplantation.

In the present study, we investigated questions relating to the feasibility of using multipotent NC-related SKPs for such practical applications. Since skin is a non-neurogenic tissue, we specifically asked to what extent SKPs have neurogenic potential and whether they produce electrophysiologically active neuronal progeny.

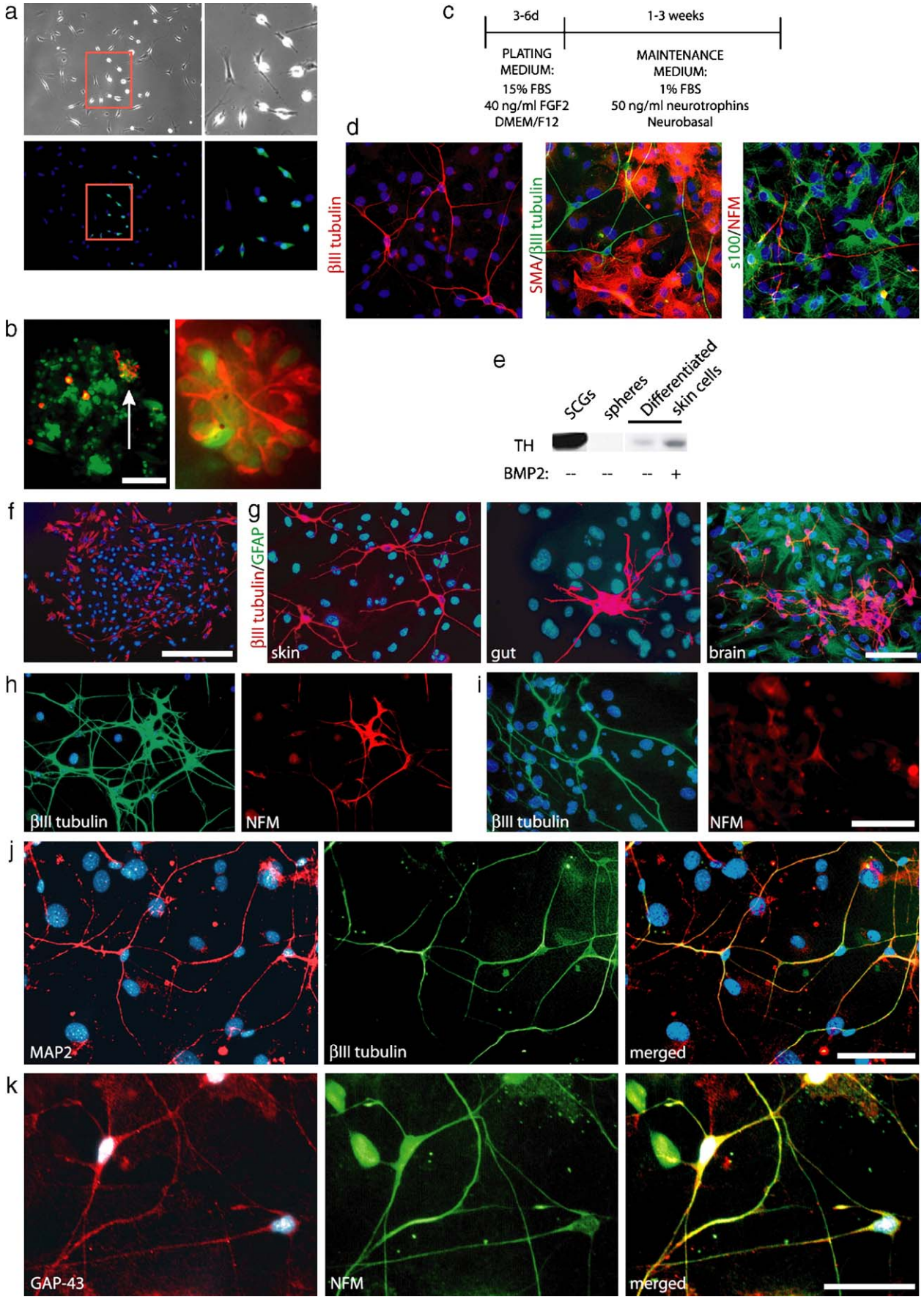
Results

In vitro neuronal differentiation conditions for SKPs

We have previously reported that FGF2 is a mitogen for nestin-expressing cells within cultures of dissociated skin cells (Fernandes et al., 2004; Toma et al., 2001). When dissociated skin cells are grown adherently in serum-containing medium, FGF2 induces rare nestin-positive cells to proliferate into clusters (Fig. 1a). Alternatively, when skin cells are grown non-adherently in serum-free medium, FGF2 promotes growth of neurosphere-like aggregates of these nestin-expressing cells (Fig. 1b). Under these non-adherent conditions, the majority of skin cells adhere to the tissue culture plastic, thereby allowing for enrichment and isolation of floating SKP spheres.

Since neurons are not normally produced within the skin in vivo, we began by testing whether a modified differentiation protocol could promote neuronal differentiation of SKPs. This protocol was based on similar differentiation protocols used for other PNS and CNS neural precursors (Fig. 1c; see Methods). We first tried differentiating skin cells immediately upon their dissociation from the backs of late embryonic mice, when the density of sphere forming cells is approximately 0.4% of total skin cells (Fernandes et al., 2004). After 2–3 weeks of differentiation using this protocol, immunocytochemical analysis revealed the differentiation of branching neuron-like cells that expressed the neuron-specific marker βIII tubulin (Fig. 1d). Approximately 472 ± 45 (n = 12, median = 469, SD = 157) βIII tubulin-expressing cells were produced from 37500 dissociated skin cells (n = 12), which would indicate that, if all precursors are indeed sphere-forming cells, each SKP generated an average of 3 neurons upon differentiation. Double label immunocytochemistry further demonstrated that these βIII tubulin-expressing cells co-expressed neurofilaments and did not co-express markers of related neural crest cell derivatives, including glial cells (S100β), smooth muscle cells (smooth muscle actin, SMA) (Fig. 1d), or melanocytes (Fernandes et al., 2004). Moreover, expression of the catecholaminergic enzyme tyrosine hydroxylase (TH) was detected in western blots, and was increased by supplementing the differentiation medium with the neurogenic factor BMP2 (Fig. 1e), as previously described for embryonic neural crest stem cells (Shah et al., 1996). Specifically, densitometry measurements indicated that BMP2-treated samples contained approximately 1.7 ± 0.1 fold (P = 0.04, paired t test, n = 2) higher TH protein levels. Thus, this neurogenic differentiation protocol promotes a neuron-like differentiation process in skin-derived precursor cells.

We also tested the effectiveness of this differentiation protocol on SKPs that were first expanded into primary spheres for 7 days using FGF2 in serum-free medium. Within the first 24 h of exposure to the plating medium, SKP spheres adhered to the substrate and nestin-expressing cells began migrating out from the spheres (Fig. 1f). Double label immunocytochemistry for neuronal (βIII tubulin) and glial (GFAP) markers after 2 weeks of differentiation demonstrated the generation of βIII tubulin-expressing cells but not mature GFAP-expressing glial cells under these conditions (Fig. 1g). Since there was abundant immunoreactivity for S100β (not shown), which labels glial precursors, and addition of heregulins results in robust Schwann cell differentiation (Fernandes et al., 2004; McKenzie and Miller, 2002), it is likely that the absence of GFAP-expressing glial cells is due to the lack of gliogenic factors required for differentiation of peripheral glial precursors. For comparison, spheres derived from embryonic gut, another neural crest-derived tissue, also differentiated into βIII tubulin-expressing presumptive neurons and not GFAP-positive cells, while spheres derived from CNS cortical cells generated both βIII tubulin-positive neuron-like cells and GFAP-expressing astrocyte-like glia (Fig. 1g). After 3 weeks of differentiation, the SKP-derived βIII tubulin-expressing cells co-expressed


a
b
c
3-6d
1-3 weeks
PLATING MEDIUM:
15% FBS
40 ng/ml FGF2
DMEM/F12
MAINTENANCE MEDIUM:
1% FBS
50 ng/ml neurotrophins
Neurobasal
d
βIII tubulin
SMA/βIII tubulin
s100/NFM
e
SCGs
spheres
Differentiated skin cells
TH
BMP2: -- -- -- +
f
g
βIII tubulin/GFAP
skin
gut
brain
h
βIII tubulin
NFM
i
βIII tubulin
NFM
j
MAP2
βIII tubulin
merged
k
GAP-43
NFM
merged

neurofilaments (Fig. 1h), as well as other classes of neuronal markers, such as the microtubule-associated protein MAP2 (Fig. 1j) and the neuronal growth-associated protein GAP-43 (Fig. 1k). Serum-derived factors were an important component of this differentiation protocol, as differentiation in serum-free conditions only produced rare βIII tubulin-positive cells, and these generally failed to mature sufficiently to express other neuronal markers, such as neurofilaments (Fig. 1i).

Time-course of early events in SKP neuronal differentiation

To examine SKP differentiation in greater detail, we monitored changes in gene expression over the course of the precursor-to-neuron transition. Spheres grown from late gestation embryonic mouse skin were plated in Plating Medium for 3 days, and then switched to Maintenance Medium. Cells were analyzed by immunocytochemistry between 1 h and 7 days of differentiation, specifically testing for expression of markers of undifferentiated SKPs (nestin, fibronectin), proliferating cells (Ki67, bromodeoxyuridine (BrdU) incorporation), and newly born neurons (neuron-specific βIII tubulin).

Differentiating SKPs gradually reduced their expression of nestin and proliferation markers. SKP spheres attached to the substrate within 1 h of plating, expressed nestin (Fig. 2a, insets), and most cells had begun dispersing by 1 day in the plating medium. Quantitation of 500–1500 cells from 2 to 4 experiments showed that at the 1 day time point, 82 ± 1% (SEM) of cells expressed nestin (Fig. 2a, left panels) and 57 ± 2% expressed the proliferation marker Ki67 (Fig. 2b, left). After 4 days of differentiation (i.e., 1 day after switching to Maintenance Medium), the proportion of cells expressing nestin (73 ± 3%) and Ki67 (47 ± 4%) were not significantly changed ($P > 0.05$, Tukey test). However, by 7 days of differentiation (i.e., 4 days in Maintenance Medium), these precursor parameters were sharply reduced. Expression of nestin was reduced to 9 ± 2% ($P < 0.005$ compared to 1 day and 4 days timepoints, Tukey test) and expression of Ki67 had decreased to 4 ± 1% ($P < 0.005$ compared to 1 day and 4 days timepoints, Tukey test) (Fig. 2b, middle and right). A similar decline in the percentage of proliferating cells was found by labeling with BrdU pulses and western blotting for cyclin dependent kinase-2 (not shown).

The differentiation-induced reduction in markers of precursors and proliferation was accompanied by the appearance of cells expressing βIII tubulin. After 7 days of differentiation, many cells had flattened into a substrate-attached layer, and nestin expression was maintained only in the small subpopulation of cells that had extended processes (Fig. 2a, right panels). Double-label immunocytochemistry revealed that βIII tubulin was expressed by these process-bearing nestin-expressing cells, a profile consistent with neuroblasts and newly born neurons (Fig. 2c). βIII tubulin-expressing cells began to appear after 4 days of differentiation, when they occasionally co-expressed Ki67 (Fig. 2b, middle) and fibronectin (not shown), but they were consistently Ki67- and fibronectin-negative after 7 days differentiation. By 2–3 weeks of differentiation, the βIII tubulin-expressing cells no longer expressed nestin. Comparable patterns of gene expression were observed for differentiated SKPs grown from postnatal skin, except that an additional population of nestin-expressing cells was also present, corresponding to the myogenic progenitors from the later developing subcutaneous muscle layer (not shown).

Collectively, the preceding results indicate that proliferating, nestin-expressing precursors isolated from skin undergo a logical precursor-to-neuron transition to produce βIII tubulin$^+$NFM$^+$MAP2$^+$GAP-43$^+$ neuron-like cells.

SKP-derived neuron-like cells survive and express a peripheral phenotype when transplanted into hippocampal slice cultures

SKP cultures differentiating in vitro express markers associated with neural crest-derived peripheral catecholaminergic neurons, such as peripherin, p75^{NTR}, and low levels of TH (Fernandes et al., 2004). We therefore tested whether exposure to the CNS environment of hippocampal slice cultures supports this peripheral neuronal phenotype, or alternatively, re-directs SKPs towards a CNS neuronal or glial phenotype. SKP spheres were prepared from EYFP-expressing transgenic mice and transplanted into rat hippocampal slice cultures (Fig. 3a). A similar model has previously been used to test the neural potential of fetal

Fig. 1. Differentiation of murine SKPs in neurogenic conditions. (a, b) FGF2 stimulates proliferation of nestin-expressing skin cells. (a) Skin cells dissociated from neonatal mouse back skin were plated in serum-containing medium supplemented with FGF2, resulting in the appearance of clusters of nestin-expressing cells within 48 h (boxed areas are enlarged to the right). The top two panels are phase micrographs and the bottom two fluorescence micrographs of the same fields immunostained for nestin (green) and counterstained with Hoechst (blue) to show all of the nuclei in the field. (b) Alternatively, when placed into serum-free suspension culture supplemented with FGF2, nestin-expressing spheres begin growing within 48 h. Panels are fluorescence micrographs of cells isolated from EYFP-expressing transgenic mice that were cultured for 48 h in FGF2 and immunostained for nestin (red) and GFP (green). The right panel shows a higher magnification micrograph of the nestin-expressing cluster indicated with arrow on the left panel. (c) Schematic of the neurogenic differentiation protocol. Further details provided in Methods. (d, e) Neurogenic differentiation of primary skin cells. (d) Fluorescence micrographs of SKPs differentiated for 3 weeks in neurogenic conditions, and then immunostained for (left panel) βIII tubulin (red), (middle panel) smooth muscle actin (red) and βIII tubulin (green) or (right panel) NFM (red) and S100β (green). All cells were counterstained with Hoechst 33258 (blue) to show nuclei. (e) Western blot analysis for expression of the catecholaminergic enzyme tyrosine hydroxylase (TH) in undifferentiated SKP spheres, in differentiated skin cells with or without BMP2 treatment, and in sympathetic superior cervical ganglion (SCG) neurons as a positive control. (f–k) Neurogenic differentiation of SKPs. (f) Immunocytochemistry for nestin (red) on a SKP sphere differentiated for 24 h. (g) Double-label immunocytochemistry for βIII tubulin (red) and GFAP (green) on primary spheres derived from embryonic skin, gut, or cortex and differentiated for 10 days in neurogenic conditions. Note that only the brain spheres differentiate into GFAP-positive cells. (h, i) Double-label immunocytochemistry for βIII tubulin (red) and NFM (green) in SKPs differentiated for 3 weeks using neurogenic conditions (h) or completely serum-free conditions (i). Note that there was abundant neuronal differentiation in neurogenic conditions (h), while (i) shows a rare cluster of βIII tubulin expressing cells generated in serum-free conditions. The latter shows little NFM immunoreactivity. (j, k) Immunocytochemical analysis for panneuronal proteins in SKPs differentiated for 3 weeks in neurogenic conditions. Cells were immunostained for (j) total MAP2 (red) and βIII tubulin (green) or (k) GAP-43 (red) and NFM (green). In all experiments, cells were counterstained with Hoechst 33258 to show all of the cells in the field.

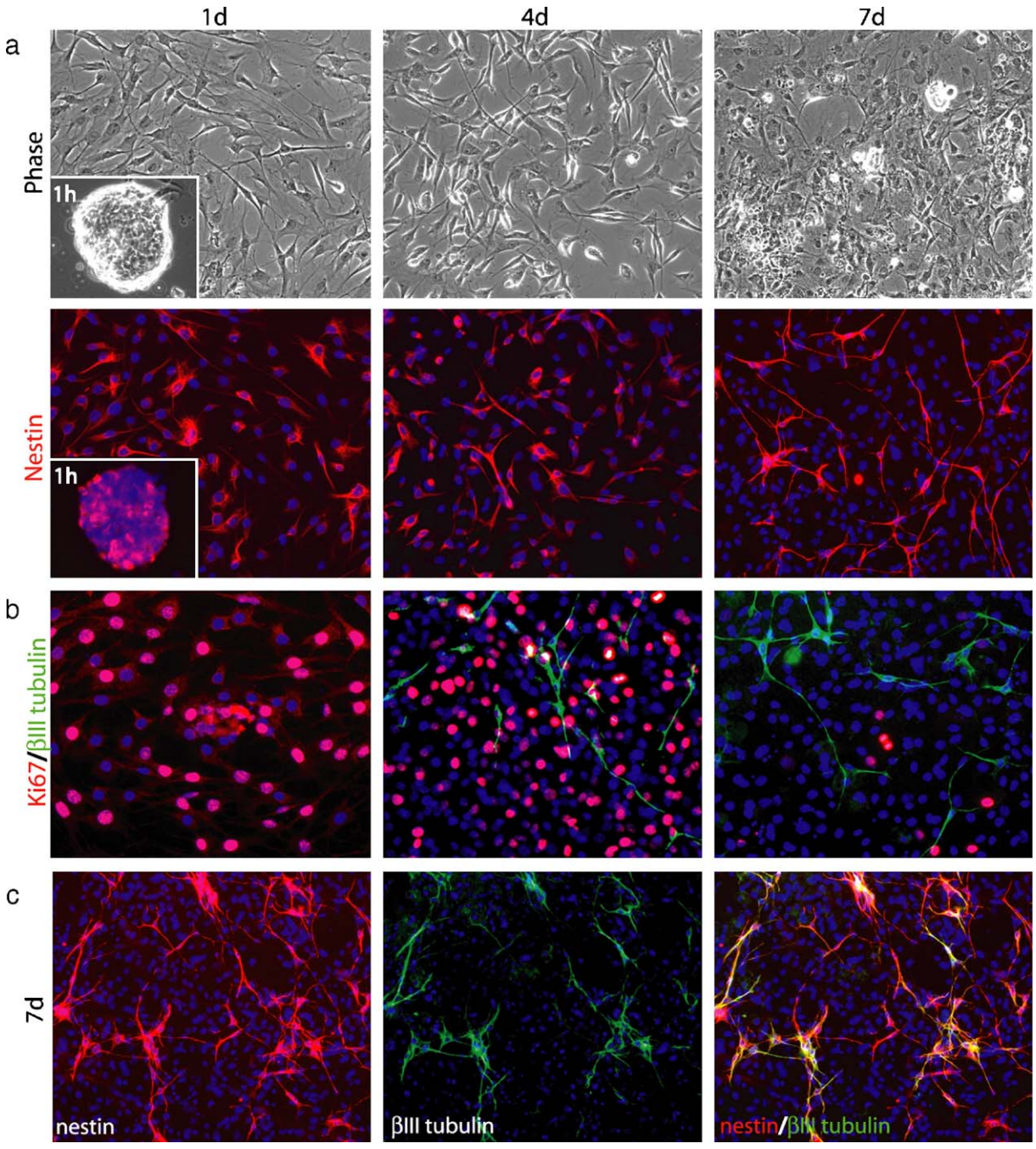


Fig. 2. Changes in neural precursor and neuronal markers over the first week of neurogenic differentiation. (a) Immunocytochemistry for the neural precursor marker nestin (red) after 1 h (insets), 1 day, 4 days, and 7 days of differentiation in neurogenic conditions. Upper and lower panels display the same fields, with the upper being phase micrographs and the lower fluorescence micrographs. Note that the proportion of nestin-expressing cells decreases significantly between 4 days and 7 days (73% vs. 9.2%, $P < 0.005$, Tukey test), so that after 7 days nestin is only maintained in cells that have extended processes. (b) Double-label immunocytochemistry for the proliferation marker Ki67 (red) and for βIII tubulin. Note that the percentage of proliferating cells at 1 day (57%), 4 days (47%), and 7 days (4%) decreases significantly ($P < 0.005$ between 4 days and 7 days time points, Tukey test) concomitant to the appearance of βIII tubulin-expressing neuron-like cells. (c) Double label immunocytochemistry for nestin (red) and βIII tubulin (green) after 7 days differentiation. Note that the βIII tubulin is expressed by cells that have maintained nestin expression (merged, right). In all experiments, cells were counterstained with Hoechst 33258 to show all nuclei. Representative images are shown from one of four experiments.

CNS precursors and embryonic stem cells (Benninger et al., 2003; Scheffler et al., 2003; Shetty and Turner, 1999). In control experiments, differentiation of EYFP-expressing spheres in vitro for 14 days generated abundant βIII tubulin-positive/EYFP-positive neuron-like cells (Fig. 3b), confirming that expression of the EYFP reporter is maintained in differentiated SKPs. However, when undifferentiated EYFP SKP spheres were transplanted into neonatal rat hippocampal slice cultures for 14 days (Fig. 3c) (prepared as described in Methods), SKPs displayed virtually no migration from the transplant site and showed little evidence of

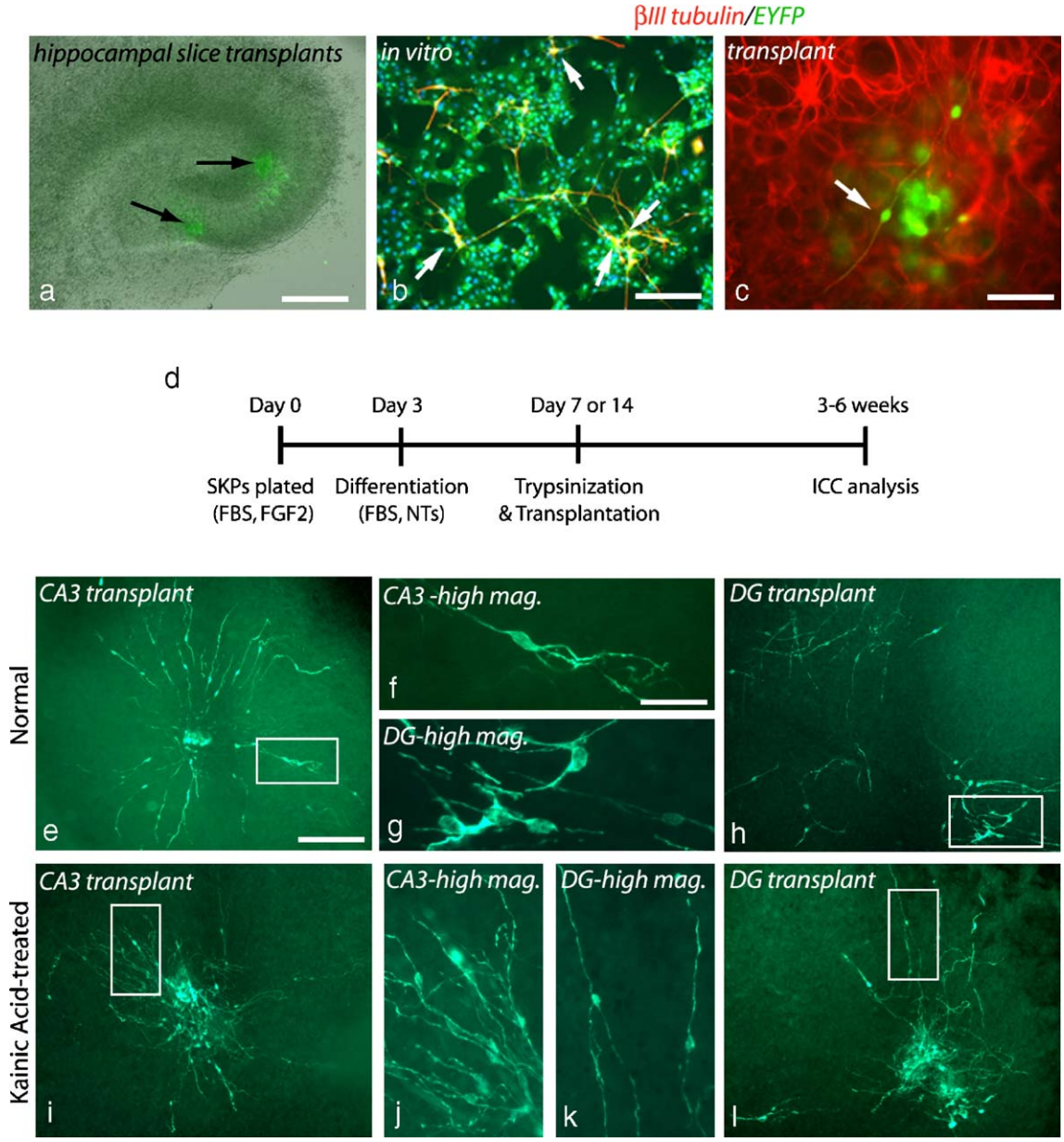


Fig. 3. Transplantation of SKPs into normal and kainic acid-treated hippocampal slice cultures. (a–c) Transplantation of naïve SKP spheres. (a) Composite of a phase image showing a neonatal hippocampal slice culture and a fluorescence image showing EYFP-expressing SKP spheres 1 week after transplantation into various locations (arrows). Scale bar = 200 μm. (b) Immunocytochemistry for βIII tubulin (red) and EYFP (green) on EYFP-expressing transgenic mouse SKPs that were differentiated for 14 days in culture. Cells were counterstained with Hoechst 33258 (blue) to show all of the nuclei. Note the generation of βIII tubulin-expressing EYFP-positive neuron-like cells (arrows). Scale bar = 150 μm. (c) Immunocytochemistry for βIII tubulin (red) and GFP (green) 14 days after transplantation of an EYFP SKP sphere. Note that naïve SKPs show minimal migration and only rarely differentiate into βIII tubulin-expressing cells when transplanted into the slice culture environment (arrow). These experiments were replicated 3 times with total $n = 18$ transplants. Scale bar = 100 μm. (d–l) Transplantation of pre-differentiated SKPs. (d) Schematic of pre-differentiation and transplantation paradigm. See Results and Methods for further details. (e–h) EYFP fluorescence of SKPs that were pre-differentiated for 7 days and then transplanted for 5 weeks into the CA3 layer (e, f) or dentate gyrus (g, h) of the hippocampus. (f) Shows a higher magnification micrograph of the boxed area in (e) and (g) shows a higher magnification micrograph of the boxed area in (h). Note the robust migration of process-bearing EYFP-expressing cells from the transplantation core (migration distances quantitated in Results). (i–l) EYFP fluorescence of SKPs predifferentiated for 14 days, and then transplanted for 4 weeks into the CA3 layer (i, j) or dentate gyrus (k, l) of neonatal hippocampal slice cultures that were pretreated with kainic acid 2 days prior to transplantation. (j) is a higher magnification micrograph of the boxed area in (i) and (k) is a higher magnification micrograph of the boxed area in (l). The experiments were repeated 3 times for the 7-day pre-differentiation experiment ($n = 24$ transplants), 6 times for the 1-day pre-differentiation experiment ($n = 144$ transplants), and 4 times for the kainic acid treatment ($n = 48$ transplants). Scale bar = 150 μm in e, h, i, l, and 50 μm in f, g, j, k.

neuronal differentiation. Specifically, only rare cells extended processes and expressed βIII tubulin (Fig. 3c), while the majority of EYFP-expressing cells disappeared over the subsequent weeks, presumably as a consequence of cell death.

Since these results suggested that the neonatal hippocampal environment does not direct undifferentiated SKPs towards a neuronal fate, we adopted a pre-differentiation strategy. Undifferentiated SKP spheres were plated and maintained in

neurogenic conditions for 1–2 weeks of pre-differentiation prior to transplantation. Aliquots containing 300–400 pre-differentiated cells were then deposited into either the dentate gyrus (DG) or CA3 layer of hippocampal slices. In striking contrast to naïve SKP spheres, SKPs pre-differentiated for 7 days rapidly emigrated from their site of transplantation, adopting a bipolar morphology reminiscent of migrating neuroblasts (Figs. 3e–h). Counts of the absolute number of EYFP-expressing cells indicated that a range of 6–11% of transplanted SKPs survived to the 5 week time point, with migration distances of up to 600 μm at both sites. For CA3 transplants, 33.7 ± 5.6 (SEM) cells survived per slice (n = 8 slices) with mean migration distances of 163 ± 12 μm (SEM) (n = 104 cells on 3 slices). For DG transplants, 31.0 ± 3.1 cells remained per slice (n = 8 slices), with a significantly greater mean migration distance of 273 ± 21 μm (n = 101 cells on 3 slices) ($P < 0.005$, Student–Newman–Keuls test). Interestingly, when pre-differentiated SKPs were transplanted into hippocampal slices pre-treated with kainic acid, which induces widespread death of host hippocampal CA3 pyramidal neurons (Routbort et al., 1999), there was no statistically significant effect on either the number of surviving SKPs or their migratory distances. Specifically, CA3 transplants had 47.4 ± 8.4 cells per slice (n = 8 slices) with migration distances of 156 ± 11 μm (n = 92 cells on 3 slices), while DG transplants had 46.5 ± 8.4 cells remaining (n = 8 slices) with mean migrations of 224 ± 13 μm (n = 86 cells on 3 slices). The differences in survival and migration compared to non-kainic acid-treated transplants were not statistically significant ($P > 0.05$, ANOVA), and migration distances for DG transplants remained greater than for CA3 transplants ($P < 0.005$, Student–Newman–Keuls test).

Immunocytochemistry 4–6 weeks following transplantation revealed that a large percentage of surviving SKPs displayed a peripheral neuronal phenotype. Approximately 78% of surviving EYFP cells were $p75^{NTR}$ immunoreactive (112/144 total EYFP cells, n = 3 slices), consistent with a peripheral phenotype (Figs. 4a–c). Moreover, 39% of the surviving EYFP cells expressed the neuronal marker βIII tubulin (53/136 total EYFP cells, n = 2 slices), and 21% of the EYFP cells also expressed the catecholaminergic marker TH (18/85 total EYFP cells, n = 3 slices) (Figs. 4d–f). Triple immunostaining confirmed that TH was expressed by βIII tubulin expressing cells (Figs. 4g–i). Transplanted SKPs also developed immunoreactivity for P/Q-type voltage-gated calcium channels (VGCCs, Figs. 4j–l), which were expressed by 23% of transplanted cells (11/47 total EYFP cells, n = 2 slices) and by host cells. Neither $p75^{NTR}$ nor TH, which are both expressed by peripheral catecholaminergic neurons, was detected within the cell bodies of host hippocampal neurons. Interestingly, there was little evidence of smooth muscle cell differentiation, with only one SMA-expressing cell found in 4 transplants (approximately 0.8% of surviving transplant cells). This suggests that pre-differentiated SKPs having a neural phenotype selectively survived within the hippocampal slice culture environment.

No evidence of SKPs differentiating into CNS neurons or glial cells was found. Immunocytochemistry for Glutamic Acid Decarboxylase (GAD, expressed by GABAergic CNS neurons) showed abundant labeling of neurons within the host hippocampal slice, but did not label transplanted EYFP SKPs (Fig. 4o). Likewise, labeling of host cells was detected with antibodies against Myelin Basic Protein (MBP, expressed by myelinating oligodendrocytes and Schwann cells) and GFAP (expressed by astrocytes and Schwann cells), but these antibodies did not label any EYFP-expressing transplanted SKPs (Figs. 4m, n).

Together, these results show that pre-differentiated SKPs mature and express a peripheral catecholaminergic phenotype following transplantation into the CNS environment of hippocampal slices.

Expanded SKPs retain neurogenic potential and responsiveness to developmental fate determinants

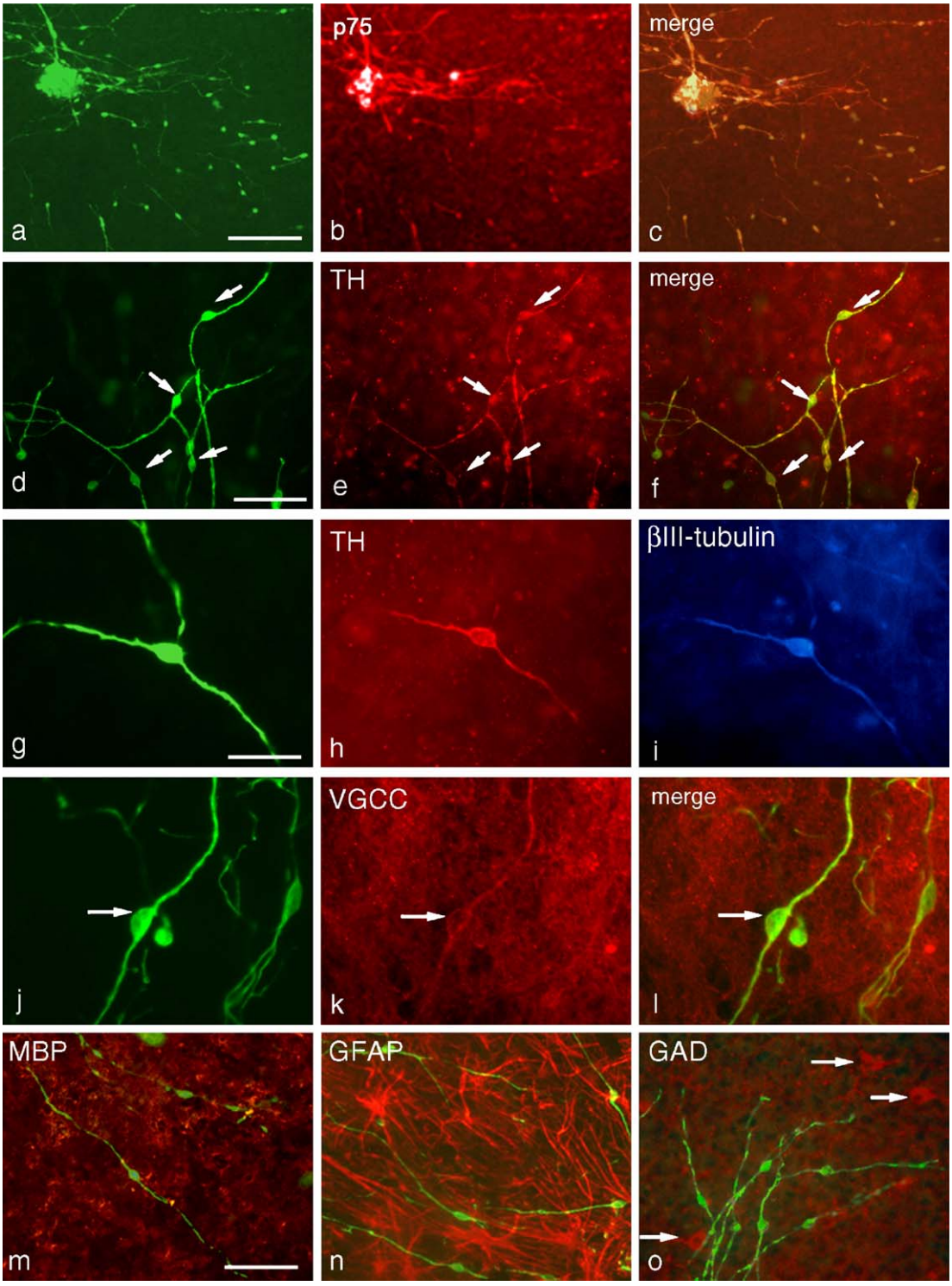
Since therapeutic applications may require considerable in vitro expansion of neural precursors, we next investigated whether expanded, multi-passage SKP cultures retain key neural precursor properties with regard to their neurogenic competence, their growth factor dependence, and their responsiveness to developmentally associated fate determinants.

Primary SKP spheres were grown from neonatal mouse back skin as previously. After 10–14 days, primary spheres were dissociated to single cells and small clusters, and grown for an additional 2 weeks to generate secondary spheres. Immunocytochemistry of secondary spheres showed that they expressed nestin but still did not express markers of differentiated neurons, glia, or smooth muscle cells (not shown). However, when plated under neurogenic differentiation conditions, nestin-expressing

Fig. 4. Immunocytochemical phenotype of pre-differentiated SKPs transplanted for 4–6 weeks on neonatal hippocampal slice cultures. (a–c) Double-label immunocytochemical analysis for (a) EYFP (green) and (b) $p75^{NTR}$ (red) in SKPs that were pre-differentiated for 14 days and then transplanted for 4 weeks on neonatal hippocampal slice cultures. The merged image is shown in (c). These low magnification images show that the majority of transplanted SKPs express $p75^{NTR}$ (78% of cells from n = 12 transplants). Scale bar = 200 μm. (d–f) Double-label immunocytochemical analysis for (d) EYFP (green) and (e) tyrosine hydroxylase (red) in SKPs that were pre-differentiated for 14 days and transplanted onto slice cultures for 4 weeks. The merged image is shown in (f). Note that many of the transplanted, EYFP-positive cells were also positive for tyrosine hydroxylase (arrows) (21% of cells from n = 16 transplants). Scale bar = 100 μm. (g–i) Triple label immunocytochemistry for (g) EYFP (green), (h) tyrosine hydroxylase (red), and (i) βIII tubulin (blue) confirms that transplanted cells that express tyrosine hydroxylase are also positive for βIII tubulin, a phenotype similar to that of peripheral catecholaminergic neurons. (j–l) Double-label immunocytochemistry for (j) EYFP (green) and (k) P/Q-type voltage-gated calcium channels (VGCC) in SKPs that were pre-differentiated for 14 days and transplanted onto slice cultures for 4 weeks. 23% of EYFP cells from n = 12 transplants were immunoreactive for VGCCs. The merged image is presented in (l). A transplanted, bipolar cell that expresses voltage-gated calcium channels is identified by the arrow. Scale bar = 50 μm in g–l. (m–o) Double-label immunocytochemical analysis for EYFP (green) and either (m) myelin basic protein (MBP, red), (n) GFAP (red), or (o) glutamic acid decarboxylase (GAD, red) in SKPs pre-differentiated for 14 days and transplanted onto slice cultures for 4 weeks (these experiments were repeated 3 times, n = 12 transplants for each marker). While there was abundant immunoreactivity for GAD, MBP and GFAP in the hippocampal slices, none of the transplanted cells expressed these proteins. Note that the apparent background staining in b, e, h, and particularly in k, is due to labeling of cells and/or processes within the host hippocampal slices. Arrows in (o) identify endogenous GAD positive neurons in the hippocampal slice. Scale bar = 100 μm in m–o.

cells migrated outwards from the spheres (Fig. 5a). After 7–10 days of differentiation, cells that had maintained nestin expression co-expressed βIII tubulin (Fig. 5a), as described previously for primary spheres. By 2 weeks after plating, secondary spheres had generated cells expressing non-overlapping neuronal, glial precursor, and smooth muscle markers (Fig. 5b), similar to primary spheres.

Separate SKP cultures were expanded in this fashion to generate several lines of multi-passage SKPs. Cytospin immunocytochemical analysis revealed that these expanded, multi-passage SKP cultures continued to possess nestin immunoreactivity (Fig. 6a). To compare their nestin expression, spheres from each line were dissociated, plated overnight, and then analyzed by nestin immunocytochemistry. Under these conditions, analysis of five separate lines of mouse and rat SKP cultures, from after 7 to 49 weeks in vitro, showed that 27 ± 2.3% (SEM) of cells were nestin immunoreactive at this time point, regardless of whether derived from mouse or rat, or from neonatal or aged skin (Fig. 6b).



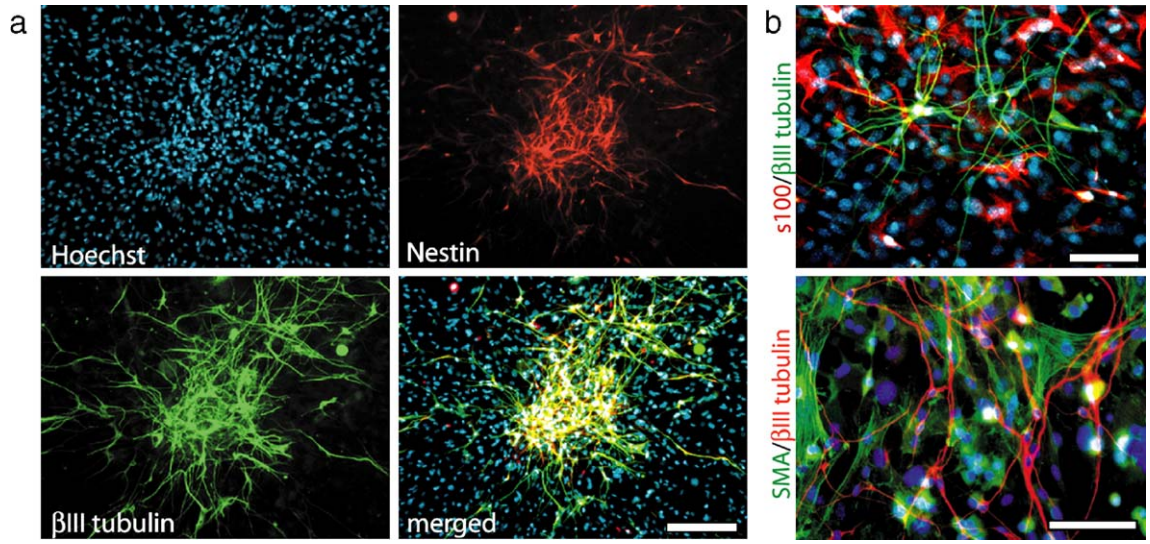


Fig. 5. Secondary SKP spheres retain neurogenic ability and multipotentiality. (a) Double-label immunocytochemistry for the precursor marker nestin (red) and neuronal marker βIII tubulin (green) in differentiating cultures of secondary SKP spheres generated as described in Methods. Note that nestin-expressing cells from secondary spheres differentiated for 7 days also express the early neuronal protein βIII tubulin. (b) Double-label immunocytochemistry for the glial marker S100β (red) and neuronal marker βIII tubulin (green) (top panel) or for βIII tubulin (red) and the smooth muscle marker SMA (green) in secondary SKP spheres differentiated for 2 weeks. Scale bars = 100 μm (a) and 50 μm (b). In all experiments, cells were counterstained with Hoechst (blue) to show all nuclei.

Since SKP lines retained robust expression of the neural precursor marker nestin following continuous expansion in FGF2 and EGF, we tested whether these expanded multi-passage SKPs also retained neurogenic competence. Multi-passage SKPs were plated and differentiated as described earlier. Nestin-expressing cells migrated outwards from plated spheres and many began to express neurofilaments within 1–3 days (Fig. 6c). After 1 week, differentiating spheres generated neurofilament-expressing neuron-like cells and SMA-expressing smooth muscle cells, the latter generally being located at the lower density periphery of the sphere-derived colonies (not shown), as previously observed for CNS precursors

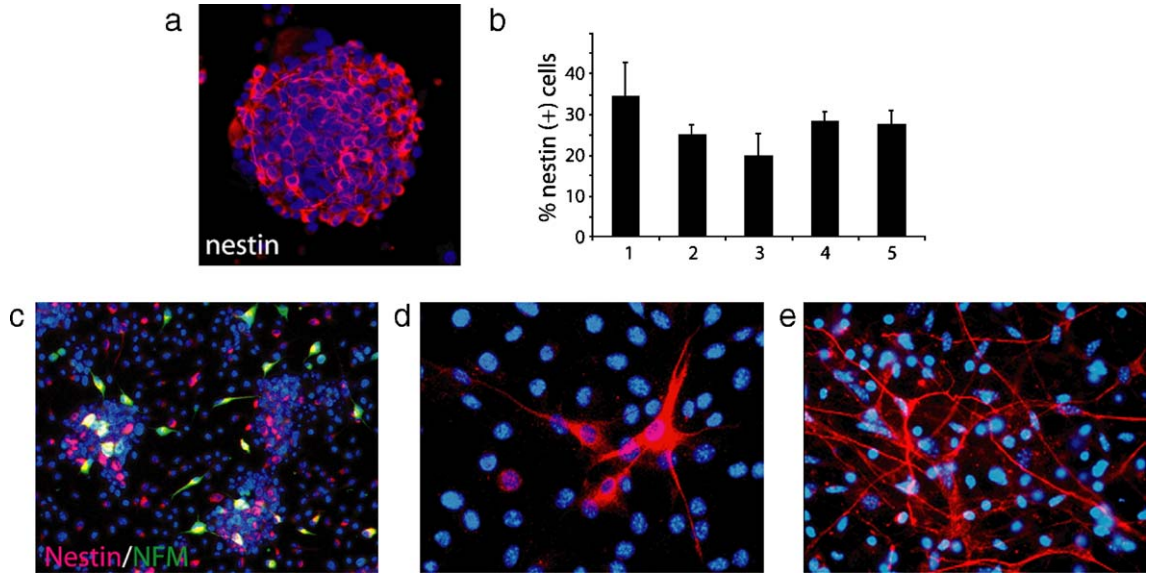


Fig. 6. Neurogenic properties of multi-passage SKP cultures. (a) Immunocytochemistry for the precursor marker nestin (red) on a cytospin of a representative SKPs sphere from a culture of neonatal CD1 back skin that was at the 28th passage in FGF2 + EGF. (b) Quantitation of the percentage of cells expressing nestin in various lines of SKPs following plating overnight on poly-D-lysine/laminin. Lines 1,2 were postnatal day 6 (P6) CD1 mouse after 9 passages, line 3 was P7 CD1 mouse skin after 22 passages, line 4 was P14 CD1 mouse skin after 13 passages, and line 5 was P14 C129 mouse skin after 8 passages. Note that no significant difference was detected ($P > 0.05$, Tukey test), and that a mean of 27 ± 2.3% of cells expressed nestin. (c) Double label immunocytochemistry for nestin (red) and NFM (green). Note that neuronal proteins have begun to be expressed in nestin-expressing cells after 4 days of differentiation (co-expressing cells are yellow). (d, e) Immunocytochemistry for βIII tubulin (red) after 2 weeks differentiation under normal neurogenic conditions (d) or high serum neurogenic conditions (e). Note that long-term expanded SKP cultures generate immature-appearing βIII tubulin-expressing cells having only short processes in normal neurogenic conditions (d), but generate cells with elaborate processes in high serum concentrations (e). In all experiments, cells were counterstained with Hoechst 33258 to show all nuclei in the field.

(Tsai and McKay, 2000). Interestingly, while differentiated neuron-like cells from high passage cultures (>8 passages) elaborated only rudimentary processes (Fig. 6d), their morphology was largely restored by increasing the serum concentrations to 20–30% during the differentiation phase (Fig. 6e), suggesting that serum contains yet-unidentified factors that promote differentiation.

We next examined the growth factor responsiveness of expanded multi-passage SKPs. Five separate lines of multi-passage SKPs were used, derived from: P7 EYFP transgenic mice (22 passages), P14 C129 mice (13 passages), P14 CD1 mice (8 passages), adult CD1 mice (9 passages), and P1 Sprague–Dawley rats (9 passages). To determine whether these expanded multi-passage lines of SKPs were still dependent on exogenous mitogens (i.e. not transformed into growth-factor independent cell lines), they were dissociated to single cells, diluted to a clonal density of 10 cells/μl, and then exposed to serum-free medium containing B27 supplement, 50% conditioned medium from the previous passage, and one of a variety of potential mitogens (EGF, FGF2, LIF, EGF +FGF2, SHH, FGF8, or BMP2). The addition of conditioned medium was necessary for cell viability under these conditions, and resulted in a low level of sphere formation by itself (presumably due to some residual FGF2). However, increased sphere formation only occurred in the presence of exogenous FGF2 (Fig. 7a). FGF2 and EGF +FGF2 treatments both significantly increased sphere formation compared to all other treatments ($P < 0.05$, Student–Newman–Keuls test), and were not significantly different from each other ($P > 0.05$, Student–Newman–Keuls test). Thus, expanded lines of SKPs remained dependent on exogenous FGF2 for sphere formation.

We also asked whether expanded SKP cultures remained sensitive to developmentally associated regulators of neural crest cells, such as differentiation-promoting bone morphogenic proteins (BMPs). Multiple members of the BMP family have previously been reported to promote differentiation of neural crest fates (Gajavelli et al., 2004). When SKPs spheres at passage 15–20 were dissociated and plated under basal differentiation conditions of 3% FBS, addition of BMP2 was observed to increase the number of neurofilament-immunoreactive cells and had a similar effect as increased serum levels (Fig. 6e) on their morphology (Fig. 7b). Similarly, densitometry of western blots demonstrated that BMP7 induced a 4.2 ± 1.5 fold increase ($n = 2$ experiments) in NCAM (120, 140, and 180 kDa isoforms, which are likewise expressed by SCGs) and a 1.6 ± 0.3 fold increase ($n = 2$ experiments) in peripherin protein levels (Fig. 7c).

Together, these results indicate that multi-passage SKP cultures remain growth factor dependent, retain neurogenic potential, and remain responsive to developmentally associated regulators of neural precursor behavior.

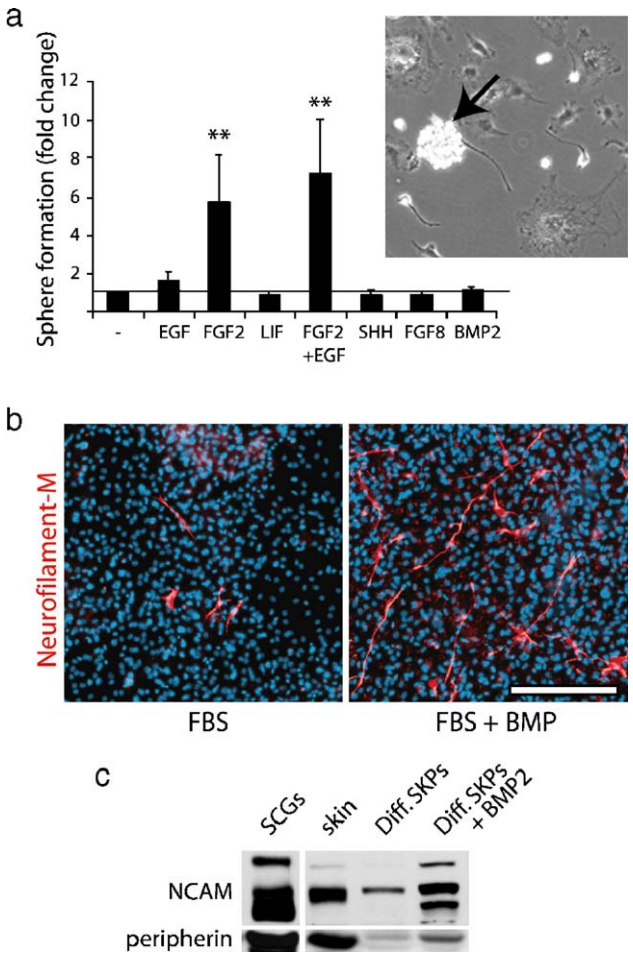


Fig. 7. Multi-passage SKP cultures remain FGF2-dependent and responsive to developmentally-associated fate determinants. (a) Spheres from five lines of multi-passage mouse and rat SKP cultures (see Results) were dissociated to single cells and plated at clonal density in serum-free medium containing B27 alone or in combination with growth factors. Bars represent the mean ± SEM of the changes from the five lines. In response to FGF2, clusters of proliferating cells were present within 3 days (arrow, inset phase micrograph). Note that FGF2 was required for multipassage SKPs to proliferate, and that no other growth factors had this ability. The effects of FGF2 and EGF + FGF2 treatments were statistically significant compared to all other treatments (**$P < 0.005$, Student–Newman–Keuls test) and were not significantly different from each other ($P > 0.05$). (b) Neonatal mouse SKP cultures passaged 5–10 times were differentiated for 7 days in low serum with and without BMP7. Note that BMP7 enhanced the generation of NFM-positive cells. (c) Western blot analysis for peripherin and NCAM in equal amounts of protein from intact neonatal skin, or from SKPs that were passaged 15–20 times and then differentiated for 3 weeks with or without BMP2. Protein from neonatal sympathetic superior cervical ganglia (SCG) was used as a positive control. Densitometry measurements indicate that BMP2 treatment enhanced expression of the peripheral neuronal marker peripherin (1.6 ± 0.3 fold, $n = 2$), and induced all three isoforms of NCAM (4.2 ± 1.5 fold increase, $n = 2$), which are likewise expressed in sympathetic superior cervical ganglion (SCG) neurons. The peripherin and NCAM present in the skin sample, which does not contain neuronal cell bodies, are presumably due to nerve terminals present in skin. Scale bar = 100 μm in (b). NCAM = neural cell adhesion molecule. In all micrographs, cells were counterstained with Hoechst 33258 to show all nuclei.

Electrophysiological properties of SKP-derived neuron-like cells

To determine the electrophysiological properties of SKP-derived neuron-like cells, SKPs were differentiated in neurogenic conditions for 26–45 days and then subjected to electrophysiological analyses. Neuronal cells were identified morphologically by their small, phase bright somata, and two or more fine processes. Patch clamp recordings were made from

Table 1
Membrane parameters of cells differentiated from skin, 1° spheres, and SCGs

	Resting membrane potential (mV)	Membrane capacitance (pF)	Input resistance (MΩ)
Skin (n = 19)	−24.3 ± 4.7	9.8 ± 3.3	675 ± 327
1° spheres (n = 18)	−26.4 ± 2.7	7.1 ± 0.9	2540 ± 768
SCGs (n = 13)	−45.8 ± 3.6	37 ± 4.4	278 ± 51.5

differentiated cells generated from two experimental sources: (i) primary spheres grown from E18 mouse skin, and (ii) dissociated skin cells from E18 mouse skin. Since SKP-derived neurons exhibit a peripheral catecholaminergic phenotype indicative of sympathetic neurons, we also used primary sympathetic neurons cultured from neonatal rat superior cervical ganglia (SCGs) as a fully differentiated positive neuronal control.

The passive membrane properties of SKP-derived neurons are summarized in Table 1. In comparison to primary SCG neurons, sphere-derived and skin-derived neuron-like cells displayed a high input resistance with relatively low membrane capacitance (Table 1). The mean resting potential of neuron-like cells from skin (−24.3 ± 4.7 mV, n = 19) and spheres (−26.4 ± 2.7 mV, n = 18) was higher than for primary SCG neurons (45.8 ± 3.6 mV, n = 18) ($P < 0.05$, Student–Newman–Keuls test). Frequency histograms indicated that the distribution of resting membrane potentials were largely overlapping, but that skin-derived and sphere-derived populations contained increased numbers of cells with weak membrane potentials in the −20 to 0 mV range (Figs. 8a–c).

To examine the electrical discharge behavior of SKP-derived neuron-like cells, current injections were made in current clamp mode (Figs. 8d–f). Step-wise current injections into primary SCG neurons induced graded depolarizations which generated robust action potentials when these depolarizations reached a threshold of approximately −30 mV (Fig. 8f). Unlike the primary SCG neurons, neuron-like cells generated in vitro from dissociated skin or from primary SKP spheres only produced graded depolarizations.

To determine whether SKPs-derived neurons had developed voltage-dependent currents, current measurements were made in voltage clamp mode. Cells were hyperpolarized to −100 mV to relieve any residual sodium channel inactivation, and were then depolarized step-wise in 20 mV increments to a maximum of +40 mV. Traces from depolarizations to −20 mV and +40 mV are selectively plotted in Figs. 8g–i. In all three populations of cells, an outwardly rectifying current that increased linearly in response to further depolarization was observed beginning at approximately −30 mV, as shown in the respective I/V curves (Figs. 8j–m) and the depolarization to +40 mV (Figs. 8g–i). However, in contrast to what was observed in primary SCG neurons, no inwardly rectifying currents were observed from dissociated skin cells and from primary spheres (depolarization to −20 mV in Figs. 8g–i). The inward currents in SCGs were blocked in the presence of 1 μM tetrodotoxin (TTX; not shown), confirming that they were mediated by voltage-gated sodium channels.

Discussion

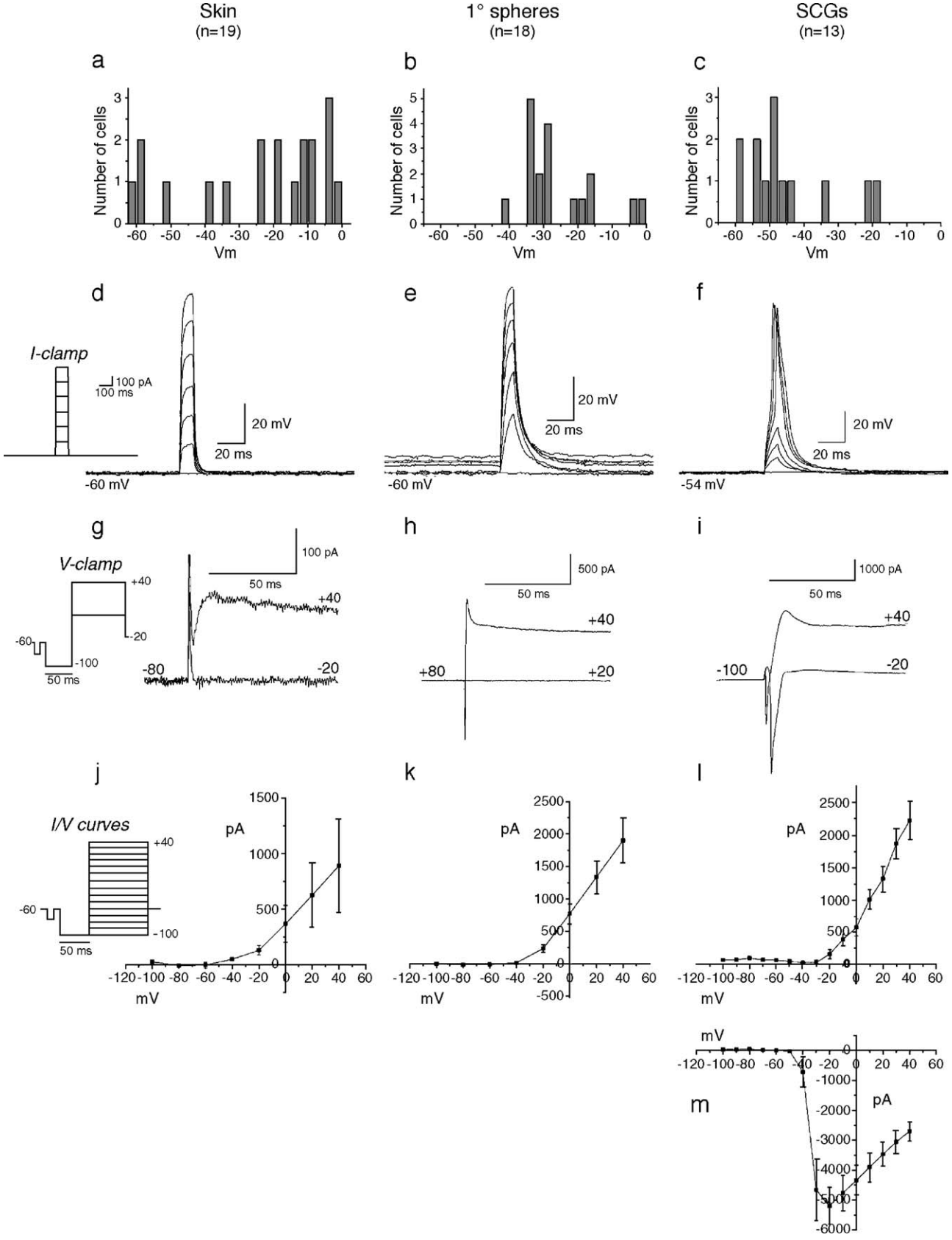
The present study explored the neural precursor properties of skin-derived precursors, focusing particularly on their neurogenic potential, and the results support four main conclusions. First, SKPs isolated from rodent back skin can be differentiated into a neuronal phenotype. Using a protocol similar to those for differentiating CNS and neural crest stem cells, SKPs follow a temporal progression from proliferating, undifferentiated nestin-expressing precursors to post-mitotic cells having a peripheral neuronal phenotype. Second, SKP-derived neuron-like cells survive and mature when transplanted into a CNS environment. These neurons stably express and maintain their peripheral catecholaminergic phenotype, even when transplanted into a kainic acid-treated degenerating CNS environment. Third, SKP cultures can be expanded for multiple passages in FGF2-containing serum-free medium. Multipassage SKP cultures continue to express the neural precursor marker nestin, retain neurogenic competence, and remain responsive to developmentally associated neural precursor fate determinants. Fourth, SKP-derived neuron-like cells display an immature electrophysiological profile that, under our in vitro conditions, lacks significant voltage-gated sodium currents. Collectively, these findings provide important baseline information for future work aimed at using peripheral neural precursors for research and therapeutic purposes.

Differentiation of SKP-derived neurons parallels early steps in sympathetic neuron development

The autonomic nervous system is developmentally derived from embryonic neural crest stem cells, and differentiating SKPs recapitulate several aspects of in vivo sympathetic neuron development. Undifferentiated SKPs initially co-express the precursor marker nestin and proliferation marker Ki67. Then, at early stages of neuronal differentiation, the nestin and Ki67-expressing SKPs begin to co-express neuron-specific βIII

Fig. 8. Electrophysiological properties of SKPs. Electrophysiological analyses were carried out by patch clamp analysis of neuron-like cells generated from E18 dissociated skin cells (left column), E18 primary SKP spheres (middle column), and cultured neonatal sympathetic neurons from the superior cervical ganglia (SCGs) (right column). (a–c) Distribution of resting membrane potentials. Note that the distribution of resting membrane potentials is shifted upward in neuron-like cells generated from skin and 1° spheres compared to cultured sympathetic neurons. (d–f) Current clamp measurements. The current clamp protocol is shown in inset in (d). Note that skin and 1° SKP spheres generated cells that responded with graded depolarizations in response to current injections (d, e), while SCG neurons began to generate action potentials once the membrane was depolarized to approximately −30 mV (f). (g–i) Voltage clamp measurements. The voltage clamp protocol is shown in inset of (j). Current traces resulting from voltage steps to −20 mV and +40 mV are selectively shown in g–i, and show that robust outward currents are generated from skin, 1° SKP spheres, and SCGs following steps to +40 mV. However, following a voltage step to −20 mV, inward currents are only produced in SCGs. The entire current–voltage relationships are shown in (j–m), showing the similar outward currents in (j–l), and the SCG inward currents in (m). The latter was TTX-sensitive (not shown), confirming that it was mediated by voltage-gated sodium channels.

tubulin, and they subsequently differentiate into post-mitotic cells that express proteins characteristic of peripheral autonomic sympathetic neurons, such as peripherin, $p75^{NTR}$, and TH. Addition of BMP2 enhances expression of TH, a marker of the catecholaminergic phenotype. These observations are consistent with in vivo sympathetic neuron development. During



embryonic development, migrating neural crest precursors express nestin and are proliferative (Lothian and Lendahl, 1997; Stemple and Anderson, 1992). Neural crest-derived sympathetic neuroblasts begin to express low levels of TH soon after aggregating in primordial ganglia (Cochard et al., 1978), and despite already expressing early neuronal markers such as βIII tubulin (Memberg and Hall, 1995), they continue proliferating for a short period (Rohrer and Thoenen, 1987; Rothman et al., 1980). In response to local factors such as BMP2 (Shah et al., 1996), differentiating sympathetic neurons rapidly increase their TH expression. Thus, development of SKP-derived neurons shares numerous characteristics with developing sympathetic neurons in vivo, including the unusual temporal overlap in proliferation and neuronal differentiation, processes that are normally mutually exclusive in other PNS neurons and in most CNS neurons (Gloster et al., 1999; Rohrer and Thoenen, 1987; Slack et al., 1998).

It remains to be determined whether SKPs can also generate other peripheral neuronal phenotypes. Until recently, neurons generated from multipotent NC-related precursors had only been demonstrated to have autonomic or gut phenotypes. For example, BMPs stimulate neural crest precursors isolated from the neural tube or from the developing sciatic nerve to generate sympathetic-like neurons (Morrison et al., 1999; Shah et al., 1996), while BMPs induce enteric NC precursors to generate gut neurons (Bixby et al., 2002; Kruger et al., 2002; Pisano et al., 2000). More recently, sensory neuron differentiation has been shown to be crucially regulated by Wnt-induced β-catenin signaling, and ectopic activation of β-catenin signaling was sufficient to direct migrating NC precursors to a sensory fate at the expense of most other neural crest derivatives, including catecholaminergic neurons (Kleber et al., 2005; Lee et al., 2004). Further experiments will be required to determine whether activation of β-catenin signaling can likewise promote sensory neurogenesis from SKPs and other NC-related precursors. In this regard, much work remains to be done to identify culture conditions that promote lineage-specific differentiation, as even conditions for promoting "default" catecholaminergic differentiation of cultured neural precursors have only recently been identified (Morrison et al., 2000; Studer et al., 2000; Yan et al., 2001).

Responses of pre-differentiated SKPs to a CNS environment

Little is known regarding how mammalian neural crest precursors might respond to a CNS environment. In birds, early migrating NC precursors are reported to generate CNS cell types when back-transplanted into the avian neural tube (Ruffins et al., 1998), but it is unclear to what extent this CNS potential might be maintained following NC migration into target tissues such as the skin. We asked this question by transplanting SKPs into hippocampal slice cultures, which are a rich source of differentiation-promoting factors for CNS neurons. Using this paradigm, we made two main observations. First, neither pre-differentiated nor naïve SKPs showed evidence of spontaneously generating CNS glial or CNS neuronal phenotypes following transplantation. Instead, pre-differentiated SKP-derived neurons matured and maintained their peripheral autonomic phenotype, arguing that their phenotype is stable once they have committed to a peripheral fate. Second, following pre-differentiation, SKP-derived neural lineage cells preferentially survived within the hippocampal slice environment. While SKPs produce large numbers of smooth muscle cells in vitro, only rare SMA-expressing cells were detected following transplantation. In contrast, 78% of surviving SKPs expressed the peripheral neural marker p75, and nearly 40% expressed the neuronal marker βIII tubulin.

Limited electrophysiological maturation of SKP-derived neurons

Despite undergoing apparently appropriate morphological and biochemical changes associated with peripheral neuronal differentiation, and not co-expressing markers of any other related neural crest cell types, SKP-derived neurons did not possess voltage-gated inward sodium currents. The reason for the absence of such currents, which are considered the hallmark of electrophysiologically functional neurons, will require further investigation. While one possibility is that SKPs are more committed to non-neuronal fates than embryonic neural crest precursors, an alternative explanation is that appropriate maturation factors for peripheral neurons have not yet been identified. In support of this possibility, previous studies have indicated that astrocytes provide essential factors that enable development of CNS stem cell-derived neurons (Song et al., 2002) and electrophysiological maturation of CNS neurons (Blondel et al., 2000). However, when CNS stem cells are differentiated using minimal culture conditions, they produce neuron-like cells that, similar to SKPs, lack inward sodium currents (Balasubramaniyan et al., 2004). Thus, it appears that morphological/biochemical and electrophysiological differentiation processes may be separable. Given the peripheral phenotype of SKPs, we speculate that satellite glial cells in developing peripheral ganglia are a candidate source of peripheral neuronal maturation-promoting factors, a testable hypothesis that can be explored further in future studies.

Neural crest precursor properties of SKPs

Previous studies indicate that neural crest-related precursors isolated from different tissues are similarly multipotent, but are specified to preferentially generate particular regional cell types (Abzhanov et al., 2003; Bixby et al., 2002; Lwigale et al., 2004). For example, NC-related precursors isolated from sensory, sympathetic, and enteric ganglia can all produce neuronal, glial, and smooth muscle cells upon differentiation in vitro, but they display significant differences in their responsiveness to fate-determining factors in vitro and in their behavior following transplantation (Bixby et al., 2002). This regional specification is further illustrated by the intrinsic differences between NC cells originating from different levels of the neuraxis, as cranial but not trunk neural crest cells have a preferential ability to generate mesectodermal derivatives (Abzhanov et al., 2003; Lwigale et al., 2004). Interestingly,

some reports suggest that culturing for extended periods may exert a homogenizing effect on the differentiation potential of distinct neural crest-derived precursors (Abzhanov et al., 2003; McGonnell and Graham, 2002).

In the case of SKPs, which can also clonally produce neuronal, glial, and smooth muscle phenotypes in vitro (Fernandes et al., 2004; Toma et al., 2001, 2005), it is interesting to note that neuron-like cells are not known to be generated within the skin. This suggests that SKPs actually possess a wider differentiation repertoire than they would normally express, and that SKP differentiation must be tightly controlled within their in vivo niche. In this respect, it is intriguing that hair follicles, the niche of endogenous SKPs, are a major site of Wnt and BMP signals, which together have recently been implicated in maintaining the undifferentiated state of embryonic neural crest stem cells (Kleber et al., 2005).

Implications for research and therapeutic applications

The ability of expanded SKPs to retain their NC precursor markers, growth factor dependence, and responsiveness to developmentally-associated NC fate determinants, suggests that they may be useful for certain research and/or therapeutic applications. For example, NC-related precursors isolated from the gut have previously been useful for modeling how genetic mutations affecting gut NC cell properties can contribute to Hirschprung's Disease (Iwashita et al., 2003). The differentiation of SKPs into peripheral neuronal and glial phenotypes may therefore enable them to be used to investigate the pathophysiology of peripheral nervous system diseases, such as genetically-based peripheral neuropathies. Alternatively, the ability to expand SKPs may allow them to be used either for high throughput screening to identify regulators of NC survival, proliferation, and differentiation, or for transplantation applications. In the latter case, a particularly promising avenue may be the differentiation of peripheral glial cells from expanded SKPs (McKenzie and Miller, 2002), as Schwann cells possess potent growth promoting properties for injured CNS neurons (David and Aguayo, 1981; Pearse et al., 2004; Richardson et al., 1980; Xu et al., 1999), which are normally refractory to axonal regeneration following spinal cord injury. Moreover, their myelination ability allows them to act as substitutes for oligodendrocytes that degenerate following spinal cord injury or in Multiple Sclerosis (Bachelin et al., 2005; Brierley et al., 2001; Halfpenny et al., 2002). Thus, since SKPs retain their multipotentiality following expansion, and can be obtained autologously from normal human skin (Toma et al., 2005), they represent an attractive source of accessible and abundant precursors for multiple basic research and potential therapeutic applications.

Methods

Cell culture

SKPs were cultured from the back skin of embryonic, neonatal, and adult rodents. CD1 mice were used for most in vitro experiments, except where otherwise indicated. For transplantation studies, SKPs were cultured from mice expressing enhanced yellow fluorescent protein were (Hadjantonakis et al., 1998). Enzymatic digestion of the back skin was performed using 0.1% trypsin (Calbiochem, San Diego, CA, USA) or, in later experiments, 1 mg/ml collagenase (Sigma Aldrich Canada, Oakville, ON, Canada), using a protocol we have described in detail elsewhere (Fernandes et al., 2004; Toma et al., 2001). Primary sphere cultures were grown at a concentration of 10–20 cells/μl. We have previously shown that culturing at this low density yields the same number of spheres from skin as when skin cells are immobilized in methylcellulose (Fernandes et al., 2004). Cultures were passaged by mechanical dissociation as previously described (Toma et al., 2001).

SKPs were differentiated by plating spheres onto chamber slides (Nalge Nunc International, Rochester, NY, USA) coated with poly-D-lysine and laminin (BD Biosciences, Mississauga, ON, Canada). Basal differentiation conditions consisted of 3–5% FBS (Cambrex Bio Science, Walkersville, MA, USA) in DMEM/F12(3:1) (Gibco-Invitrogen Canada, Burlington, ON, Canada). Neurogenic differentiation conditions consisted of an initial Plating Medium containing 2% B27 (Invitrogen Canada), 40 ng/ml FGF2 (BD Biosciences) and 15% fetal bovine serum (FBS) in DMEM-F12 (3:1) (to promote overall cell survival, and proliferation/differentiation of neural cells), and a long-term Maintenance Medium containing 2% B27, 1% N2 (Invitrogen Canada), 50 ng/ml nerve growth factor (NGF; prepared from male mouse submandibular glands, Cedarlane Laboratories, Hornby, ON, Canada), brain-derived neurotrophic factor (BDNF; recombinant human, PeproTech Inc., Rocky Hill, NJ, USA), neurotrophin-3 (NT-3; recombinant human, PeproTech) and 1% FBS in Neurobasal Medium (Gibco-Invitrogen) (to promote neuronal survival and growth). Cells were maintained in the Plating Medium for 3–6 days, until cultures neared confluence. Approximately one third of the Plating Medium and Maintenance Medium was replaced with fresh medium and growth factors every 2 days.

Cultures of neonatal rat sympathetic neurons were performed as described previously (Majdan et al., 2001; Vaillant et al., 2002).

Immunocytochemistry

Immunocytochemistry was performed as previously described (Barnabé-Heider and Miller, 2003). Primary antibodies used were: mouse anti-nestin (1:400; BD Biosciences), mouse anti-Ki67 (1:200; BD PharMingen, Mississauga, ON, Canada), mouse anti-βIII tubulin (1:400; Tuj1 clone, Babco-Covance, Evanston, IL, USA), rabbit anti-βIII tubulin (1:800; Babco-Covance), rabbit anti-NFM (1:200; Chemicon International, Temecula, CA, USA), rabbit anti-GFAP (1:200; DakoCytomation Inc, Mississauga, ON, Canada), rabbit anti-$p75^{NTR}$ (1:500; Promega, Madison, WI, USA), mouse anti-SMA (1:400; Sigma), rabbit anti-fibronectin (1:400; Sigma-Aldrich), mouse anti-S100β (1:1000; Sigma-Aldrich), rabbit anti-MBP (1:100; Chemicon), mouse anti-TH (1:200; Chemicon), rabbit anti-TH (1:1000; Pelfreeze), rabbit anti-α1A voltage gated calcium

channel (1:1000; Chemicon), chicken anti-GFP (1:1000; Molecular Probes, Eugene, OR, USA), rabbit anti-GAD (1:800; Chemicon), mouse anti-GAP-43 (1:400; Sigma-Aldrich), and mouse anti-MAP2a,b,c (1:200; Sigma-Aldrich). Fluorescent secondary antibodies used were Cy3-conjugated anti-mouse or anti-rabbit IgG (1:800) and FITC-conjugated anti-mouse or anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA), or Alexa 488 anti-chicken IgG, Alexa 555 anti-rabbit or anti-mouse IgG, Alexa 350 anti-mouse IgG (1:1000; Molecular Probes). To ensure the specificity of staining, we (i) omitted the primary antibodies from the procedure, which resulted in the loss of all fluorescence signals; and (ii) tested the antibodies on control cell types that did not express the proteins of interest, which likewise resulted in the loss of fluorescence signals.

Western blotting

Western blotting procedures were performed as described in detail in Barnabé-Heider and Miller (Barnabé-Heider and Miller, 2003). For biochemical analysis of primary skin cells, SKP spheres, and differentiating SKPs, equal amounts of protein were resolved using 7.5% or 10% polyacrylamide gels. Control lysates were from cultured superior cervical ganglion neurons (SCGs). Primary antibodies used for Western blots were mouse anti-NCAM (1:800; Chemicon), rabbit anti-peripherin (1:1000; Chemicon), and mouse anti-TH (1:800; Chemicon). Secondary antibodies used were HRP-conjugated goat anti-mouse or goat anti-rabbit (1:10,000; BioRad).

To quantitate changes in protein levels, densitometry was performed on between 2 and 4 blots for each experiment, and the mean changes expressed as fold increase/decrease.

Transplantation into hippocampal slice cultures

Hippocampal slice cultures were prepared based on the method described by Stoppini et al. (1991). In brief, hippocampi from postnatal days 7 to 9 Wistar rat brains were carefully dissected out in cold Hank's balanced salt solution and sliced at 400 μm on a tissue chopper (McIlwain, Campdan Instruments, Lafayette, IN). The slices were transferred onto tissue culture membrane inserts (Millicell, Millipore, Billerica, MA) and placed into 6 well culture plates containing 25% horse serum (Gibco-Invitrogen), 50% MEM (Gibco-Invitrogen), 25% HEPES buffered salt solution (Gibco-Invitrogen) and 1% Penicillin-streptomycin (PS; 100 and 50 units, respectively). The medium was changed every 3 days for the duration of the experiment (4–6 weeks).

Suspension cultures of SKPs were prepared from E19-P5 mice expressing EYFP as described above. Primary spheres or secondary spheres were plated in laminin and poly-D-lysine coated 6-well plates and cultured for 7 or 14 days in Neurobasal medium (Gibco-Invitrogen) supplemented with 1% B27, 1% N2, 1% FBS, and 1% PS. On days 7 or 14, monolayer SKP cultures were lifted with 0.25% trypsin for 3–5 min, washed twice with HBSS and single cell suspensions were prepared at a concentration of 50, 000–100,000 cells/μl. 50 nl of this cell suspension was transplanted through micro glass capillary (Nanoject, Drummond Scientific Company, Broomall, PA) to the vicinity of CA3 pyramidal and dentate granule cell layers on each hippocampal slice. Transplanted SKPs were observed routinely for their viability and migration with an inverted fluorescence microscope (Axiovert, Zeiss) for 4–6 weeks. They were then fixed with 4% paraformaldehyde for 30 min for immunohistochemistry.

Cell survival was measured by counting the absolute number of GFP-immunoreactive cells present at the end of the analysis period. Preliminary Z-stack analysis using a confocal microscope indicated that the deepest migration into the hippocampal slices was 50–60 μm. Cell counts were subsequently performed using a fluorescence upright microscope to capture images at multiple focal planes for each transplant. Lateral migration distances were determined using a 10× objective and Adobe Photoshop to measure the distance from the transplantation core to the center of each GFP-immunoreactive cell body.

Electrophysiology

Cells for electrophysiology were plated onto 35 mm dishes coated with either collagen for superior cervical ganglion cells, or poly-D-lysine/laminin for skin cells and SKPs spheres. Differentiated cells were continuously perfused with extracellular recording media containing (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.3, $MgCl_2$ 1, D-Glucose 10, HEPES 25 (pH 7.35, osmolarity: 320). Experiments were carried out at room temperature. For patch clamp experiments, borosilicate glass pipettes were pulled to between 5 and 7 MΩ. Standard intracellular recording solution contained (in mM): K-Gluconate 130, EGTA 10, HEPES 10, $MgCl_2$ 1, MgATP 4, NaGTP 0.3.

An axopatch 1D patch clamp amplifier was used in conjunction with Clampex 9.2 software to inject current or voltage pulses. Data were sampled at 10 kHz and were filtered at 2 kHz through the amplifier and saved to a PC. All data analyses were performed off-line using Clampfit 9.2 software (Axon instruments).

Voltage clamp experiments were carried out in whole-cell mode. Following breakthrough membrane potential, series resistance and whole cell capacitance were measured. Whole cell capacitance was compensated while series resistance was not. In voltage clamp experiments, cells were held at −60 mV. To reveal active currents, cells were stepped to −100 mV for 50 ms to relieve inactivation of potential sodium channels, followed by a series of 10 mV voltage steps to progressively more positive potentials between −100 and +40 mV for 100 ms. In current clamp experiments, current was injected in increasing steps of 150 pA and was held for 10 ms.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM), unless otherwise noted. Analyses were performed using SigmaStat version 3.1 (Systat Software, Inc). Comparisons were made using paired *t* tests, and post hoc pairwise multiple comparisons using Tukey and Student–Newman–Keuls tests.

Acknowledgments

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