STEM CELLS AND DEVELOPMENT 17:1–8 (2008)

DOI: 10.1089/scd.2008.0008

Neural Precursors from Canine Skin: A New Direction for Testing Autologous Cell Replacement in the Brain

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Recent work indicates that neural progenitors can be isolated from the skin of rodents and humans. The persistence of these cells in accessible adult tissue raises the possibility of their exploitation for research and therapeutic purposes. This study reports on the derivation, culture, and characterization of homogenous canine skin-derived neuroprecursor cells (SKiNPs) from mature animals. Canine tissue was used because naturalistic brain diseases in community-dwelling dogs are emerging as ecologically sound models for a range of neurological conditions. Adult SKiNPs were initially isolated as neurospheres and then cultured for 10–15 passages in an adherent monolayer assay. Serumfree expansion conditions contained B-27, 20 ng/mL EGF, and 40 ng/mL bFGF. Gene expressions by PCR indicated expression of *nestin*, *CD133*, *NCAM*, and *FGF2R*, but not *GFAP*. Highly uniform expression of *nestin* (76 ± 8.3%), NCAM (84 ± 3.3%), *βIII-tubulin* (96 ± 4.3%), and *CD133* (68 ± 13.5%) was also observed. Directed differentiation of SKiNPs in the presence of serum induced *βIIItubulin*, *NSE*, *NCAM*, and *MAP2* in >90% of differentiated cells by immunophenotype analysis. Our culture system rapidly induces canine skin cells into neural precursors, maintains *nestin* expression in more than 75% of proliferating cells, and generates an almost universal neuronal-like phenotype after 7 days of *in vitro* differentiation. Their biological characteristics are suggestive of transiently amplifying fate-restricted neuroprecursors rather than true neural stem cells. This system may be an effective alternative for autologous neurorestorative cell replacement in canine models for further translational research.

Introduction

Throughout adulthood mammalian skin is constantly regenerated due to an ongoing high rate of cell loss. Skin stem cells are therefore highly proliferative, a characteristic readily evident in *in vitro* culture [1]. In human skin, different stem cell niches are thought to persist into adulthood. A highly regulated "bulge" microenvironment located adjacent to the hair follicle shaft functions to maintain the cells of the epidermis [2], while a subpopulation with a latent multi-lineage potential resides at the base of the dermal papillae [3]. The proper physiological function of this niche remains unclear.

Like our own skin, the brain's three major cell types arise from the embryonic ectoderm. The shared ontogenic origin of both skin and brain has therefore spurned interest in the possibility of inducing skin stem cells down the neural pathway. In this study, canine skin was investigated because of increasing recognition that dogs develop many of the same important neurological diseases that afflict humans in a naturalistic fashion. Canine equivalents of spinal cord injury [4], stroke [5], epilepsy [6], neurometabolic disorders [7], Alzheimer's disease [8], parkinsonism [9], and muscular dystrophy [10] are prime examples.

For future clinical purposes, a skin-derived neural precursor (SKiNP) system is an attractive option because it would allow for an autogenic graft source free from ethical controversy and involving minimal invasiveness [11]. Initial reports from murine tissue found that EGF/FGF-dependent neurospheres could be induced and propagated for over 80 population doublings, were able to proliferate from single-cell clonal conditions, expressed the neural precursor marker *nestin*, and differentiated in the absence of mitogenic

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factors to produce both neural and glial cells [12]. Neuronal yields from skin-based protocols, however, have been low—varying from 6 to 15% *β-tubulin*-positive cells when derived from either mouse [12] or infant human skin [13,14), or from adult human skin when supplemented with undefined factors from glia-conditioned media [15].

One possible reason for the low neuronal yield is that skin-based protocols have so far relied exclusively upon the neurosphere assay for propagation. The neurosphere assay is a highly effective means of generating, amplifying, and studying neural precursors [16,17). Potential clinical applications are however limited by the inherent cellular heterogeneity that characterizes neurosphere cytoarchitecture [18]. Quantitative analysis suggests that less than 1% of a given neurosphere's constituent cells are bona fide neural stem cells [17]. Moreover, complex and dynamic biological interactions continue to occur within the cytosphere mass, including phagocytosis and apoptosis as well as *de novo* mitosis [19]. By contrast, the two-dimensional simplicity of the adherent monolayer assay appears to favor culture of a more uniform neural precursor population [20,21).

Our *in vitro* strategy aims to take advantage of the strengths of both stem cell culture approaches by first inducing neurosphere formation from a skin biopsy followed by transfer to the monolayer assay under serum free expansion conditions. This dual-assay system allows for the efficient and effective homogenous culture of canine SKiNPs. This system (1) rapidly induces canine skin cells into neural precursors, (2) doubles cell numbers every 7 days, (3) maintains *nestin* expression in more than 75% of proliferating cells, and (4) under standard differentiation conditions, generates an almost universal neuronal-like phenotype. Trial of cell replacement strategies using SKiNPs cultured in this way in dogs affected by naturally prevalent neurological conditions may provide valuable insights for the design and conduct of future human studies.

Materials and Methods

Animals

All results are based on three replications from independent cell lines derived from two different domestic dogs. Animals were community-based dogs that underwent routine surgery at Struggletown Veterinary Hospital in Sydney and who had waste skin tissue (1–2 cm^2) opportunistically harvested and donated by consenting owners. Skin came from a 2-year-old female Rottweiler and a 4.5-year-old male Blue Terrier and these animals had surgery for conditions unrelated to the skin. The use of canine tissue was approved by the Animal Care and Ethics Committee of The University of New South Wales.

SKiNP isolation and culture

Skin biopsies were immediately transferred in PBS (Gibco,[2] Melbourne, Victoria, Australia) and processed within 1 h. The specimen was chopped into 2–3 mm^2 pieces and washed twice with PBS containing 1% penicillin and streptomycin (Gibco) followed by fresh PBS alone. SKiNPs were isolated according to the modified protocol of Toma et al. [12]. Briefly, tissue was digested with 0.1% DNAse (Roche Applied Science, Castle Hill, NSW, Australia) for 1 min followed with 0.1% trypsin for 40 min at 37°C, then mechanically dissociated and filtered through a 40 µm cell strainer (Falcon, BD Biosciences, San Diego, CA). Dissociated cells were placed in serum-free DMEM-F12 (3:1; Invitrogen, Carlsbad, CA), which contained 20 ng/mL EGF (BD Biosciences), 40 ng/mL bFGF (Invitrogen), and 2% B27 (Gibco) at 37°C/5% CO_2. SKiNPs were initially cultured as neurospheres, which formed within 1–5 days after isolation (<20 µm). When most neurospheres reached 70 µm in diameter, they were mechanically dissociated to form single cells, which were seeded onto gelatin-coated flasks and grown as an adherent monolayer. When 75% confluence was reached (usually within 1–2 weeks), SKiNPs were passaged using TripLE Select (Gibco). SKiNPs were cryopreserved and thawed successfully by using a standardized in-house slow freezing procedure [22].

Directed differentiation of canine SKiNP towards a neuronal lineage

After a different number of passages, dog SKiNPs were grown on glass cover slips pre-coated with laminin (24 h, 5 µg/cm^2; Invitrogen) in 24-well culture plates (initial plating density 2×10^4 cells/well). SKiNPs were allowed to attach and form a monolayer using serum-free DMEM-F12 (3:1) media supplemented with 20 ng/mL EGF and 40 ng/mL bFGF before being subject to the differentiation protocol. After 3 days, cells were allowed to differentiate by removing EGF and bFGF and supplementing the media with 3% FBS (Invitrogen) for 7 days or 3% FBS plus 10 ng/mL BDNF (Invitrogen) for 21 days. Media were changed every 3 days.

RT-PCR analysis

Total RNA was extracted from canine SKiNP at different passages by using RNeasy Minikit (Qiagen, Doncaster, Australia) with DNase 1 treatment (Qiagen), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA in 20 µL reaction mixture using Superscript III RT First Strand Synthesis System (Invitrogen) using oligo(dT) as a primer according to the manufacturer's instructions. PCR amplification was carried out using the Platinum *Taq* DNA Polymerase (Invitrogen). Primer sequences, lengths of amplified products, and amplification conditions are listed in Table 1. Products were analyzed on a 1% agarose gel (Ultrapure agarose; Invitrogen) and visualized by SYBR green (Invitrogen).

Immunofluorescence analysis

SKiNPs on laminin-coated cover slips were washed with PBS (Gibco), and then fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature. Cells were then washed twice in PBS/0.1% Tween-20 (PBST) solution and permeabilized with 0.1% Triton X-100/PBS for 10 min, as described previously [23]. The cells were subsequently washed twice in PBST solution, and non-specific sites were blocked with 2% bovine serum albumin (BSA;

Table 1. Primer sequence for gene expression analysis

Primer	*Sequence 5′ –3′*	*PCR conditions*	*Product size (bp)*	*Accession no.*
Nestin	F gagaaccaggagcaagtgaa R tttccagaggcttcagtgtc	94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; 72°C for 5 min	328	XM_547531
CD133	F ggacacaaaagccaacaatc R atcttgacccattgcaggta	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	315	XM_545934
FGF2R	F ggacacagaatggataagccag R ggtgaatactgttcgagaggttg	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	153	NM_022969
NCAM	F aggcagagcatagtgaatgc R aggcttcacaggtcagagtg	94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min; 72°C for 5 min	343	NM_001010950
βIII-tubulin	F gcacactgctcatcaacaag R tcttgctctccttcatggac	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	357	XM_848641
NSE	F gagaacagtgaagccttgga R accaatctggttgaccttga	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	390	XM_534902
GFAP	F cgagttaccaggaggcacta R tccacggtctttaccacaat	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	277	XM_843285
β-actin	F acggcatcgtcaccaact R aggaaggaaggctggaagag	94°C for 2 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 1 min; 72°C for 5 min	503	NM_001101

Sigma, St. Louis, MO) in PBST for 1 h at room temperature. Cells were incubated with primary antibodies for 1 h at room temperature. After washing with PBST solution three times for 2 min each, the secondary antibody conjugated to either FITC or TRITC (1:500, AlexaFluor 488 or 594; Molecular Probes, Melbourne, Australia) was applied for 30 min at room temperature. The cells were washed with PBST three times at 2 min each wash, and the nuclei stained with 4′ 6-diamidino-2-phenylindole (Prolong Gold anti-fade reagent with DAPI; Invitrogen) for 5 min. Omitting the primary antibodies were used as negative controls. Primary antibodies and dilutions used were *βIII-tubulin* rabbit polyclonal antibody (1:1000; Covance, Berkeley, CA), *CD133* rabbit polyclonal antibody (1:100; Abcam, Cambridge, United Kingdom), *MAP2* (1:1000; Covance), *NCAM* mouse monoclonal antibody (1:500; Covance), *nestin* mouse monoclonal antibody (1:200; Chemicon, Boronia, Australia), and *NSE* rabbit polyclonal antibody (1:200; Abcam).

Images were collected using a digital camera mounted onto an immunofluorence microscope (Carl Zeiss MicroImaging GmbH, Sachsen, Thüringen, Jena, Germany) and analyzed using the image software Axiovision (v. 4.6, Carl Zeiss Vision). Semi-automated cell counting was carried out using CellProfiler [24], an open source freeware package (v. 1.0.4828 <www.cellprofiler.com>). For each phenotype marker, at least three random frames (each with more than 20 cells) from two different experiments on independent cell lines were analyzed. CellProfiler allows for the automatic calculation of cell number via nuclei extraction and quantification. An optimized cell outline is then generated for each nucleus based on the intensity and distribution of peri-nuclear immunostaining. Individual framewise thresholds for overall immunostain intensity and immunostain intensity/cell area were then established on the basis of a consensus selection of representative cells considered to be either immuno-positive or -negative. Application of these thresholds across the entire frame then allowed automatic quantification of positive and negative cells.

Statistical analysis

All data are presented as mean ± standard deviation. Comparison between two groups was performed using two-sample student's *t*-test, with $p < 0.05$ being statistically significant. Statistical analyses were performed using SPSS (v. 14.0 for Windows software).

Results

Morphology

SKiNPs were initially cultured as small neurospheres (diameter <20 μm), which appear within 1–5 days after isolation. When most neurospheres reach 70 μm in diameter (Fig. 1A), they were dispersed enzymatically and grown as an adherent monolayer that showed a typical bipolar "batwing" formation (Fig. 1B).

These SKiNPs have now been passaged up to 12 population doublings, with passages 1–7 exhibiting a doubling time of ~7 days, while later passages slowed to 14 days (Fig. 3F). Adherent SKiNPs that are enzymatically dissociated and suspended in expansion media in uncoated flasks readily reform neurospheres (Fig. 1C).

Gene expression

RT-PCR analysis of SKiNPs across passages 1–11 reveal expression of a number of neural precursors: *NCAM*, *βIII-tubulin*, and *NSE*, as well as stem cell markers *nestin, CD133, and FGF-2* receptor (Fig. 2). Expression of *GFAP* was absent in expansion cultures taken from passages 1 to 11. These results have been replicated across two different cell lines, with PCR performed three times for each sample.

Homogeneity and immunofluorescent studies

Figure 3 shows the immunofluorescent staining and quantification of SKiNPs in adherent expansion culture.

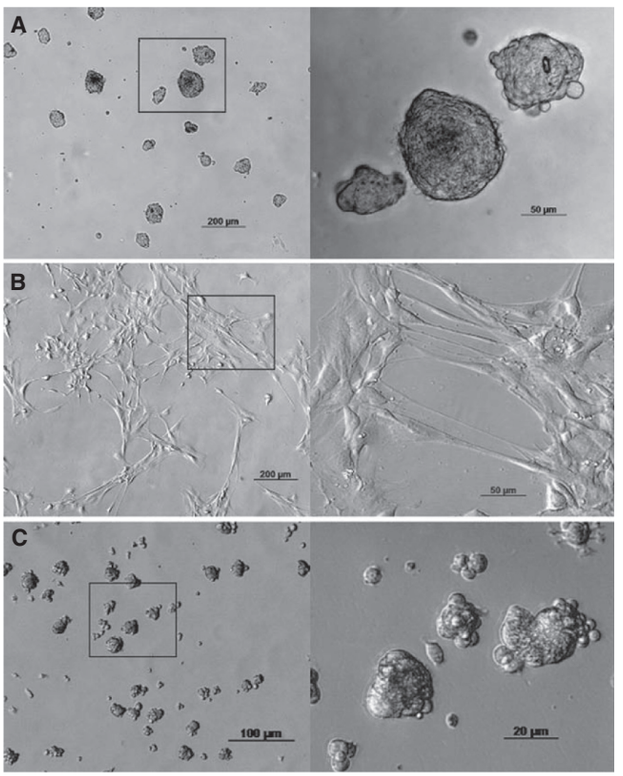

FIG. 1. Morphology of canine SKiNPs under light microscopy. (**A**) Single cell suspension of SKiNPs produced many freefloating spheres with a typical morphology of neurospheres within 1–5 days. (**B**) These cells can also be grown as adherent cultures with typical bipolar or "bat wing" projections. (**C**) Adherent SKiNPs that were dissociated into single cells and suspended in expansion media readily reform neurospheres.

More than 76% (±8.3) of SKiNPs were immuno-positive for *nestin*, with similar results obtained from phenotype cell counting of *NCAM* (84 ± 3.3%), *βIII-tubulin* (96 ± 4.3%), and *CD133* (68 ± 13.5%).

In vitro *differentiation*

SKiNPs at passage 6 were differentiated on laminincoated cover slips for 7 days by adding 3% FBS and removing EGF and bFGF from the DMEM-F12 media (Fig. 4). Quantitative phenotyping after 7 days of differentiation showed that >90% of the SKiNPs were positive for *NCAM* (97 ± 0.2%; Fig. 4A), *βIII-tubulin* (90 ± 5.3%; Fig. 4B), *MAP2* (92 ± 0.8%; Fig. 4C), and *NSE* (97 ± 0.2%; Fig. 4D).

In order to assess the characteristics of a more mature neural phenotype, SkiNPs at passage 7 were differentiated for 21 days after the addition of 10 ng/mL BDNF to the differentiation protocol. On the basis of immunotypic quantitation, there was no change in the percentage of cells expressing *MAP2* (91 ± 5.1%, t value = 0.3, p = 0.78).

Discussion

We report here for the first time the isolation, uniform expansion, and characterization of neural precursors from mature adult dog skin. Self-renewal of canine SkiNPs propagated with our dual-assay system was clearly evident across more than 10 passages. Importantly, proliferation appeared to be efficient enough to be clinically practical: an initial tissue sample of ~3 cm^3 generates the first confluent adherent culture of ~10^6 cells after 10 days, which then continues to double every week. There was, however, evidence that proliferation rates slowed after passage 7. Given transplantation of pluripotential embryonic stem cells in the mammalian brain has seen development of uncontrolled growth [25] and teratoma formation [26], the onset of terminal differentiation within a circumscribed number of cell cycles may prove advantageous.

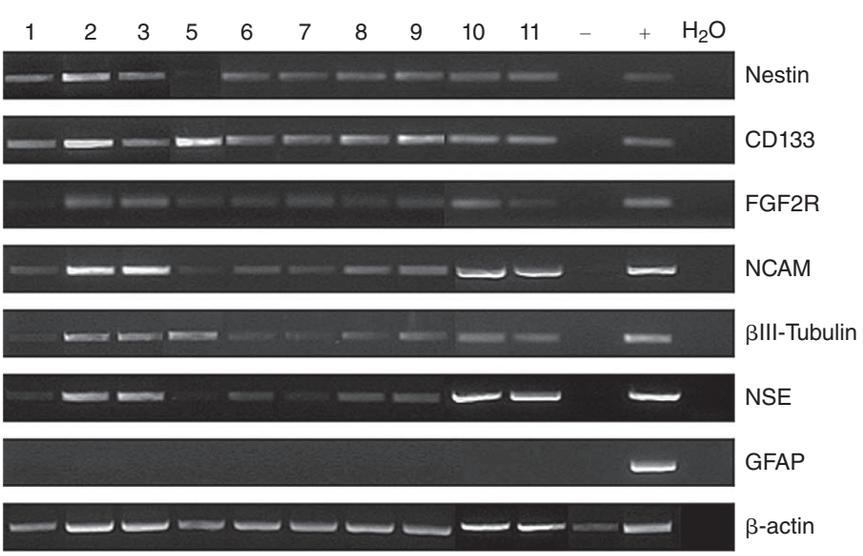

FIG. 2. Gene expression profile of undifferentiated canine SKiNPs as analyzed by RT-PCR. Lanes 1–11 correspond to the passage number of cultured SKiNPs. For controls, dog fibroblast (−) and brain (+) cDNA were used. RT-PCR was performed on each sample three times.

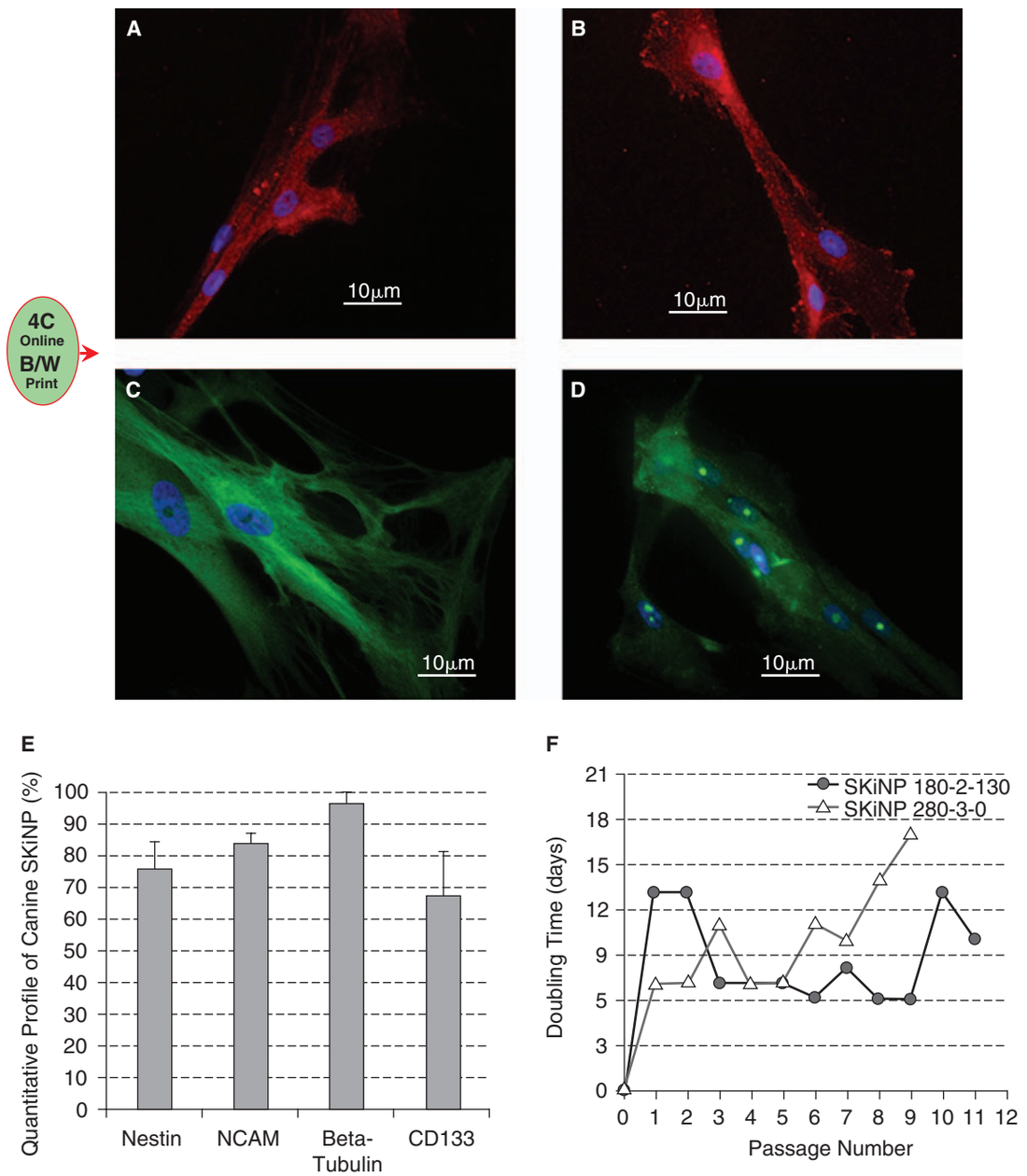

FIG. 3. Immunofluorescent staining and quantification of canine SKiNPs in expansion culture. SKiNPs at passage 6 were immuno-positive for *nestin* (**A**), *NCAM* (**B**), *βIII-tubulin* (**C**), and *CD133* (**D**) with the nuclei stained with *DAPI* (blue) (all at ×40 magnification). (**E**) Quantitative profile of SKiNP expansion culture based on cell counts analyzed using CellProfiler software. **(F)** Doubling time of two canine SKiNP cell lines grown using monolayer assay as a function of passage number.

Gene expression of our canine SKiNPs was similar to the profile noted for neural stem cells derived from conventional sources, including *nestin* and *CD133* markers [27–29]. *Nestin* and *CD133* were also highly conserved across passage number, with phenotypic expression rates between 67 and 76%. Expression of the immature neural protein, *βIII-tubulin*, was particularly high at over 95% under serum-free expansion conditions. Quantitative immunocytochemistry therefore suggests that our system favors propagation of a more uniform neuroprecursor population. As mentioned, this has been a potential limitation of previous skin-to-neuron studies, which have relied on neurosphere propagation. This may also explain why previous reports have yielded neuronal differentiation levels below 16%. By contrast, our SKiNP culture method produced over 90% neural-like cells after 7 days of differentiation. This result was underscored

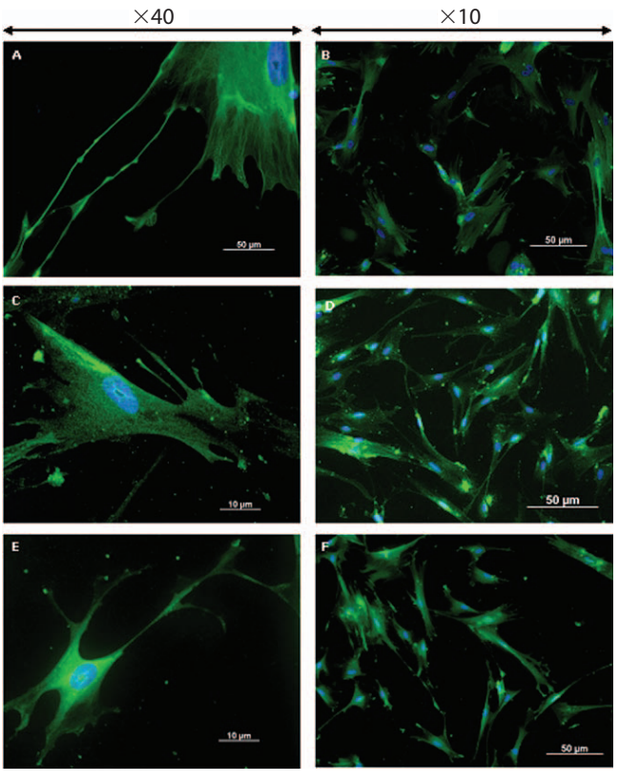

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FIG. 4. Immunofluorescent staining of canine SKiNPs differentiated for 7 days. SKiNPs at passage 6 differentiated on laminincoated cover slips using 3% FBS. Cells were immuno-positive (green) for *βIII-tubulin* (**A and B**), *MAP2* (**C and D**), and *NSE* (**E and F**) with the nuclei stained with *DAPI* (blue).

by phenotypic cell profiles of over 90% from three different neural markers, including *βIII-tubulin, NSE*, and *MAP2*.

Thus our SKiNP results differ from previous studies in a number of ways. Firstly, proliferative senescence appears to be more marked. Second, during propagation there appeared to be no glial cell expression. Rather, a homogenous population of cells expressing neural stem cell markers was readily amplified and produced a neuronal-like yield 7–10 times more efficient than previously reported after a standard differentiation protocol. Next, evidence of neural precursor markers and neural-like cells were obtained from trunk skin as opposed to facial skin of neural crest origins [12,30]. Finally, the multi-lineage potential reported in previous differentiation studies does not appear to be recapitulated; neural-like cells were almost exclusively generated. The biological nature of the SKiNP cells expanded in our dual-assay system would therefore appear to more closely resemble a transiently amplifying fate-restricted neuroprogenitor cell than a bona fide neural stem cell [31]. Why our results differ in these important ways are unclear; however the novel species' origins of the adult canine skin that was used, and the application of our two-step assay system, are suggested as possible factors.

The high neurogenic potential that we observed was primarily based on PCR expression and immunocytochemical phenotype analyses. These results require further elaboration via electrophysiological and receptor expression studies. While a clinically practical, autologous, homogenous, and efficient system of neural precursor culture that avoids glial differentiation would be of great significance, it is unclear whether the neuronal-like phenotype witnessed *in vitro* will translate into a greater level of neuronal differentiation *in vivo* until transplantation studies are completed. However as a general principle, a greater level of neuronal yield *in vitro* is preferable. Belicchi and co-workers [14] have previously propagated CD133+ neurospheres clonally derived from mouse skin and found that 15% of cells were positive for *nestin* and *βIII-tubulin* and 75% positive for *GFAP* after 7 days of differentiation *in vitro*. Two months after intraventricular transplantation into immunodeficient mice, donor cells migrated throughout the striatum and cortex with a predominant (>75%) glial *GFAP* positive phenotype. By contrast, our SKiNPs did not express GFAP mRNA at any stage and showed a high degree of viability over an extended *in vitro* differentiation period. There was also no evidence of alteration to the high neuronal profile after longer-term differentiation. It is unknown whether a homogenous SKiNP cell transplant would remain viable in the adult CNS, nor integrate into complex *in situ* neural networks. This is an area of obvious future interest.

The wider implication of a serum-free technique for the efficient isolation and propagation of neural precursors from canine skin is the opportunity to test their therapeutic potential in community-based dogs. Interest in canine stem cell science is increasing [32]. Unlike rodents, dogs develop many of the same neurological injuries and diseases as humans in a naturalistic manner [33]. Like their human masters, dogs are also living longer and suffering from more age-related neurological conditions. Concerned dog owners may therefore be open to the possibility of testing cell-based

interventions when certain criteria are met, including: (1) when supported by a clear scientific rationale, (2) when the alternative is likely to be euthanasia, (3) when treatment success will extend quality of life, and (4) when chances of further pain and injury is low. Therefore, the establishment of a reliable protocol for the efficient and predictable culture of neural precursors from adult canine skin may accelerate clinical neuroscience interest in working with dogs as translational models for test of cell-based therapy, and so expedite the success of future human clinical trials.

Acknowledgments

Kind thanks to Professor Brent Reynolds (Queensland Brain Institute, University of Queensland, Australia) who commented on this manuscript and to Associate Professor Rosanne Taylor (Faculty of Veterinary Science, University of Sydney) who provided valuable advice along the way, as well as practical help in the form of control canine brain tissue. Warm thanks to Dr. Simon Roberts and the staff of the Struggletown Veterinary Hospital in Sydney, and to Dr. Geraldine Hunt at the University of Sydney Veterinary Clinic, for their help with obtaining skin tissue, as well the dogs and dog-owners who participated and gave their consent. Thanks to Mr. Lawrence Choi who optimized the CellProfiler software used in this study and assisted with cell counting. Thanks to Mr. Mark Lutherborrow for his generous gift of the FGF2R primers. We also appreciate Dr. Holly Zhao's pilot work prior to these studies.

This research was funded by program grant #350833 of the National Health and Medical Research Committee of Australia and the Rebecca Cooper Medical Research Foundation. MV is a University of New South Wales Dean of Medicine Research Fellow.

References

1. Rheinwald J and H Green. (1975). Serial cultivation of strains of human keratinocytes: the formation of keratiniz ing colonies from single cells. Cell 6:331–344.
2. Alonso L and E Fuchs. (2003). Stem cells of the skin epithelium. Proc Natl Acad Sci doi:10.1073(/pnas. 1734203100).
3. Fernandes KJ, IA McKenzie, P Mill, KM Smith, M Akha- van, F Barnabe-Heider, J Biernaskie, A Junek, NR Kobayashi, JG Toma, DR Kaplan, PA Labosky, V Rafuse, CC Hui and FD Miller. (2004). A dermal niche for multi- potent adult skin-derived precursor cells. Nat Cell Biol 6:1082–1093.
4. Laverty P, A Leskovar, GJ Breur, JR Coates, RL Bergman, WR Widmer, JP Toombs, S Shapiro and RB Borgens. (2004). A preliminary study of intravenous surfactants in paraplegic dogs: polymer therapy in canine clinical SCI. J Neurotrauma 21:1767–1777.
5. Platt S and L Garosi. (2003). Canine cerebrovascular disease: do dogs have strokes? J Am Anim Hosp Assoc 39: 337–342.
6. Volk H, K Handler, R Cappello and G Cherubini. (2005). Molecular basis of canine epilepsies. Vet Rec 157:752.
7. Taylor R, B Farrow, G Stewart and P Healy. (1986). Enzyme replacement in nervous tissue after allogenic bonemarrow transplantation for fucosidosis in dogs. Lancet 2:772–774.
8. Cummings B, J Su, CW Cotman, R White and M Russell. (1993). b-Amyloid accumulation in the aged canine brain: a model of early plaque formation in Alzheimer's disease. Neurobiol Aging 14:547–560.
9. O'Brien DP, GS Johnson, RD Schnabel, S Khan, JR Coates, GC Johnson and JF Taylor. (2005). Genetic mapping of canine multiple system degeneration and ectodermal dysplasia loci. J Hered Adv 96:727–734.
10. Sampaolesi M, S Blot, G D'Antona, N Granger, R Tonlorenzi, A Innocenzi, P Mognol, JL Thibaud, BG Galvez, I Barthelemy, L Perani, S Mantero, M Guttinger, O Pansarasa, C Rinaldi, M Cusella G De Angelis, Y Torrente, C Bordignon, R Bottinelli and G Cossu. (2006). Mesangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature 444 doi:10.1038/nature05282.
11. Valenzuela M, K Sidhu, S Dean and P Sachdev. (2007). Neural stem cell therapy for neuropsychiatric disorders. Acta Neuropsychiat 19:11–26.
12. Toma J, M Akhavan, K Fernandes, F Bernabe-Heider, A Sadikot, D Kaplan and F Miller. (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. Cell Biol 3:778–784.
13. Toma J, I McKenzie, D Bagli and F Miller. (2005). Isolation and characterization of multipotent skin-derived pre cursors from human skin. Stem Cells 23:727–737.
14. Belicchi M, F Pisati, R Lopa, L Porretti, F Fortunato, M Sironi, M Scalamogna, EA Parati, N Bresolin and Y Torrente. (2004). Human skin-derived stem cells migrate throughout forebrain and differentiate into astrocytes after in jection into adult mouse brain. J Neurosci Res 77:475–486.
15. Joannides A, P Gaughwin, C Schwiening, H Majed, A Compston and S Chandran. (2004). Efficient generation of neural precursors from adult human skin: astrocytes promote neurogenesis from skin-derived stem cells. Lancet 364:172–178.
16. Reynolds B and S Weiss (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.
17. Reynolds B and R Rietze (2005). Neural stem cells and neurospheres--re-evaluating the relationship. Nat Methods 2:333–336.
18. Singec I, R Knoth, R Meyer, J Maciaczyk, B Volk, G Nikkhah, M Frotscher and E Snyder. (2006). Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods 3:801–806.
19. Bez A, E Corsini, D Curti, M Biggiogera, A Colombo, RF Nicosia, SF Pagano and EA Parati. (2003). Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization. Brain Res 993:18–29.
20. Cattaneo E and R McKay (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. Nature 347:762–765.
21. Conti L, S Pollard, T Gorba, E Reitano, M Toselli, G Biella, Y Sun, S Sanzone, QL Ying, E Cattaneo and A Smith. (2005). Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3(9):e283.
22. Sidhu K. (2005). Growing Human Embryonic Stem Cells. A Class Manual. Sydney, Australia, The Diabetes Transplant Unit Publication.
23. Lim U, K Sidhu and B Tuch (2006). Derivation of motor neurons from three clonal human embryonic stem cell lines. Curr Neurovasc Res 3:281–288.
24. Carpenter AE, TR Jones, MR Lamprecht, C Clarke, IH Kang, O Friman, DA Guertin, JH Chang, RA Lindquist, J Moffat, P Golland and DM Sabatini. (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol 7:R100.
25. Roy N, C Cleren, S Singh, L Yang, M Beal and S Goldman. (2006). Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with teleromase-immortalised midbrain astrocytes. Nat Med Online 12:1259–1268.

26. Wang Q, Y Matsumoto, T Shindo, K Miyake, A Shindo, M Kawanishi, N Kawai, T Tamiya and S Nagao. (2006). Neural stem cells transplantation in cortex in a mouse model of Alzheimer's disease. J Med Invest 53:61–69.
27. Uchida N, D Buck, D He, M Reitsma, M Masek, T Phan, A Tsukamoto, F Gage and I Weissman. (2000). Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci USA 97:14720–14725.
28. Cummings BJ, N Uchida, SJ Tamaki, DL Salazar, M Hooshmand, R Summers, F Gage and AJ Anderson. (2005). Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci USA 102:14069–14074.
29. Lendahl U, L Zimmerman and R McKay. (1990). CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595.
30. Wong C, C Paratore, M Dours-Zimmermann, A Rochat, T Pietri, U Suter, DR Zimmermann S Dufour, JP Thiery, D Meijer, F Beermann, Y Barrandon and L Sommer. (2006). Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. J Cell Biol 175:1005–1015.
31. Seaberg R and van der KD. (2003). Stem and progenitor cells: the premature desertion of rigorous definitions. Trends Neurosci 26:125–131.
32. Schneider M, H Adler, J Braun, B Kienzle, E Wolf and HJ Kolb. (2007). Canine embryo-derived stem cells--towards clinically relevant animal models for evaluating efficacy and safety of cell therapies. Stem Cells 25:1850–1851.
33. Neff M and J Rine. (2007). A fetching model organism. Cell 124:229–231.

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Received for publication January 16, 2008; accepted after revision February 18, 2008.