

Expression of Neurodevelopmental Markers by Cultured Porcine Neural Precursor Cells

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ABSTRACT

Despite the increasing importance of the pig as a large animal model, little is known about porcine neural precursor cells. To evaluate the markers expressed by these cells, brains were dissected from 60-day fetuses, enzymatically dissociated, and grown in the presence of epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor. Porcine neural precursors could be grown as suspended spheres or adherent monolayers, depending on culture conditions. Expanded populations were banked or harvested for analysis using reverse transcription–polymerase chain reaction (RT-PCR), immunocytochemistry, microarrays, and flow cytometry, and results compared with data from analogous human forebrain progenitor cells. Cultured porcine neural precursors widely expressed neural cell adhesion molecule (NCAM), polysialic acid (PSA)–NCAM, vimentin, *Ki-67*, and *Sox2*. Minority subpopulations of cells expressed

doublecortin, β -III tubulin, synapsin I, glial fibrillary acidic protein (GFAP), and aquaporin 4 (AQP4) consistent with increased lineage restriction. A human microarray detected porcine transcripts for *nogoA* (RTN4) and stromal cell–derived factor 1 (SDF1), possibly *cyclin D2* and *Pbx1*, but not *CDI33*, *Ki-67*, *nestin*, or nucleostemin. Subsequent RT-PCR showed pig forebrain precursors to be positive for *cyclin D2*, nucleostemin, *nogoA*, *Pbx1*, vimentin, and a faint band for *SDF1*, whereas no signal was detected for *CDI33*, fatty acid binding protein 7 (FABP7), or *Ki-67*. Human forebrain progenitor cells were positive for all the genes mentioned. This study shows that porcine neural precursors share many characteristics with their human counterparts and, thus, may be useful in porcine cell transplantation studies potentially leading to the application of this strategy in the setting of nervous system disease and injury. STEM CELLS 2005;23:1286–1294

INTRODUCTION

The devastating consequences of central nervous system (CNS) diseases have motivated the search for effective treatments, with much attention devoted to the possibility of neuronal replacement. One strategy is neural transplantation, in which developmentally immature donor tissue is grafted to selected sites within the host CNS. This approach has been shown to restore a number

of visual functions in rodent models using grafts of fetal neural tissue [1, 2]. A major limitation of neural transplantation, however, has been the difficulty of extending these results to adult rodents [3] and large mammals [4], including humans [5]. Recent studies suggest that this challenge may be surmountable through the use of cultured neural stem or progenitor cells instead of solid tissue grafts [6].

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It is now well established that developmentally immature precursor cells can be isolated from the CNS of developing rodents and propagated for extended periods in culture using mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [7, 8]. When shown to meet specific criteria for self-renewal and multipotency, these cells are referred to as neural stem cells (NSCs) or multipotent neural progenitor cells (NPCs) [9]. In cases in which the cultured population has been less well defined, or appears to be particularly heterogeneous, use of the broader term neural precursors is often preferred. For example, when growing these cells from large mammals, cellular heterogeneity and senescence are characteristic findings. Furthermore, detailed characterization in terms of gene expression and marker studies is limited by the availability of reagents that work reliably in the particular species under investigation. Neural progenitors or precursors have now been derived from the brains of a number of mammalian species, including mouse [7, 8], rat [10], dog [11], pig [12], and human [13]. In addition, we have previously shown that NPCs can be cultured from human CNS tissue after relatively prolonged postmortem intervals [14–16]. The degree to which these various cultured neural populations have been characterized, however, varies considerably.

A number of previous studies have demonstrated that NPCs can be derived from the pig brain and propagated in culture using EGF and bFGF as mitogens [12, 17–19]. The expanded porcine neural precursors have been used as donor cells and are capable of engrafting into the mammalian CNS after transplantation [17–21]. These cells therefore provide a large animal comparison for human NPCs (hNPCs) and have been considered as a potential alternative source for donor material, yet relatively little is known about these cells. Porcine neural precursors reportedly express markers of neural lineage under differentiation conditions; however, apart from nestin, little has been reported with respect to marker expression under baseline proliferation conditions. Major disadvantages of working with these novel cells are the paucity of pig-specific antibodies, together with the lack of a comprehensive database for the porcine genome. Here we apply reagents derived for use in other mammalian species to the further characterization of porcine neural precursors and identify a number of markers also expressed in hNPC cultures. Evidence of similarities between human and porcine NPCs is relevant when interpreting the results of pig transplantation studies in the context of potential clinical trials in humans.

MATERIALS AND METHODS

Donor Animals and Human Subjects

Porcine fetuses were obtained off-site from a pregnant sow placed under general anesthesia and terminated prior to waking, according to a protocol approved by the Institutional Animal Care and Use Committee. Informed consent for the donation of human

brain tissue was acquired prior to tissue acquisition, under the auspices of the protocol for the National Human Neural Stem Cell Resource as approved by the Children's Hospital of Orange County Institutional Review Board. All tissues, both porcine and human, were acquired in compliance with National Institutes of Health and institutional guidelines.

Cells

The techniques used for cell isolation of the hNPCs used in this study were described previously [14–16]. The isolation of porcine cells followed a similar protocol, albeit with fetal pigs collected at 60 days of gestational age (total gestational period ~114 days). Briefly, the cranium was opened, brains removed, and the forebrain separated from the cerebellum and brainstem. Neural tissue was enzymatically digested, and the resulting cell suspension was washed repeatedly and cultured at high density in fibronectin-coated flasks containing Dulbecco's modified Eagle's medium/F-12 with high glucose (Irvine Scientific, Santa Ana, CA, <http://www.irvinesci.com>), L-glutamine (200 mM), BIT9500 (10% by volume; StemCell Technologies, Vancouver, British Columbia, Canada, <http://www.stemcell.com>), EGF (20 ng/ml), bFGF (40 ng/ml), platelet-derived growth factor-AB (20 ng/ml), and antibiotics. Fetal bovine serum (10% by volume) was included overnight to promote adherence, and the media was completely changed the next day. Subsequently, cells were fed by 50% media exchange every 2–3 days and passaged at confluence using Cell Dissociation Buffer (Gibco, Grand Island, NY, <http://www.invitrogen.com>) and gentle trituration.

Candidate Markers and Genes

The markers and gene products examined in this study (Table 1) were suggested either by previous work with mammalian CNS progenitor cells from species other than pig or, in some cases, by analysis of human gene microarray data.

Immunocytochemistry

Live cells were fixed for 10 minutes in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (Irvine Scientific). Fixed cells were washed with PBS with 0.05% (w:v) sodium azide. A blocking solution of tris-buffered saline (TBS) + 0.3% Triton X-100 + 3% donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, <http://www.jacksonimmuno.com>) was applied for 15 minutes. Cells were then rinsed twice in 0.1M TBS buffer. Primary antibodies were diluted in 250 μ l of antibody buffer (TBS + 0.3% Triton-X100 + 1.0% donkey serum) at concentrations determined through experimentation (Table 2). Primary antibodies were applied to the samples and kept at 5°C overnight. The following day the cells were rinsed twice with TBS. All secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) were donkey-derived and diluted 1:100 in antibody buffer. Secondary antibodies were applied to

samples and kept at 5°C overnight. The next day, samples were rinsed three times with TBS for 5 minutes. Slides were mounted with Prolong® Antifade Kit (Molecular Probes, Inc., Eugene, OR, <http://probes.invitrogen.com>), and digital images were obtained using an IX70 Microscope (Olympus, Melville, NY, <http://www.olympusamerica.com>) and a QuantiFIRE CCD

camera (Optronics, Goleta, CA, <http://www.optronics.com>). Image files were managed with Image-Pro Plus 4.0 software (Media Cybernetics, Inc., Silver Spring, MD, <http://www.mediacy.com>) with AFA plugin 4.5.

Table 1. Candidate markers and genes

Marker/gene	Method of identification	Description
A2B5	ICC	Neuroglial precursor marker
Aquaporin 4	ICC	Water channel, surface marker
β ₂ -Microglobulin	FC	Immune surface marker
β-III Tubulin	ICC	Microtubule protein
<i>CCND2</i>	GMA	Cell cycle protein
CD133	RT-PCR	Prominin-like 1, surface marker
CD15	ICC	Lewis antigen, surface marker
CD54 (ICAM)	FC	Adhesion molecule, surface marker
CD56 (NCAM)	FC, GMA, ICC	Adhesion molecule, surface marker
CD81	FC	Tetraspanin, surface marker
CD9	FC	Tetraspanin, surface marker
<i>CXCR4</i>	GMA	Cytokine receptor, surface marker
<i>DACH1</i>	RT-PCR	Dachshund, transcription factor
<i>DCX</i>	ICC, RT-PCR	Doublecortin, neuroblast marker
<i>DLX2</i>	GMA	Distal-less homeobox
<i>DLX5</i>	GMA	Distal-less homeobox
<i>EGFR</i>	RT-PCR	EGF receptor, surface marker
<i>FABP7</i>	GMA	Fatty acid binding protein
<i>GAD1</i>	GMA	Glutamate decarboxylase
<i>GD2</i>	FC, ICC	Ganglioside, surface marker
<i>GFAP</i>	ICC, RT-PCR	Intermediate filament
<i>Hes1</i>	RT-PCR	Transcription factor
HLA-1	FC	Immune surface marker
HLA-2	FC	Immune surface marker
Ki-67	ICC, RT-PCR	Cell cycle protein, proliferation marker
<i>LHX2</i>	GMA	Homeobox transcription factor
<i>LIFR</i>	GMA	LIF receptor, surface marker
<i>MELK</i>	GMA	Leucine zipper kinase
<i>NANOG</i>	GMA	Transcription factor
<i>Nestin</i>	GMA, ICC, RT-PCR	Intermediate filament
<i>NeuN</i>	ICC	Neuronal nuclear protein
<i>NogoA</i>	RT-PCR	Reticulon 4, surface protein
Nucleostemin	GMA, RT-PCR	Nucleolar protein
<i>OLIG2</i>	GMA	Oligodendrocyte lineage
<i>PAX6</i>	GMA, ICC, RT-PCR	Paired box
<i>PBX1</i>	RT-PCR	Transcription factor
<i>POU5F1</i>	GMA	POU transcription factor
Recoverin	ICC, RT-PCR	Phototransduction-related protein
<i>SDF-1</i>	RT-PCR	CXCL12, chemokine
<i>SIX3</i>	GMA, RT-PCR	Sine oculis homeobox
<i>SIX6</i>	RT-PCR	Sine oculis homeobox
<i>SOX2</i>	ICC, RT-PCR	Transcription factor
Synapsin 1	ICC	Synaptic protein
Vimentin	ICC, RT-PCR	Intermediate filament

Abbreviations: FC, flow cytometry; GMA, gene microarray; ICC, immunocytochemistry; RT-PCR, reverse transcription–polymerase chain reaction.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from cultured progenitor cells after 3 weeks in culture (passage 4), using Purescript RNA Isolation Kit (Gentra Systems, Inc., Minneapolis, <http://www.gentra.com>) according to the manufacturer's protocol. Any residual genomic DNA was eliminated by treatment with DNase (DNA-free™; Ambion, Inc., Austin, TX, <http://www.ambion.com>). RNA was reverse-transcribed with Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (RT) (Invitrogen Corporation, Carlsbad, CA, <http://www.invitrogen.com>). Negative controls contained RNA, but no M-MLV RT, to further ensure that polymerase chain reaction (PCR) product did not result from amplified genomic DNA. PCR was carried out in a final volume of 50 µl with 3 µl of cDNA template, 0.75 µl of forward and reverse primers (0.5 µg/µl) (Qiagen, Inc., Valencia, CA, <http://www.qiagen.com>) (Table 1), and 1.25 units of *Taq* DNA Polymerase (Amersham, Piscataway, NJ, <http://www.amersham.com>) on a Genius thermocycler (Techne Corporation, Minneapolis, <http://www.techne-corp.com>). Initial denaturation for 4 minutes at 94°C was followed by 30 cycles of 1 minute at 94°C, 1 minute at the corresponding annealing temperature (Table 3), and 1 minute at 72°C. The final step consisted of 7 minutes of extension at 72°C. PCR products were run on 2% agarose gels and visualized with ethidium bromide against a 100-bp ladder.

Microarray Analysis

Porcine neural precursor cells were grown to confluence as an adherent culture and harvested for microarray analysis at passage 4. A total of three hNPC cultures, grown under similar conditions, were harvested at passages 3–5 for the same purpose. In each case, total RNA was extracted from cultured cells, using Purescript RNA Isolation Kits (Gentra Systems, Inc.) according to the manufacturer's protocol. In the absence of an available porcine microarray, all RNA was examined using the HG-U133 human GeneChips (Affymetrix, Santa Clara, CA, <http://www.affymetrix.com>) at the University of California, Irvine DNA Microarray Facility. A minimum of 10 µg total cRNA was used for each analysis, as measured by spectrophotometry (NanoDrop Technologies, Wilmington, DE, <http://www.nanodrop.com>). A bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA, <http://www.agilent.com>) was used to ensure 28S/18S ratios above 1.6 and 260/280 ratios above 1.8, and samples were further purified using Qiagen RNeasy columns as needed. Microarray Suite 5.0 software (Affymetrix) was used to analyze the data, using default set-

Table 2. Primary antibodies

Antigen	Species (subtype)	Manufacturer	Product code	Dilution
AQP4	Rabbit	Chemicon International	AB3594-50ul	1:100
DCX	Goat (IgG)	Santa Cruz Biotechnology	sc-8066	1:100
GFAP	Guinea pig	Chemicon International	AB1540	1:200
Ki-67	Mouse (IgG)	BD Biosciences Pharmingen	556003	1:200
NCAM	Rabbit	Chemicon International	AB5032	1:100
Nestin	Goat (IgG)	Santa Cruz Biotechnology	sc-21248	1:100
Nestin	Mouse (IgG)	Chemicon International	MAB5326	1:200
Nestin	Rabbit (IgG)	Chemicon International	AB5922	1:100
PSA-NCAM	Mouse (IgM)	Chemicon International	MAB5324	1:100
Sox2	Goat (IgG)	Santa Cruz Biotechnology	sc-17320	1:50
Synapsin I	Rabbit	Sigma	S193	1:100
β -III Tubulin	Mouse (IgG)	Chemicon International	MAB1637	1:100
Vimentin	Mouse (IgG)	Sigma	V 6630	1:1,000

Abbreviation: IgG, immunoglobulin G.

Table 3. Reverse transcription–polymerase chain reaction primers

Gene	5' primer	3' primer	Annealing temperature (°C)	Size (bp)
β -Actin	CGTGCTGCTGACCGAGGCC	TTCGTGGATGCCACAGGAC	68	522
<i>CD133</i>	CTGGGGCTGCTGTTTATTATTCTG	ACGCCTTGTCCTTGAGTAGTGTG	56	336
<i>CyclinD2</i>	CTGGCCATGAACCTACCTGGA	CATGGCAAACCTAAAGTCGG	50	353
<i>DCX</i>	AATCCCAACTGGTCTGTCAAC	GTTTCCCTTCATGACTCGGCA	62	405
<i>FABP7</i>	TGCCACCCCTC TTCCAAA	CCACCTCCACACCAAGGATA	52	646
<i>GFAP</i>	ACATCGAGATCGCCACCTAC	ACATCACATCCTTGCTGCTCC	64	219
<i>Hes1</i>	CAGCCAGTGTCACACGACAC	TCGTTCATGCACTCGCTGA	56	307
<i>Ki-67</i>	GGAGGCAATATTACATAATTTCA	CAGGGTCAGAAGAGAAGCTA	50	232
<i>Nestin</i>	GGCAGCGTTGGAACAGAGGTTGGA	CTCTAAACTGGAGTGGTCAGGGCT	65	718
<i>NogoA</i>	TGGAGGTGCTGGAGAGGAA	GATGCACAGGAAGAGCAA	54	432
<i>Nucleostemin</i>	CATGACCTGCCATAAGCGGT	CAATTACTCCAACCCGAATGGC	55	745
<i>Pbx1</i>	CCATCTCAGCAACCCCTTAC	CCATGGGCTGACACATTG	49	181
<i>SDF-1</i>	TGCCAGAGCCAACGTCAAG	CAGCCGGGCTACAATCTGAA	55	73
<i>Sox2</i>	GGCAGCTACAGCATGATGCAGGAGC	CTGGTCATGGAGTTGTACTGCAGG	73	131
<i>Vimentin</i>	TGGCACGTCTGACCTTGAA	GGTCATCGTGATGCTGAGAA	53	750

tings, with expression designated as “present” or “absent” by the software algorithms together with semiquantitative numerical assessment of expression levels. Of the large number of gene loci assessed in this manner, particular attention was directed to a circumscribed set of genes, determined to be of interest based on relevance to neural ontogeny and comparison between human and porcine data sets (Table 1).

Flow Cytometry

Samples were handled in the manner described previously for flow cytometry, using the same antibodies [15]. Briefly, cells were harvested enzymatically as a single cell suspension, incubated with labeled-primary or primary and labeled-secondary antibodies, and analyzed on a FACS Vantage cell sorter with an Enterprise 488-nm argon laser (BD Biosciences, San Jose, CA, <http://www.bdbiosciences.com>). The isotype control used was mouse IgG, clone MOPC. Up to 30,000 events per sample were collected for analysis.

RESULTS

Proliferative cultures were obtained from fetal pig brain, including both forebrain and pooled brainstem/cerebellum samples. These cultures could be grown as adherent monolayers (Fig. 1) or as suspended spheres. Both adherent cells and spheres exhibited morphological characteristics consistent with other mammalian neural progenitor cultures, including analogous cells from humans [13–15].

Immunocytochemistry

Further analysis of marker expression by porcine CNS precursors was carried out using immunocytochemical (ICC) techniques. Pig forebrain and brainstem/cerebellum cultures appeared to exhibit equivalent staining patterns for the markers examined, and therefore the combined ICC data are presented here (Fig. 2). These studies showed the expression of doublecortin (DCX), glial fibrillary acidic protein (GFAP), and Sox2, and also demonstrated the presence of neural cell adhesion molecule (NCAM),

polysialic acid (PSA)–NCAM, synapsin I, β -III tubulin, and vimentin. Other proteins detected included the membrane channel aquaporin 4 (AQP4) and the proliferation marker Ki-67. All these markers have been identified in previous studies of human CNS progenitor cells [15, 16]. Table 3 lists all the gene products investigated, grouped by techniques used, as well as their cellular functions. As with the RT-PCR results, however, there was no clear signal to indicate expression of nestin with any of the three different antibodies tried.

RT-PCR

As an initial step toward characterization of these cells, total RNA from adherent forebrain-derived porcine precursors was examined by RT-PCR for the expression of established neurodevelopmental markers. Findings from the pig were compared with results obtained from brain-derived hNPCs. The pig cells were clearly positive for *DCX*, *GFAP*, *Hes1*, and *Sox2*, whereas the signal for *nestin* was very faint (Fig. 3). In contrast, hNPCs were clearly positive for expression of all five of these genes.

Gene Microarray

In an effort to increase the number of potential targets for additional marker studies, total RNA from porcine forebrain precursors was analyzed using an available microarray designed for human samples. The resulting data from the pig were then compared with that from hNPCs. With respect to expression data as a whole, the degree of variance in reported expression levels was considerably larger between the two species than seen for comparisons of human samples to each other (data not shown). In addition, the proportion of transcripts detected in human, but not pig, samples was large. Conversely, the proportion of genes reported as expressed in pig, but not human, was small (Fig. 2H).

A number of specific genes were then chosen, based on positive expression in hNPCs [15, 16], and the expression data from the

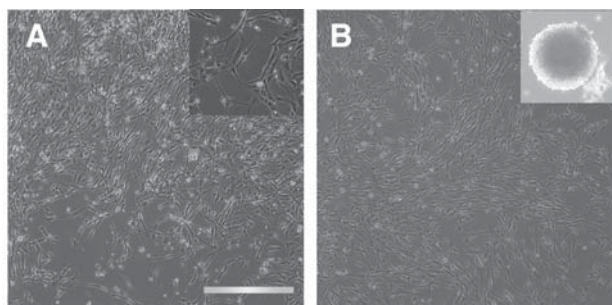


Figure 1. Porcine neural precursor cells in culture. (A): Cultures derived from forebrain and plated on fibronectin grew as an adherent monolayer under proliferation conditions. Inset shows enlarged view of field from same image. (B): Equivalent cultures derived from cerebellum/brainstem, grown under identical conditions, also formed an adherent monolayer. Inset shows low-power view of neurosphere from separate culture of the same cells. All images are phase-contrast; scale bar = 50 μ m.

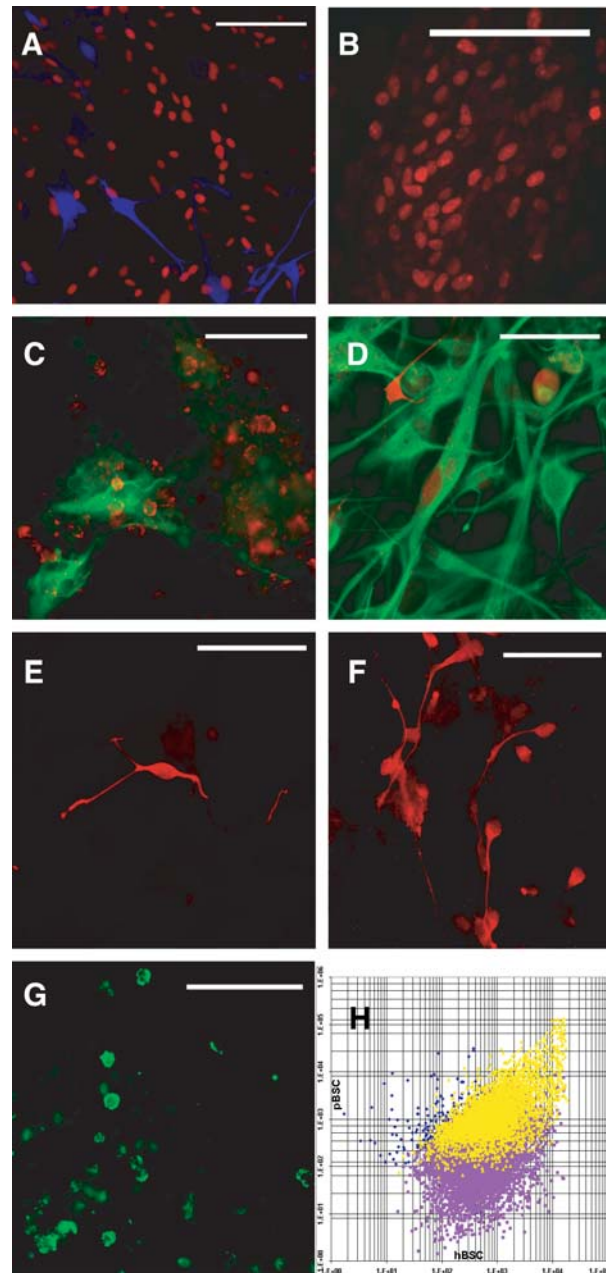


Figure 2. Immunocytochemical analysis of porcine CNS precursors. (A): Widespread nuclear staining for *Sox2* (red), subset of cells with cytoskeletal staining for *GFAP* (blue). (B): Widespread nuclear staining for *Ki-67* (red). (C): Surface staining of numerous small rounded profiles for *PSA-NCAM* (red), subset of broader profiles staining for *GFAP* (green). (D): Widespread cytoskeletal staining of broad profiles for vimentin (green), subset of smaller profiles with fine processes staining for synapsin I (red). (E): Subset of small bi- or tripolar profiles stain for *DCX* (red). (F): Profiles with long, thin processes staining for β -III tubulin (red). (G): Subset of small, rounded profiles with punctate surface staining for *aquaporin-4* (green). (H): Scatter plot of human microarray data from pig and human brain stem cell (pBSC, hBSC) cultures showing relative proportion of genes “present” in human only (purple), pig only (blue), or both (yellow). Scale bars = 50 μ m. Abbreviations: CNS, central nervous system; DCX, doublecortin; GFAP, glial fibrillary acidic protein; PSA-NCAM, polysialic acid–neural cell adhesion molecule.

pig were examined with respect to these loci. Of this subset, genes reported as positive in the pig included *Hes1*, *Sox2*, and vimentin, consistent with the RT-PCR and ICC studies reported above, as well as the new targets *nogoA* (RTN4) and stromal cell–derived factor 1 (*SDF1*). Genes not detected in the pig by human microarray, despite having been demonstrated by the initial RT-PCR and ICC studies, included *DCX* and *GFAP*. Also not detected by microarray were transcripts for the commonly used markers *CD133*, *Ki-67*, *nestin*, and nucleostemin. Closer examination of the data revealed an additional subset of genes showing some oligomers as positively or marginally detected, yet others absent. Examples include *CXCR4* (CD184, fusin), *cyclin D2*, fatty acid binding protein 7 (*FABP7*), and *Pbx1*. A number of these candidate genes were selected for further analysis using RT-PCR with human primer sequences.

Additional RT-PCR

The second set of RT-PCR experiments was directed toward validating the microarray data. This work revealed positive expression by pig forebrain precursors of *cyclin D2*, nucleostemin, *nogoA*, *Pbx1*, and vimentin (Fig. 4). No signal was detected for *CD133*, *FABP7*, *Ki-67*, or *SDF1*. Positive expression of all nine of these genes was verified in human forebrain NPCs.

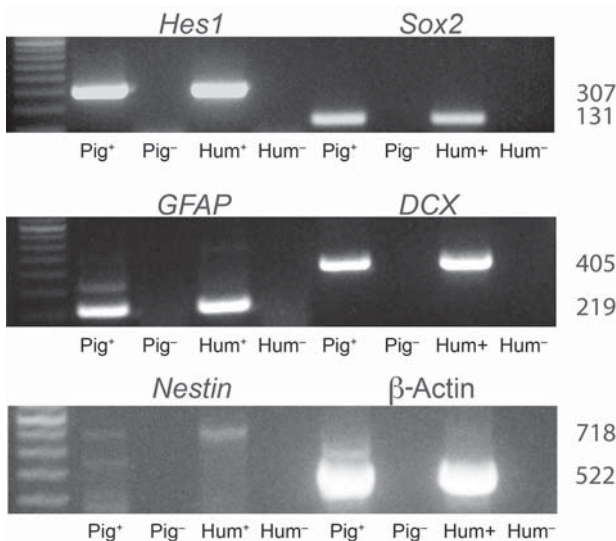


Figure 3. Neurodevelopmental markers by RT-PCR: pig versus human. Primers designed for detection of human transcripts (Table 1) were used to evaluate proliferative porcine and human forebrain cultures. For each gene, four alternating lanes from left to right contained porcine product (Pig⁺), porcine negative control (Pig⁻), human product (Hum⁺), and human negative control (Hum⁻). Porcine precursors showed expression of *DCX*, *GFAP*, *Hes1*, and *Sox2*, yet there was little signal for *nestin*. Human progenitors clearly expressed all five genes. Positive control was β -actin. Products are shown together with a 100-bp ladder at the left of the gel and predicted product sizes are in the right margin. Abbreviations: DCX, doublecortin; GFAP, glial fibrillary acidic protein; RT-PCR, reverse transcription–polymerase chain reaction.

Flow Cytometry

Porcine forebrain precursors were also examined by flow cytometry. This study confirmed expression of the neural adhesion molecule *NCAM* (CD56) on the surface of these cells, as compared with IgG control (Fig. 5). Other cell surface markers known to be highly positive in hNPCs [15] but showing no staining in porcine forebrain precursors included CD29, CD44, CD81, CD90, CD133, and CD184.

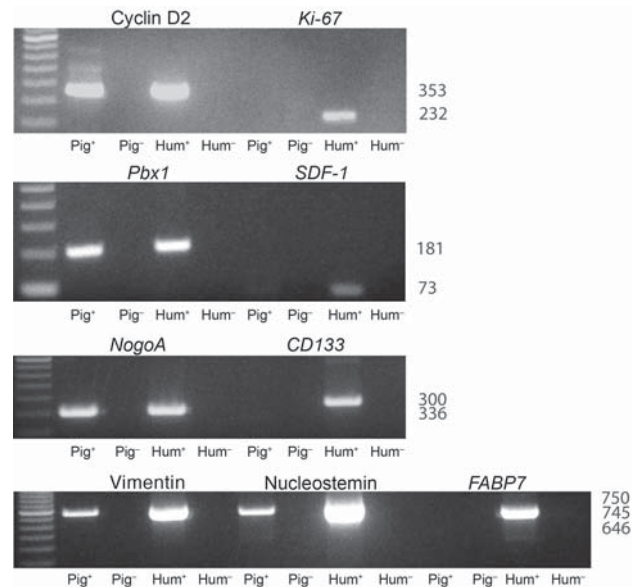


Figure 4. RT-PCR evaluation of candidate genes: pig versus human. Human primers (Table 1) were used to evaluate proliferative porcine and human forebrain cultures. For each gene, four alternating lanes from left to right contained porcine product, porcine negative control, human product, and human negative control, all labeled as in Figure 3. Porcine precursors showed expression of cyclin D2, *Pbx1*, *nogoA*, vimentin, and nucleostemin, but no product was detected for *Ki-67*, *SDF1*, *CD133*, or *FABP7*. Human progenitors clearly expressed all nine genes. A 100-bp ladder is at the left of the gel and predicted product sizes are in the right margin. Abbreviations: FABP7, fatty acid binding protein 7; RT-PCR, reverse transcription–polymerase chain reaction; SDF1, stromal cell–derived factor 1.

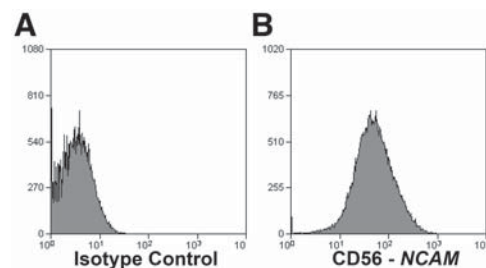


Figure 5. Flow cytometric analysis of porcine forebrain precursors. (A): Baseline fluorescence of isotype control. (B): Positive expression of *NCAM* by pig forebrain precursors. Horizontal axis = relative fluorescence; vertical axis = counts. Abbreviation: *NCAM*, neural cell adhesion molecule.

Novel Markers Expressed by hNPCs

Of the markers found to be positively expressed by hNPCs, some have not been previously reported for these cells. These markers include *FABP7*, *Hes1*, *nogoA*, *Pbx1*, and *SDF1*. The remainder have been mentioned in previous studies [15, 16].

DISCUSSION

Previous work on the characterization of porcine neural precursor cells has been limited in terms of marker studies, in part because of a shortage of pig-specific genetic data and reagents. In particular, previous molecular characterization of these cells in the undifferentiated state has been limited to evaluation of nestin expression. The current study shows that detection of nestin in pig cells can itself be problematic when using a number of readily available reagents, but that a range of additional markers can be detected and that these are consistent with immature CNS precursors, including cytoskeletal proteins, surface markers, cell cycle-related proteins, and nuclear transcription factors. This study, therefore, increases our understanding of neural precursors in large animals and provides data bearing on the validity and limitations of available techniques for further characterizing these cells.

The intermediate filament protein nestin is highly expressed during development of the vertebrate CNS and has been considered a useful, if imperfect, marker of NSCs. For instance, nestin is not a surface marker and can be expressed by a number of normal and abnormal CNS cell types [22–24]. Previous studies have reported nestin expression in proliferative precursor populations cultured from the porcine brain [19, 20]. These studies used ICC techniques to demonstrate the presence of nestin-associated epitopes. In contrast, the current study was unable to demonstrate substantial nestin expression, either by immunocytochemistry or RT-PCR. Because nestin expression is a hallmark of vertebrate CNS development, this result can be reasonably attributed to inadequate detection, presumably as a result of the particular antihuman reagents used here, despite the fact that several different antibody preparations were tried. Whereas many proteins can be detected across a range of species by a given antibody, nestin is more specific, generally requiring the use of different monoclonal antibodies for detection in rodents and humans. Similarly, detection of nestin transcripts by RT-PCR benefits from species-specific primers. Because of the noted tendency toward species-related restriction in the specificity of nestin epitopes and primers, the discrepancy in the nestin findings between the current study and previous reports is most likely related to the use of different antibodies. In this case, the development of pig-specific antibodies and primers would provide the optimal solution to reliable nestin detection in porcine cells and tissues.

In addition to nestin, a number of other markers have been associated with neural stem and progenitor cells. The present study shows expression of the transcription factors *Sox2* and *Hes1*, the surface marker PSA-NCAM, and the intermediate

filament vimentin, all of which have been reported as expressed by CNS precursor cells, or subsets thereof [25, 15]. In addition, the present study provides evidence from RT-PCR for expression of *Pbx1*, a homologue of the *Drosophila* extradenticle (exd) homeodomain protein [26]. Pbx proteins are known to act as cofactors for other homeodomain transcription factors [27]. *Pbx1* has not been previously identified in cultured neural precursor populations; however, it is known to be expressed at high levels in the developing nervous system [28]. Studies in the rat have shown that Pbx1 is expressed by proliferating cells of the subventricular zone, as well as their neuronal progeny, but not by glia [29]. In the context of previous *in vivo* work, the results of the current study showing *Pbx1* expression in cultured neural precursors from the pig and human forebrain suggest a significant role for this gene in mammalian neurogenesis.

Another feature of immature precursor cell cultures is proliferative activity, reflected in the expression of genes involved in the cell cycle and its regulation. In this context, the nucleolar marker nucleostemin [30] was expressed by both pig and human neural precursors, as was *cyclin D2*. In contrast to the findings with *nestin*, the commonly used proliferation marker *Ki-67*, readily detected in human cells by ICC, microarray, and RT-PCR, was identified in porcine cells, albeit by immunocytochemistry alone. No signal was obtained from porcine samples using the RT-PCR primers or the human gene microarray. Again, this seemingly paradoxical finding presumably relates to differences in the human and porcine coding sequences for this gene, together with conservation of the immunologically recognized epitope of the expressed protein.

In terms of phenotypic potential, porcine neural precursor cultures included subsets of cells that expressed markers of either neuronal or glial lineages. The neural cell adhesion molecule *NCAM* (CD56) is widely expressed throughout the developing and mature CNS. *DCX*, β -III tubulin, and synapsin I are expressed by neuronal precursors, whereas *GFAP* is associated with astroglia, as well as neural precursors. The expression of this latter intermediate filament protein by cultured porcine neural precursors has been previously reported [20] and is replicated here. *AQP4* is a water channel found in the plasma membrane, particularly of glial vascular processes (endfeet) and may play a role in the formation of the blood–brain barrier [31, 32].

Cross-species hybridization on microarrays has been reported previously, including work with pig RNA on human arrays [33]. Cross-species comparisons are known to be associated with increased variability of the expression data, as was the case here. Some of this variability may reflect actual differences in relative gene expression between species; however, a substantial artifactual component is likely as well. Similarly, the large number of porcine transcripts undetected using the human array might be anticipated as a result of hybridization failures secondary to species-specific differences in sequence. Whereas this large body of negative data are inherently difficult to interpret, the subset of

genes reported as positively expressed by both human and porcine cells represents a source of candidate genes with a high likelihood of verification upon further analysis. Indeed, the cross-species microarray analysis performed here led to the presumptive identification of *cyclin D2*, *nogoA* (RTN4), and *Pbx1* expression by porcine neural progenitors, all of which were subsequently confirmed by RT-PCR (Fig. 5).

An additional consideration with respect to differences found in the data from porcine and human cells is that the cell populations studied are not entirely equivalent. In the present study, samples were taken at different gestational ages and different cerebral locations in pigs and humans. In this respect, differences in the structure and developmental time-course of the brain in these two species would appear to make sample nonequivalence unavoidable. This fact therefore makes the similarity of findings between the two species seen here all the more striking, while also reaffirming that negative marker expression in the pig might be real in some instances because of species differences or nonequivalence of cells. Additional cross-species work will be necessary to clarify these issues.

CONCLUSION

The present study expands considerably the number of markers known to be expressed by cultured porcine neural precursors. Of particular interest are nuclear transcription factors, both because of their highly conserved sequences, as well as their prominent role in

phenotypic plasticity and lineage specification. The expression of *Hes1*, *Pbx1*, and *Sox2* by forebrain precursors of both pig and human argues for an important role for these genes in the neural development of large mammals. The markers examined here were chosen based on positive expression by human forebrain progenitors and, with that in mind, the results are quite similar between the two species. Given this degree of similarity, it would seem probable that the frequent failure to detect porcine markers using human-specific primers, oligomers, and antibodies reflects specificity issues more than biological differences in expression. The development of pig-specific reagents would be helpful and is likely to increase as interest grows in the use of the pig as a large animal model in a variety of experimental paradigms, including stem cell transplantation [34].

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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