

Matrix Cells from Wharton's Jelly Form Neurons and Glia

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ABSTRACT

We have identified an easily attainable source of primitive, potentially multipotent stem cells from Wharton's jelly, the matrix of umbilical cord. Wharton's jelly cells have been propagated in culture for more than 80 population doublings. Several markers for stem cells, including c-kit (CD117), and telomerase activity are expressed in these cells. Treatment with basic fibroblast growth factor overnight and low-serum media plus butylated hydroxyanisole and dimethylsulfoxide induced Wharton's jelly cells to express a neural phenotype. Within several hours of this treatment, Wharton's jelly cells developed rounded cell bodies with multiple neurite-like extensions, similar to the morphology of neural stem cells. Neuron-specific enolase (NSE), a neural stem cell marker, was expressed in these cells, as shown by immunocytochemistry. Immunoblot analysis showed similar levels of NSE expression in both

untreated and induced Wharton's jelly cells. After 3 days, the induced Wharton's jelly cells resembled bipolar or multipolar neurons, with processes that formed networks reminiscent of primary cultures of neurons. The neuron-like cells in these cultures stained positively for several neuronal proteins, including neuron-specific class III β -tubulin, neurofilament M, an axonal growth-cone-associated protein, and tyrosine hydroxylase. Immunoblot analysis showed increasing levels of protein markers for mature neurons over time postinduction. Markers for oligodendrocytes and astrocytes were also detected in Wharton's jelly cells. These exciting findings show that cells from the matrix of umbilical cord have properties of stem cells and may, thus, be a rich source of primitive cells. This study shows their capacity to differentiate into a neural phenotype in vitro. *Stem Cells* 2003;21:50-60

INTRODUCTION

The two most basic properties of stem cells are the capacities to self-renew indefinitely and to differentiate into multiple cell or tissue types. Embryonic stem cells proliferate indefinitely and can differentiate spontaneously into all tissue types [1]. Adult stem cells are tissue-specific, may

have less replicative capacity, and, until recently, were thought to have restricted developmental fates [2]. The "plasticity" of adult stem cells relies on their ability to transdifferentiate into tissues different from their origin and, perhaps, across embryonic germ layers. The potential of stem-cell-based therapies for treating a myriad of human

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and animal diseases emphasizes the importance of comparing the various sources of stem cells and seeking a better understanding of their proliferative capacity and differentiation potential. Here, we describe a unique site of potential stem cells in the umbilical cord.

The ability of stem cells to self-renew is critical to their function as a reservoir of primitive cells. In contrast, most somatic cells have a limited capacity for self-renewal due to telomere shortening [3]. Telomerase is a ribonucleoprotein that replicates telomeres during the S phase of mitosis [4]. Telomerase activity is found in human germ, tumor, and embryonic cell lines and is thought to be responsible for the unlimited capacity for self-renewal of these cell types [5, 6]. Telomerase activity is, thus, one marker for stem cells. Another stem cell marker is the stem cell factor receptor, c-kit (CD117). c-kit is a receptor tyrosine kinase that is expressed in many types of tissue-specific stem cells and is essential for many developmental processes, including hematopoiesis, melanogenesis, and fertility [7]. The c-kit receptor ligand, stem cell factor, is a member of the PDGF family of growth factors and is secreted by epithelial cells, leukocytes, fibroblasts, and myofibroblasts [8].

Embryonic stem cells are totipotent and can be maintained in culture in the presence of the cytokine, leukemia inhibitory factor (LIF) [9-11]. When LIF is withdrawn, aggregates, called embryoid bodies, form. A wide variety of cell types migrate away from the embryoid bodies, including some that appear neuronal [12]. Importantly, it has been shown that embryonic stem cells can be induced to differentiate into neurons and glia by treatment with retinoic acid [13-15] or basic fibroblast growth factor (bFGF) [16]. Embryonic-stem cell-derived neurons express neurofilaments, neuron-specific class III β -tubulin (TuJ1) [13-16], and a number of neuron-specific microtubule-associated proteins, including growth-cone-associated protein (GAP-43), which is localized to axons [17].

Neural stem cells are immature, uncommitted cells that exist in both the developing brain and in the adult nervous system [18-21]. Neural stem cells can undergo expansion and can differentiate into neurons, astrocytes, and oligodendrocytes [22, 23]. However, neural stem cells from the central nervous system are rare, require an invasive procedure to obtain, and may have a more limited potential to proliferate in culture than embryonic stem cells.

Postnatal stem cells from other sources, such as bone marrow stromal cells, may offer an easily attainable source of cells for therapeutic purposes. Recent studies have indicated that adult stem cells derived from bone marrow and skin can be expanded in culture and can give rise to multiple lineages [24-26]. Further evidence of stem cell plasticity comes from a study that showed that bone marrow

stromal cells injected into lateral ventricles of neonatal mice differentiated into astrocytes and neurofilament-expressing cells [27]. Bone marrow stromal cells have also been induced to differentiate into neurons in vitro [28, 29]. Woodbury *et al.* [29] showed that bone marrow stromal cells could be induced to differentiate into neurons, almost exclusively, by using a variation of the bFGF method that induces embryonic stem cells to form neural cell types. When treated in this manner, the induced bone marrow stromal cells developed a highly differentiated, neuron-like morphology, with processes that had primary and secondary branches, and expressed markers for neural precursors, mature neurons, and glial cells.

Wharton's jelly is the gelatinous connective tissue from umbilical cord and is composed of myofibroblast-like stromal cells, collagen fibers, and proteoglycans [30]. Here, we present our findings about proliferative cells that we have isolated from Wharton's jelly. Our results show that Wharton's jelly cells are easily attainable and can be expanded in vitro, maintained in culture, and induced to differentiate into neural cells. They are a potential source of multipotent stem cells that may serve many therapeutic and biotechnological roles.

MATERIALS AND METHODS

Initiation of Wharton's Jelly Matrix Cell Cultures

Umbilical cords were aseptically collected from porcine reproductive tracts obtained from a commercial abattoir at gestational day 45-60. Human umbilical cords were obtained from a local obstetrician from full-term Caesarian section births. Umbilical arteries and vein were removed, and the remaining tissue was transferred to a sterile container in Dulbecco's modified essential media (DMEM) (Invitrogen Life Sciences; Carlsbad, CA; <http://www.invitrogen.com>) with antibiotics (penicillin 100 μ g/ml, streptomycin 10 μ g/ml, and amphotericin B 250 μ g/ml; Invitrogen Life Sciences) and was diced into small fragments. The explants were transferred to 6-well plates containing the DMEM along with 20% fetal bovine serum (Invitrogen Life Sciences). They were left undisturbed for 5-7 days to allow migration of cells from the explants, at which point the media was replaced. They were re-fed and passaged as necessary.

Induction of Neural Cells from Wharton's Jelly Matrix Cells

Wharton's jelly cells were induced by the method of Woodbury *et al.* [29] to become neural stem cells and neuronal cells. Briefly, Wharton's jelly cells were preinduced by overnight treatment with bFGF (10 ng/ml) in DMEM and 20% fetal bovine serum. Neuronal differentiation was induced with 2% dimethylsulfoxide (DMSO) and 200 μ M butylated hydroxyanisole (BHA) in DMEM plus 2% fetal

bovine serum. After 5 hours, the medium was modified for long-term induction by adding 25 mM KCl, 2 mM valproic acid, 10 μ M forskolin, 1 μ M hydrocortisone, and 5 μ g/ml insulin. Matrix coated plates and tissue culture slides were obtained from BD Biosciences.

Immunocytochemistry

Immunocytochemistry was done by immunoperoxidase using a commercial kit (VectaStain; Vectur Laboratories; Burlingame, CA) or immunofluorescence staining. For immunofluorescence, cells were washed with phosphate buffered saline (PBS) and fixed by treating with methanol at -10°C . This was followed by washing with three changes of PBS and air drying. Slides were blocked with 10% normal blocking serum (derived from same species as the secondary antibody) in PBS for 20 minutes, washed with PBS, and incubated with primary antibody in 1.5% normal blocking serum in PBS for 60 minutes (0.1 to 2.0 μ g/ml). The slide was then washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology) for 15 minutes. Resulting immunoreactive cells were visualized by fluorescence microscopy. For immunoperoxidase, cells were fixed by treating with 10% buffered neutral formalin overnight. Slides were blocked with 5% normal blocking serum (derived from same species as the secondary antibody) in PBS for 30 minutes, followed by incubation with primary antibody for 60 minutes. The slide was incubated with horseradish-peroxidase-linked secondary antibody and developed according to kit instructions.

Preparation of Whole-Cell Lysates

Whole-cell lysates were made from Wharton's jelly cells by standard techniques using a lysis buffer consisting of PBS with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (1:500) (Sigma P8340; St. Louis, MO). Lysis buffer was added to the culture dish with Wharton's jelly cells after washing with ice-cold PBS three times. The culture dishes were scraped, and the lysate was aspirated into a syringe with a 21-gauge needle to shear DNA. The lysates were rocked at 4°C for 1 hour and centrifuged for 10 minutes at 10,000 *g* to remove insoluble material. Protein concentrations were determined by the Micro BCA assay (Pierce; Rockford, IL). Typically, a protein concentration of 1 μ g/ μ l was obtained by this protocol.

Immunoblotting

Solubilized proteins were separated by SDS-PAGE on 8%-16% continuous gradient gels under reducing conditions and transferred to nitrocellulose membranes by electrophoretic transfer in a tank system with plate electrodes. The membranes were blocked for 1 hour at room temperature

with 5% nonfat milk in Tris-buffered saline (TBS: 100 mM Tris, 0.9% NaCl, pH 7.5) containing 0.1% Tween 20. Membranes were incubated with primary antibody for 1 hour at room temperature followed by three washes with 0.1% Tween/TBS. Membranes were incubated for 1 hour at room temperature with the appropriate horseradish-peroxidase-conjugated secondary antibody (Pierce) diluted in 0.1% Tween/TBS at (1:50,000). After four additional washes with 0.1% Tween/TBS, the blots were visualized by chemiluminescence (Super Signal; Pierce) and recorded on radiographic film.

Antibodies

Antibodies were used at the following dilutions for immunoblotting and immunocytochemistry, respectively: neuron-specific enolase (NSE) (1:2,000, 1:500; Chemicon; Temecula, CA; <http://www.chemicon.com>); neurofilament M (NFM) (immunocytochemistry only 1:500; Chemicon); TuJ1 (1:2,000, 1:1,000; Covance; Princeton, NJ); glial fibrillary acidic protein (GFAP) (1:2,000, 1:500; Chemicon); 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase) (1:2,000, immunoblot only; Chemicon); smooth muscle actin (1:2,000, immunoblot only; Research Diagnostics; Flanders, NJ); c-kit (1:2,000, 1:200; Research Diagnostics); tyrosine hydroxylase (TH) (1:1,000, immunoblot only; East Acres Biologicals; Southbridge, MA); and GAP-43 (1:2,000, 1:200; Santa Cruz Biotechnology; Santa Cruz, CA).

Telomerase Assay

The TRAPEze[®] XL Telomerase Detection Kit (Intergen; Norcross, GA) was used according to the manufacturer's instructions to measure the telomerase enzyme activity of porcine Wharton's jelly cells. The TRAPEze[®] XL Telomerase Detection Kit uses a modified TRAP (telomerase repeat amplification protocol) assay to detect telomerase activity through the amplification of telomeric repeats using fluorescence energy transfer primers (Amplifluor[™]) that produce measurable fluorescence only when incorporated into TRAP products.

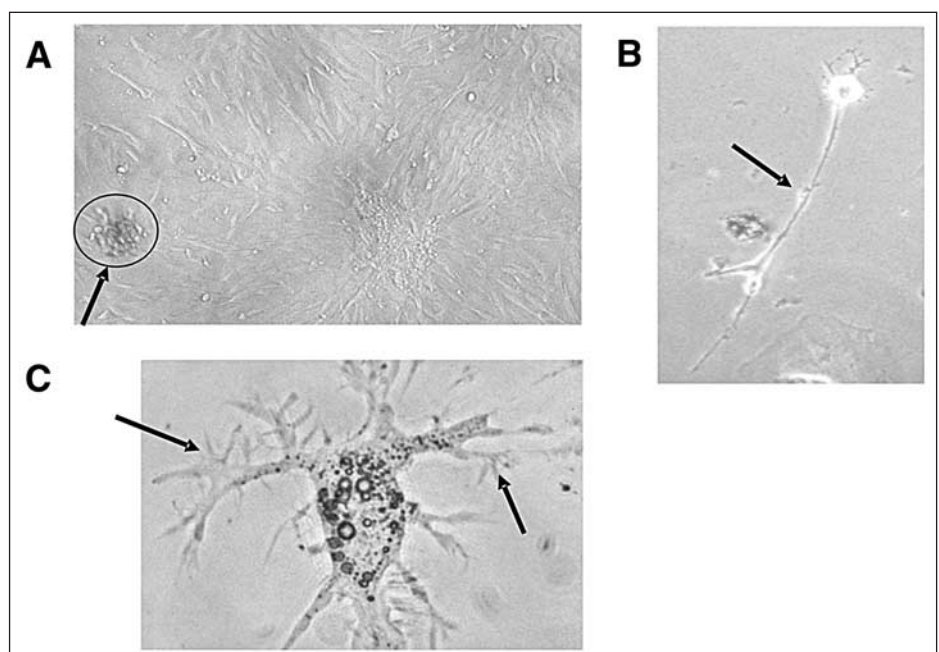
Porcine Wharton's jelly cells that were grown in culture for 45 passages were washed twice with PBS and then frozen at -80°C for 30 minutes. The cells were resuspended in 100 μ l of CHAPS XL Lysis Buffer (included in the telomerase detection kit) with 20 U ribonuclease inhibitor (Promega; Madison, WI; <http://www.promega.com>). This suspension was incubated on ice for 30 minutes. The extract was pelleted (12,000 *g* at 4°C), and the supernatant was frozen at -80°C until assayed for telomerase. Human carcinoma cells (included in the telomerase detection kit) were used as a positive control. For telomerase quantification, 50- μ l reactions were prepared containing 10 μ l of the 5X TRAPEze XL[®] Reaction Mix, 2 U of Taq Polymerase (Promega), 38 μ l of sterile polymerase chain reaction

(PCR) water, and 2 μ l of the sample cell extract. This mixture was then incubated at 30°C for 30 minutes to allow for the telomerase enzyme to synthesize telomeric repeats. PCR amplification of the telomeric repeats was performed on a Touchgene gradient thermocycler (Techne; Princeton, NJ) using a three-step PCR at 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute for 35 cycles, followed by a 55°C/25-minute extension step. Following a 5-minute incubation at 5°C, the fluorescence of each reaction was measured with a Fluoroskan Ascent FL fluorescent plate reader (Labsystems; Farnborough, UK). The telomerase activity of each sample was determined by calculating the ratio of the increase in fluorescein absorbance (produced by the amplification of telomeric repeats) divided by the increase in sulforhodamine absorbance.

RESULTS

We reasoned that umbilical cord matrix could be a rich source of multipotent stem cells based on the primitive cell types seen in Wharton's jelly. Wharton's jelly cells have a vesicular nucleus with multiple long processes reminiscent of mesenchymal cells. Wharton's jelly matrix has very little collagen, another indicator of the primitive state of this tissue. Moreover, during embryogenesis, totipotent cells, such as primordial germ cells, and multipotent cells, such as hematopoietic stem cells, migrate from the yolk sac through this region to populate target tissues in the embryo and fetus. Wharton's jelly cells were successfully isolated from porcine umbilical cord explants and expanded as primary cultures. The morphology of the heterogeneous population of Wharton's jelly cells isolated from explants included mesenchymal-like cells with a fusiform or stellate appearance and individual round cells (Fig. 1A). As Wharton's jelly cells reached confluency, colonies of round cells began to form, reminiscent of neurospheres.

Figure 1. Neuron-like morphology of Wharton's jelly cells after induction. A) Uninduced colonies of Wharton's jelly cells (arrow). B) Typical neuron-like cell with long axon-like process (arrow) 3 days after long-term induction. C) High magnification (40 \times) phase-contrast view of a long-term induced Wharton's jelly cell after 3 days. Note the multiple neurites with primary and secondary processes (arrows).



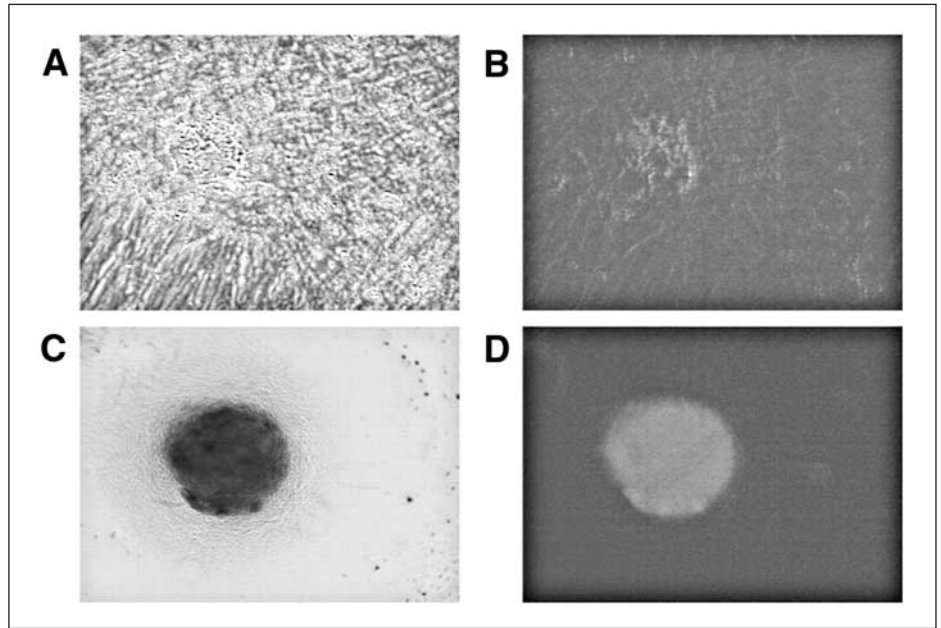
WHARTON'S JELLY CELLS DIFFERENTIATE INTO NEURAL CELLS

A number of postnatal mesenchymal stem cells have been shown to differentiate into neurons and glia. We wanted to determine if the primitive mesenchymal cells from Wharton's jelly had a similar potential. Wharton's jelly cells were grown to near confluency and treated with bFGF overnight and then low-serum media plus BHA and DMSO, a neural-inducing protocol described by *Woodbury et al.* [29]. This treatment caused Wharton's jelly cells to undergo profound changes in morphology (Fig. 1B, 1C), with some cells developing multiple neurites extending from the cell body. Single long axon-like processes developed (Fig. 1B), and granular structures reminiscent of Nissl substance were also observed in many of the Wharton's jelly cells (Fig. 1C).

Characteristics of Undifferentiated Wharton's Jelly Cells

Wharton's jelly cells were examined for expression of cell markers that have been identified in other postnatal mesenchymal stem cells. We tested for expression of c-kit, the stem cell factor receptor, which is expressed in bone marrow stromal cells and hematopoietic stem cells. c-kit expression was very high in Wharton's jelly colony-forming cells (Fig. 2) and in individual round undifferentiated cells that were plated on matrix-coated plates with a combination of poly-D-lysine (PDL) and laminin or laminin alone, even after neural induction (Fig. 3). The expression of c-kit by Wharton's jelly cells was greatly diminished after induction into neural cells, and expression was detected only in cells plated on laminin (Fig. 3C).

Figure 2. Wharton's jelly cells are positive for *c-kit*, the stem cell factor receptor. Untreated Wharton's jelly cells grown on PDL/laminin-coated culture plates were fixed in cold methanol and probed with rabbit polyclonal *c-kit*, followed by incubation with FITC-labeled donkey anti-rabbit secondary antibody. Wharton's jelly cells with newly forming colonies: A) brightfield and B) fluorescence. Colonies formed by untreated Wharton's jelly cells: C) brightfield and D) fluorescence.

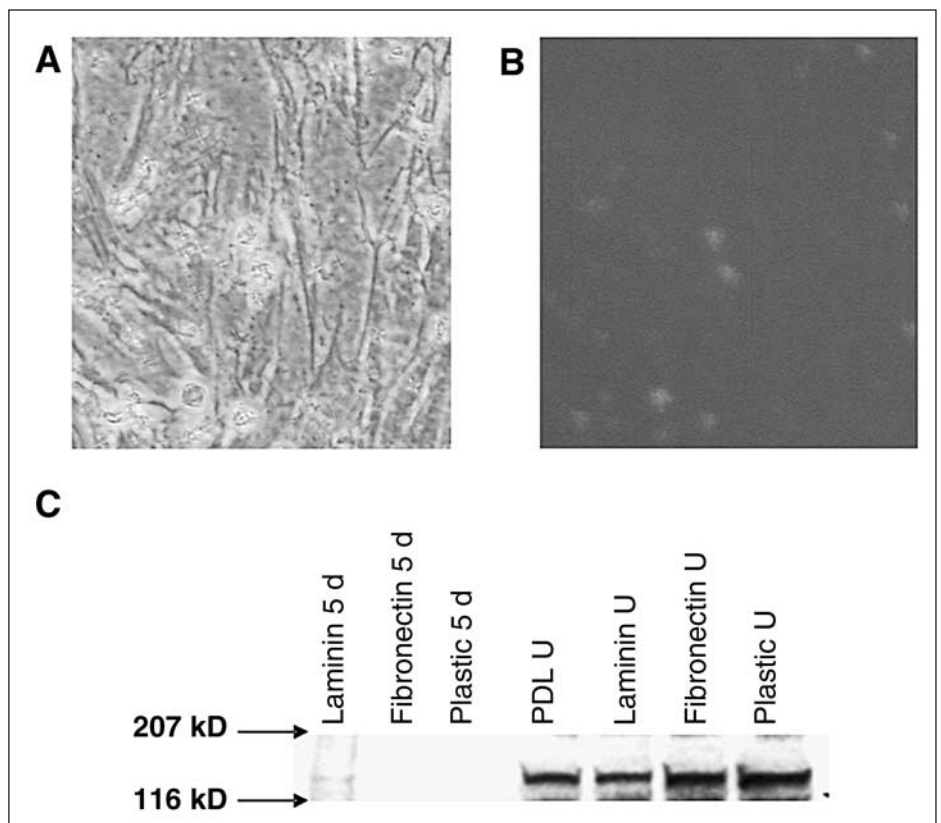


Porcine Wharton's jelly cells have been maintained in culture for more than 80 population doublings with no decrease in proliferative capacity. Telomerase activity is found in embryonic stem cells and may contribute to their proliferative capacity by maintaining telomere length. To determine if this characteristic of embryonic stem cells was shared by Wharton's jelly cells, we assayed telomerase activity using a fluorescence-based modified TRAP assay. An aliquot of each sample was heated to 85°C for 20 minutes to inactivate the telomerase enzyme and serve as a negative control. In order to assure that the measured telomerase activity was not affected by the presence of PCR inhibitors, a sample of 500 porcine Wharton's jelly cells was "spiked" with 50 positive-control cells. Wharton's jelly cells expressed telomerase activity that was about 10% of that expressed by a positive-control carcinoma cell line (Fig. 4). The telomerase activity was inactivated by heating, as expected. Minimal, if any, PCR inhibition was detected, as indicated by the greater telomerase activity measured in the sample that included Wharton's jelly cells and positive-control cells. The

sum of the telomerase activities measured for the two separately was approximately the same as the combined sample.

Wharton's jelly was previously shown to be composed of smooth-muscle-actin-positive, myofibroblast-like stromal cells [30]. To determine if Wharton's jelly cells, after being propagated in culture, maintained the phenotype described previously for Wharton's jelly stromal cells, we measured

Figure 3. *c-kit*-positive cells persist in porcine Wharton's jelly cells after induction. Micrographs 10 days after neural induction and grown on PDL/laminin-coated plates: brightfield (A) and anti-*c-kit* immunofluorescence (B). C) Western blot showing *c-kit* immunoreactive bands (145 kD) in cell lysates 5 days postinduction, plated on PDL/laminin. U = untreated.



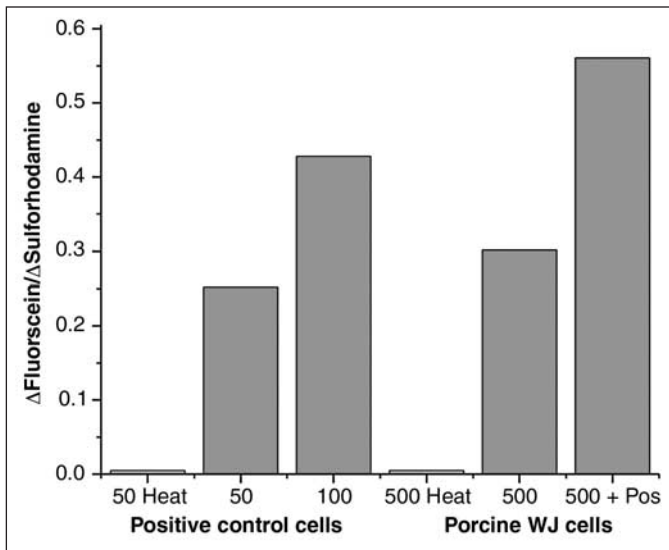


Figure 4. Telomerase activity of porcine Wharton's jelly cells. Telomerase activity (Δ fluorescein/ Δ sulforhodamine) from left to right: 50 positive-control cells heat-treated to inactivate the telomerase enzyme, 50 positive-control cells, 100 positive-control cells, 500 Wharton's jelly cells heat-treated to inactivate the telomerase enzyme, 500 Wharton's jelly cells, and 500 Wharton's jelly cells "spiked" with 50 positive-control cells, indicating the absence of PCR inhibitors in the 500-cell sample.

smooth muscle actin expression by immunoblotting (Fig. 5). Smooth muscle actin was expressed at similar levels in Wharton's jelly cells grown on PDL/laminin matrix and in Wharton's jelly cells grown on plastic. Thus, Wharton's jelly cells that were maintained in culture for extensive doublings continued to express this myofibroblast marker. Growth on matrix did not appear to select for a different population of cells. However, smooth muscle actin expression was much

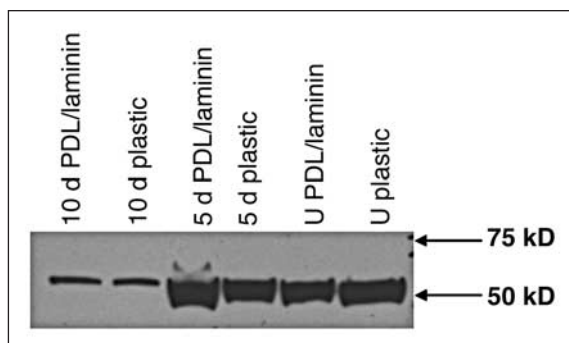


Figure 5. Smooth muscle actin, a marker for myofibroblasts, is expressed by Wharton's jelly cells. Whole-cell lysates of Wharton's jelly cells grown on plastic or PDL/laminin, which were either left untreated (U) or treated to induce neural differentiation for 5 and 10 days were resolved by SDS-PAGE on 8%-16% gradient gels and transferred to nitrocellulose. The blots were probed for the presence of smooth muscle actin. Smooth muscle actin expression was much lower by 10 days postinduction.

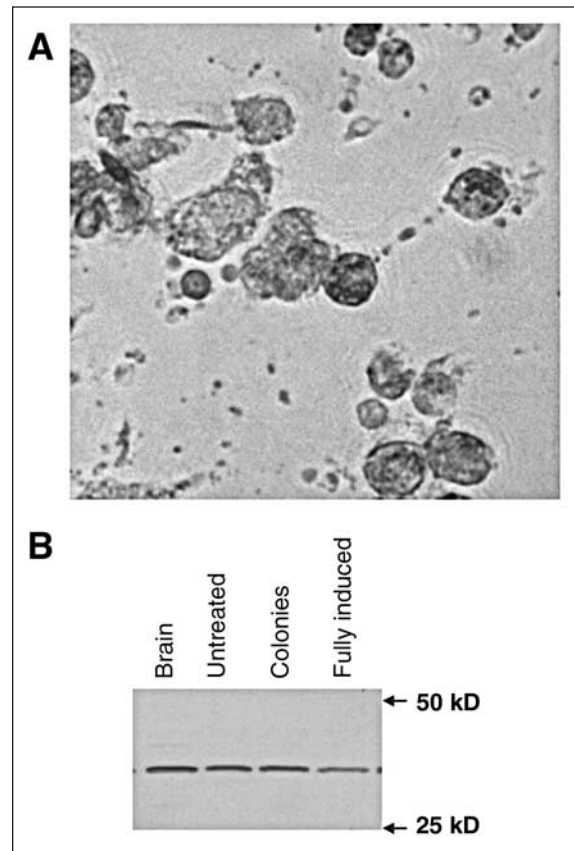


Figure 6. Wharton's jelly cells express NSE. A) Immunocytochemical detection of NSE expressed 1 hour after induction treatment. B) Whole-cell lysates of Wharton's jelly cells that were pre-confluent untreated, isolated colonies from confluent untreated, or fully induced for 5 hours were resolved by SDS-PAGE on 8%-16% gradient gels and transferred to nitrocellulose. The blots were probed for the presence of NSE (predicted mass 38 kDa). Rat brain was used as a positive control.

lower by 10 days after neural induction, suggesting that the phenotype of the majority of the induced Wharton's jelly cells changed to neural from myofibroblast.

Expression of Neural Stem Cell Markers

To determine if the Wharton's jelly cells expressed a marker for neural stem cells, NSE, immunocytochemistry was done within 1 hour after treatment with the inducing agents BHA and DMSO. The induced Wharton's jelly cells showed positive immunostaining for NSE at that time point, and were round and blast-like in appearance with a few neurites beginning to form (Fig. 6A). Immunoblots of whole-cell lysates were done to assess whether NSE was expressed in untreated Wharton's jelly cells or in the neurosphere-like colonies. NSE was expressed in both untreated Wharton's jelly cells and in colonies (Fig. 6B). However, there was slightly less NSE expression in Wharton's jelly cells 5 hours postinduction (Fig. 6B).

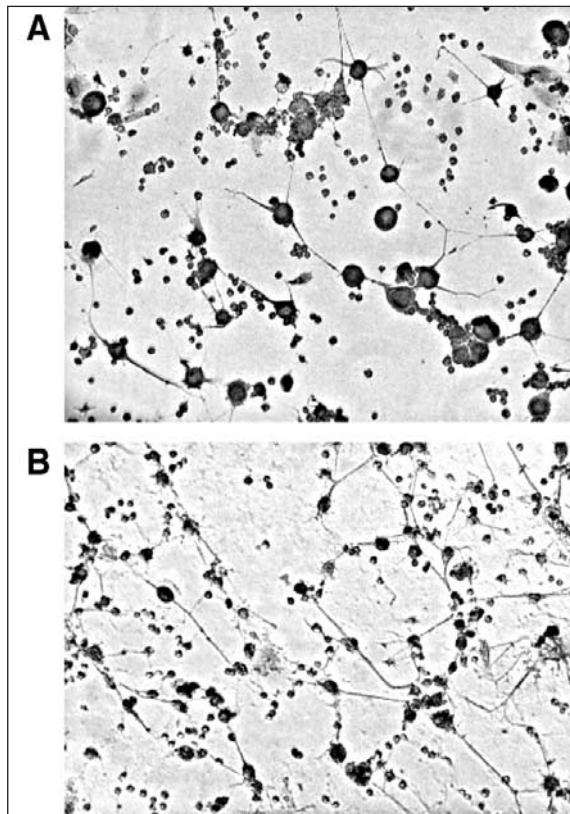


Figure 7. Wharton's jelly cells express NFM, a neuron-specific intermediate filament. NFM-positive cells 1 (A) and 3 (B) days after long-term induction. The induced Wharton's jelly cells showed an increasingly extensive network of processes on day 1 and day 3.

Expression of Mature Neuronal Proteins

Expression of mature neuronal markers was determined to assess the extent of differentiation of the Wharton's jelly cells after induction. NFM, a neuron-specific intermediate filament, was expressed at 1 (Fig. 7A) and 3 (Fig. 7B) days postinduction. Note the long processes that were revealed by NFM immunostaining, and the formation of networks that increased in complexity from day 1 to day 3 postinduction. TuJ1, a class III neuron-specific β -tubulin, is another marker for neuronal differentiation. The immunoblot in Figure 8A shows that increasing levels of TuJ1 were expressed during the course of differentiation from day 1 to day 10 postinduction. Interestingly, a low level of TuJ1 was expressed in Wharton's jelly cells treated only with bFGF overnight. Fully induced Wharton's jelly cells showed positive immunostaining for TuJ1 primarily in the soma and proximal part of the axon-like structure (Fig. 8B).

To determine whether the Wharton's jelly cells could become fully differentiated into a specific neuronal phenotype, we looked for expression of TH, a marker for catecholaminergic neurons (Fig. 8C). Immunoblot analysis showed that TH was expressed in neurosphere-like colonies

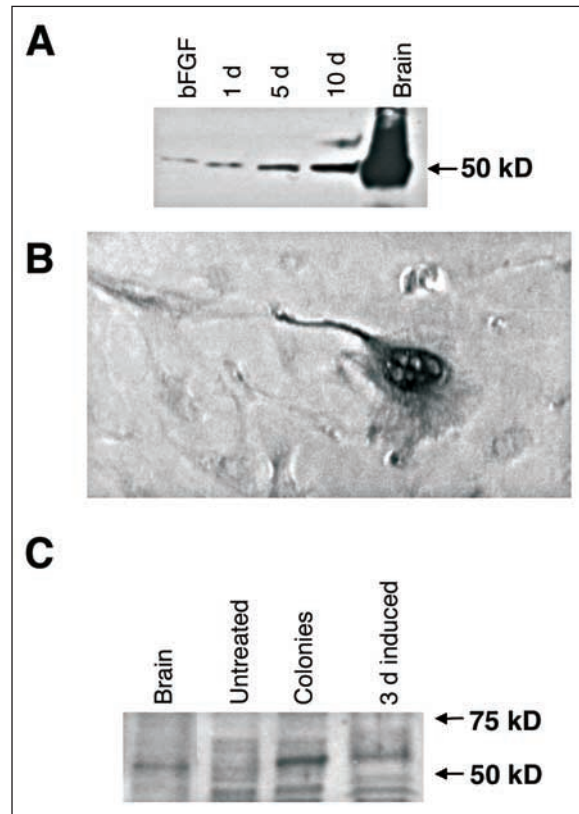


Figure 8. TuJ1 is expressed after full induction of Wharton's jelly cells. A) Immunoblot probed with TuJ1 of whole cell lysates from bFGF-treated (lane 1) and fully induced Wharton's jelly cells after 1 day (lane 2), 5 days (lane 3), and 10 days (lane 4). Rat brain tissue lysate was used as a positive control (lane 5). Predicted molecular mass 50 kDa. B) Immunocytochemistry for TuJ1 after full induction of Wharton's jelly cells. C) Immunoblot probed with TH, a marker for catecholaminergic neurons. Expected molecular mass 56 kDa. Rat brain (positive control, lane 1); untreated pre-confluent Wharton's jelly cells (lane 2); colonies of Wharton's jelly cells (lane 3); and Wharton's jelly cells 3 days postinduction (lane 4).

and in fully induced Wharton's jelly cells, but not in untreated cells. GAP-43, a neuron-specific microtubule-associated protein that localizes to axons, was also expressed in Wharton's jelly cells after they differentiated (Fig. 9). The long processes of the induced Wharton's jelly cells showed positive staining for GAP-43. Immunoblot analysis confirmed expression of GAP-43 10 days postinduction in cells grown on either plastic or PDL/laminin but not in untreated Wharton's jelly cells (Fig. 9C) although the level of expression was higher in the cells grown on PDL/laminin.

Expression of Glial Markers

To determine whether Wharton's jelly cells differentiated into glial cells, as was observed for induced bone marrow stromal cells [28, 29], expressions of GFAP and CNPase, astrocyte and oligodendrocyte markers, respectively, were

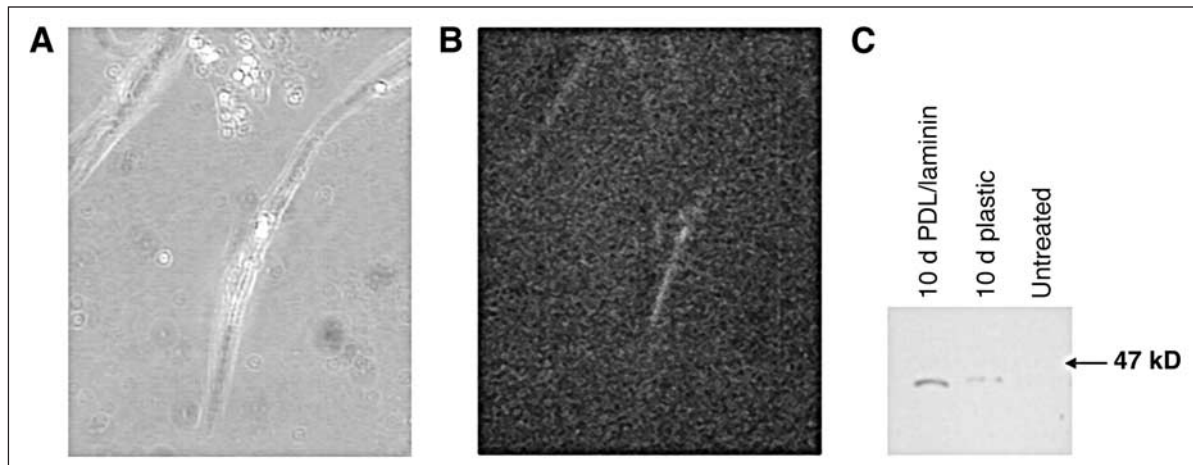


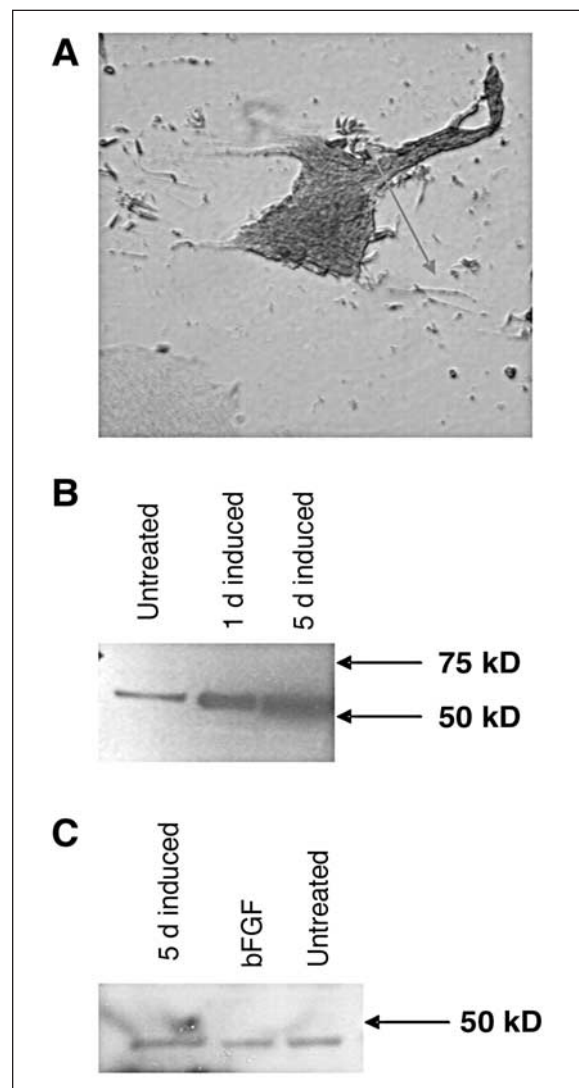
Figure 9. Wharton's jelly cells express axon-specific protein, GAP-43, after induction. Brightfield (A) and immunofluorescence (B) micrographs of Wharton's jelly cells grown on PDL/laminin for 10 days in long-term induction media. Cells were fixed and probed with anti-GAP43 and detected with FITC-conjugated secondary antibody. (C) Immunoblot probed with GAP-43 of lysates of WJ cells grown on plastic or PDL/laminin 10 days postinduction or untreated Wharton's jelly cells grown on plastic. Expected molecular mass 46 kDa.

determined (Fig. 10). GFAP-positive cells were identified in Wharton's jelly cultures after full induction. The morphology of the GFAP-positive cells was stellate and lacked the long processes of the cells that were positive for neuronal markers (Fig. 10A). GFAP expression was observed in untreated Wharton's jelly cells but was expressed at slightly higher levels after induction (Fig. 10B). In contrast, expression of CNPase, a marker for oligodendrocytes, was nearly identical in untreated, bFGF-treated, and fully induced Wharton's jelly cells (Fig. 10C).

Human Wharton's Jelly Cells Differentiate into Neurons

Cultures from human umbilical cord matrix recently have been established (Fig. 11). Initial studies to determine whether there were different populations of cells that arose from different regions of the umbilical cord indicated that the placental end may be a richer source of cells. Whether there are differences in the characteristics of the cells from the placental versus fetal end is currently under investigation. Like porcine Wharton's jelly cells, at least some human cells were positive for smooth muscle actin (data not shown).

Figure 10. Expression of astrocyte (GFAP) and oligodendrocyte (CNPase) markers by Wharton's jelly cells. A) Stellate morphology of anti-GFAP-reactive Wharton's jelly cells 3 days postinduction. B) Immunoblot probed for GFAP of whole-cell lysates of Wharton's jelly cells that were either untreated or treated with neural-inducing reagents 1 and 5 days postinduction. Expected molecular mass 50 kDa. C) Immunoblot probed for CNPase of whole-cell lysates of Wharton's jelly cells that were either untreated, treated with bFGF alone overnight, or 5 days after treatment with neural-inducing reagents. Expected molecular mass of 48 kDa.



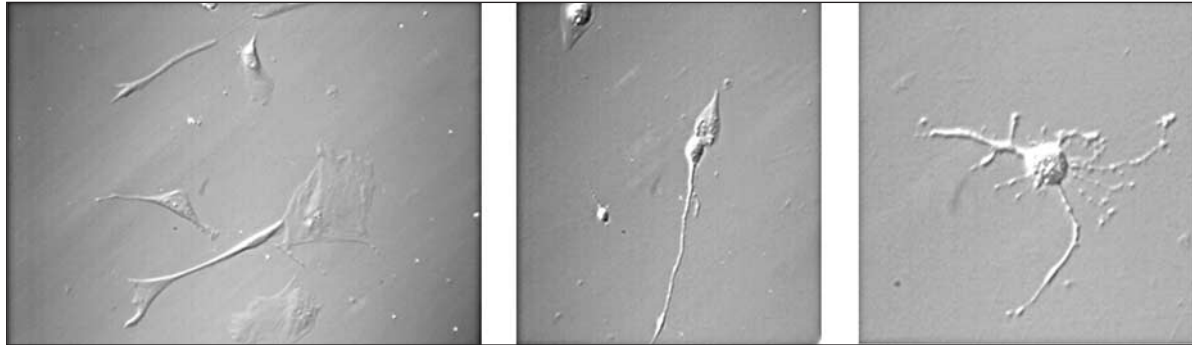


Figure 11. Human Wharton's jelly cells differentiate into neurons. (Left) Hoffman micrograph of untreated Wharton's jelly cells from passage 11, grown on plastic, showing both round and flattened stellate cells (100× magnification). (Center and right) Human Wharton's jelly cells after treatment with the induction protocol and culture in long-term induction media for 1.5 days have a neuron-like morphology with long processes (center, 100× magnification) and multiple neurites (right, 200× magnification). Immunocytochemical analysis showed that induced human Wharton's jelly cells were positive for TuJ1 and NFM (data not shown).

Human Wharton's jelly cells can be induced to form neurons by the method used for porcine Wharton's jelly cells. Figure 11 shows the changes observed in morphology of human Wharton's jelly cells after treatment with bFGF, BHA, and DMSO and after long-term induction media treatment for 1.5 days. Nearly 100% of the fully induced cells were positive for TuJ1 and NFM by immunocytochemistry. These studies are currently being extended to examine characteristics of longer-term cultures of induced human Wharton's jelly cells.

DISCUSSION

We isolated cells from Wharton's jelly, the gelatinous connective tissue from the umbilical cord. Our reasoning for looking at Wharton's jelly as a source of primitive cell types was based on the low levels of collagen expressed in gelatinous connective tissue and the fact that, during embryogenesis, totipotent cells, such as primordial germ cells and hematopoietic stem cells, migrate from the yolk sac through this region to populate target tissues in the embryo and fetus. Wharton's jelly cells have been cultured for more than 80 population doublings with no indications of senescence, changes in morphology, increased growth rate, or change in ability to differentiate into neurons. These observations suggest that the chromosomal makeup has not been altered in these cells, although karyotype analysis is needed to confirm this. Thus, Wharton's jelly cells possess one of the defining characteristics of stem cells, the ability to self-renew.

Wharton's jelly cells share characteristics with other types of stem cells as well. Importantly, Wharton's jelly cells have telomerase activity, which is found in human embryonic stem cells [11]. In addition, at least a subpopulation of Wharton's jelly cells cultured from umbilical cord

explants expressed the c-kit receptor. In this study, we showed that Wharton's jelly cells underwent changes in morphology and expressed neural-specific proteins when induced by an adaptation of the method of Woodbury *et al.* [29]. Therefore, cells from the gelatinous connective tissue of umbilical cord matrix may be an easily attainable source of multipotent stem cells that can be expanded in vitro, maintained in culture, and induced to differentiate into neural cells.

We found that NSE, a marker for neural stem cells, was expressed at nearly equal levels in treated and untreated Wharton's jelly cells. While this result was somewhat surprising, it is consistent with results from bone marrow stromal cells, a source of adult mesenchymal stem cells [28, 29]. Woodbury *et al.* [29] also found that NSE was expressed in untreated bone marrow stromal cells, and that expression of NSE in those cells was increased by the induction protocol we used in the present study. In other work with bone marrow stromal cells, Sanchez-Ramos *et al.* [28] found that NeuN and GFAP, markers for early mature neurons and astrocytes, respectively, were expressed at similar levels in uninduced and bone marrow stromal cells induced to form neural cells by retinoic acid. Likewise, we found that the glial cell markers, GFAP and CNPase, were expressed at equivalent levels in treated and untreated Wharton's jelly cells. Our results, along with the bone marrow stromal studies, suggest that mesenchymal stem cells express a number of neural proteins spontaneously and, perhaps, are primed to differentiate along a neural program.

Colonies of Wharton's jelly cells also expressed NSE, c-kit, and even more intriguing, TH, a marker for catecholaminergic neurons. Therefore, we suggest that the colonies that arose spontaneously after Wharton's jelly cells

grew past confluency were neurosphere-like masses of cells. Further work is being done to characterize the types of cells that exist in these colonies and to explore whether they are neurospheres with a variety of different neural cell types present. *c-kit* is expressed in melanocyte precursors from neural crest [31], so expression of *c-kit* by colonies does not preclude their being neurospheres. Whether there is expression by the colonies of markers for non-neuronal cell lineages has yet to be determined.

Recently, other sources of postnatal stem cells have been identified that are capable of multipotential differentiation. Multipotent stem cells isolated from the dermis of mammalian skin can be expanded in culture, and differentiated into neurons and glia [26]. Astrocytes have also been isolated, expanded in culture and shown to form neurospheres composed of neurons, oligodendrocytes, and astrocytes [32]. Are these postnatal stem cells related? Wharton's jelly was previously shown to be composed of myofibroblast-like stromal cells [30]. Our findings confirm this based on smooth muscle actin expression by the Wharton's jelly cells. Myofibroblasts are important cells in growth, development, and repair found throughout the body and include bone marrow stromal cells, astrocytes, and pericytes [8]. This suggests that myofibroblasts may be a source of postnatal stem cells and/or they may be involved in secretion of cytokines and growth factors involved in proliferation and differentiation of closely associated stem cells. Importantly, the mixed population of cells in Wharton's jelly may provide both stem cells and feeder cells, thus explaining why LIF is not required, as is the case for maintaining mouse embryonic stem cells in an undifferentiated state [9-11].

Work is under way to characterize the cell types present in Wharton's jelly and to determine whether clonal lines of Wharton's jelly cells can be established that maintain their capacity to proliferate and to differentiate into neural cells as well as other tissue types. Importantly, this will also establish whether neurons and glial cells can arise from a single clonal cell or whether they arise from different progenitor cells potentially found in the mixed population of cells derived from Wharton's jelly. Also yet to be addressed is whether the telomerase activity of Wharton's jelly cells results in tumorigenicity, as is the case for embryonic stem cells. Preliminary studies are under way to assess this potential by injecting Wharton's jelly cells into nude mice. Thus far, there is no evidence of tumor formation by Wharton's jelly cells injected into brain after 6 weeks (Weiss, submitted).

The potential for exploiting the capacity of stem cells to differentiate into mature neural cells holds much promise for treating a number of devastating neurological diseases.

This premise is based upon a number of preliminary studies that use either neural cells derived from embryonic stem cells or neural stem cells. Embryonic-stem-cell-derived neurons that were transplanted into the injured adult striatum were found to differentiate into both dopaminergic and serotonergic neurons [33]. *Studer et al.* [34] reported similar results with neural stem cells after they were expanded in vitro. The neural stem cells were transplanted into the brain of a Parkinsonian rat, and they subsequently differentiated into dopaminergic neurons, resulting in alleviation of behavioral deficits. Indeed, neural stem cells appear to show tropism for pathology. In a study by *Aboody et al.* [35], neural stem cells targeted intracranial gliomas of adult brains when either implanted at areas distant from the tumor or applied intravascularly. Neural stem cells have also been shown to supply myelin basic protein to the shiverer mouse, which lacks this protein, when intracerebroventricular implantations of neural stem cells were done [36].

Although these initial studies are promising, there are drawbacks with the use of both embryonic stem cells and neural stem cells. The use of human embryonic stem cells is controversial and will likely remain so. Human neural stem cells are not readily accessible and grow slowly in culture [37]. These limitations emphasize the importance of identifying alternative sources of stem cells for treatment of neurological disorders. Based on the ability of Wharton's jelly cells to differentiate into neural cells in vitro, as described here, and the ability of Wharton's jelly cells to differentiate into neurons in vivo (Weiss *et al.*, submitted), Wharton's jelly cells may be an easily attainable and non-controversial source of stem cells for the treatment of neurological diseases.

In summary, we show here that cells from Wharton's jelly are a rich source of primitive cells that are readily expanded in culture and that can be induced to form neurons and glia. Clonal populations of human umbilical cord matrix cells are being established. The clonal cells will be characterized in terms of proliferative capacity, karyotype analysis, and expression of HLA antigens as well as their ability to differentiate into ectodermal, mesodermal, and endodermal lineages.

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