Effect of Umbilical Cord Matrix Stem Cells on Parkinson's Disease Model Rats

By

Satish Medicetty

B.V. Sc & A.H., A. N. G. R. Agricultural University, India, 2001

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
College of Veterinary Medicine

Kansas State University
Manhattan, Kansas

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Abstract

Umbilical cord matrix or Wharton's Jelly is a mucous connective tissue ensheathing the cord blood vessels and contains mesenchymal-like stem cells. Previously, we have shown that pig umbilical cord matrix stem (pUCMS) cells transplanted into normal rat brain were recovered up to 6 weeks post-transplantation, where a sub-population of pUCMS cells exhibited neuronal morphology and expressed a variety of neuronal markers. Here, approximately 150 pUCMS cells were transplanted into non-immunesuppressed rats that previously received a brain lesion by neurotoxin, 6-hydroxydopamine (6-OHDA), which specifically affects midbrain dopaminergic neurons, leading to pathologic findings similar to that of Parkinson's disease (PD). The pUCMS cells proliferated up to 8 weeks post-transplantation and there was a significant increase in the percentage and number of pUCMS cells expressing tyrosine hydroxylase (TH), which is a marker for dopaminergic cells. We conclude that 1. Xenotransplants of pig UCMS cells are not rejected by rats at least up to 8 weeks after transplantation and 2. The pig UCMS cells proliferate and differentiate after transplantation into PD model rats.

The surface antigen and gene expression profile of human umbilical cord matrix stem (hUCMS) cells resemble that of mesenchymal stem cells. Apomorphine-induced rotatory behavior was used to analyze the motor deficits of the PD model rats. In different experiments 1000, 2500 and 25000 hUCMS cells were transplanted into the brain of non-immunesuppressed PD model rats. There was

a dose-dependent decrease in apomorphine-induced rotations; the maximum benefit was found in the rats that received 1000 hUCMS cells. The graft cells were recovered at 2 days and 1 week, but not at 6, 10 or 12 weeks post-transplantation. Quantitative assessment of host TH-positive midbrain dopaminergic neurons revealed a positive correlation between the behavioral improvement and TH-positive cell number in the low-density (1000 cells) transplant group, showing that the hUCMS cells may play a role in rescuing damaged host dopaminergic neurons and promote improvement of motor deficits in PD-model rats. In summary, hUCMS cells appear to be mesenchymal stem cells that can be harvested in great numbers from a non-controversial, inexhaustible source. Human UCMS cells show therapeutic benefit in PD model rats, but the mechanism by which they promote improvement is presently unknown.

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Satish Medicetty

DEDICATION

This dissertation is dedicated to my family, all my teachers, and to all of my friends who believed in me, and provided love and support with out which I would have not been able to complete this degree**.**

PART I

BACKGROUND AND SIGNIFICANCE

PARKINSONS DISEASE

Parkinsons disease (PD) is a chronic, progressive neurodegenerative disease of the central nervous system that belongs to a group of conditions called as movement disorders. PD was first described in 1817 by Dr. James Parkinson, a London physician, who described the disease as 'Shaking Palsy'. The degeneration of neurons in this disease is largely limited to dopaminergic (DA) neurons in substantia nigra (SN) and ventral tegmental area (VTA) of the midbrain, and their projections to the forebrain, leading to the deficiency of neurotransmitter, dopamine (see figure 1.1). Dr. Arvid Carlsson, who was a leader in the development of these ideas, has won a Nobel prize for his valuable contributions. The primary symptoms of PD are motor symptoms: muscle rigidity – stiffness of muscles, resting tremor – trembling at rest, and bradykinesia – slowness of movement (Lang and Lozano, 1998b;Lang and Lozano, 1998a). Figure 1.2A shows the normal circuitry that involves the midbrain DA neurons and their projections to the forebrain, and figure 1.2B indicates the hypokinetic changes in the circuitry that lead to the motor symptoms in PD. As the disease is progressive, the symptoms become worse with time involving multiple neurotransmitter systems (Beal, 2001). Although there are exceptions of early onset, the symptoms in PD generally appear late, in the fifth or sixth decade of life. An estimated 1.5 million Americans suffer from PD, with an estimated 60, 000 new cases diagnosed each year.

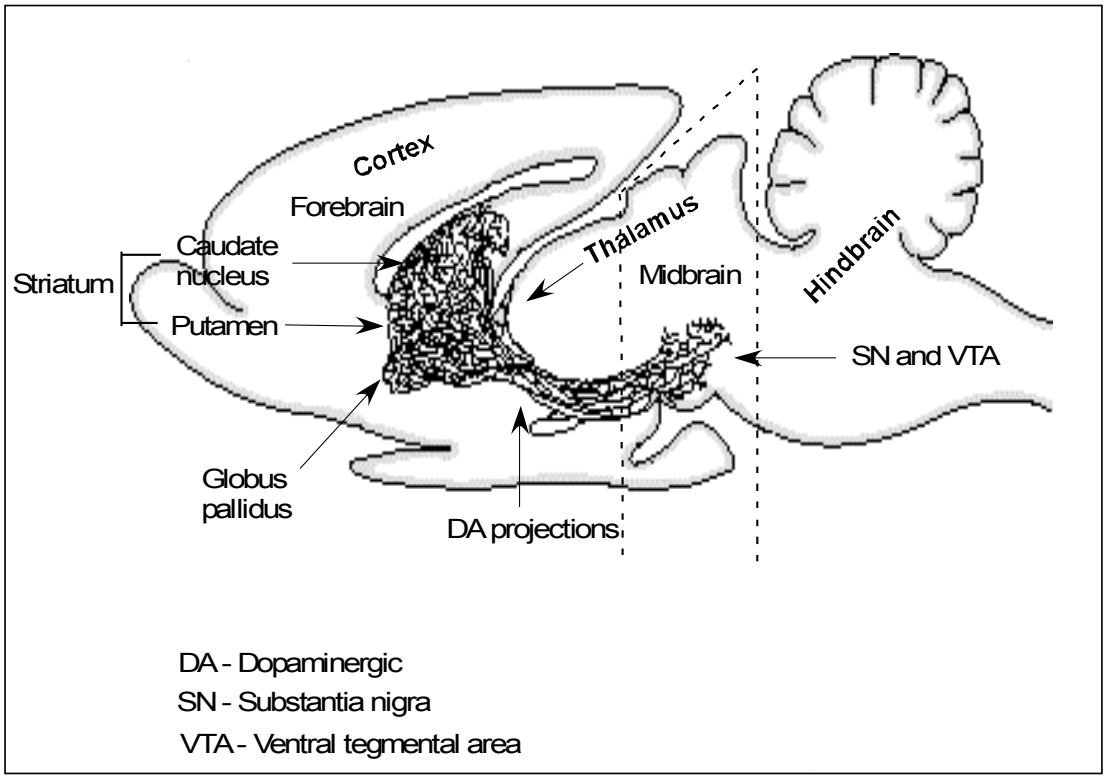

Figure 1.1 Illustration of nigrostriatal tract in parasagittal section of a rat brain: Midbrain dopaminergic (DA) neurons in substantia nigra (SN) and ventral tegmental area (VTA) and their axonal projections to striatum (caudate nucleus and putamen), globus pallidus, and thalamus, constitute the nigrostriatal pathway, which is mainly affected in Parkinson's disease.

Approximately 5 years before the development of the symptoms, the levels of dopamine in SN are significantly reduced suggesting that the symptoms develop only after a threshold is reached. The major neuropathologic findings in PD are loss of DA neurons in ventral midbrain and presence of Lewy bodies (mis-folded protein aggregates). The loss of DA neurons is also seen in normal individuals, where there is a steady decrease of about 4% per decade beginning at about the age of 20. Based on the etiology, there are two forms of PD; sporadic and familial (see figure 1.3). The normal age-dependent loss as well as death of DA neurons in both forms of PD is thought to be due to oxidative stress (Beal, 2003b;Dawson and Dawson, 2003;Zhang et al., 2000). In PD, there is a clear evidence of oxidative stress in the following situations: glutathione depletion – one of the primary antioxidants, iron deposits – impairs the ability of the cells to detoxify hydrogen peroxide, lipid peroxidation, oxidative damage to DNA (mitochondria), protein nitration, and decreased mitochondrial function (Dawson and Dawson, 2003;Zhang et al., 2000;Beal, 2003b). This may be due to an increase in the production of free radicals as well as decreased detoxification. As the level of MAO-A (mono amino oxidase – A) is high in DA neurons, they produce high amount of hydrogen peroxide, and thus any compromise in the oxidative state will lead to toxicity.

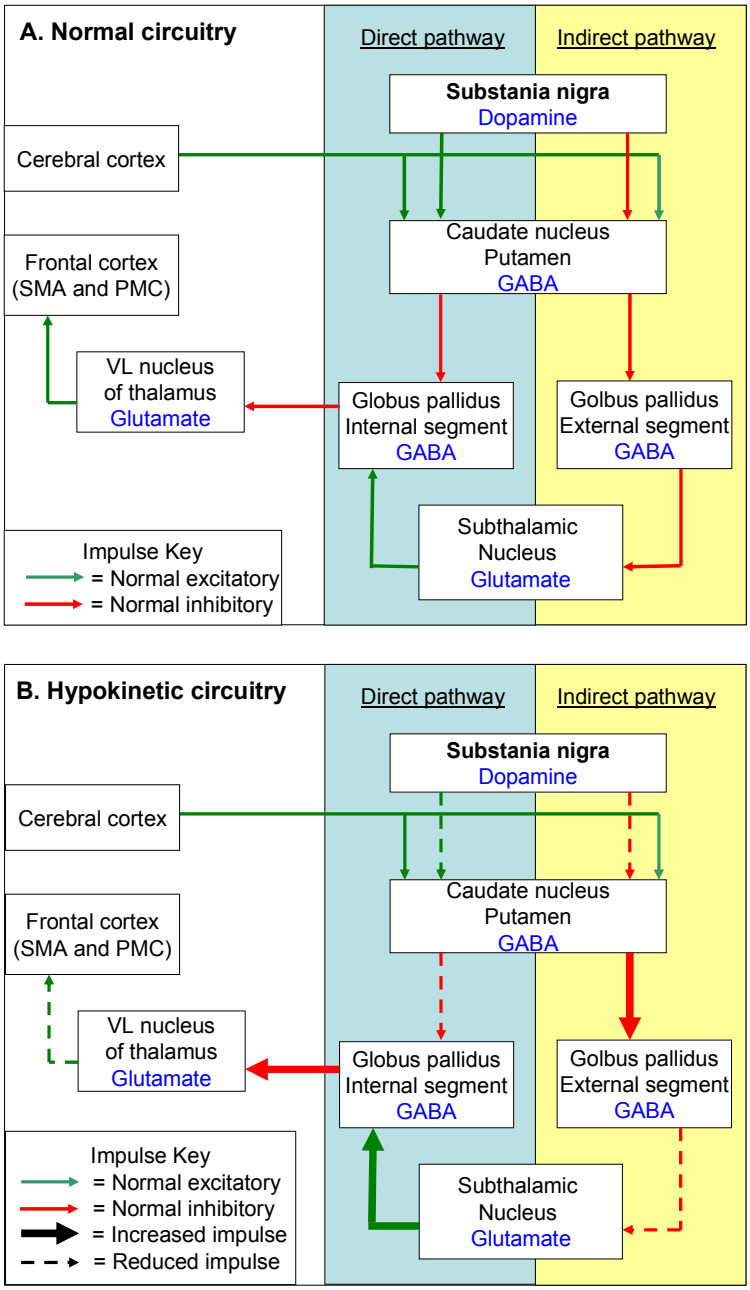

Figure 1.2 Normal and Hypokinetic circuitry in Parkinson's disease

A. Normal circuitry (connections) in the corto-basal ganglia loop.

B. Abnormal hypokinetic motor loop in Parkinson's disease.

GABA: γ-aminobutyric acid, SMA: smooth muscle afferents, PMC: peripheral muscle contractions. Neurotransmitters are indicated in blue.

A large number of PD cases are identified as sporadic form, with environmental causes being implicated, but additional genetic involvement has been identified as a predisposing factor (Dawson and Dawson, 2003;Zhang et al., 2000). An example of an environmental cause, which has been well documented, is the toxicity of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyrodine) – a synthetic heroin analog. MPTP induces irreversible and severe motor abnormalities that are similar to that of PD (Beal, 2001). A neurotoxin, 6-hydroxydopamine (6-OHDA) selectively accumulates in DA neurons, and it then kills these neurons primarily owing to oxidative stress as described above (Beal, 2003b;Beal, 2001;Kim et al., 2002). Selectivity of DA neurons appears to be due to high affinity of these toxins for dopamine transporters, and once inside the neuron they act by inhibiting the mitochondrial complex I, resulting in energy failure and increased formation of superoxide anion (Beal, 2004;Beal, 2003a). Most of the toxicity due to superoxide anion is believed to be due to its reaction with other reactive oxygen species such as nitric oxide (NO), resulting in a potent oxidant peroxynitrate, which has been implicated in many neurologic diseases. It has been shown that transgenic mice which over express superoxide dismutase (SOD-1) were more resistant to toxin-induced DA neurotoxicity (Przedborski et al., 1992).

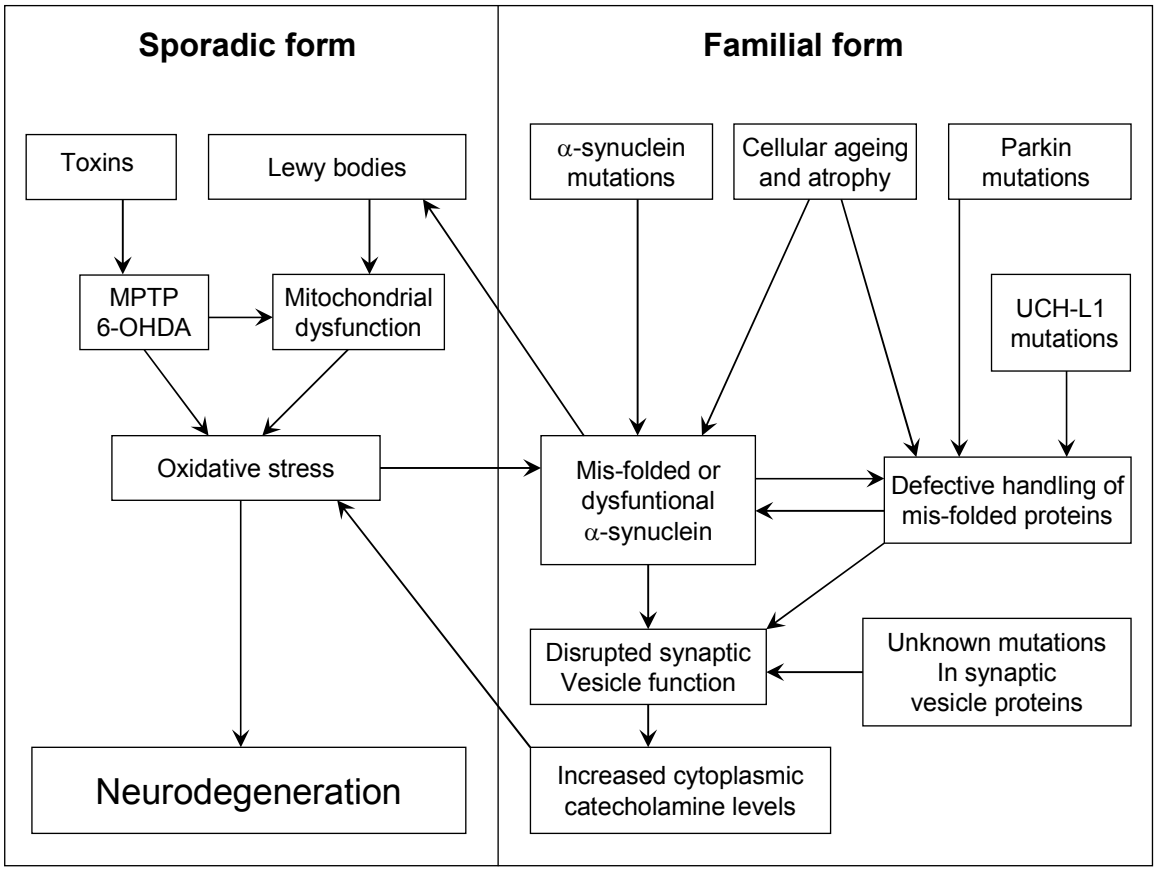

Figure 1.3 Pathogenesis of Parkinson's disease (modified from *Lotharius, 2002*)

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrodine

6-OHDA: 6-hydroxydopamine.

Although different etiological factors lead to sporadic and familial forms of Parkinson's disease, all the different pathways may result in oxidative stress and followed by neurodegeneration.

In 1997 the gene for one form of inherited PD was identified and the protein was identified as α-synuclein, which is located on chromosome 4 and belongs to a family of structurally related proteins that are prominently expressed in the CNS. Mutations in α-synuclein (A53T and A30P) cause autosomal dominant familial PD (Lotharius and Brundin, 2002b;Lotharius and Brundin, 2002a). After the identification of α-synuclein gene, the protein was identified as a component of Lewy bodies. It has also been shown that the α-synuclein in the Lewy bodies is nitrated, which is a clear indication that the cells are experiencing oxidative stress. A second gene for PD, Parkin, was identified in 1998 and is located on chromosome 6, and causes autosomal recessive juvenile parkinsonism (AR-JP) (Dawson and Dawson, 2003;Lotharius and Brundin, 2002b). Mutations in other gene, UCH-L1, which affect the ubiquitin proteosome pathway that helps in degradation of protein aggregates, have been reported to cause autosomal dominant PD (Dawson and Dawson, 2003). The interaction of these three genes and their role in PD is illustrated in figure 1.3.

Symptomatic treatments such as, drugs that replace dopamine and specific surgeries (Obeso et al., 2000), are currently used to reverse the symptoms of PD, but a therapeutic agent to prevent or cure PD is yet to be discovered. The current (symptomatic) and future therapeutic strategies for PD are listed in figure 1.4. The future therapeutic interventions in PD primarily include the replacement and rescue strategies (fig 1.4), which shows that stem cells can be used to replace or rescue the damaged DA neurons in PD.

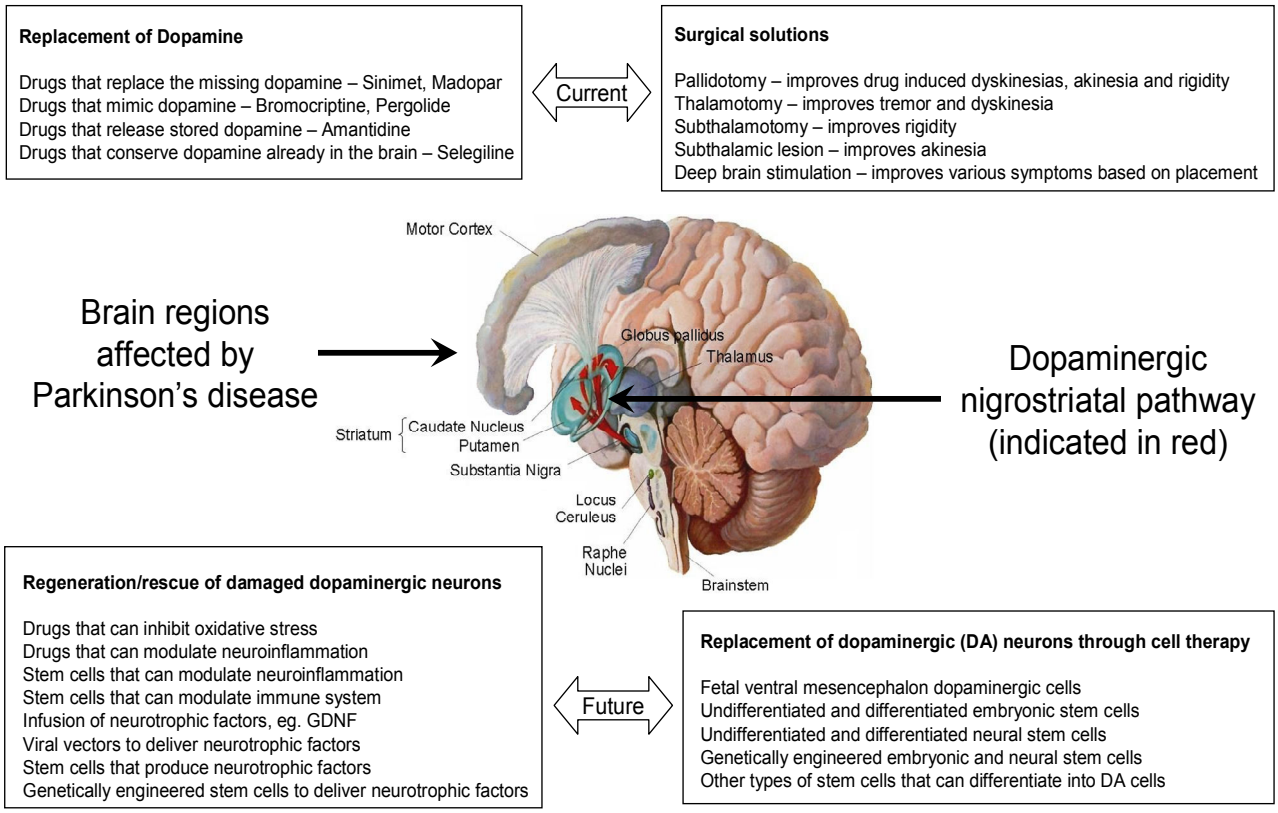

Figure 1.4 Current (symptomatic) and future therapeutic strategies for Parkinson's disease.

The current (symptomatic) therapy for Parkinson's disease (PD) is mainly targeted to replace the dopamine or surgically manipulate/stimulate specific brain areas to modulate the motor deficits of PD. The future therapeutic strategies including the stem cell therapy is targeted to either replace or rescue damaged dopaminergic neurons.

STEM CELLS

Stem cells are defined as a population of primitive undifferentiated cells, which are capable of self-renewal and differentiate into multiple cell or tissue types. Figure 1.5 illustrates various sources of human stem cells. Embryonic stem cells (ESCs), which are derived from the inner cell mass of the blastocyst (Evans and Kaufman, 1981), are pluripotent cells, meaning that they can give rise to cell-types of all the three germlayers (ectoderm, endoderm and mesoderm) in the embryo (Nagy et al., 1990;Nagy et al., 1993). Although there are additional markers, the markers listed in table 1.1 are commonly used to identify pluripotent stem cells. Among the markers listed in table 1.1, Oct-4, Sox-2 and Nanog are often considered as standard markers for undifferentiated pluripotent stem cells. However, it has been shown that stem cells that do not express all the pluripotency markers may still have the potential to give rise to cells/tissues of all the three germ layers, thus proving their pluripotency (Jiang et al., 2002a). ESCs that can generate terminally differentiated neuronal and glial cells could provide an unlimited source of these cells, which can be used for replacement therapy in various neurodegenerative diseases. Embryonic germ cells (EGCs) are pluripotent stem cells that can be harvested from a developing embryo (See figure 1.5), and were shown to have the potential of differentiating into neurons *in vitro* (Pan et al., 2005;Park et al., 2004;Turnpenny et al., 2005). Embryonic carcinoma (EC) cells, derived from tumors called teratocarcinomas, are pluripotent stem cells that are known to be as malignant counterparts of ESCs (Przyborski et al., 2004). Teratocarcinomas are thought to originate from

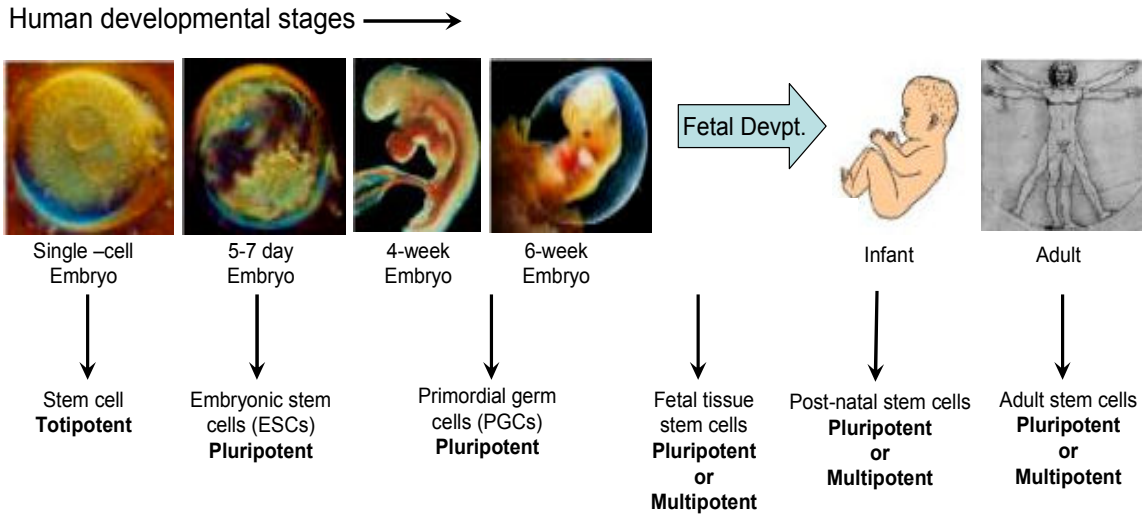

Figure 1.5 Sources of human stem cells

Totipotent: potential to form any cell/tissue type in the embryo including extraembryonic membranes.

Pluripotent: potential to form any cell/tissue type in the embryo, but not extraembryonic membranes.

Multipotent: potential to form cell/tissue type of more than one germ layer, but not all.

transformed germ cells and are observed in both young and adult individuals. Human EC cells were suggested to be useful as a model to study human development (Przyborski et al., 2004). Previously, it has been shown that EC cells are capable of differentiating into neurons *in vitro* (Pleasure and Lee, 1993).

Although they are described as fetal stem cells, neural stem cells (NSCs) are immature, uncommitted cells that exist in both the developing and adult brain (Morrison et al., 1997;Palmer et al., 1997;Armstrong and Svendsen, 2000;Svendsen and Caldwell, 2000). NSCs can be expanded *in vitro* and differentiated into neuronal and glial cells *in vitro* (Reynolds and Weiss, 1992;Vescovi et al., 1993) and *in vivo* (Armstrong et al., 2000;Ostenfeld et al., 2000). However, adult NSCs in the central nervous system require an invasive procedure to obtain, and have a limited potential of expansion *in vitro*. Different types of neuronal and glial cells that can be obtained from NSCs can be useful for replacement therapy in various neural diseases (Armstrong and Svendsen, 2000). Similar to NSCs, hematopoietic stem cells (HSCs) can be extracted from fetal and adult tissues. Although bonemarrow is the classic source, umbilical cord blood is a rich source of HSCs. Transplantation of HSCs is shown to be effective in treating many metabolic diseases and malignancies (Consolini et al., 2001;Mayhall et al., 2004;Wright-Kanuth and Smith, 2001). In addition, it has been shown that HSCs may play a role in repairing the neural tissues in traumatic and ischemic brain damage and neurodegenerative diseases (Nan et al., 2005;Newman et al., 2004;Sanchez-Ramos et al., 2000).

Marker	Significance
Oct-4	Transcription factor essential for establishment and maintenance of undifferentiated PSCs
Sox-2	Transcription factor identified in PSCs and developing nervous system
Nanog	Transcription factor essential for maintenance of undifferentiated PSCs
SSEA-1	Glycoprotein specifically expressed in early embryonic development and by undifferentiated PSCs (specific to mouse)
SSEA-3	Glycoprotein specifically expressed in early embryonic development and by undifferentiated PSCs (specific to human)
SSEA-4	Glycoprotein specifically expressed in early embryonic development and by undifferentiated PSCs (specific to human)
Telomerase	Enzyme uniquely associated with immortal cell lines or proliferating cells
Alkaline phosphatase	Elevated expression of this enzyme is observed in undifferentiated PSCs
ABCG2 (BCRP)	Multidrug resistance transporter found in PSCs and cancer cells.
TRA-1-60	Extra-cellular molecule synthesized by undifferentiated PSCs
TRA-1-81	Extra-cellular molecule synthesized by undifferentiated PSCs

Table 1.1 Typical markers to identify undifferentiated pluripotent stem cells (PSCs)

Bone marrow stromal cells (BMSCs), which are a type of mesenchymal stem cells, are harvested from fetal or post-natal or adult sources. Previous studies have indicated that BMSCs can be expanded in culture to a limited extent and give rise to multiple cell-types of mesodermal origin (Pittenger et al., 1999;Prockop et al., 2001). BMSCs were shown to be capable of differentiating into neural cells *in vitro* (Sanchez-Ramos et al., 2000;Woodbury et al., 2000), and *in vivo* following transplantation into rodents (Kopen et al., 1999). Another type of adult stem cell that raised public interest is the multipotent adult progenitor cell (MAPC), which is isolated from bone marrow (Reyes and Verfaillie, 2001). Recent studies with MAPCs have shown that they are pluripotent and can be extracted from muscle and brain in addition to bone marrow, suggesting that MAPCs may be involved in plasticity (capability of regeneration) of various adult tissues (Jiang et al., 2002b;Jiang et al., 2002a).

Whether the post-natal and adult stem cells are undifferentiated stem cells that can differentiate into other cell types, or they are differentiated cells that have the capability to form other cell types, has been an interesting debate among various research groups. Although it is contentious, the differentiation capability of post-natal and adult stem cells is attributed to various mechanisms, such as transdifferentiation, dedifferentiation and cell-fusion (Shen et al., 2003;Tosh and Slack, 2002). Transdifferentiation is described as conversion of one differentiated cell type into other, for example, BMSCs were shown to differentiate into a neural cells (Woodbury et al., 2000). Dedifferentiation is

progression of cells from a more differentiated to less differentiated or progenitor state, for example, during regeneration of the lens fibers in the eye, pigment epithelial cells were shown to dedifferentiate into intermediate progenitor cells before differentiating into lens fibers (Burke and Tosh, 2005). Cell-fusion is a phenomenon where donor stem cells fuse with the resident cells and differentiate or attain the phenotype of the recipient cells, for example, bone marrow cells have been shown to fuse spontaneously with ESCs *in vitro* to attain the phenotype of the recipient cells (Terada et al., 2002). However, dedifferentiation and cell-fusion are sometimes considered as a part of transdifferentiation.

Stem cells, with their unique ability of self-renewal and differentiation, may have a great potential to be used as therapeutic agents in various debilitating diseases including neurological diseases. For example, ESCs, which are immortal (have the capability of indefinite self-renewal) and pluripotent (Dinsmore et al., 1996;Lee et al., 2000;Thomson et al., 1998a;Thomson et al., 1998b) have been shown to be beneficial in various disease models (Auerbach et al., 2000;Johkura et al., 2003;McDonald et al., 1999). However, each of the well documented stem cell types has its own limitations: the moral and ethical concerns of harvesting and using ESCs, difficulty in maintaining ESCs in culture and problems associated with their transplantation, difficulty in maintenance and expansion of neural stem cells, difficulty in expansion and limited use of HSCs for transplantation into a heterogeneous tissue or a mismatched individual. Although some EC cells were shown to be phenotypically similar to ESCs and

exhibit pluripotency, their basic malignant nature, altered genotype (Duran et al., 2001) and inconsistent differentiation potential are major limitations for these cells to be used for research and clinical purposes. The various limitations associated with the established stem cell types emphasize the importance of exploring and evaluating other sources of stem cells. For example, adult stem cells from bone marrow have been shown to be multipotent or pluripotent (Jiang et al., 2002b;Jiang et al., 2002a;Schwartz et al., 2002). Other sources of post-natal and adult stem cells, such as skin, liver, muscle, intestine, adipose tissue etc., have been reported (Safford et al., 2004;Dotto and Cotsarelis, 2005;Tang, 2005;Bjerknes and Cheng, 2005).

STEM CELL THERAPY IN PARKINSON'S DISEASE

Although PD animal models do not show similar symptoms as seen in human patients, the pathology and related motor deficits seen in these animals provide a good model to evaluate various therapeutic agents including stem cells. Drug-induced rotatory behavior is an easily quantifiable motor deficit that is often used to evaluate the behavioral improvement in the rodent PD models (Beal, 2001). Figure 1.6 shows an illustration of an experimental model to test the potential of stem cells in promoting behavioral recovery in PD model rats. As mentioned earlier, stem cells can be used for replacement or rescue of DA cells in PD (see figure 1.7). Ventral midbrain cells from human fetus that are transplanted into the striatum of PD model animals have resulted in functional recovery by restoring DA neurotransmission (Lindvall et al., 1990). Cells derived from the fetal

midbrain can modify the course of neurodegenerative diseases, but they are inadequate source of dopamine synthesizing neurons because their ability to generate these neurons is unstable (Brundin et al., 2000). Ventral midbrain cells obtained from fetal pigs were used for therapeutic transplantation in PD patients, which is not yet approved for clinical purposes (Bjorklund and Lindvall, 2000;Deacon et al., 1997). In addition, a major concern with xenotransplantation is graft versus host disease (GVHD) and immune rejection (Larsson et al., 2000). Although immunesuppression increases the survival of transplanted cells (Deacon et al., 1997), it was estimated that only 5-10% (Bjorklund, 1991) or about 25% (Brundin et al., 2000) of the harvested cells survive transplantation. Moreover, serious complications (nephrotoxicity, cardiovascular toxicity, neurotoxicity, etc.) can arise as secondary effects of the immunosuppressive drugs, such as cyclosporine-A (Li et al., 2004;Rezzani, 2004;Serkova et al., 2004;Miller, 2002). Although adult CNS stem cells can be expanded and differentiated into dopaminergic (DA) neurons, these are tough to harvest and have only a limited ability to expand *in vitro* and to adopt a dopaminergic fate (Gage et al., 1998;Gage et al., 1995).

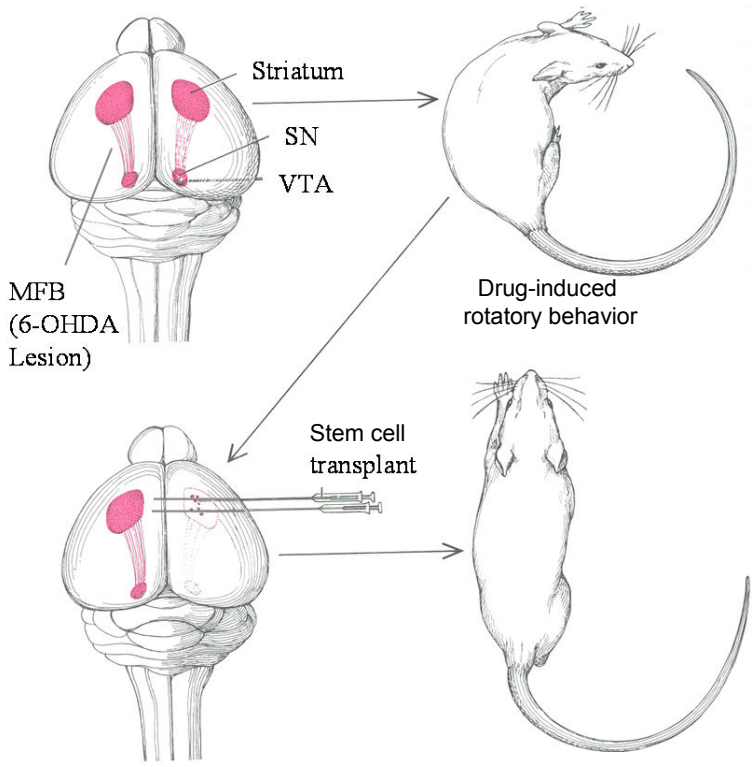

Figure 1.6 Experimental model to evaluate the effect of stem cell therapy in a Parkinson's disease (PD)-model rat.

MFB: medial forebrain bundle (projections of dopaminergic neurons)

6-OHDA: 6-hydroxydopamine, SN: substantia nigra, VTA: ventral tegmental area.

Parkinson's disease model rats can be created by injecting a neurotoxin, 6-OHDA, into the nigrostriatal pathway (either in the dopaminergic neurons in SN and VTA or their projections). Drug-induced rotatory behavior can be quantified and followed over time after stem cell transplantation to evaluate the potential of stem cells to promote behavioral recovery. Further, histological analysis of the rat brain would elaborate on the potential of stem cells to replace or rescue damaged DA neurons in the PD model rat.

Embryonic stem cells (ESCs) can be expanded *in vitro* and differentiated into DA neurons both *in vitro* and *in vivo* (Morizane et al., 2002;Kim et al., 2002;Lee et al., 2000). The DA neurons derived from ESCs have been shown to promote therapeutic benefit in PD model rats and mice (Kim et al., 2002;Nishimura et al., 2003). When small numbers (1000-2000 cells) of undifferentiated ESCs were transplanted to rat striatum, they proliferate and differentiate into DA neurons (Bjorklund et al., 2002). Dopamine neurons derived from ESCs function in an animal model of PD (Bjorklund et al., 2002;Nishimura et al., 2003). The problems with transplanting ESCs are formation of teratomas (due to uncontrolled proliferation) and immune rejection. Although NSCs and ESCs have been big contenders for replacement therapy, adult stem cells such as BMSCs were reported to be a potential source of replacement cells that can promote the desirable therapeutic effect in the PD animal models (Dezawa et al., 2004). In addition, stem cells that were genetically engineered to enhance DA neuronal differentiation have been shown to be effective in rescuing PD model animals (Kim et al., 2002;Chung et al., 2005). Thus, the ideal characteristics of cell grafts for replacement therapy would be, 1. The capacity to engraft and differentiate into the appropriate replacement cells, 2. The ability to not stimulate a robust immune rejection response, 3. Non-tumorigenic, 4. Non-controversial and 5. Economic feasibility.

Applications of stem cells in Parkinson's disease.

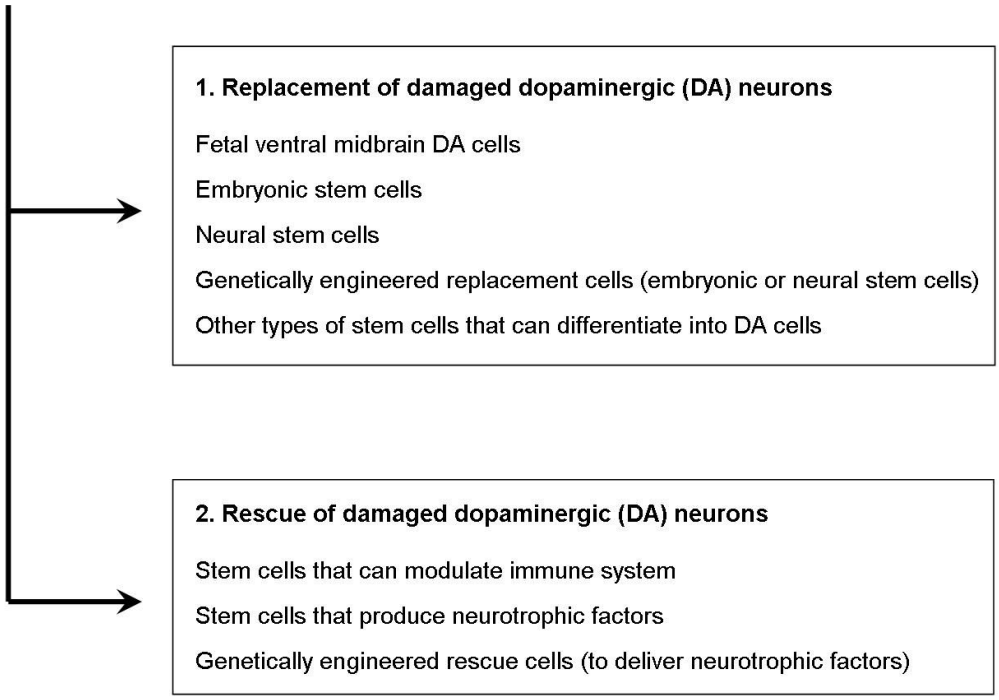

Figure 1.7 Applications of stem cells in Parkinson's diseases

Recently, neuroprotective strategies to regenerate/rescue damaged neurons have received much attention. As PD is characterized by progressive degeneration of DA neurons, it may be possible to intervene the degeneration process to rescue and revitalize the cells. It has been reported that some stem cells may have an inherent ability to rescue damaged neurons by releasing neurotrophic factors (Ourednik et al., 2002;Borlongan et al., 2004). In addition, the use of stem cells to deliver neurotrophic factors as well as non-pathogenic viral vectors as gene delivery vehicles (to increase the expression of neurotrophic factors) has been developed and tested on animal models of PD (Bjorklund and Lindvall, 2000;Bjorklund, 1991;Bjorklund et al., 2000). According to Dr. Ron D. McKay, "Cells are the ultimate device for delivering substances to the brain, so this could become one of the most widely used therapies in medical research (1998)." Stem cells that are genetically engineered *ex vivo* with specific therapeutic molecules are capable of delivering those substances effectively to the desired target (Yoshimoto et al., 1995;Lu et al., 2005). For example, it has been shown that mesenchymal stem cells engineered with tyrosine hydroxylase (TH – rate limiting enzyme in dopamine synthesis) show therapeutic benefit in PD model rats by restoring the dopamine levels in the striatum (Lu et al., 2005). Glial cell line derived neurotrophic factor (GDNF) is a neurotrophic factor that has been shown to rescue damaged DA neurons *in vitro* and *in vivo* (Arenas et al., 1995;Ding et al., 2004). GDNF has been used to rescue PD animal models through genetically engineered stem cells that produce GDNF or non-pathogenic viral vectors containing GDNF transgene (Bjorklund et al., 1997;Bjorklund et al.,

2000;Kirik et al., 2000a;Kirik et al., 2000b). In both approaches substantial prevention of neurodegeneration was observed. However, the recent clinical trial in humans with GDNF has been discontinued for unknown reasons.

In addition to the rescue mediated by neurotrophic factors, it has been reported that stem cells can mediate regeneration of neural tissue by immune modulation (Pluchino et al., 2005b;Pluchino et al., 2005a). As it has been shown previously that immune cells may play a role in PD (Block and Hong, 2005;Wu et al., 2005;Zhang et al., 2005), it is possible that the immune modulatory effects of stem cells may be useful to promote therapeutic benefit in PD. Recently, it has been shown that mesenchymal stem cells possess immunosuppressive capabilities (Djouad et al., 2003;Ryan et al., 2005), which may play a direct role in promoting rescue of the damaged neural tissue or they may play an indirect role as co-grafts with other replacement cell type and modulate the immune response and facilitate engraftment and differentiation of the replacement cell (Le and Ringden, 2005a;Le and Ringden, 2005b). This approach of neuroprotection to regenerate/rescue damaged neurons might lead to other interesting therapy for neurodegenerative diseases as well as suggesting a fundamental change in some of our concepts in neurobiology about the brain. In addition to the replacement and rescue from transplanted stem cells, it has been reported that endogenous stem cells in the brain can be recruited to the site of repair and contribute to either replacement or rescue of the damaged cells (Ferretti, 2004).

UMBILICAL CORD MATRIX STEM CELLS

Umbilical cord is a flexible cord-like structure found in amniotes. In placental mammals, umbilical cord connects the fetus to its placenta (tissue that connects fetus to the uterus and mediates the exchange of nutrients, oxygen, hormones, and waste products throughout the pregnancy). The umbilical cord primarily comprises umbilical vessels and supporting connective tissue (Figure 1.8 shows the H&E stained cross section of human umbilical cord showing two arteries and a vein surrounded by connective tissue). In addition, the umbilical cord may contain the remnants of yolk sac (omphalomesenteric duct) and allantois (urachus). The umbilical vessels are formed by the extraembryonic mesoderm that connects the embryo to the trophoblast (outermost layer of cells of blastocyst that helps in implantation of early embryo in the uterine wall). Although the origin of the connective tissue that supports these umbilical vessels is unclear, it closely resembles mesenchyme, an embryonic tissue of mesodermal origin that is characterized by a loose aggregation of cells surrounded by extracellular matrix. The extracellular matrix contains abundant collagen and glycosaminoglycans (GAGs), mainly hyaluronic acid, which constitutes 70% of the total GAGs (Bankowski et al., 1996).

Umbilical cord was once regarded as a biological waste and discarded after the fetus is delivered. Collection of umbilical cord blood in mid-20th century to isolate hematopoietic mononuclear cells has ascertained importance to this tissue, which is followed by reports of various types of stem cells being harvested from

the umbilical cord. It has been shown that umbilical cord blood is a source of HSCs (Consolini et al., 2001;Wright-Kanuth and Smith, 2001) and mesenchymal stem cells (Kogler et al., 2004;Sanberg et al., 2005). In addition, it has been reported that umbilical cord blood may be used to treat various diseases including neural disorders (Wright-Kanuth and Smith, 2001;Borlongan et al., 2004;Nan et al., 2005;Newman et al., 2004). Several labs have reported mesenchymal stem cells that are pluripotent or multipotent from various parts of the umbilical cord (Sarugaser et al., 2005;Kogler et al., 2004;Lee et al., 2004). Although stem cells from umbilical cord are regarded as post-natal stem cells, the fact that umbilical cord is formed much earlier during embryonic development (fig 1.5 shows umbilical cord in a 4 week human embryo) shows that it may be a source of more primitive cells. In addition, it has been shown that fetal HSCs and mesenchymal stem cells exhibit more primitive stem cell characters than their adult counterparts (Gotherstrom et al., 2005;Huang et al., 1998). During development, primitive stem cells, such as EGCs and HSCs migrate from the secondary yolk sac region through the umbilical cord region to the embryo.

The umbilical cord matrix (UCM) or Wharton's jelly is a completely different part of the umbilical cord than cord blood that is collected from the umbilical vessels. The UCM is a mucous connective tissue of umbilical cord that encircles the umbilical vessels and consists of myofibroblast-like stromal cells, collagen fibers and proteoglycans (Kobayashi et al., 1998;McElreavey et al., 1991). In addition,

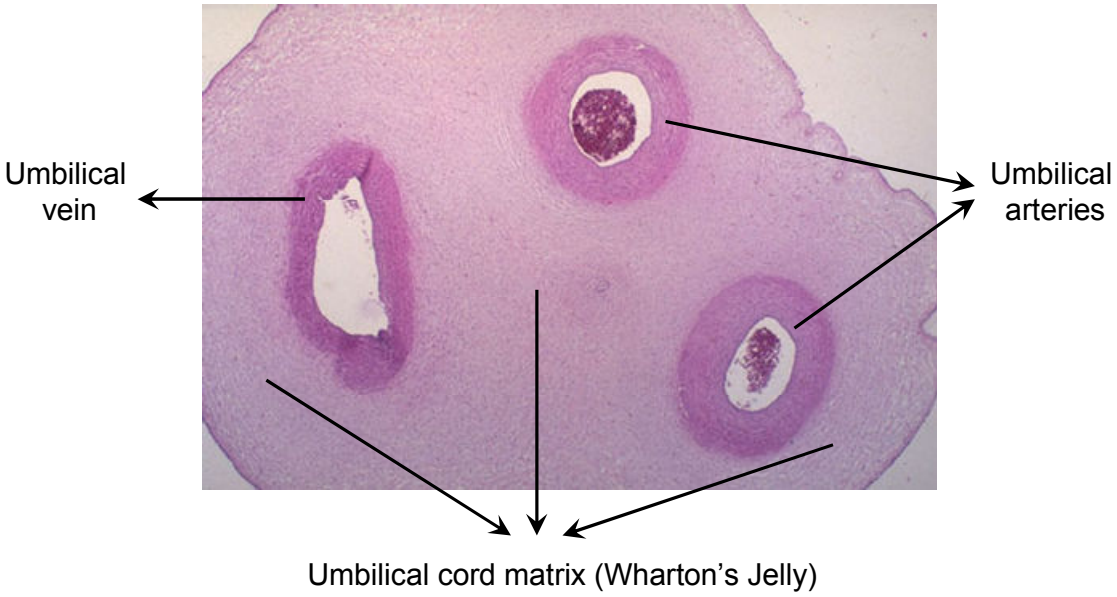

Figure 1.8 H&E stained cross-section of human umbilical cord

UCM has been identified as a source of growth-regulatory factors that are constitutively produced by the UCM cells (Irvine et al., 1984). UCM is a source of post-natal stem cells that, in contrast to other sources, is amenable to rapid isolation of large numbers of cells. Moreover, the cells can be harvested non-invasively from this inexhaustible and non-controversial source.

The pig umbilical cord matrix stem (pUCMS) cells exhibit stem cell characteristics and can be differentiated into neural-like cells that express neural and glial markers *in vitro* (Mitchell et al., 2003). Two to six weeks following transplantation into rat brain, a subset of pUCMS cells were found to morphologically resemble neurons and express pig-specific neuronal markers (Weiss et al., 2003). In addition, cells were isolated from the human umbilical cord matrix (hUCM) that can be expanded in culture and respond to *in vitro* neural differentiation signals (Mitchell et al., 2003). Recently, other labs were able to harvest multipotent mesenchymal stem cells from Wharton's jelly (Wang et al., 2004) and reported that these cells from the umbilical cord matrix may have therapeutic potential in PD (Fu et al., 2005). Moreover, mesenchymal stem cells from bone marrow have been shown to be capable of differentiating into neurons *in vitro* and *in vivo* and exhibit immunosuppressive capabilities, and thus may be useful in treating PD either by replacement or rescue of damaged DA cells (Dezawa et al., 2004;Prockop et al., 2001;Woodbury et al., 2000). These UCMS cells are non-controversial and inexhaustible stem cell source that may be useful to treat neurodegenerative disorders, such as PD.

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PART II

Transplantation of pig stem cells into rat brain: Proliferation during the first 8 weeks.

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Abstract

Previous work indicated that pig umbilical cord matrix (pUCM) cells are a type of primitive stem cell and that these cells could be recovered after central or peripheral injection into rats that did not receive immune suppression therapy. To determine the safety and proliferation potential of pUCM cells after brain transplantation, approximately 150 pUCM cells were transplanted into the brains of rats that previously received a striatal injection of the neurotoxin 6-hydroxydopamine (6-OHDA). The pUCM cells were previously engineered to express enhanced green fluorescent protein (eGFP); in this way the graft cells were identifed. The rats did not receive immune suppression therapy. There were no post-surgical complications and the animals thrived following transplantation. At 2, 4, 6, and 8 weeks after transplantation, two rats were sacrificed and the morphology, size and number of graft cells and the percentage of tyrosine hydroxylase (TH)-positive graft cells was determined. The size distribution of the grafted pUCM cells was unimodal and normal and the average size increased significantly over the 2-8 week survival period. The number of pUCM cells increased from approximately 5,400 cells at the 2 week survival period post-transplantation to approximately 20,000 cells at the 8 week survival period. There was an increase in the percentage of TH-positive pUCM cells from approximately 1% at the 2 week survival period to approximately 6% at the 8 week survival period. There was no evidence of a significant host immune response at any time e.g. no accumulation of CD-4, CD-8, CD-11b, CD-161 cells

in the transplantation site. These results suggest that pUCM cells engraft and proliferate without requiring immune suppression. These findings also suggest that a subset of pUCM cells can differentiate into TH-positive cells within 8 weeks after transplantation into the 6-OHDA lesioned rat brain.

Keywords: umbilical cord matrix, green fluorescent protein, tyrosine hydroxylase, Wharton's jelly, xenotransplantation, 6-hydroxydopamine brain lesion

Introduction

In Parkinson's disease (PD), human fetal mesencephalic transplants were once used as a source of replacement cells. Moral / ethical concerns associated with the use of fetal tissue, the scarcity of that tissue and other problems associated with obtaining enough tissue were barriers to the widespread use of human fetal mesencephalic transplantation. Mesencephalic cells obtained from fetal pigs are one treatment modality used for therapeutic transplantation in PD patients, which is yet to be approved for clinical purposes [2;7]. Xenotransplantation has problems such as graft versus host disease (GVHD) and immune rejection [13]. For therapeutically useful numbers of the xenografted cells to survive, the host's immune system must be suppressed [7]. Despite immune suppression, it was estimated that about 5-10% [1] or about 25% [5] of the harvested cells survive transplantation. Experimental work with fetal pig mesencephalic tissue grafted into the rat brain indicates that the grafted tissue is rejected in about 4-6 weeks [14]. The rejection response affects the function of the engrafted tissue because the grafts with a lower immune rejection response have better survival and more extensive fibers compared to animals with stronger immune rejection response [14]. Furthermore, immunosuppression treatment slows but does not prevent the rejection of pig tissue grafts in rat brain [8]. At the same time serious complications can arise from immunosuppression and from the secondary effects of the immunosuppressive drugs, such as cyclosporine-A. Thus, two ideal characteristics of therapeutic grafts would be 1) the capacity to treat the neurodegenerative disease, i.e., differentiate into the appropriate replacement

cells or produce factors that slow or reverse the disease process and 2) the ability to not stimulate a robust immune rejection response.

The umbilical cord matrix, or Wharton's jelly, is a gelatinous connective tissue of umbilical cord, which consists of myofibroblast-like stromal cells, collagen fibers and proteoglycans [12;18]. The umbilical cord matrix is a source for primitive stem cells and differentiation signals cause pUCM cells to exhibit a neuronal morphology and express neural and glial markers *in vitro* [19]. Two to six weeks following transplantation into rat brain, a subset of pUCM cells were found to morphologically resemble neurons and to express pig-specific neuronal markers [33]. Here, the safety of pUCM cells for transplantation is evaluated by testing their potential to proliferate and the ability of pUCM cells to express tyrosine hydroxylase (TH) following transplantation into the brain of rats with a previous catecholaminergic (6-OHDA) lesion, a rodent model of PD.

Materials and Methods

Cell culture and counting: Pig UCM cells were cultured and maintained as described previously [19;33] Manipulation of pUCM cells to express enhanced green fluorescent protein (eGFP) has been described previously [33]. Briefly, pUCM cells that were cultured *in vitro* 60 passages were transfected to express eGFP. After several selection passages, the eGFP expressing pUCM cells were lifted by a trypsin solution. The cells were counted by a hemocytometer and were adjusted to a final concentration of approximately 150 cells per microliter. The number of cells in one microliter was verified by spreading a 1 microliter drop on a plastic petri dish and manually counting the cells at 10x in bright field of a light microscope. The cell concentration was confirmed before and after the injection to insure that approximately 150 cells were delivered.

Transplantation procedure: A guide cannula was implanted in the brain of anesthetized male Lewis rats (Harlan) via stereotaxic surgery into the right striatum (Bregma +0.5, Lateral 3.4, Ventral 5.0 mm from the surface of the brain)[25]. The cannula was attached to the skull with screws and dental acrylic. At least three days later, anesthetized animals received a single injection of 10 μl of 7mg/ml 6-hydroxydopamine (6-OHDA, Sigma) over 5 min. At least one week after 6-OHDA injection, approximately 150 eGFP-pUCM cells in 1 μl of the sterile media were injected over 5 min. Rat which received guide cannula, but did not receive a transplant or 6-OHDA, served as control. At 2, 4, 6 and 8 weeks post-transplantation, two rats were randomly selected, anesthetized and sacrificed by

transcardial perfusion with heparinized isotonic saline rinse followed by 10% buffered neutral formalin. The brains were removed, postfixed, and cryoprotected in 20% sucrose overnight. Frozen sections of the brains were cut coronally at 40 µm and the sections were collected into three sets of adjacent sections, each set consisting of every third serial section.

Tissue processing and Immunocytochemical processing (IC):

Immunocytochemical (IC) detection of a single antigen was performed on one set of sections as previously described [33] and the adjacent sets of sections were held in reserve in a cryoprotectant solution [32]. The free-floating tissue sections were stained with primary antibodies for GFP (rabbit host, 1:1000, Santa Cruz Biotechnology, Inc), TH (rabbit host, 1:2000, East Acres Biologicals), CD-4 (mouse host, 1:500, Serotec), CD-8 (mouse host,1:500, Serotec), CD-11b (mouse host, 1:250, serotec), CD-161(mouse host, 1:250, Serotec) . The antigens were visualized either with diaminobenzidine (DAB) and hydrogen peroxide using a commercially available ABC kit (Vectastain) or with immunofluorescence. For immunofluorescence localization, 7-amino-4-methylcoumarine-3-acetic acid (AMCA)- Avidin D (Vector Laboratories) was used with the biotinylated secondary antibody. The IC-stained sections were mounted on subbed microscope slides, air-dried, and rinsed with distilled water. To detect the immunofluorescence and eGFP staining, the sections were observed using epifluorescence illumination with the appropriate filter combinations on a Leica DMRD microscope after clearing and coverslipping with glycerol containing N-

propyl gallate (3 parts 2% N-propyl gallate in 0.1M Tris buffer, pH 9.0 and 7 parts glycerol). IC-stained cells were considered positive if: 1) the signal in the cytoplasm is above background and 2) if the signal was absent in tissues in which the primary antibody had been omitted. To be considered double-labeled, the morphology and location of the cells must appear identical in both brightfield (DAB) and fluorescence (eGFP) for immunoperoxidase detected cells, or in both filter combinations for immunofluorescence (UV filter set for AMCA versus FITC filter set for eGFP).

Cell size and number: To measure cell size and number, one set of sections were stained with anti-GFP antibody and localized with DAB. A design-based unbaised stereological method is used for counting the cells. Individual cells were identified in brightfield illumination and measured using an morphometry/image analysis system (Bioquant Nova Prime, R&M Biometrics). Graft cells that were identified in both brightfield (IC for GFP) and epifluorescence (GFP fluorescence) were measured. With the experimenter-blind to survival group, the area of at least 75 cells (cells which did not overlap with any other graft cell and had an obvious nucleus) was measured (this area represents cell size and the term "cell size" is used for cell area throughout the manuscript) and the cell size distribution was analyzed for outliers and normality (StatView 5.0) and a frequency histogram was generated for each individual animal. To estimate the number of transplanted cells, the morphometry/image analysis software was used to measure the area occupied by graft cells in each

section (the area occupied by the graft cells in a particular section is assumed to be the volume of the graft in that section). After measuring the graft cell area in all sections containing GFP IC-positive cells, the total area of the graft was represented as the sum of the area in each section. No correction factors were applied. The total area of the graft was divided by the average cell size to yield an estimate of the number of graft cells detected in each set of sections. Because there are 3 sets of brain sections for each animal, the estimate of the total number of graft cells in the respective animal was three times the total number of graft cells in one set of sections.

Assessment of TH-positive cells: A second set of brain sections was IC-stained for tyrosine hydroxylase (TH) and the TH antibodies were localized using AMCA. The TH-positive graft cells appeared in both the FITC filter set (eGFP) and the UV filter set (AMCA). Again, with the experimenter-blind, an estimate of the percentage of graft cells that stained for TH was made by evaluating a minimum of ten fields per animal (fields were selected for counting based upon the distribution of cells in the field, e.g., fields were selected that did not have clumps of graft cells). In each field, the number of eGFP cells (FITC) and the eGFP-TH positive cells (AMCA and FITC) were counted. The percentage of TH-positive graft cells was calculated and averaged to yield an estimate of the percentage of TH-positive graft cells in each animal. After the analysis was completed, the individual animals were assigned to the appropriate survival groups. To estimate

the total number of TH cells, the percentage of TH graft cells was multiplied by the graft cell number for each animal.

Assessment of Host immune response: The frozen brain sections were IC-stained using primary anti-rat CD4, CD8, CD11b or CD161 antibodies. These primary antibodies were localized using biotinylated secondary antibodies and Avidin-AMCA.

Statistical analysis: All tissue manipulations were conducted in large batches to avoid batch to batch differences in tissue IC-staining. Tissue processing and data collection were conducted in an experimenter-blind fashion. After measurements and counting, the survival group status was decoded prior to statistical analysis. The histogram of the cell size was inspected for outliers (visual inspection of the distribution compared to a normal one). If no outliers were observed, then the Kolmogorov-Smirnov (K-S) test was used to compare the distribution of measured cells size to an idealized normal distribution (Statview 5.0). When outliers were observed, the Mann-Whitney U test was used to compare the distributions. In all cases, no outliers were observed, thus the K-S test was used here. The K-S test revealed that all distributions were normal. Thus, ANOVA was used to test interactions between the independent variable (survival period after grafting) and the dependent variables (cell size, graft cell number, percentage of TH graft cells and number of TH graft cells). Significance for ANOVA was set at $p<0.05$ (two tailed). Following significant ANOVA, post

hoc analysis using Scheffe's F test was used to examine planned comparisons. Significance for post hoc testing was set at $p < 0.05$ (two tailed). The mean plus or minus one standard error are presented on graphs.

Results

Cannula placement

In one of the eight animals, the guide cannula was misplaced. This animal was excluded from further analysis (six week survival). Because the six week survival period had only one animal with a good cannula placement, no data from that survival period is included. Thus, the results presented are from the 2, 4 and 8 week survival periods averaged from two animals at each survival period.

Behavioral and histological findings

None of the animals showed any behavioral abnormalities following the implantation of cannula. Following 6-OHDA lesion, the animals demonstrated rotation (spontaneous) towards the damaged hemisphere during periods of excitement. Rotational behavior was not quantified. Following 6-OHDA lesion, the animals did not show other behavioral signs or changes in their health status. After the transplantation with eGFP-pUCM cells, the animals acted normally and appeared in robust health throughout the 2-8 week survival period (there was no indication of sickness behavior, weight-loss, etc). Midbrain sections, when stained for TH, revealed destruction of most of the TH-positive dopaminergic cells in substantia nigra of the ipsilateral side of the 6-OHDA lesion, but not on the contralateral side (Fig. 2.1).

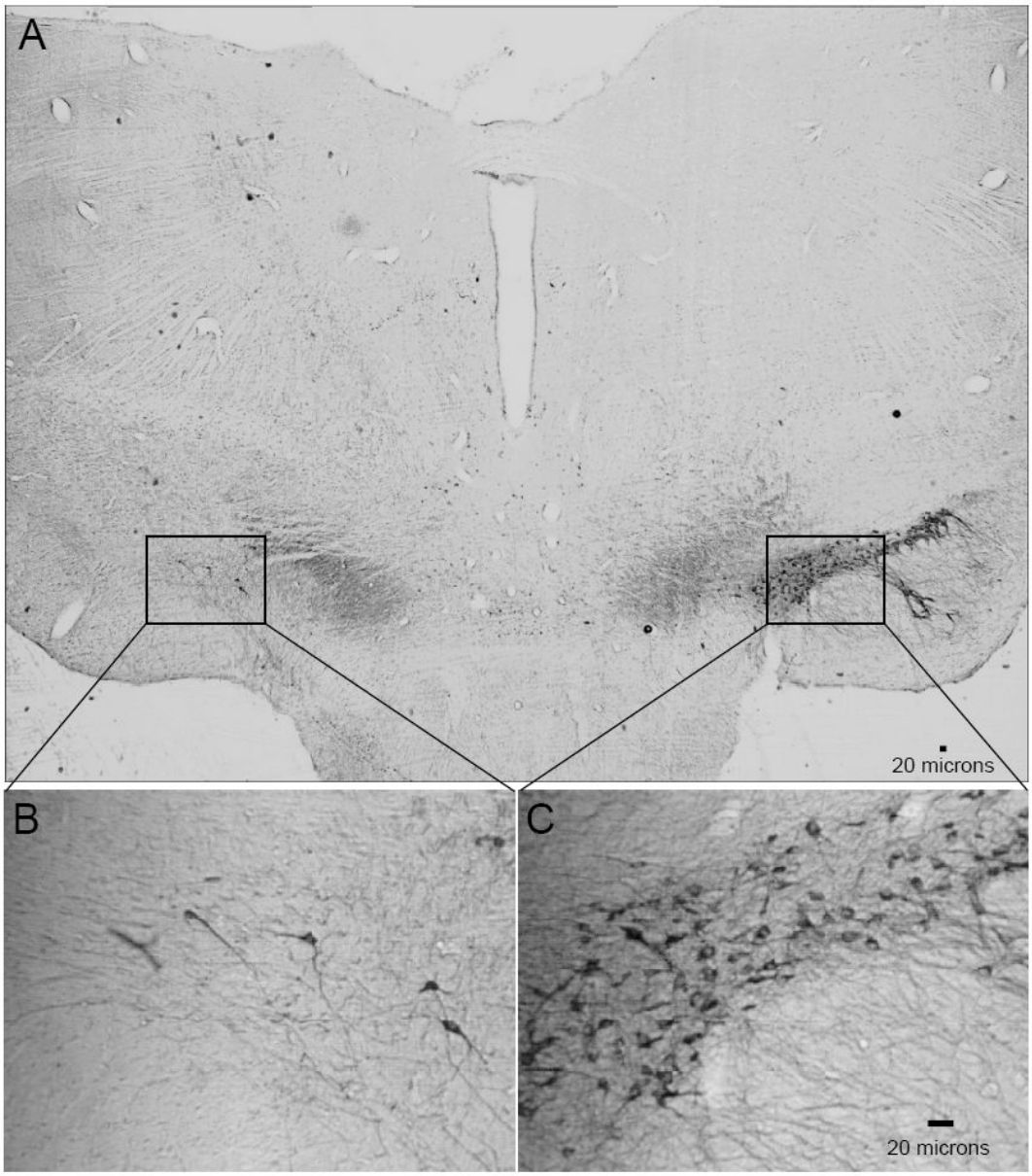

Figure 2.1: Histological findings in substantia nigra (SN) of the lesioned rats. A unilateral 6-OHDA lesion is created by injecting 6-OHDA in to the right striatum of all the rats under study. The mid brain sections were stained for TH to evaluate the effect of the lesion. Most of the TH-positive dopaminergic neurons in the ipsilateral (right) SN are destroyed (A; magnified view in B), but not in the contralateral (left) SN (A; magnified view in C).

Histological findings within the graft

There was no evidence of tumor, teratoma or scar formation in the transplant recipients. Withdrawal of host tissue was noted around the cannula implantation site in both control and grafted animals. The pUCM-eGFP cells were identified by their endogenous candy apple green fluorescence (FITC filter cube, see Fig. 2.2A). To control for the possibility of auto-fluorescence by host cells, the sections were IC-stained using an antibody to GFP and visualized with AMCA. These results are shown in Fig 2.2B. Virtually all the graft cells exhibiting GFP fluorescence were localized with AMCA. When the primary antibody is omitted, the graft cells were not observed using the UV filter set (Figs. 2.2C & D).

The graft cells were localized by IC staining for GFP followed by DAB visualization. Two weeks after transplantation, the graft cells were found along the sides and at the tip of the guide cannula tract. Most of the graft cells were clustered. Occasionally, individual cells were observed; most individual graft cells appeared small and spherical with a granular cytoplasm (Fig. 2.3A). Four weeks after transplantation, the graft cells were found farther from the guide cannula tract in the surrounding host brain tissue. At this time, more of the graft cells were dispersed and a greater percentage of the graft cells were elongated or bipolar in appearance. Some of the graft cells had short, primary processes attached to the cell body (Fig. 2.3B). Eight weeks after transplantation, the GFP staining in the graft cells was less intense and the graft cells appear larger and morphologically more complex when compared to the graft cells recovered 2

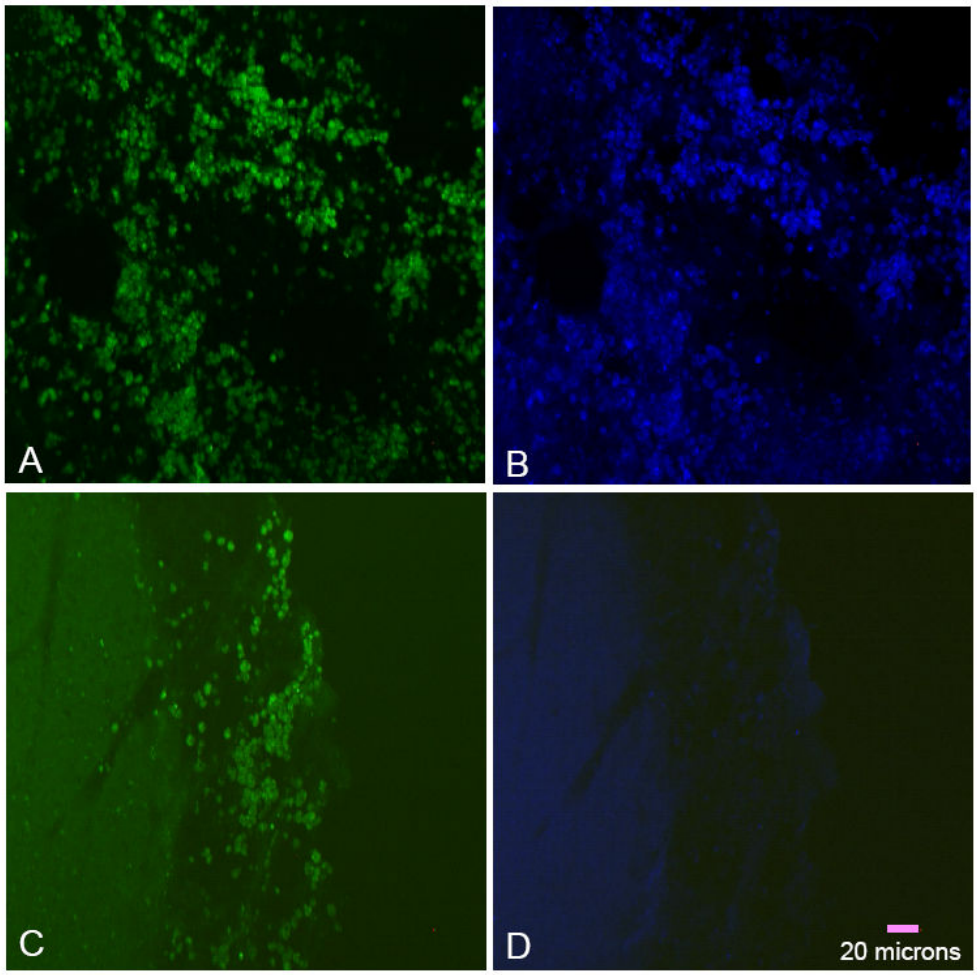

Figure 2.2: Appearance of pig UCM cells after transplantation into rat brain. Pig UCM cells that were engineered to express eGFP were transplanted into the brains of rats that had a previous unilateral 6-OHDA lesion. The pUCM graft cells were recovered after 2, 4 and 8 weeks post-transplantation. A. The eGFP-pUCM graft cells were identified by the epifluorescence (FITC filter set). B. The brain sections were immunostained with anti-GFP serum and visualized using AMCA. The co-localization of AMCA with eGFP confirms the recovery of the graft cells. Virtually all of the graft cells show positive reaction with AMCA. C, D. Control staining performed without the primary antibody for GFP shows no reaction for AMCA.

weeks post-transplantation (Fig. 2.3C). No further migration was noticed from 4 weeks to 8 weeks post-transplantation. Short processes were observed on a subset of the population of pUCM cells 4, 6 and 8 weeks after transplantation (see Fig. 2.4).

Cell size

Graft cells were identified by IC staining for GFP (visualized with DAB) and epifluorescence of GFP (FITC filter set). From 75-110 cells per animal were measured and a frequency histogram of the cell size was created. No outliers were observed. All distributions were normal (K-S normality test) and unimodal. Next, the size distributions between animals at a given survival period were compared; there was no significant difference between cell size distributions at each survival period; those data were blocked for subsequent analysis by survival period (data not shown). At 2 weeks post-transplantation, the average size of the graft cells was 140.0 ± 3.7 sq. microns. At 4 weeks post-transplantation, the average size of the graft cells was 160.2 ± 12.1sq. microns. At 8 weeks post-transplantation, the average size of the graft cells was 171.9 ± 2.3 sq. microns. Thus, the graft cells at 8 weeks after transplantation were 22% larger than those at 2 weeks; this represents a significant increase in cell size (see Figure 2.5).

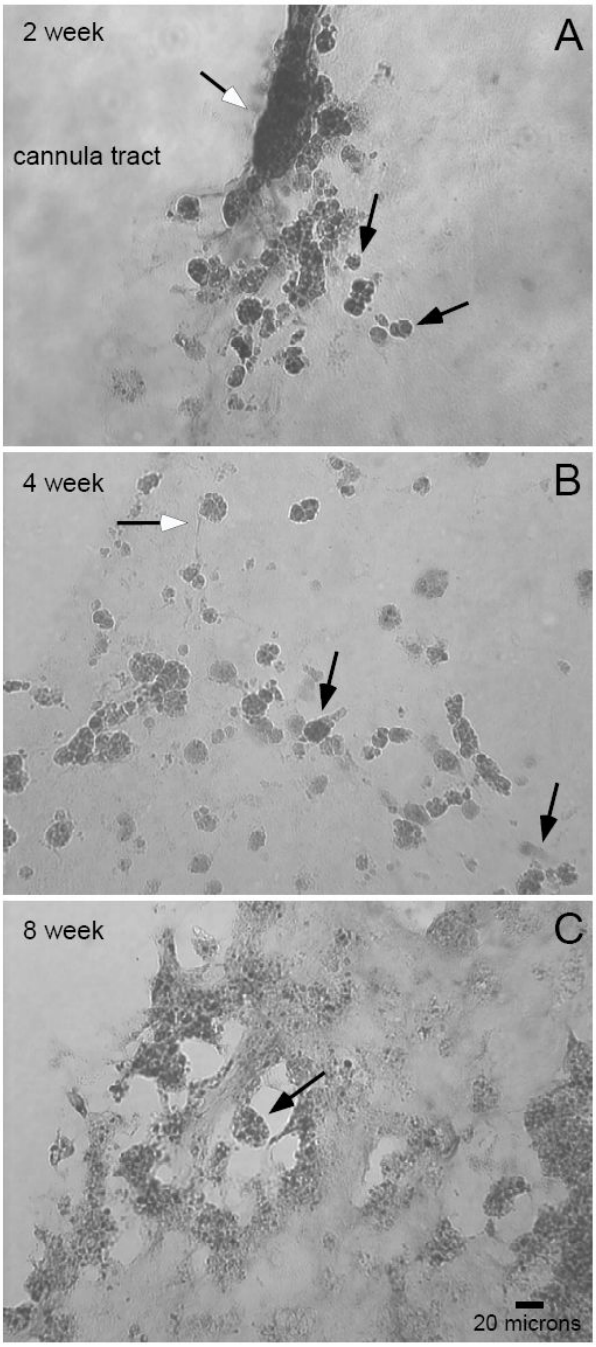

Figure 2.3: Pig UCM graft cells after 2, 4 and 8 weeks post-transplantation. The frozen brain sections of all the rats were immunostained with GFP antibody and localized with DAB. A. After 2 weeks post-transplantation, the pUCM cells can be recovered and they appear round (black arrow), granular, tend to form clumps (white arrow) and are located just next to the cannula tract. B. After 4 weeks post-transplantation, the pUCM cells can be recovered. Some of the cells appear elongated (black arrow) and some appear to be extending small processes (white arrow). The graft cells are more scattered into the brain tissue around the cannula tract. C. After 8 weeks post-transplantation, the pUCM cells can be recovered and appear to be bigger in size (black arrow) than after 2 and 4 weeks. A more diffused staining is noticed in the brain tissue around the cannula tract.

Figure 2.4: Pig UCM cells extend processes. A subset of pUCM graft cells extend processes (black arrow) into the host's brain. This may indicate that the graft cells respond to the local cues and differentiate into a neural phenotype.

Number of graft cells

To estimate the proliferation of the graft cells in each individual, the total GFP-IC positive area in one set of sections (set A) was divided by the average cell size for that individual. The estimated number of graft cells at each survival period is shown in figure 2.4. At 2 weeks after transplantation, the estimated number of graft cells was 1825 ± 163 (yields a total of about 5475 graft cells per animal). At 4 weeks after transplantation, the estimated number of graft cells was 5758 ± 400 (yields a total of 17274 graft cells per animal). At 8 weeks after transplantation, the number of graft cells was estimated to be 6904 ± 1000 (yields an estimated total of 20712 graft cells per animal). The number of graft cells increases significantly from 2 to 4 weeks and 2 to 8 weeks (see Figure 2.5). This indicates that there is roughly a 5-6 fold expansion 2 weeks after transplantation and a 7-8 fold expansion of the original 150 graft cells by 8 weeks after transplantation.

TH-positive graft cells

The sections in set B were IC-stained using anti-TH antibody and visualized with AMCA. The graft cells that co-localized with green fluorescence for GFP (FITC filter set) and blue fluorescence for AMCA (UV filter set) are considered to be TH-positive graft cells (Fig. 2.6A & B). When the TH primary antibody was omitted, no positive staining for TH was observed (Figs. 2.6C & D). The percentage of TH-positive graft cells is shown in figure 2.6. Two weeks post-transplantation, 1.0 ± 0.6 % of the graft cells were positive for TH. Four weeks post-transplantation,

3.4 ± 0.6 % of the total graft cells were positive for TH. Eight weeks post-transplantation, 6.0 ± 0.3 % of the total graft cells were positive for TH. Thus, there was a significant increase in the percentage of TH-positive graft cells at the 8 week survival period compared with the 2 week survival period. The estimate of total number of TH-positive graft cells at each survival period is shown in figure 2.7. The total number of graft cells previously calculated was multiplied by the percentage of TH-positive graft cells. It was estimated that a total of 54 TH-positive graft cells were found in the two week survival animals, 587 TH-positive graft cells in the four week survival animals, and approximately 1242 TH-positive graft cells in the 8 week survival animals (see Figure 2.7). Thus, there was a significant increase in the number of TH-positive graft cells at the 8 week survival period compared with the 2 week survival period.

Host immune response

There was no gross or significant histological evidence of immune rejection in the brain of the animals 2-8 weeks following transplantation, e.g., there was no vacuolization, perivascular cuffing, or cellular infiltrate. In addition, the frozen brain sections were IC-stained using anti-rat CD4, CD8, CD11b and CD161 antibodies, and visualized with AMCA. IC-staining for anti-rat CD4 showed negligible staining; in contrast, the control animal, which received a cannula only, had variable and conspicuous staining for anti-CD4 antibody (see Figure 2.8). Similar results were observed when the sections were IC-stained using primary antibodies directed at CD8, CD11b, and CD161 (data not shown).

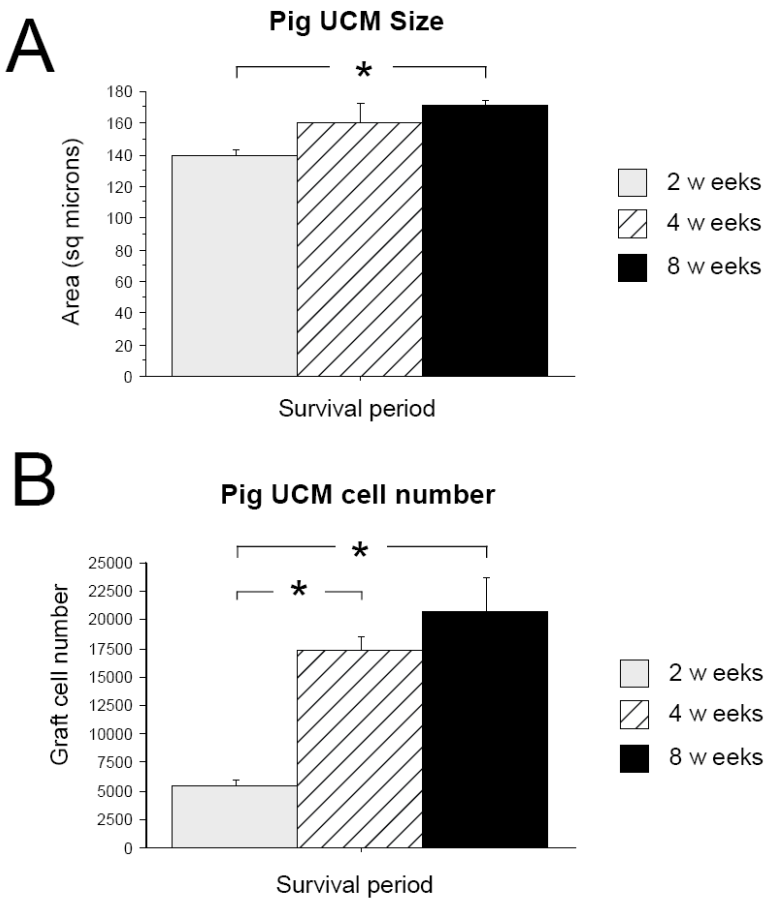

Figure 2.5: Pig UCM cell size and cell number. 150 pUCM cells were transplanted into rats with previous 6-OHDA lesions. The cell size and number of grafted cells was evaluated at 2, 4 and 8 weeks post-transplantation. A. Cell size measured in 75-110 graft cells. The distribution of size was tested and found to be normal with a single mean (data not shown). The cell size is calculated for each animal and cell size at each survival period was calculated. B. The number of pig UCM cells was estimated in each animal. The graft cells undergo about a five fold expansion in the first two weeks and increase to a maximum of about a seven fold expansion by the eighth week. N=2 at 2, 4 and 8 weeks. * $P<0.05$

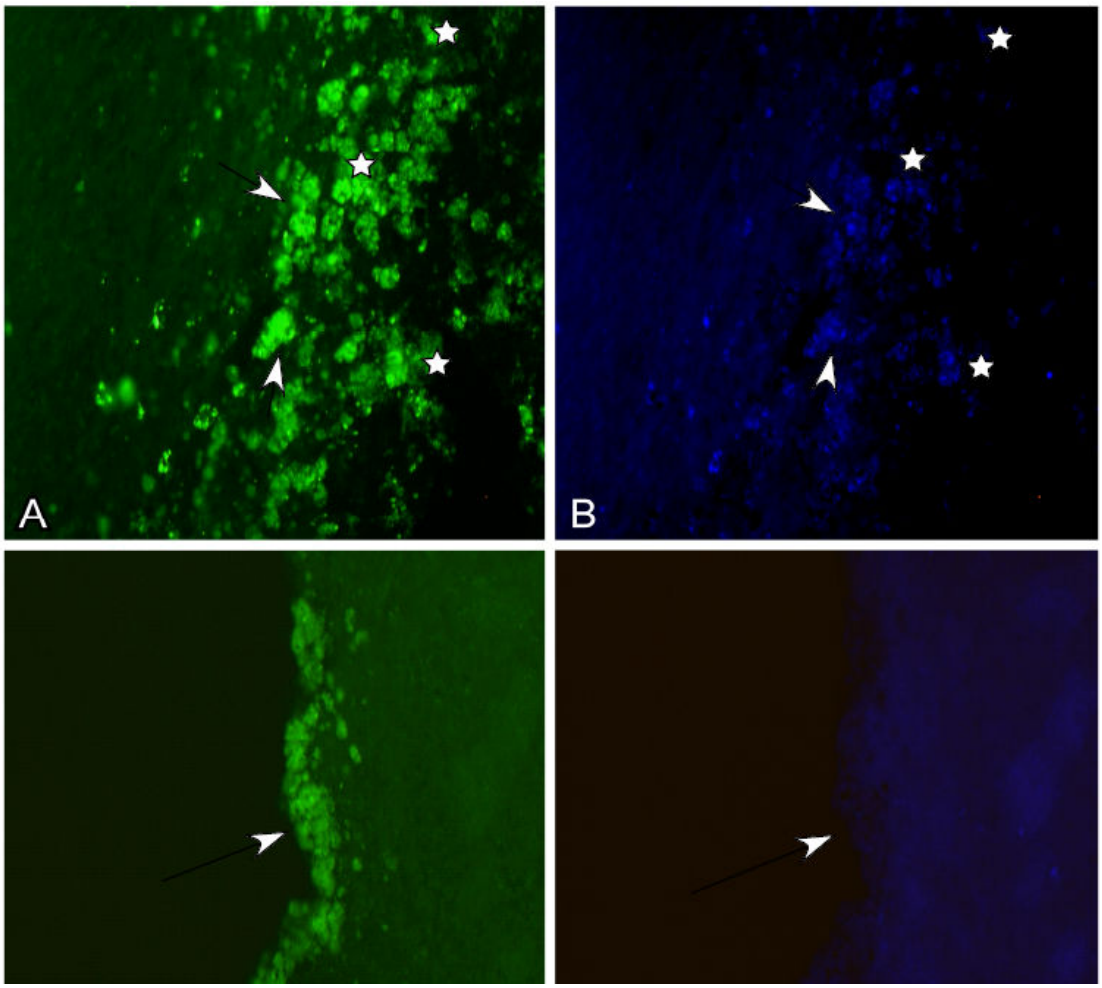

Figure 2.6: Pig UCM cells stain for TH. Approximately 150 pig UCM cells expressing eGFP are transplanted into rats with a previous unilateral 6-OHDA striatal lesion. The pUCM graft cells were recovered after 2, 4 and 8 weeks post-transplantation. A. The pUCM graft cells can be identified by the green fluorescence of the GFP. B. The frozen brain sections were IC-stained using anti-TH serum and visualized by AMCA. The co-localization of AMCA with eGFP confirms the TH-positive graft cells (white arrows). TH-negative graft cells are indicated by white asterisks. C, D. Control staining performed without the primary antibody for TH shows no reaction for AMCA (white arrowheads).

Discussion

Here, approximately 150 pig UCM cells were transplanted into the brains of previously 6-OHDA lesioned rats and characteristics of the graft cells were determined over a 2-8 week survival period. Specifically, the size and morphology of the graft cells, the total number of graft cells, and the percentage and number of pig UCM cells that synthesize tyrosine hydroxylase (TH) was determined at 2, 4 and 8 weeks after transplantation. Four observations were made. First, pig UCM cells increased in size over the 2-8 week period and a subset of the grafted cells extended short processes into the parenchyma of the host's brain. Second, pig UCM cells proliferated rapidly in the first four weeks after transplantation, but the proliferation rate appears to slow over the next four weeks after transplantation (weeks 4-8 after transplantation). Third, the percentage and total number of pig UCM cells that stain for TH significantly increased over the 2-8 week survival period. Fourth, there was no apparent evidence of a significant host immune response to the transplantation of pig UCM cells. These results indicate that pig UCM cells proliferated after transplantation and that a subset of the grafted cells that synthesize TH increased over the 2-8 week survival period. Also, the pig UCM cells appear to engraft without stimulating a significant immune rejection response over the 2-8 week period.

Pig UCM cells were detected by GFP fluorescence and IC staining for GFP. No positive GFP-IC staining, nor GFP fluorescence was found in the control animal which had a cannula but no graft cells (data not shown). The expression of a

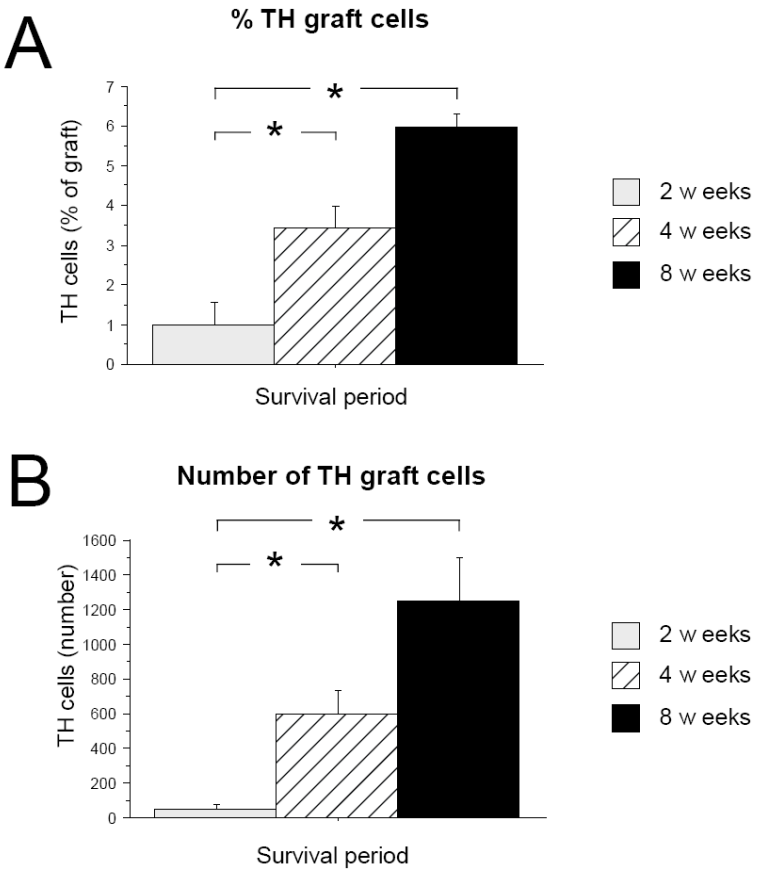

Figure 2.7: Percentage and number of TH-positive graft cells. About 150 pig UCM cells were transplanted into the striatum of rats with 6-OHDA lesion. The percentage and number of TH-positive graft cells are evaluated at 2, 4 and 8 weeks post-transplantation. A. The percentage of TH stained graft cells was determined in at least ten fields per animal. The percentage of TH-positive graft cells increases over the time (1% at 2 weeks to about 6% at 8 weeks post-transplantation). B. Based on the total number of graft cells and the percentage of TH stained graft cells, the number of TH-positive graft cells was calculated. The number of TH-positive graft cells increases from about 50 at 2 weeks to about 1200 cells at 8 weeks post-transplantation. * $P<0.05$

marker protein such as GFP over the 2-8 week survival period following transplantation supports the notion that pig UCM cells have not been scavenged by the immune system. In previous work, lysed pig UCM cells were injected into the rat brain and no stained cells (neither host or graft cells) were detected at the injection site [33]. This indicates that either cellular debris or the leakage of GFP from dead or dying cells does not produce non-specific staining. It was observed that GFP staining was less intense in animals with the longest survival times compared to those animals that survived two weeks. The reason for the decrease in intensity is unknown. Also, the grafted cells increased in average size over time; the reason for this is not understood.

Here, 150 cells were transplanted because previous work has shown that the number of cells in a graft may influence graft survival or function. For example, Ostenfeld et al. compared human neural precursor cells that were transplanted at 200,000 cells (low density), or high-density grafts (1-2 million cells)[21]. They reported that low-density grafts had significantly fewer host immune cells compared to animals with high-density grafts. They found that the low-density grafts have a higher proliferation rate 2-6 weeks after transplantation, and compared to the high-density grafts, the low-density grafts had more fiber outgrowth [21]. Bjorklund et al. transplanted 1000-2000 undifferentiated ES cells and found proliferation in the rat brain [3]. These authors noted that when a high concentration of ES cells were transplanted, there was a greater likelihood of teratoma formation [3]. They speculated that when cells are transplanted at low

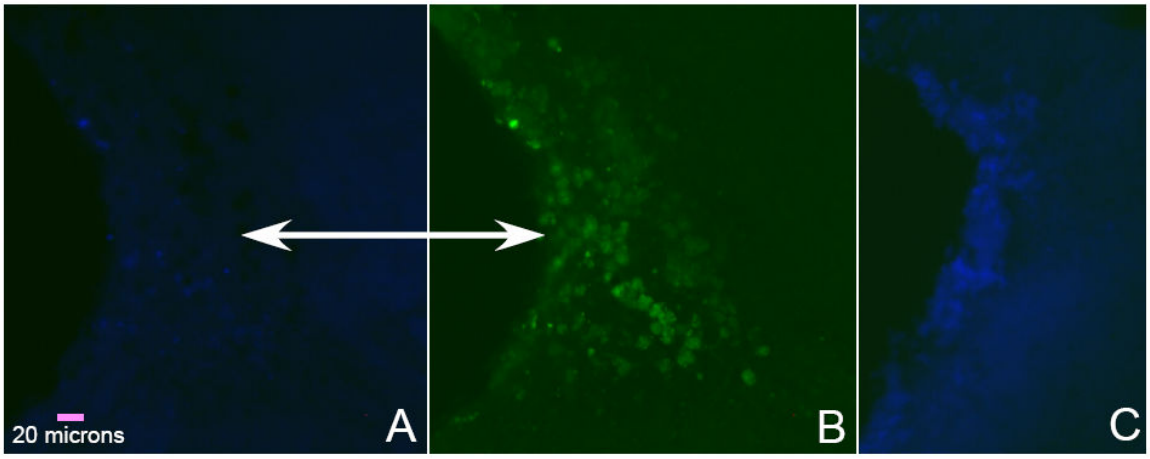

Figure 2.8 Host immune cell infiltration into the injection site. The frozen brain sections were IC-stained using anti-CD4 antibody and visualized by AMCA. A. No significant evidence of immune cell infiltration; few CD4-positive cells are found in the site of pig UCM cell transplants. B. Pig UCM graft cells expressing GFP. C. A variable and conspicuous staining for anti-CD4 antibody was found around the cannula tract of control animal which had no graft cells. Similar staining was obtained for CD8, CD11b and CD161 (data not shown).

concentration, the cell-to-cell contact will be less and the cells will be influenced by the local host signals leading to increased differentiation to neural cells. In their work, 2000-2500 TH-positive cells were recovered 14 – 16 weeks after transplantation [3]. Thus, similar to the present findings, previous work demonstrates that stem cells can proliferate and differentiate into TH-positive cells following xenografting. In contrast to the present findings, the work reviewed above was conducted in animals that were receiving immune suppression therapy.

We have shown that UCM cells are a type of primitive stem cell that can differentiate into neural cells *in vitro* [19] and pig UCM cells engraft following central or peripheral injection of 10,000 cells into rats without requiring immune suppression [33]. Pig UCM cells, after transplantation into rat brain, were shown to stain for pig-specific neurofilament and other neural markers [33]. In those experiments, the number of cells in the graft could not be determined because of the high-density grafts and wide distribution of the cells in the host brain tissue. The present work follows the previous work and demonstrates that UCM cells apparently proliferate during the first 4 weeks following transplantation, but this growth rate tapers apparently during the next 4 weeks. The decrease in the rate of proliferation after 4 weeks allows us to speculate that the cells might stop proliferating after a while, and we intend to address this speculation in our future work with longer survival period. Further, over the 2-8 week survival period, the percentage and number of TH-positive graft cells increases. In future work, the

number of graft cells undergoing apoptosis and division will be assessed using TUNEL staining and Ki67 staining over time.

Tissue transplantation has associated complications such as graft versus host disease (GVHD) and immune rejection of the graft by the host. The rat is a model system to study rejection of grafted tissue. For example, transplantation of fetal pig brain tissue into rat brain results in a significant host immune response and the grafted material is cleared from the rat brain in 2-6 week period after transplantation [4;9;10;13-15;34]. The clearance of the grafted tissue is correlated with an infiltration of host immune cells. In contrast, transplantation of tissues that are generated from the same species, an allograft, has less tissue rejection problems than a xenograft [10]. In the rat, treatment with immune suppressive drugs is not successful at blocking the immune response to a xenograft [8], but immune suppression does increase the survival rate of the graft significantly [4;23]. Rejection of the grafts remains a hurdle to therapeutic neural xenografting [4].

In contrast to the rejection problems observed during xenotransplantation of tissue, here, approximately 20,000 GFP-positive graft cells were recovered 8 weeks after transplantation with no significant host immune response. There are several possible explanations for this observation. First, pig UCM cells may have immunosuppressive effects like another type of stem cell [16]. Second, the low number of graft cells used here may not provide a sufficient stimulus for rejection.

Indeed, previous work has indicated that the number of cells in the transplant can affect differentiation to neurons and survival [3;20]. Third, in humans, cells located at the feto-maternal interface contain HLA-G which has been suggested to play a role in dampening the mother's inflammatory and immune response against the fetus [6;17;24;26-29]. In preliminary work, human UCM cells stained for HLA-G [31]. Hypothetically, pig UCM cells may synthesize an analogue of HLA-G which may dampen the rat's immune response. Further work is needed to understand how UCM cells avoid immune surveillance.

Therapeutic potential of stem cells for neurodegenerative diseases has been demonstrated in rodent models [11]. One hypothesis for their therapeutic role is that stem cells are attracted to the neurodegenerative environment, where they replace dead cells [2] or rescue sick or dysfunctional cells [22]. Here, rats with a previous unilateral 6-OHDA lesion received 150 pig UCM cells. From these 150 transplanted cells, the number of TH-positive pig UCM cells increased from about 50 at 2 weeks post-transplantation to more than 1200 cells after 8 week survival. Exceeding 1000 TH-positive cells may be significant because it was previously estimated that 1000 dopaminergic cells are needed for behavioral recovery in a rat model of Parkinson's disease [30]. This finding, together with the lack of significant host immune response to the xenograft, would suggest that transplantation of pUCM cells may provide a therapeutic approach for the treatment of neurodegenerative diseases such as Parkinson’s disease.

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PART III

Ms: *Stem Cells*: In press

Human Umbilical Cord Matrix Stem Cells: Preliminary Characterization and Effect of Transplantation in a Rodent Model of Parkinson=s Disease.

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Abstract

The umbilical cord contains an inexhaustible, non-controversial source of stem cells for therapy. In the United States, stem cells found in the umbilical cord are routinely placed into biohazardous waste after birth. Here, stem cells derived from human umbilical cord Wharton=s Jelly, called umbilical cord matrix stem (UCMS) cells, are characterized. UCMS cells have several properties that make them of interest as a source of cells for therapeutic use. For example, they: 1) are isolated in large number; 2) are negative for CD34 and CD45, 3) grow robustly and can be frozen/ thawed, 4) can be clonally expanded, and 5) can easily be engineered to express exogenous proteins. UCMS cells have genetic and surface markers of mesenchymal stem cells (positive for CD10, CD13, CD29, CD44, CD90, and negative for CD14, CD33, CD56, CD31, CD34, CD45 and HLA-DR), and appear to be stable in terms of their surface marker expression in early passage (passages 4-8). Unlike traditional mesenchymal stem cells derived from adult bone marrow stromal cells, small populations of UCMS cells express endoglin (SH2, CD105) and CD49e. UCMS cells express growth factors and angiogenic factors suggesting that they may be used to treat neurodegenerative disease. To test the therapeutic value of UCMS cells, undifferentiated human UCMS cells were transplanted into the brains of hemiparkinsonian rats that were not immune suppressed. UCMS cells ameliorated the apomorphine-induced rotations in the pilot test. UCMS cells transplanted into normal rats did not produce brain tumors, rotatory behavior or a frank host immune rejection response. In summary, the umbilical cord matrix appears to be a rich, non-controversial and inexhaustible source of primitive mesenchymal stem cells.

Key words: Wharton's jelly, flow cytometry, regenerative medicine, in vitro expansion, non-controversial source of stem cells

Introduction

Stem cells derived from embryos (embryonic stem cells, ESCs) or fetal tissues have the potential for therapeutic use. Both ESCs and fetus-derived stem cells have significant problems which impede their adaptation into the clinic, not the least of which are the moral/ethical issues surrounding their derivation (1-7).

With regard to moral/ethical issues, postnatal stem cells offer fewer concerns. Dogma has it that, in contrast to fetus-derived stem cells or ESCs, postnatally-derived stem cells have less broad developmental potential. This dogma has no real teeth: several labs have shown that postnatal stem cells have wide potential, e.g., (8-11).

At least two types of stem cells have be found in umbilical cord/placental tissues: blood-forming stem cells (hematopoietic stem cells, HSCs) and mesenchymal stem cells (MSCs). Umbilical cord blood (UCB) is a source of a population of pluripotent, mesenchymal-like stem cells (8) and hematopoietic stem cells (HSC) for transplantation. UCB is rapidly gaining attention for its therapeutic value for several reasons. First, UCB has more primitive HSC per volume than bone marrow (12). Second, there is a lower incidence of rejection after UCB transplantation (13-15). Third, unlike bone marrow transplants, UCB transplantation does not require perfect antigen matching (13). Fourth, UCB transplantation has been useful for the treatment of inborn errors in metabolism (16). Finally, the methods for collecting, storing and freezing human blood were developed in the 1940s, so no new technology is needed to save the mononuclear cells from UCB. This has led to the establishment of cord blood banks

and the increased use UCB for transplantation (17,18). Interestingly, non-hematopoietic stem cells derived from umbilical cord blood have been shown to produce cytokines that may facilitate engraftment and ex vivo expansion and are more effective than bone marrow derived mesenchymal stem cells for *in vitro* expansion of HSCs from CB (19).

The umbilical cord vessels and surrounding mesenchyme (including the connective tissue matrix that becomes Wharton's jelly) are derived from extraembyronic mesoderm and/or embryonic mesoderm. Thus, these tissues, as well as primordial germ cells, differentiate from the proximal epiblast around the time of primitive streak formation (20). There are several reports of mesenchymal stem cells (MSCs) or somatic stem cells with pluripotent differentiation potential from various sites in the umbilical cord (8,21-25). Thus, these tissues are rich sources of stem cells that may be useful for a variety of therapeutic purposes. We speculate that the umbilical cord matrix material derives from primitive mesenchyme that is in a transition state to the mesenchyme found in the bone marrow niche of adult animals. Here, we investigate this hypothesis by characterizing the human umbilical cord matrix stem cells and comparing them with stem cells derived from other sources.

As mentioned above, umbilical cord tissues contain pluripotent stem cells. We speculated that these stem cells may be useful for treating neurodegenerative disease, such as Parkinson's disease. Previous work has indicated that cells derived from embryonic or fetal sources may be useful when transplanted into PD rodents (26-30). In our previous work, we found that pig umbilical cord matrix stem cells engrafted and

expanded significantly without forming teratomas, when transplanted into rats with 6-hydroxydopamine lesions of the striatal-nigral tract (31,32). Here, to test the value of human umbilical cord matrix stem cells, we expanded upon our previous work by testing the behavioral effects of human umbilical cord matrix cells in the PD rat model.

Material and Methods

Human subjects:

The work was conducted following approval of the Kansas State University human subjects board (IRB approval # 3515).

Generation and expansion of UCMS cells:

The collection and expansion of UCMS cells was described previously (24). Briefly, human umbilical cords from both sexes were collected from full-term births (two sets of fraternal twins) with informed consent of the mother after either C-section (11 of 14) or normal vaginal delivery and aseptically stored at 4^{o}C in sterile saline until processing. To isolate UCMS cells, the cord was rinsed several times with sterile saline and cut into 2-4 cm lengths. The interval between collection and isolation of UCMS cells ranged up to 48 hr. To isolate UCMS cells, the cord blood was drained and clots flushed from the vessels. Next, the vessels were stripped manually from cord segments, the wall of the cord was opened and the tissue immersed in an enzyme cocktail (such as Hyaluronidase, trypsin and Collagenase) for 45-60 min at 37^{o}C. This tissue was then crushed with forceps to release individual UCMS cells and large pieces of tissue were removed. The cells were pelleted by low speed centrifugation (250g for 5 min), suspended in fresh media and plated onto hyaluronic acid (HA)-coated plastic plates. Pilot work indicated that HA-coated plates optimized the attachment of UCMS cells.

Several different media support the growth of the adherent UCMS cells: a low serum, Adefined media@ (56% low glucose DMEM (Invitrogen), 37% MCBD 201 (Sigma), 2% fetal bovine serum (FBS, Mediatech, MTT 35-010-CV), 1x insulin-transferrin-selinium-A (ITS, Invitrogen), 1x ALBUMax1 (Invitrogen), 1x Pen /Strep (Invitrogen), 1x Amphotericin-B (ICN-1672348), 10 nM dexamethasone (Sigma), 50 µM ascorbic acid 2-phosphate (Sigma), 1 ng/ml epidermal growth factor (EGF, R&D systems), 10 ng/ml platelet derived growth factor-BB (PDGF-BB, R&D systems)) or the same media without ascorbic acid and dexamethasone. This media was used for all the experiments described here. Cells were incubated at 37^{o}C in an incubator with 5% CO_2 at saturating humidity. When cells reached 70-80% confluency or when numerous colonies were observed, the cells were detached with 0.25% trypsin-EDTA (Invitrogen), the trypsin was inactivated with fresh media, centrifuged for 250g for 5 min, and and replated on HA-coated plates 1:3. UCMS cells could be stored frozen at -135^{o}C in a freezing media containing 1:1 defined media: 80% FBS, 20% DMSO, or in 93% FBS, 7% DMSO, or 93% FBS and 7% glycerol, alone, after removal of the media. To demonstrate that human UCMS cells have the capacity for clonal expansion, clonal lines were established by the removal of a single cell to an individual well of a 24 well plate. The addition of a single cell per well was confirmed visually for each well using phase contrast microscopy. To determine whether human UCMS cells were karyotypically stable in culture, human UCMS cells from various passages (2-13) were selected for karyotypic analysis as described (33,34).

Total RNA

Total RNA was collected from human UCMS cells (various passages from 2-8). Human bone marrow mesenchymal stem cells (MSCs) were purchased from Cambrex and grown using the manufacturer=s protocol. Total RNA from MSCs was collected from passage 5. Undifferentiated human ESCs were obtained from WyCell (H1) using the supplied protocol. RNA was isolated either with TRIZOL LS using manufacturer=s protocol or by Qiagen Rneasy kit using manufacturer=s protocol. The RNA was treated with Dnase and stored at -80^{o}C. Total RNA from USSC and from nestin-positive islet precursors (NIPs) was supplied from ViaCell (Andrei Kritsov and Elizabeth Abraham, respectively).

Flow cytometric analysis of UCMS cells

The cells were analyzed by flow cytometry in two independent labs (KSU and ViaCell SRC). To stain the UCMS cells, the cells were lifted with trypsin and the trypsin was inactivated with fresh media. Approximately 10^6 cells were pelleted and resuspended in PBS and fixed with 4% buffered paraformaldehyde for 5 min at room temperature. To stain, the non-specific binding was blocked with PBS with 2% normal serum for 5 min, and then the cells were incubated with primary antibody for 45 min at room temperature. The cells were stained with a fluorescent secondary antibody for 30 min. Control cells were prepared by incubation with the secondary antibody alone. In each case, the cells were gently pelleted and washed with PBS rinses between each incubation step. For cytoplasmic antigens, the cells were permeablized by 100% cold methanol for 5 min. Antibodies used are shown in supplemental table 3.1.

RT-PCR

The cDNA was synthesized using 0.2-1 µg total RNA in the presence of Superscript II and oligo(dT)12B18 (both from Invitrogen). The DNA sample was treated with Rnase H and stored frozen until use. PCR was performed in a 20-µL reaction solution containing 2 µL 10x PCR buffer, 150 µmol $MgCl_2$, 10 µmol dNTP, 20 pmol primer, 1 µL 10 ç diluted cDNA and 1 U RedTag DNA polymerase (Sigma). The PCR conditions were as follows: 5 min at 94°C followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and final extension for 10 min at 72°C. Primer sequences are provided in supplemental table 3.2.

Focused Gene array analysis.

The non-radioactive GEArray cDNA expression array filters (HS-601.2; SuperArray Inc.) were used and hybridization procedures were as described by the manufacturer. Total RNA was checked spectrophotometrically or by gel. Biotin dUTP-labeled cDNA probes were specifically generated in the presence of a designed set of gene-specific primers using total RNA (4 µg per filter) and 200 U Moloney Murine Lukemia Virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). The array filters were hybridized with biotin-labeled probes at 60°C for 17 h. The filters were then washed twice with 2x saline sodium citrate buffer (SSC)/1% sodium dodecyl sulfate (SDS) and then twice with 0.1x SSC/1% SDS at 60°C for 15 min each. Chemilumilescent detection steps were performed by subsequent incubation of the filters with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate. The microarray experiments were performed multiple times with new filters and with RNA isolated from different passages from the

same umbilical cord and from different umbilical cords. The Z transformed data from all samples are in supplemental table 3.3. Here, the averaged z-transformed data from the fifty most highly expressed genes are presented (table 3.2)

Testing therapeutic effect of UCMS cells in Parkinsonian rats.

The hemi-parkinsonian rat model:

Brain damage was induced by a single stereotaxic injection of 6-hydroxydopamine (6-OHDA) as previously described (35). Anesthetized female Sprague-Dawley rats received a 4.0 μl injection of 32 mM 6-OHDA HCl (Sigma) dissolved in 0.02% ascorbate at 1 μl/min into the left medial forebrain bundle (Bregma -2.8; lateral 2.0; ventral 8.4mm; incisor bar 3.9mm ventral to interaural axis) or an injection of vehicle (sterile saline) four weeks prior to transplantation. The lesioned animals showed spontaneous rotations upon recovery from lesion surgery. Following surgery, rats were monitored for behavioral changes, weight loss and overall health. Four weeks following lesion surgery the animals received the human UCMS cell transplant or sham transplant (sterile saline). The efficacy of the 6-OHDA lesion was assessed by apomorphine-induced rotations every two weeks following the lesion and also by characterizing the staining for TH in the ipsilateral and contralateral striatum and ventral midbrain after sacrifice (sees below).

Behavioral Assessment

To test, rats were placed in round, opaque testing chambers and allowed to acclimate for 5 minutes prior to receiving the apomorphine injection (0.1 mg/kg, sc). The number

of rotation numberrotations and the direction was counted for a 30 minute period after giving apomorphine injection. Four weeks following the lesion, rats that did not exhibit at least 200 rotations/30 minutes toward the contralateral side after apomorphine treatment were excluded from the experiment. Recovery of function was evaluated over time (repeated measures) and pooled across groups to compare treatments.

UCMS transplantation.

At four weeks after brain lesion, animals were behaviorally tested and randomly assigned to transplantation or sham-transplantation groups. Approximately 1000 human UCMS cells in 1µl of the sterile PBS were injected over 5 min into the striatum (Bregma +0.5, Lateral +3.4, Ventral -5.0 mm from the surface of the brain). All animals received UCMS cells from the same passage (passage 9) on the same day. Before transplantation, the cells were lifted by a trypsin solution and were counted by a hemocytometer, and were adjusted to a final concentration of approximately 1000 cells per microliter. The cell concentration was confirmed before and after the injection to insure that approximately 1000 cells were delivered. Multiple control groups were used such as, 1. Lesioned rats with sham transplant (saline), 2. Non-lesioned rats with hUCMS cell transplant, 3. Non-lesioned rats with sham transplant (saline). At 6 and 12 weeks post-transplant, 12 rats were randomly selected from the experimental groups, anesthetized and sacrificed by transcardial perfusion with heparinized isotonic saline rinse followed by 10% buffered neutral formalin. The brains were removed, postfixed, and cryoprotected in 20% sucrose overnight. Frozen sections of the brains were cut

coronally at 40 ςm and the sections were collected into six sets of adjacent sections, each set consisting of every sixth serial section.

Tissue processing and Immunocytochemistry

Immunocytochemical (IC) detection of a single antigen was performed on one set of sections as previously described (32) and the adjacent sets of sections were held in reserve in a cryoprotectant solution(36). The free-floating tissue sections were stained with primary antibodies for anti-human nuclear antigen (mouse host, 1:30, Chemicon), TH (rabbit host, 1:2000, East Acres Biologicals), anti-rat CD-4 (mouse host, 1:500, Serotec), anti-rat CD-8 (mouse host, 1:500, Serotec), and anti-rat CD-11b (mouse host, 1:250, Sserotec), anti-rat CD161 (mouse host, 1:250, Serotec). The antigens were visualized either with diaminobenzidine (DAB) and hydrogen peroxide using a commercially available ABC kit (Vectastain) or with immunofluorescence. For immunofluorescence localization, 7-amino-4-methylcoumarine-3-acetic acid (AMCA) - Avidin D (Vector Laboratories) was used with the biotinylated secondary antibody. The IC-stained sections were mounted on subbed microscope slides, air-dried, and rinsed with distilled water. Immunofluorescence in the sections was observed using epifluorescence illumination with the appropriate filter combinations on a Leica DMRD microscope after clearing and coverslipping with glycerol containing N-propyl gallate (3 parts 2% N-propyl gallate in 0.1M Tris buffer, pH 9.0 and 7 parts glycerol).

Counting TH-positive cells in SN and VTA

The TH-positive DA neurons in substantia nigra (SN) and ventral tegmental area (VTA) were counted using a design-based unbiased stereological method and a

morphometry/image analysis system (Bioquant Nova Prime, R&M Biometrics). One set of brain sections of all the experimental animals were stained with anti-TH antibody and visualized with DAB. In each section the region of interest was outlined (SN and VTA), and the number of TH-positive cells per sq. mm. of that region were selected and semi-automatically counted. The SN and VTA TH-positive cells were counted, and the average number of TH-positive DA neurons per mm^2 was calculated for each animal.

Statistical analysis

All tissue manipulations were conducted in large batches to avoid batch to batch variability in tissue IC-staining. Data collection was conducted in experimenter-blind fashion. Following data collection, the group status was decoded prior to statistical analysis. In general, analysis of variance (ANOVA) was used to evaluate group differences. ANOVA was followed by post hoc testing using Scheffe=s test. Significance was set at $p<0.05$. Data is presented as mean c standard error of the mean (SEM) throughout.

Results:

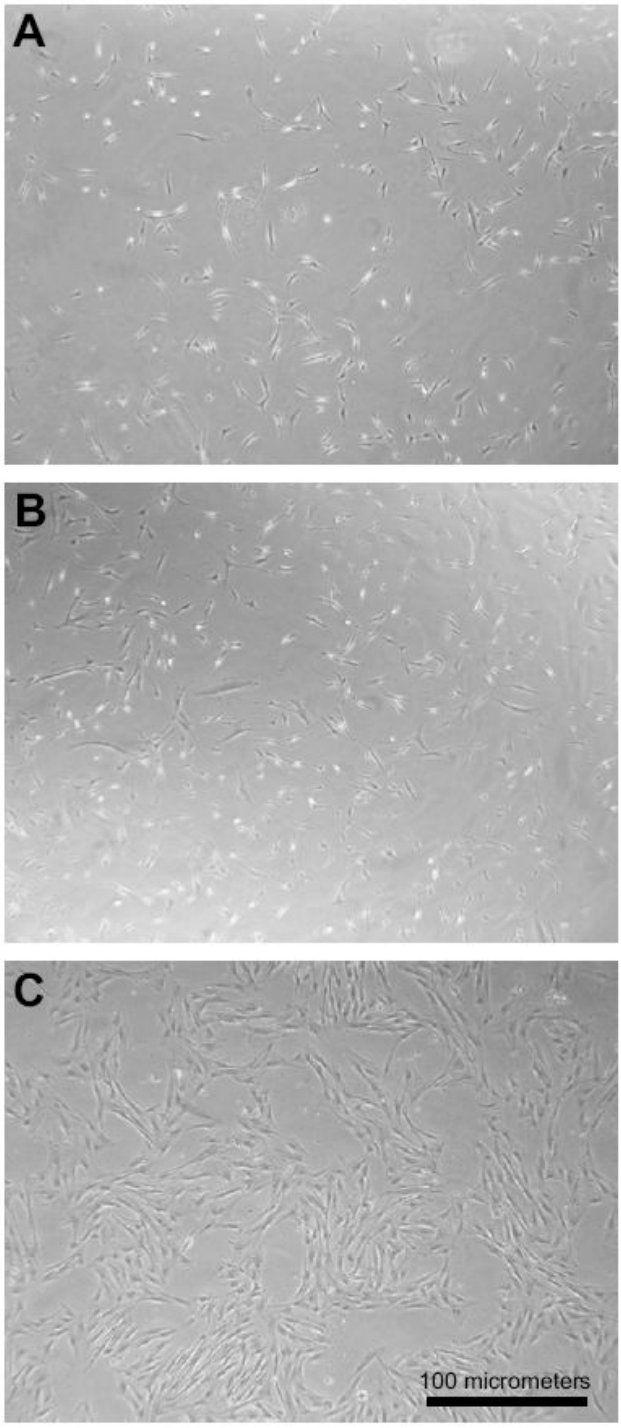
Characterizing UCMS cells.

Culture:

Human UCMS cells were isolated from 15 of 17 cords. We isolated 15-17x10^3 cells per cm of cord length (range: 10-50 x 10^3 cells per cm). The primary isolates were heterogeneous with mesenchymal-like cells possessing short and long processes as well as small round cells with a high nuclear to cytoplasm ratio (see supplemental figure 3.1). Individual cells could be clonally expanded. Through 10 passages, the populations are morphologically and immunophenotypically similar to the parent (data not shown). Clonally derived lines of human UCMS cells were confirmed by visual inspection that a single UCMS cell was delivered per well of a 24 well plate. The clonal capacity is estimated at a rate of 1-4 clonal populations derived per 48 attempts (data not shown). The clonal lines were not analyzed further. Human UCMS cells from four isolates were GTG-banded and chromosomes evaluated. None of the isolates were abnormal; the karyotype of the cells was stable through 13 passages (approximately 30 population doublings) (supplemental figure 3.2).

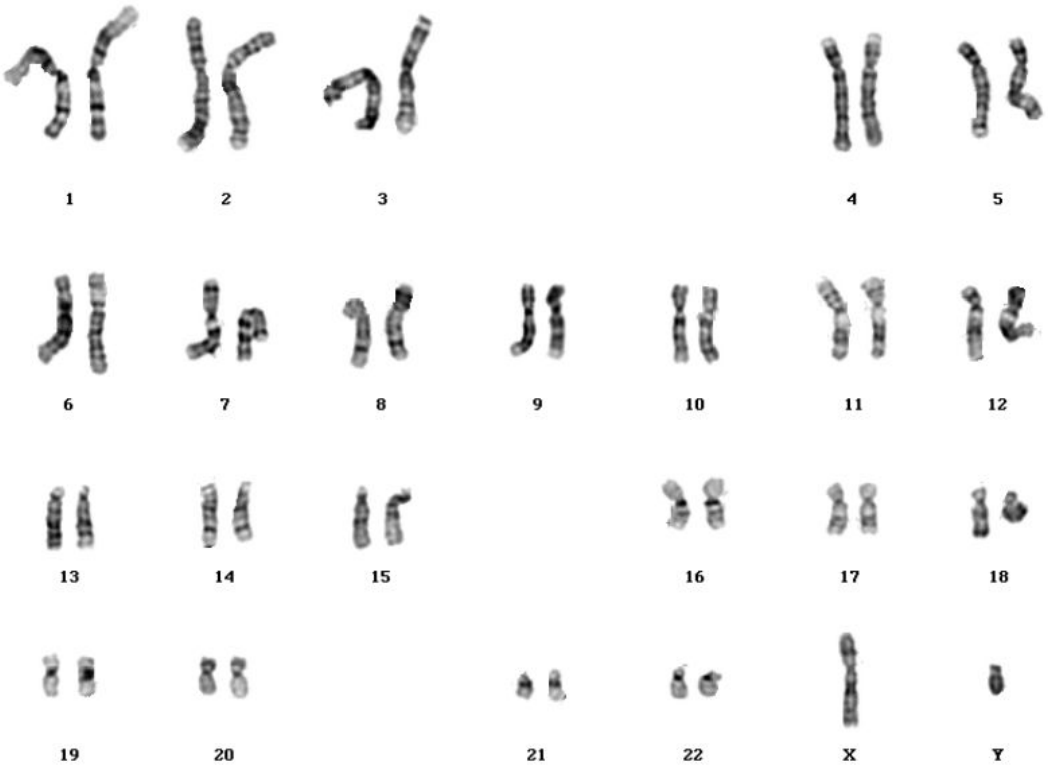
Flow cytometry and sorting of Hoechst dim cells:

A relatively large percentage of UCMS cells exclude Hoechst dye (Hoechst-low, average of 23%, N=4, range 8-32%). About 85% of the Hoechst-low stained are also CD44 positive (figure 3.1). Interestingly, sorting dim Hoechst cells did not enrich for CD44 stained cells (data not shown). To determine whether the Hoechst-low/CD44 positive population could be enriched further and expanded, the Hoechst-low



Supplemental Figure 3.1: Morphology of HUCMS cells. Most cells are mesenchymal in appearance, with a minor population of attached, small, round cells.

Supplemental Figure 2



Supplemental Figure 3.2: Representative HUCMS karyotype. Four HUCMS isolates were karyotyped (P10-P14), all were normal. This karyotype was isolated from a term human umbilical cord and passaged 11 times.

population was sorted using FACS. The sorted Hoechst-low staining cells appeared morphologically distinct from the Hoechst-bright population, with a higher percentage of the cells being the small round cells (see figure 3.1, middle). The enriched population of Hoechst-low cells was expanded through 5 passages and the percentage of Hoechst-low and CD44-positive cells was again assessed. At this time, the two populations looked morphologically similar and 23.5% of the cells were Hoechst-low sorted population and 20% in the Hoechst-bright population. Presently, enrichment for Hoechst dim cells by flow sorting is not maintained after passage. Previous work has indicated that Hoechst low staining is due to the presence of the ABCG2 receptor (37,38). As shown in figure 3.1, bottom, human UCMS cells showed the characteristic surface staining for the ABCG2 receptor and CD44. Verapamil (VP, 10 μM) was used as a non-specific blocker of the Hoechst dye exclusion channels. VP treatment partially blocked Hoechst dye exclusion from 24% dye excluding cells without VP to 7% dye excluding cells with VP (data not shown).

Flow cytometry:

Human UCMS cells from passage 4 and passage 8 from two different cord samples were characterized by flow cytometry (see Table 3.1; representative histograms of this data can be found in supplemental figure 3.3). UCMS cells stained positively for CD10 (average of 58% in P4 and 64% in P8), CD13 (average of 58% in P4 and 79% in P8), CD29 (average of 89% P4, 83% P8), CD44 (average of 85% P4, 74% in P8), CD90 (average of 70% in P4 and 41% in P8), HLA-1 (average 43% P4, 55% P8). A smaller number of UCMS cells express CD49e (average of 5% P4 and 5% P8) and CD105

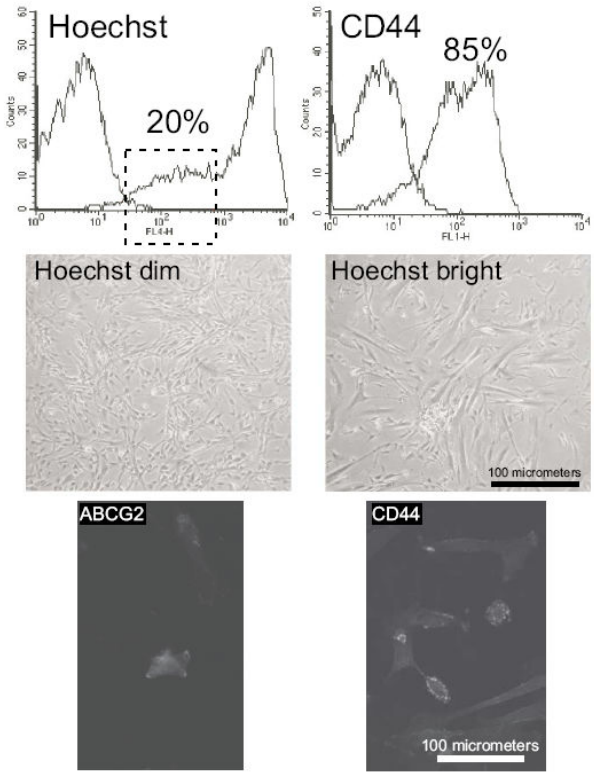
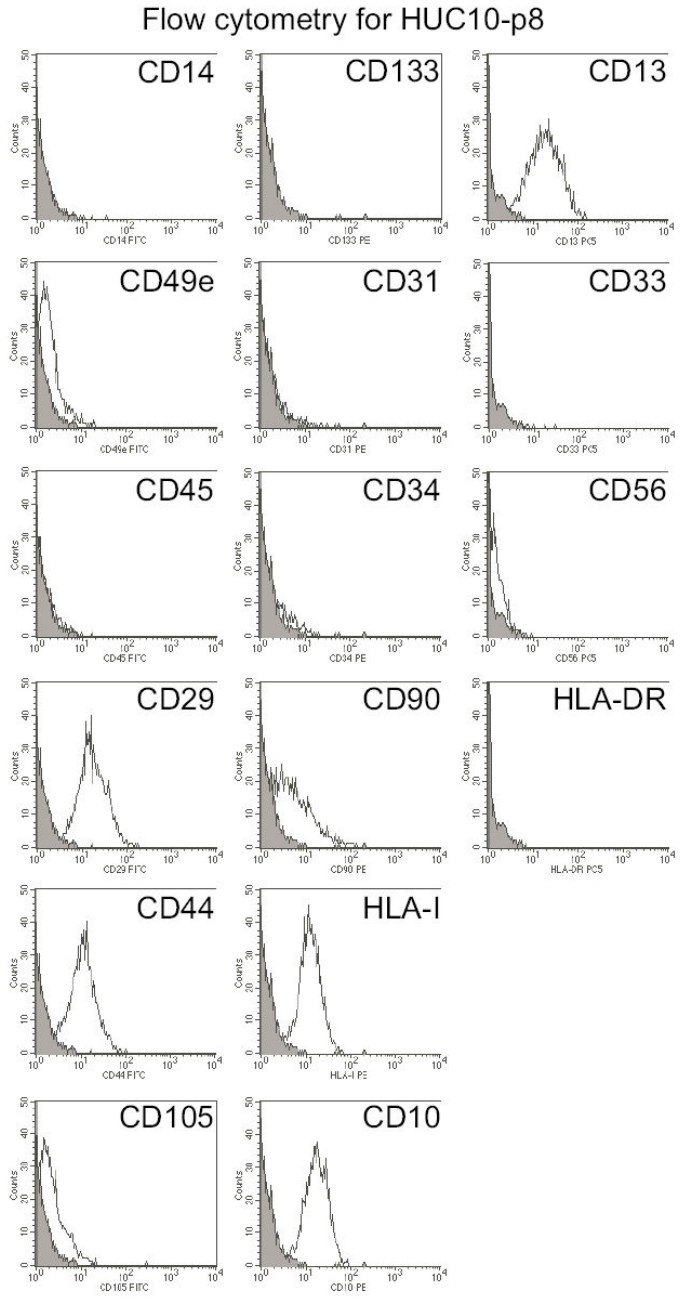


Figure 3.1: Flow cytometry and FACS of HUCMS cells. TOP LEFT: HUCMS cells stained for Hoechst 33342 revealed a population showed low fluorescence (see box) that comprised about 20% of the cells. When this population was sorted off and stained for CD44 expression, about 85% of the Hoechst-low cells express CD44. Hoechst-low (TOP RIGHT). Middle panel: The two populations, Hoechst-dim and Hoechst-bright were expanded in vitro. The morphology of the two populations is shown in the middle two panels: Hoechst low (LEFT) and Hoechst bright (RIGHT). Following expansion and passage the two populations were again Hoechst and CD44 stained. Immunocytochemistry was used to verify ABCG2 (the transporter thought to be responsible for Heoscht dye efflux)(Bottom left) and CD44 (a mesenchymal cell marker that is the hyaluronate receptor)(Bottom right).

Antibody	HUC10-P4	HUC10-P8	HUC11-P4	HUC11-P8	ave p4	ave p8
CD14	neg	neg	neg	neg	neg	neg
CD49e	6%	1%	4%	8%	5%	5%
CD45	neg	neg	neg	neg	neg	neg
CD29	93%	85%	85%	80%	89%	83%
CD44	81%	68%	88%	80%	85%	74%
CD105	10%	3%	4%	15%	7%	9%
CD133	neg	neg	neg	neg	neg	neg
CD31	neg	1%	neg	neg	neg	1%
CD34	neg	neg	neg	neg	neg	neg
CD90	84%	29%	55%	52%	70%	41%
HLA-1	58%	82%	27%	28%	43%	55%
CD10	35%	87%	80%	40%	58%	64%
CD13	49%	89%	66%	68%	58%	79%
CD33	neg	neg	neg	neg	neg	neg
CD56	neg	neg	neg	neg	neg	neg
HLA-DR	neg	neg	neg	neg	neg	neg

Table 3.1: Flow cytometry data



Supplemental Figure 3.3: Representative flow cytometry results. Flow cytometry data for one cord sample (HUC 10) at passage 8. The gray histogram is the cell staining following isotype control, the white (unfilled) histogram is the antibody-stained cells. The percentage of cells staining for each surface marker is provided in table 3.2.

(average of 7% in P4 and 9% in P8). CD14, CD31, CD33, CD34, CD45, CD56, CD133, and HLA-DR were not detected (Table 3.1). Note that the percentage of cells staining was fairly stable over four passages examined with no clear trends (Table 3.1).

Neural potential of hUCMS Cells.

A majority of undifferentiated UCMS cells stain for glial-cell line derived neurotrophic factor (GDNF, see Figure 3.2, top left). Immunocytochemistry for GDNF showed the staining evenly distributed through the cytoplasm of the cells (see Figure 3.2, top right). Neural induction, using the methods described by Woodbury et al. (39), resulted in a decreased number of cells expressing the marker for early neural progenitors, e.g., nestin, and an increased number of cells expressing the marker for catecholaminergic cells, tyrosine hydroxylase, a mature neural marker (see figure 3.4, bottom).

Gene array results for undifferentiated hUCMS cells.

Human UCMS cells express many different genes, suggesting that they are promiscuous in the production of genes as has been described for other stem cells such as hematopoietic stem cells and embryonic stem cells (40,41). The fifty genes that were most highly expressed by human UCMS cells are shown in table 3.2(note: the z-transformed data for all array elements is provided in supplemental table 3.1). Of the top fifty genes, genes found expressed in undifferentiated embryonic stem cells (ESCs) that were also expressed in the UCMS were: LIF receptor pathway, ESG1, SOX2, and TERT. Human UCMS cells express genes for proteins associated with morphogenesis: SHH, neuregulin1 and 4, Patched, SNA2, and WNT4. In addition, human UCMS cells express extracellular adhesion molecules: N-cadherin, V-cadherin, R-cadherin, integrin

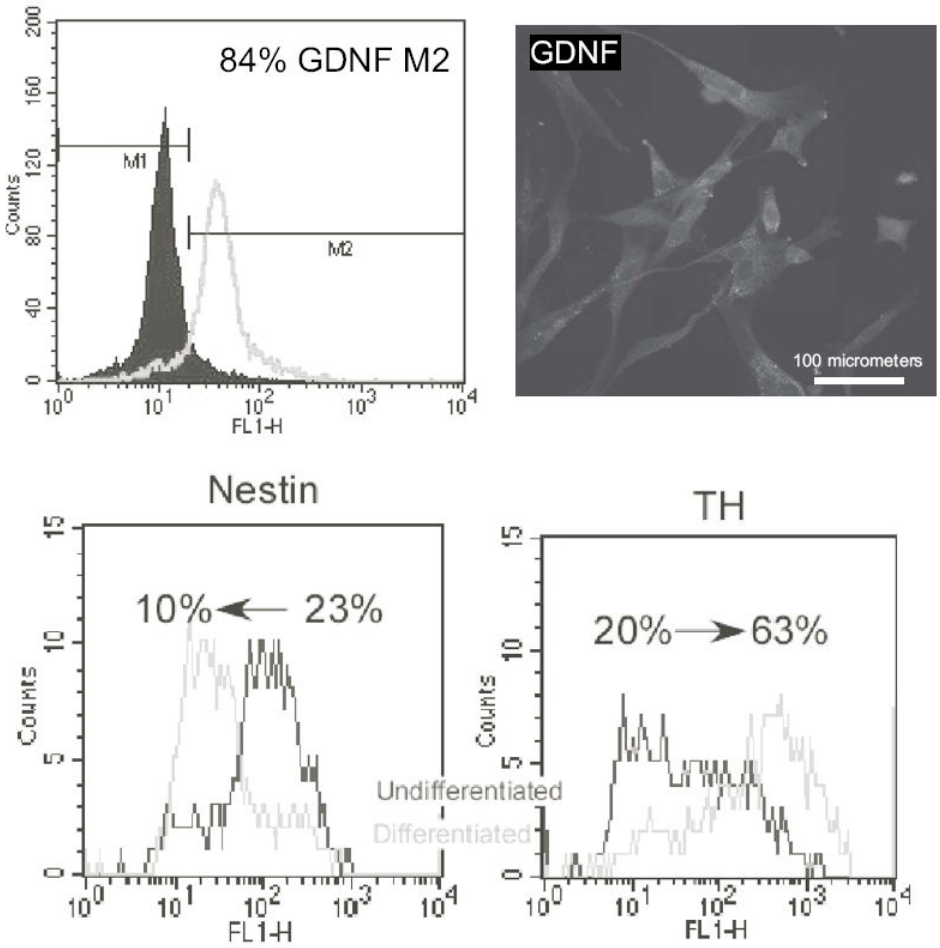


Figure 3.2: A. Production of glial cell line-derived neurotrophic factor (GDNF) by HUCMS cells. More than 80% of the cells are positive as revealed by flow cytometry (left). The immunocytochemical staining confirmed the flow cytometry findings (right). B. Effect of differentiation of UCM cells on expression of nesting and tyrosine hydroxylase staining. The expression of nestin, a marker of the primitive neural stem cells, decreases in the differentiated cells (10.03%) compared to undifferentiated cells (23.41%). The expression of tyrosine hydroxylase (TH), a marker of catecholaminergic neurons, increased in differentiated cells (63.23%) compared to the undifferentiated cells (20.1%).

Table 2. Fifty most highly expressed genes in human UCMS cells

Symbol	Description	Gene name	UCMS	NIP	MSC
ACTB	Beta Actin	b-Actin	-7.567	-5.690	-3.620
CDH2	Cadherin 2, N-cadherin (neuronal)	Cadherin 2	-6.597	-6.029	-4.494
PPIA	Homo sapiens peptidylprolyl isomerase A	Cyclophilin A	-5.753	-4.808	-3.060
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Integrin b1	-4.313	-4.418	-3.717
VEGF	Vascular endothelial growth factor	VEGF	-3.842	-5.368	-2.802
ACTG2	Actin, gamma 2, smooth muscle, enteric	ACTG2	-3.600	-2.890	-3.897
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	GP130	-2.987	-1.252	-2.399
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Integrin a5	-2.957	-0.607	-0.467
ACTA2	Actin, alpha 2, smooth muscle, aorta	ACTA2 (a-actin)	-2.754	-2.134	-4.757
SNAI2	Snail homolog 2 (Drosophila)	SNAI2	-2.155	-3.763	-2.433
ESG1	ESTs, Weakly similar to embryonal stem cell specific gene 1 [Mus musculus] [M.musculus]	ESG1	-1.958	-4.033	-2.173
INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	-1.830	-2.685	-1.966
GAPD	Glyoeraldehyde-3-phosphate dehydrogenase	GAPDH	-1.736	-2.624	-2.293
CTNND2	Catenin (cadherin-associated protein), delta 2 (neural plakophilin-related arm-repeat protein)	Catenin delta 2	-1.723	-2.392	-0.341
NRG1	Homo sapiens neuregulin 1 (NRG1), transcript variant HRG-beta2	Neuregulin 1	-1.647	-2.758	-0.728
VCAM1	Vascular cell adhesion molecule 1	VCAM-1	-1.580	-1.184	-1.027
CDH4	Cadherin 4, R-cadherin (retinal)	Cadherin 4	-1.312	-1.250	-0.458
SOX2	SRY (sex determining region Y)-box 2	SOX2	-1.235	-3.084	-0.931
TERT	Telomerase reverse transcriptase	TCS1	-1.221	-0.729	-0.013
TGFBR3	Transforming growth factor, beta receptor III (betaglycan, 300kDa)	TGFBR3	-1.203	-0.517	-0.073
CNTF	Ciliary neurotrophic factor	CNTF	-1.077	-2.526	-1.818
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	LFA1b	-1.051	-0.102	0.120
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	p27Kip1	-1.032	-0.393	-0.755
RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A	-0.851	-0.954	-1.090
PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGF2/SIS	-0.814	-0.851	-0.643
CD34	CD34 antigen	CD34	-0.687	-0.417	-1.883
HSPA9B	Heat shock 70kD protein 9B (mortalin-2)	Mortalin-2	-0.676	-0.643	-1.711
CCNE2	Cyclin E2	Cyclin E2	-0.649	-0.536	-1.118
EGR2	Human early growth response 2 protein (EGR2)	EGR2	-0.541	-0.873	0.172
LOC145957	Neuregulin 4	Neuregulin 4	-0.500	-0.514	-0.589
KRT8	Keratin 8	KRT8	-0.473	0.127	0.005
TGFBR1	Transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kD)	ALK-5/ACVRLK4	-0.385	-1.252	-0.175
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	Integrin aV	-0.351	-0.195	-0.866
IGF2R	Insulin-like growth factor 2 receptor	IGFR-2	-0.277	-0.193	0.009
WNT4	Wingless-type MMTV integration site family, member 4	WNT4	-0.261	-0.767	-0.175
IL6	Interleukin 6 (interferon, beta 2)	IL-6	-0.251	-0.589	-0.195
FGFR2	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor)	FGFR2	-0.251	-0.211	-0.180
ITGB5	Integrin, beta 5	Integrin b5	-0.211	-0.540	-0.058
CDH5	Cadherin 5, VE-cadherin (vascular epithelium)	Cadherin 5	-0.205	0.421	-0.059
CDK4	Cyclin-dependent kinase 4	Cdk4	-0.195	-0.657	-1.581
BMP1	Bone morphogenetic protein 1	BMP1	-0.169	-0.197	-0.600
INS	Insulin	INS	-0.115	-0.603	-0.875
CTNNA2	Catenin (cadherin-associated protein), alpha 2	CTNNA2	-0.107	-0.047	-0.032
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	Integrin a4 (VLA-4)	-0.106	0.097	0.221
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	Integrin a3	-0.081	0.021	0.154
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase (CNP)	CNP	-0.080	0.043	-0.208
FGF20	Fibroblast growth factor 20	FGF20	-0.076	-0.348	-0.831
PTCH2	Patched (Drosophila) homolog 2	Patched 2	-0.067	-0.236	-0.813
SHH	Sonic hedgehog homolog (Drosophila)	SHH	-0.064	-0.658	-0.516
NTRK3	neurotrophic tyrosine kinase, receptor, type 3	Trk C	-0.057	-0.387	-0.095

beta 1, integrin alpha 5, VCAM 1, integrin alpha 2 (CD49b), integrin alpha V, integrin beta 5, integrin alpha 4, and integrin alpha 3. Human UCMS cells express genes of proteins shown to have a neurotrophic effect: CNTF, VEGF, FGF20 and TRKC. Human UCMS cells express markers of three germ layer derivatives: mesoderm: ACTG2, ACTA2, BMP1, PDGFB; ectoderm: keratin 8, SHH, endoderm: insulin. In contrast to what was seen with gene array, neither flow cytometry (table 3.1) nor RT-PCR (data not shown) detected CD34.

RT-PCR:

The gene expression profile of UCMS cells and two other cell types, Nestin-positive Islet Precursor cells (NIP, a generous gift from Elizabeth Abraham, ViaCell, Cambridge MA) and bone marrow-derived mesenchymal cells (MSCs, Cambrex), and positive human control RNA (liver, placenta and muscle, BioChain, Hayward CA) was evaluated using RT-PCR to confirm or expand the gene array results. As shown in figure 3.3(left), human UCMS cells express markers of the undifferentiated state, Oct4, FGFR4, LIFR, Glut-1, ABCG2, Nanog, and Rex-1 as detected by RT-PCR. Human UCMS cells also express genes of proteins from all three germ layers and trophectoderm. Trophectoderm markers were Bex1/Rex3, Hand1, and HEB (figure 3.3, middle panel). Neurectoderm markers were nestin. Mesoderm markers were CXCR4, vimentin, CD44, collagen X, and Flk-1 (figure 3.3center and right panel). Endoderm markers were PDX1 and islet-1. Figure 3.3, right panel shows the expression of UMCS cells compared with MSCs. The two cell types were similar in the expression of OPN, ABCG2, CD44, LIFR, and collagen I, alpha1. One notable difference was the UMCS cells expressed Flk-1.

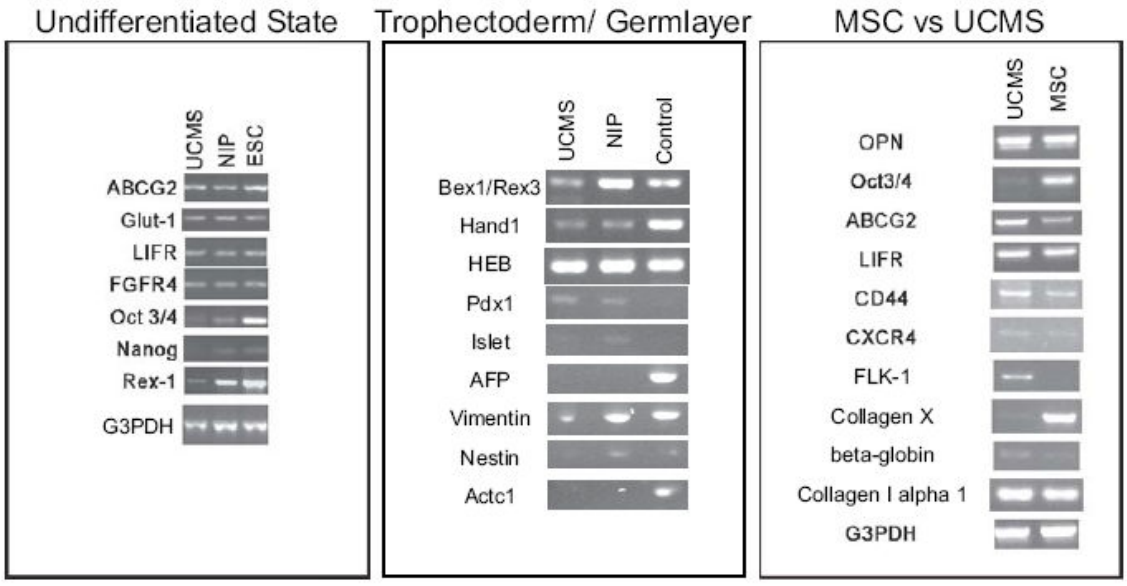


Figure 3.3 RT-PCR data comparing human UCMS cells to various other cell types with regard to markers for pluripotent cells (left), trophectoderm or germ layers (middle), and some miscellaneous markers (right). NIP: nestin positive islet percursors (ViaCell, Inc); Control RNA: placenta (top three rows), liver (rows 4-8), heart muscle (bottom row); MSCs: bone marrow mesenchymal stem cells (Cambrex), ESC: a mixed total RNA from several approved human embryonic stem cell lines (supplied by MSR).

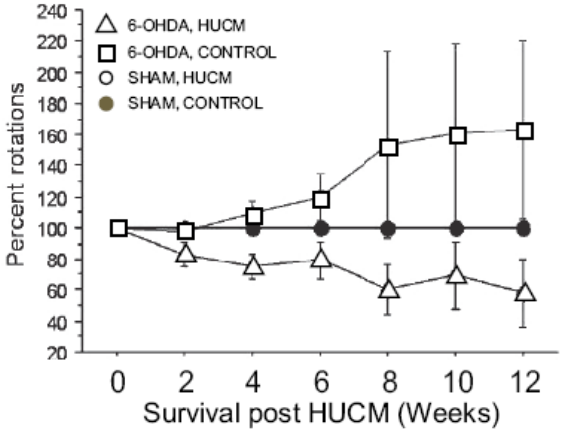
Effect of UCMS cells in Parkinsonian rats.

Behavioral assessment

The PD model rats that received the human UCMS cell transplant showed a significant decrease in the number of rotations compared to the sham transplanted animals (see Figure 3.4). Non-PD model animals with and without human UCMS cell-transplant did not rotate following apomorphine treatment (these data point overlay each other in Figure 3.4). In retrospect, we concluded that the grafts were unlikely to be effective in all animals, so post hoc, the animals that responded to the transplant, e.g., decreased their rotations following transplant of hUCMS cells, were identified (indicated by boxed region on right of Figure 3.4). The data from individual animals is shown in figure 3.4, right side. This data shows that at six weeks post transplant 2 out of 4 animals responded (decreased rotations by about 60%). At 12 weeks survival, 3 out of 4 animals responded to the transplant (decreased rotations by about 60%). One animal in the 12 week survival group decreased rotations by 90%. In contrast, in this experiment, the PD animals that were not transplanted with UCMS cells all showed a progressive degradation in performance following 6-OHDA. Behavior of the non-responder animals and the control animals was not significantly different.

Histological assessment of 6-OHDA lesion

Unilateral staining of TH in the dopaminergic (DA) neurons of SN and VTA on the contralateral side of the lesion shows the evidence of the toxic effect of 6-OHDA on the DA neurons of the ipsilateral side of the injection site (see figure 3.5, panel A). The loss of DA neurons is restricted to the SN and VTA. In addition, unilateral TH staining was



Lesion + HUCM	% rotations 0wk	6wk	12wk
SC 45	100	59	-
46	100	61	-
55	100	115	-
59	100	134	-
47	100	88	56
57	100	26	8
60	100	62	52
62	100	87	115

Figure 3.4: Effect of HUCMS cells on PD disease. LEFT: Apomorphine-induced rotations (percent change after transplantation surgery) in animals that received HUCM or sham transplants. The number of rotations made at the time of transplantation is taken as 100%. A significant decrease in the number of rotations was observe in the PD model rats (6-OHDA) that received HUCMS cell transplant compared to the control PD rats, that received saline injection. The lesion control rats (no lesion), both with HUCM cell transplant or saline injection, did not show rotatory behavior. RIGHT: Inspection of the rotatory behavior of individual PD model rats that received HUCMS cells revealed two populations: One population decreased their rotations (responders, indicated in box), the other population did not. Note that at 6 weeks, 2 of 4 rats responded and at 12 weeks, 3 of 4 rats responded to HUCMS cell transplant.

observed in the striatum indicating that the ipsilateral nigro-striatal tract is destroyed by 6-OHDA (not shown). Figure 3.5 (panel A) shows the TH-staining from the experimental groups at the short and long survival period. In the PD model rats, 6 weeks after human UCMS cell transplantation there was no TH-positive staining in the ipsilateral SN and VTA, and a slight degree of TH-immunoreactivity was observed at 12 weeks after transplantation (Fig. 3.5, panel A lower left). The PD model rats that received sham transplant did not show any changes in TH staining for TH at the longer survival period. In intact, non-PD model rats with or without UCMS cell-transplant, TH staining was observed in SN and VTA at both survival periods and no enhanced TH staining was observed around the transplant site (data not shown).

Counting TH-stained cells

We quantified the number of TH-positive cells in SN and VTA; these results are shown in figure 3.5 (panel B). There is a correlation with the behavioral recovery at twelve weeks, but not six weeks following surgery. At six weeks, there was no effect of the transplant on the number of TH cells in the SN or VTA on either the ipsi- or contralateral side (see figure 3.5, panel B). In contrast, at 12 week survival, the animals that responded to the transplant had significantly more TH stained cells in the SN and VTA ipsi- and contralateral to the transplant. In the non-responder animals, there was no difference in the numbers of TH-stained cells compared with the sham transplanted controls.

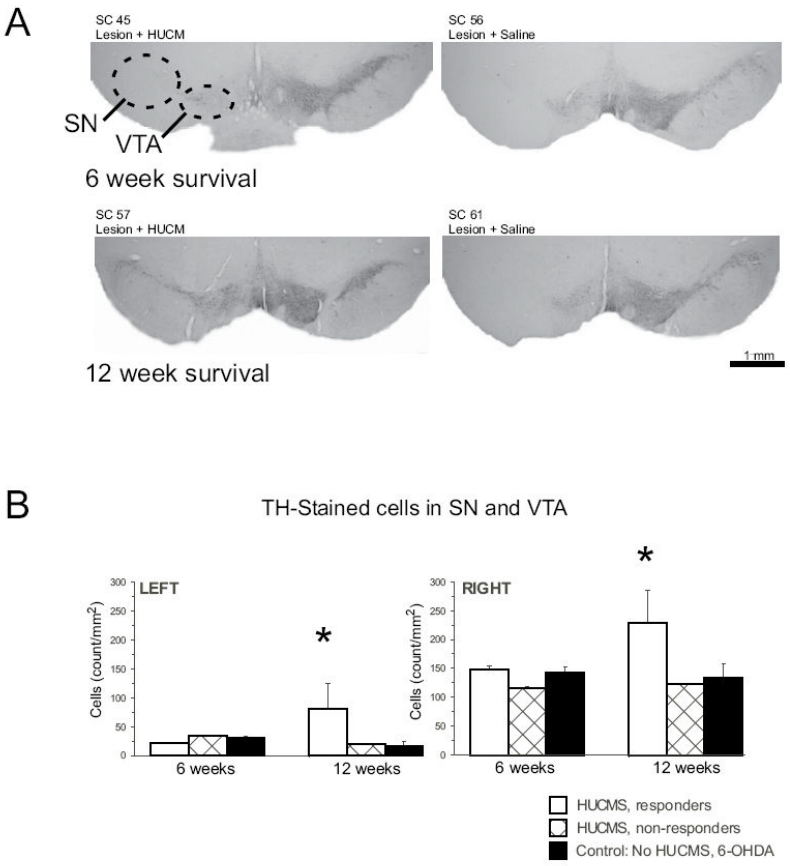


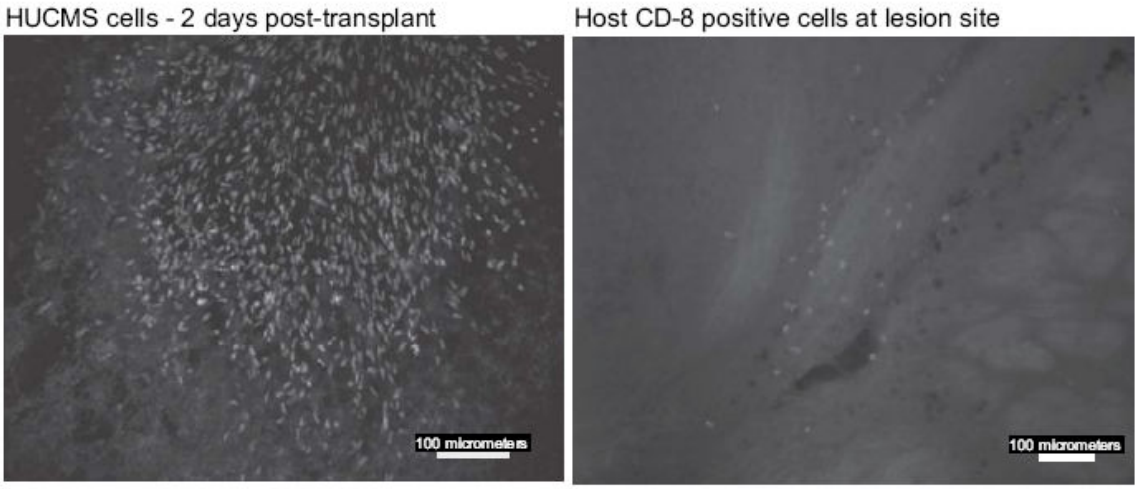
Figure 3.5: Effect of transplant on tyrosine hydroxylase staining. Panel A: Immunocytochemical staining for tyrosine hydroxylase in ipsi- (left) and contralateral (right) SN/VTA of transplanted and control animals at 6 week (top) and 12 week (bottom) survival periods. Panel B: Effect of HUCMS cell transplant on the number of tyrosine hydroxylase-positive (TH-positive) cells in midbrain of PD model animals. LEFT: In responder animals (animals demonstrating behavioral recovery following HUCMS cell transplant), but not in control animals or in non-responder animals, more TH-positive cells were found in the ipsilateral (LEFT) and contralateral (RIGHT) side 12 weeks following transplantation. This largest effect was seen in SN, but increases were also seen in VTA (data not shown).

Recovery of the transplanted cells

Human UCMS cells were recovered 2 days following transplantation by staining the brain sections with anti-human nuclear (AHN) antigen (see supplemental Fig. 3.4, left). However, no positive staining human cells were found at 6-weeks or 12-weeks following UCMS cell-transplant.

Immune-cell infiltration

One set of brain sections from selected animals of 6-week and 12-week survival groups were stained with anti-CD8 antibody (recognizes T-cytotoxic cells) and visualized by AMCA. Brightly stained CD-8 cells were seen in the lesion site (supplemental Fig. 3.4, right), but not in the transplantation site. Similar staining was observed with CD-4 (recognizes T-helper cells) and CD11b (recognizes activated microglial cells) antibodies.



Supplemental Figure 3.4: Immunocytochemical staining for human nuclei (LEFT) and host immune cells (RIGHT). LEFT: Two days following transplanting 50,000 HUCMS cells, many cells positively stained for human nuclear antigen were recovered surrounding the injection site. In contrast, no staining was found in the animals that received 1,000 HUCMS cells 6 or 12 weeks following transplantation. This observation suggested that the transplanted cells may be cleared by the host=s immune system. (RIGHT) To investigate the role of the host immune system, the brain sections surrounding the transplant and the lesion site were stained for immune cells (CD4, CD8, CD11b). From previous work (see background), we speculated that the immune response would be apparent at 6 weeks. Very few immune cells were detected in the transplantation site, more were found in the lesion site (CD8-positive cells shown at right).

Discussion

Here, human umbilical cord matrix cells were characterized *in vitro* and tested for their therapeutic potential in a Parkinson=s Disease rodent model. Three novel observations were made. First, UCMS cells have surface markers that indicate that they maybe a primitive MSC population. Second, UCMS cells express genes found in early development and genes associated with the three principal germinal layers, e.g., ectoderm, mesoderm and endoderm. Third, UCMS cells can reverse partially the Parkinsonian behavioral phenotype in rats. Taken together, these results suggest that the umbilical cord matrix may be an important, non-controversial source of primitive stem cells that may be harvested at low cost and in large numbers for cryogenic banking.

During development, primordial germ cells and hematopoietic cells migrate from the secondary yolk sac through the umbilical region into the embryo proper. Thus, the cell described here may be germ or hematopoietic type cells that were retained in the hyaluronic acid-rich extracellular matrix. Alternatively, the cell described here may be a support cell (stroma cell) like those found adjacent to other stem cells (as is in the bone marrow cavity, intestinal crypts, etc). It is important to note that other labs have isolated stem cells from various umbilical or placenta structures as well as the amniotic fluid. Clearly, these primitive structures, like umbilical cord blood, are important, non-controversial sources of stem cells. From the characterization of the cells derived from other sources , e.g., umbilical vein (42,43), perivascular region (22), cord blood (8,25,44), amniotic fluid (45,46) and placenta (47-51), we hypothesize the cells

described here may be recovered in smaller number and less frequently from other primitive fetal structures.(47-51) Further work will be needed to confirm this hypothesis.

Properties of human UCMS cells.

Previously we reported that UCMS cells could be started via explant culture of the minced cord matrix. Here we report a more efficient method of starting the cultures via enzymatic degradation of the extracellular matrix to release the cells from the Wharton=s jelly. UCMS cells were isolated from 78% of the cords, including two cords that were refrigerated for 24 hrs prior to isolation (cells were isolated from one of the two refrigerated cords). Since the improved procedure has been used, the isolation of UCMS cells has been 100%. Many cells are found in the initial extraction from the cord and, this provides the opportunity to sort off special populations early on. Adherent cells were obtained, expanded, passaged and could be frozen, thawed and cultured. The attached UCMS cells had fairly uniform morphology that is similar to MSCs isolated from bone marrow. Flow cytometry revealed that 20-30% of low passage UCMS cells exclude Hoechst dye. The Hoechst dye exclusion was reduced by verapamil treatment, an inhibitor of some of the ABCG protein family. Flow sorting of Hoechst low (also called side population cells) is a technique used to isolate primitive hematopoietic stem cells (HSCs) and other stem cells (52,53). Thus, Hoechst staining, like aldehyde dehydrogenase staining, may be a vital staining marker of primitive stem cell populations. Our attempts to further enrich a primitive population by flow sorting and in vitro expansion was only partially successful: after five passages we failed to increase in the number of SP cells beyond 32%. This suggests that the current culture conditions

can not maintain UCMS cells in the most primitive state or, perhaps, some factor is needed to maintain the undifferentiated state. Work by Sarugaser et al. on perivascular umbilical cord cells suggested that their grown was regulated by paracrine factors (22). In contrast to what is found with UCMS cell culture, the percentage of population (SP) cells in Lin- adult peripheral blood is about 0.1% (54) and the percentage in unfractionated umbilical cord blood is about 0.2% (53). Thus, cord matrix has the highest percentage of side population cells known (ranging from 8-32%). Flow cytometry of the UCMS cells revealed about 85% of UCMS cells stain CD44, the hyaluronic acid (HA) receptor. CD44 is a marker found on other stem cell populations including MSCs and neural stem cells.

Flow cytometric analysis indicates that UCMS cells have MSC markers.

As reported by Pittenger et al.(55) and subsequently by others, MSCs have a characteristic set of surface markers including cluster of differentiation makers (CD), for example, CD29, CD44, CD71, CD90. The consensus marker set for MSCs include SH2 (endoglin, CD105), SH3 (CD73). UCMS cells have many but not all the surface markers on adult MSCs. For example, UCMS cells stain for CD13, CD29, CD44, CD90, and CD10. In contrast, UCMS cells do not stain (or stain at a low proportion) for SH2 (average of 7%, range of 3-15%), or CD49e (average of 4%), or HLA-I (43%). Saraugauser et al. (22) reported that perivascular cells isolated from the umbilical cord had higher expression of SH2 and SH3 antigens compared to what we report here. This difference may be due to the different growth conditions used between the two laboratories, differences in the antibodies used, or technical differences in gating.

Alternatively, two different populations of cells are isolated (as suggested by Sarugauser et al.). To summarize, a significant percentage of UCMS cells lack surface markers found on bone marrow derived MSCs, e.g., SH2 and HLA-1. This suggests that UCMS cells may be a more primitive population of MSC cell and are perhaps similar to the more rare multipotent adult progenitor cell described by Verfaillie=s lab at the University of Minnesota (9,10).

RT-PCR results.

UCMS cells are pleiotropic, expressing many genes, such as those found in ESCs and other primitive stem cells, and genetic markers of all three germ layers, ectoderm, endoderm and mesoderm. This finding is similar to what has been reported for other primitive stem cells and suggests that gene silencing or DNA methylation maybe important mechanisms to regulating UCMS differentiation. Currently, we have no data to address the mechanisms regulating UCMS differentiation.

Gene Array results

Of the 50 most highly expressed genes, the genes expressed by UCMS cells fall roughly into several categories: those associated with the undifferentiated state of ESCs, extracellular adhesion molecules, neurotrophic effects, morphogenesis and those associated with the three germ layers. Mesenchymal cells derived from umbilical cord blood have recently been analyzed by microarray and express genes often found in mesenchymal stem cells derived from other sources (56,57). Technical differences such as media used to expand the cells, length of time in culture, differences in gene

array methodologies prevent the comparison of extant data sets. Such difficulties have been noted previously. To our thinking, comparisons of gene array data are best made from data collected within a single laboratory until such time that there is agreement on standardized media, serum, growth conditions, etc.

Transplantation of UCMS into PD rats

As widely reported previously, unilateral injection of 6-OHDA into the medial forebrain bundle caused destruction of the nigrostriatal dopaminergic neurons in the ipsilateral SN and VTA. This destruction produced motor abnormalities analogous to those seen in Parkinson=s disease (PD). The hemi-parkinsonian rats had motor deficits indicated by rotatory behavior following apomorphine injection. The rats had to meet a criterion of 200 apomorphine-induced rotations for inclusion in our experiment. The groups were randomly divided into those that received a transplantation of hUCMS cells and a control group. Approximately 1000 hUCMS cells were transplanted into PD model rats in a single day from a single aliquot of freshly prepared cells. None of the animals received immunosuppression therapy because our preliminary work where we transplanted pig UCMS cells showed that the transplanted cells were not rejected (31,32). Moreover, pig UCMS cells survived and proliferated up to 8 weeks after transplantation.

Here, the transplanted animals showed a significant recovery as a group. As individuals, when the data was inspected post hoc, it was revealed that more than 50% of the transplanted animals demonstrated a behavioral recovery of about 50% reduction

in the rotatory behavior. One of the animals showed a greater reduction of rotatory behavior. Following sacrifice, immunocytochemistry for TH revealed no changes in the number of cells in the SN or VTA at six week survival periodB this is despite the fact that behavior recovery was observed and accounting for the animals that responded to the transplantation. In contrast, the responding transplanted animals that survived 12 weeks, there was a significant increase in the number of TH-stained cells recovered in the SN and VTA. Most interestingly, the number of TH-stained cells was increased ipsilateral and contralateral to the lesioned nigrostriatal pathway. The mechanism that underlies this observation is unknown. Hypothetically, a rescue of dying nigrostriatal TH neurons may be stimulated by growth and neurotrophic factors released by the UCMS cells transplant. The hUCMS cells produce significant amounts of GDNF (figure 3.4), one of the most potent trophic factors for dopaminergic neurons, and fibroblast growth factor 20 (table 3.3), which is thought to be an important survival factor for dopaminergic neurons that is preferentially expressed in rat brain substantia nigra (58). On the other hand, UCMS cells may have properties similar to bone marrow stromal MSCs to inhibit the host immune response. Dampening the host immune response may limit secondary damage and permit moribund neurons to recover.

We could not recover the transplanted hUCMS cells; the various reasons for this could be 1. The graft cells are cleared by the host immune system and 2. The low-density graft was not able to support itself and the cells could not survive for long time. Due to the fact that we found immune cells at the lesion site, but not the transplantation site, it is not clear if the transplanted cells were scavenged by immune cells. Although, there is

a possibility that the transplanted cells were cleared and the immune cells left the area, as previous work with fetal pig mesencephalic tissue grafted into the rat brain indicates that the grafted tissue is rejected in about 4-6 weeks (59). We have not direct or indirect evidence for a host immune response to the transplanted cells.

That the low-density graft was not recovered may also be due to death of the grafted cells. Again, we have no direct evidence that the graft cells died, e.g., there was no cellular debris, increase astrocyte infiltration, etc, that would indicate that the graft cells died. That we can recover human graft cells up to one week following transplantation and recover pig umbilical cord matrix cell micrograft up to 8 weeks following transplantation into rat (31,32) suggests that our transplantation protocols are responsible for the problems recovering the grafted cells.

We observed a significant decrease in apomorphine-induced rotations in the PD model rats. There was a significant decrease in the number of rotations 4 weeks following transplantation that continued to 12 weeks after transplantation. Irrespective of the survival period, out of 8 PD models that were transplanted with hUCMS cells, 4 animals showed 40-50% decrease in number of rotations, 1 animal showed 90% decrease and 3 did not respond. The 3 animals that did not respond to the transplantation therapy showed a slight increase in the number of rotations, which can be attributed to the clearance of the graft cells earlier than the others. The TH-positive DA neurons in SN and VTA show a valid correlation between the number of cells and apomorphine-induced rotations i.e. more the number of TH-positive cells in SN and VTA lesser the

number of rotations. Therefore, the behavioral recovery of the PD model animals could be due to rescue of the degenerating DA neurons of SN and VTA, which could be mediated by various trophic factors (60-63). Further analysis is due to prove this hypothesis. The above data from UCMS cells indicate that these cells may be therapeutically useful in treating central nervous system disorders.

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Supplemental Table 1. RT-PCR Primers

Gene	AA#	Size	FPrimer	Rprimer	AT	Source
ABCG2	NM_004827	684	F: 747 gtttatccgtggtgtgtctgg 767	R: 1398 ctgagctatagaggcctggg 1379	55	Laboratory of MSR
Nanog	NM_024865	158	F: 432 caaaggcaaacaacccactt 451	R: 589 tctgctggaggctgaggtat 570	55	Laboratory of MSR
OCT3/4	NM_002701	171	F: 662 cttgctgcagaagtgggtggaggaa 686	R 830 ctgcagtgtgggtttcgggca 810	55	Laboratory of MSR
Rex-1	NM_174900	559	F: 1283 tgaaagcccacatcctaacg 1302	R: 1839 caagctatcctcctgctttgg 1819	55	Laboratory of MSR
Glut-1	P11166	416	F: 2182 atctcttcctacccaaccac 2201	R: 2597 tctcactcccatccaaacc 2579	55	Weiss
FGFR-4	NM_002011	194	F: 1049 gtttcccctatgtgcaagtcc 1069	R: 1243 gcgctgctgcggtccatgt 1225	55	(Tartaglia et al., 2001).
AFP	NM_001134	416	F: 1254 aaatacatccaggagagcca 1273	R: 1670 ctgagcttggcacagatcct 1651	55	(Lafuste et al., 2002)
Actc1	J00071	296	F: 1319 cactgaatccgcctacctcc 1338	R: 1615 tcgtgcctctacaccagacc 1596	55	(Karkkainen et al., 2002)
human islet-1	NM_002202	174	F: 940 gcaaggacaagaagcgaag 958	R: 1114 ttccaaggtggctggtaac 1096	55	Weiss
Nestin	NM_006617	338	F: 852 872 cagcgttggaacagaggttgg 872	R: 1240 tggcacaggtgtctcaagggtag 1218		(Ignatova et al., 2002)
Pdx1	NM_000209	359	F: 421 gtcctggaggagcccaac 438	R: 780 gcagtcctgctcaggctc 763	55	(Itkin-Ansari et al., 2000)
vimentin	BC000163	417	F: 1202 gacactattggccgcctgcggatgag 1220	R:1619 ctgcagaaaggcacttgaaagc 1598	55	(Nishio et al., 2001).
Bex1/Rex3	NM_018476	269	F: 390 acaggcaaggatgagagaag 409	R: 659 cccacgtaaacaagtgacag 640	55	Laboratory of MSR
Hand1	NM_004821	273	F: 1362 tgcctgagaaagagaaccag 1381	R: 1635 atggcaggatgaacaaacac 1616	55	(Knofler et al., 2002)
HEB	NM_003205	165	F: 1771 actgaaaacaaagaaaaggatgaaacc 1798	R: 1936 ccctttctatcttctgttcagggttc 1911	55	(Knofler et al., 2002)
LIFR	NM_002310.2	496	F: 365 ctggaacaggccgtggtac 383	R: 861 actccactcttogagaccag 842	55	(Knight et al., 1999)
CXCR4	NM_003467	508	F: 239 actggcattgtgggcaatgg 258	R: 747 caatagcaggacaggatgac 728	58	Lu et al., Exp. Hematol. 30:58-66, 2002
Flk-1	NM_002253	794	F: 887 ctggcatggtcttctgtgaagca 909	R: 1681 aataccagtggatgtgatgcgg 1660	55	Meister B, Eur J Cancer. 1999 35(3):445
collagen X	NM_000493.	588	F: 1529 ctggcatagcaactaagggc 1548	R: 2116 ctgagaaagaggagtggacatac 2094	55	Weiss
collagen I, alpha1	NM_000088	108	F: 3899 tgacctcaagatgtgccactc 3919	R: 4006 ccagtctccatgttgcagaag 3986	55	Weiss
OPN	NM_000582	318	F: 307 ccatctcagaagcagaatctcc 328	R: 624 cagtccataaaccacactatcacc 601	55	Weiss
CD44	NM_000610	688	F: 1535 ttcttcaacccaatctcacacc 1556	R: 2222 gcttttcttctgcccacac 2203	55	Weiss
beta-globin	NM_000518	376	F: 112 tggatgaagttggtggtgagg 132	R: 487 tacttgtgggccagggcatta 467	58	Lu et al., Exp. Hematol. 30:58-66, 2002
G3PDH	NM_002046	890	F: 86 tgaaggtcggagtcaacggatttggt 111	R: 1068 catgtgggccatgaggtccaccac 1045	55	Laboratory of MSR

Supplemental Table 2. Antibodies

Primary antibody	Source	Catalog no.
CD14 -FITC	BD	555397
CD49e -FITC	Beckman	IM1854
CD45 -FITC	BD	555482
CD29 -FITC	Beckman	IM0791
CD44 -FITC	Beckman	IM1219
CD105 -FITC	Serotec	MCA1557F
CD133 -PE	MACS - Miltenyi Biotec	130-080-801
CD31 -PE	Beckman	IM2409
CD34 -PE	BD	555822
CD90 -PE	Beckman	IM3600
HLA-1 -PE	BD	555553
CD10 -PE	Beckman	IM1915
CD13 - PC5	Beckman	IM2639
CD33 - PC5	Beckman	IM2647
CD56 -PC5	BD	555517
HLA-DR -PC5	Beckman	IM2659
TH	Biogenesis	9360-0004
GDNF	Chemicon	ab5252p
Nestin	Chemicon	ab5922
ABCG2	Chemicon	MAB4155

Supplemental Table 3. Focused gene array

Symbol	Gene name	UCMS 8 p5	UCMS 8 p5	UCMS 8 p5	UCMS 9 p2, M	UCMS 9 p2, F	UCMS 9 p4, M	AVE UCMS	NIP p2	MSC p4
ACTB	b-Actin	8.1071	6.3971	5.4179	9.3387	11.7821	4.3602	7.5672	5.6902	3.6198
CDH2	Cadherin 2	7.4977	7.7578	8.1848	6.0011	4.8276	5.3104	6.5966	6.0286	4.4944
PPIA	Cyclophilin A	7.2789	5.4833	4.0024	5.9424	7.2225	4.5889	5.7531	4.8076	3.0599
ITGB1	Integrin b1	4.8330	5.6106	4.2132	3.5086	4.9059	2.8079	4.3132	4.4178	3.7174
VEGF	VEGF	4.0533	2.9016	1.8096	6.1146	4.4501	3.7244	3.8423	5.3675	2.8019
ACTG2	ACTG2	2.6870	2.1962	1.3390	5.0263	5.4871	4.8658	3.6002	2.8903	3.8965
IL6ST	GP130	3.6396	4.5927	3.9890	1.9870	1.1726	2.5398	2.9868	1.2524	2.3987
ITGA5	Integrin a5	4.2446	2.8709	1.3230	3.9215	3.7181	1.6621	2.9567	0.6067	0.4667
ACTA2	ACTA2 (a-actin)	2.2917	2.6109	1.8583	3.2761	3.5432	2.9447	2.7541	2.1337	4.7571
SNAI2	SNAI2	1.9290	1.6867	2.2618	2.1340	1.7419	3.1785	2.1553	3.7633	2.4331
ESG1	ESG1	1.3070	1.2749	2.7225	0.9255	2.2793	3.2409	1.9583	4.0325	2.1727
INHBA	INHBA	1.7208	2.4230	2.2321	0.7581	1.8256	2.0207	1.8301	2.6845	1.9658
GAPD	GAPDH	2.3948	2.0772	1.1511	1.7130	1.4291	1.6508	1.7360	2.6242	2.2930
CTNND2	Catenin delta 2	2.4595	1.8566	1.4417	1.4857	1.2516	1.8426	1.7229	2.3919	0.3406
NRG1	Neuregulin 1	0.9171	1.2180	0.7312	1.6763	1.9624	3.3762	1.6469	2.7579	0.7279
VCAM1	VCAM-1	3.2772	2.7061	1.5851	0.7271	-0.2419	1.4251	1.5798	1.1643	1.0273
CDH4	Cadherin 4	0.8815	1.4347	0.8330	1.7802	1.4803	1.4648	1.3124	1.2497	0.4583
SOX2	SOX2	0.9106	0.8767	2.0546	0.9787	0.6606	1.9311	1.2354	3.0644	0.9308
TERT	TCS1	2.0394	1.1201	0.8262	1.3391	1.3171	0.6810	1.2205	0.7291	0.0133
TGFBR3	TGFBR3	1.3563	1.1103	0.9986	1.6750	0.8869	1.1908	1.2030	0.5170	0.0733
CNTF	CNTF	1.1343	0.7278	0.5632	0.9581	1.5819	1.4994	1.0774	2.5255	1.8175
ITGA2	LFA1b	1.1706	1.1438	0.4935	0.7518	0.6756	2.0682	1.0506	0.1022	-0.1196
CDKN1B	p27Kip1	0.8590	1.1000	0.4745	0.6889	1.8511	1.2180	1.0319	0.3930	0.7552
RPL13A	RPL13A	0.6527	0.5987	0.0823	1.4278	1.1985	1.1473	0.8512	0.9541	1.0897
PDGFB	PDGF2/SIS	1.2951	1.5128	1.3884	0.6053	-0.2211	0.3008	0.8135	0.8512	0.6426
CD34	CD34	0.8660	0.4193	0.3148	0.9653	0.9727	0.5844	0.6871	0.4169	1.6829
HSPA9B	Mortalin-2	0.8639	0.9029	0.0543	0.9476	0.8713	0.4174	0.6763	0.6434	1.7110
CCNE2	Cyclin E2	0.2907	0.3206	0.1693	0.9902	1.0675	1.0531	0.6486	0.5355	1.1181
EGR2	EGR2	1.0196	0.6318	0.1949	0.4139	0.5866	0.3965	0.5406	0.8731	-0.1722
LOC145957	Neuregulin 4	0.0658	0.2674	0.0785	0.6836	0.6741	1.2313	0.5001	0.5137	0.5886
KRT8	KRT8	0.1194	0.2402	0.0422	0.8010	1.3310	0.3044	0.4731	-0.1274	-0.0053
TGFBR1	ALK-5/ACVRLK4	0.8143	0.4104	0.5457	0.0693	0.1082	0.3608	0.3848	1.2524	0.1751
ITGAV	Integrin aV	0.2013	0.8486	0.4338	0.3203	0.1630	0.1393	0.3511	0.1946	0.8663
IGF2R	IGFR-2	0.6798	0.5204	-0.0345	0.1024	0.4277	-0.0335	0.2771	0.1932	-0.0092
WNT4	WNT4	1.0309	0.6832	0.1312	-0.1835	-0.1216	0.0238	0.2607	0.7667	0.1754
IL6	IL-6	-0.1615	-0.2220	-0.2406	0.0120	-0.0512	2.1701	0.2511	0.5893	0.1950
FGFR2	FGFR2	0.2225	0.4325	0.0408	0.1750	0.2533	0.3799	0.2507	0.2112	0.1804
ITGB5	Integrin b5	0.3861	0.5178	0.0335	0.1377	0.1055	0.0842	0.2108	0.5402	0.0582
CDH5	Cadherin 5	0.1472	0.0862	-0.0800	0.5150	0.3856	0.1785	0.2054	-0.4210	0.0590
CDK4	Cdk4	0.1895	0.2240	0.4571	0.1971	-0.1006	0.2033	0.1950	0.6568	1.5812
BMP1	BMP1	0.0197	-0.0436	0.0089	0.1737	0.5753	0.2823	0.1694	0.1971	0.5996
INS	INS	-0.0760	0.1269	0.4382	0.0392	0.0955	0.0684	0.1154	0.6032	0.8746
CTNNA2	CTNNA2	-0.1469	-0.0113	-0.0343	0.3273	0.3742	0.1314	0.1067	0.0471	0.0317
ITGA4	Integrin a4 (VLA-4)	0.1260	0.1629	-0.2527	0.3921	0.4158	-0.2098	0.1057	-0.0972	-0.2209
ITGA3	Integrin a3	-0.0550	-0.0588	-0.1328	0.4253	0.2986	0.0092	0.0811	-0.0207	-0.1539
CNP	CNP	0.0151	0.0407	0.0125	0.1696	0.1452	0.0949	0.0797	-0.0434	0.2075
FGF20	FGF20	0.1154	0.0175	-0.1156	-0.1004	0.2657	0.2704	0.0755	0.3475	0.6312
PTCH2	Patched 2	0.1539	0.0098	-0.0476	0.1965	0.0194	0.0670	0.0665	0.2356	0.8125
SHH	SHH	-0.0893	0.0897	0.1253	-0.0029	0.1260	0.1340	0.0638	0.6580	0.5159
NTRK3	Trk C	0.1109	-0.1035	0.0319	0.2378	0.1328	-0.0703	0.0566	0.3872	0.0954
MDM2	Mdm2	-0.1339	-0.0879	0.0481	0.1530	0.2303	0.1086	0.0530	0.4464	-0.0015
CXCL12	SDF1	-0.0492	0.1266	-0.0646	0.0121	-0.0130	0.3022	0.0524	0.4150	0.1191
FOXH1	FOXH1	0.0189	-0.0239	-0.1117	0.2249	0.1904	-0.0418	0.0428	-0.1591	-0.1158
THY1	THY1	0.0305	-0.1394	0.0844	0.0572	-0.0139	0.0588	0.0129	-0.0848	0.1441
C3orf4	C3orf4	0.0164	0.0302	-0.1388	0.0438	-0.0301	0.1286	0.0083	0.1139	0.3501
TGFB1	TGFb1	-0.2116	-0.4692	-0.3398	0.2852	0.7870	-0.1330	-0.0136	0.0200	-0.0078
PTPRC	cd45	0.0236	0.0097	0.0231	-0.0676	-0.1500	0.0623	-0.0165	0.0968	0.0374
WNT5A	WNT5A	0.0019	-0.1123	-0.1775	-0.0556	0.1011	0.0677	-0.0291	-0.0415	-0.0494
PUM1	PUM1	-0.0342	-0.1787	0.0926	-0.0803	-0.1787	0.1084	-0.0451	0.1676	0.2230
LIF	LIF	-0.0939	0.0434	-0.0925	-0.2244	-0.0697	0.1064	-0.0551	0.2298	-0.1626
BMP10	BMP10	0.0298	-0.0443	-0.2065	-0.0483	-0.0807	0.0149	-0.0559	-0.0934	-0.0932
MAP2	MAP2	-0.1513	-0.2771	0.1240	-0.0235	-0.0333	0.0095	-0.0586	0.0035	0.5002
FZD7	FZD7	-0.0999	0.0054	-0.0011	-0.1823	-0.0929	-0.0355	-0.0677	-0.1798	-0.1101
SOX3	SOX3	0.0179	-0.0119	0.0588	-0.2009	-0.2463	-0.0261	-0.0681	-0.0404	0.2612
CCNE1	Cyclin E1	-0.0338	-0.1034	-0.1722	-0.0347	-0.0287	-0.0860	-0.0765	-0.0684	0.1029
TGFB3	TGF b3	0.0042	-0.1091	-0.0854	-0.0292	-0.1543	-0.1156	-0.0815	-0.0159	0.2483
CD24	CD24	-0.2399	-0.2314	-0.1077	0.0498	0.1787	-0.1460	-0.0827	-0.1416	0.1584
MYH6	MYH6	-0.2155	-0.0927	-0.1383	-0.1120	0.0692	-0.0861	-0.0959	-0.2092	-0.1600
TINF2	TINF2	-0.1686	-0.2222	-0.0118	-0.1522	-0.1445	0.1198	-0.0966	-0.0463	-0.0557
PAX6	PAX6	-0.1457	-0.1942	-0.1598	-0.0731	-0.0034	-0.0455	-0.1036	-0.0961	-0.1125
RBL2	P130/Rb2	-0.1267	-0.0997	0.0088	-0.1387	-0.1943	-0.0733	-0.1040	-0.0610	-0.0458
SLC1A6	SLC1A6	-0.1333	-0.1870	0.0477	-0.2162	-0.0903	-0.0519	-0.1052	-0.1155	-0.2120

Supplemental table 3.

FGF8	FGF8	-0.0966	-0.0380	0.0256	-0.2016	-0.2430	-0.1207	-0.1124	0.1383	2.9587
FZD4	FZD4	-0.1343	-0.0786	-0.0339	-0.1844	-0.1212	-0.1355	-0.1147	-0.1428	-0.1030
PDGFRA	PDGFR2	-0.0673	-0.1543	-0.0630	-0.0881	-0.2406	-0.0841	-0.1162	-0.0014	-0.1056
FOXO1A	FOXO1A	-0.1621	-0.1622	0.0475	-0.1730	-0.1207	-0.1335	-0.1173	-0.0827	-0.0105
FZD1	FZD1	-0.1594	-0.1403	-0.0318	-0.1129	-0.0992	-0.1792	-0.1204	0.0219	-0.1762
DNMT3B	DNMT3B	-0.0791	-0.1347	-0.1219	-0.1979	-0.1710	-0.0308	-0.1226	-0.1765	-0.1400
TGFBR3	TGFb RIII (Betaglycan)	-0.0900	-0.0774	-0.1252	-0.1870	-0.2724	0.0108	-0.1235	-0.0226	-0.1549
ICAM1	ICAM-1	-0.2682	-0.2484	-0.1273	-0.1090	-0.1372	0.1477	-0.1237	-0.1476	-0.1078
SLC2A1	GLUT1	-0.1052	-0.1000	-0.0561	-0.2961	-0.1485	-0.0717	-0.1296	0.0840	0.4408
GDF1	GDF1	-0.2077	-0.0956	-0.1471	-0.0748	-0.2174	-0.0668	-0.1349	-0.1351	-0.1665
FGF6	HST2	-0.0983	-0.2084	-0.0253	-0.2361	-0.1912	-0.0529	-0.1354	0.0718	0.0331
NOTCH1	NOTCH1	-0.2087	-0.1531	-0.1009	-0.0653	-0.3043	0.0080	-0.1374	-0.1096	0.0925
ITGB7	Integrin b7	-0.1805	-0.1634	-0.0940	-0.1630	-0.1180	-0.1520	-0.1452	-0.2010	-0.1646
KRT14	KRT14	-0.1443	-0.2501	-0.1399	-0.0342	-0.0686	-0.2363	-0.1456	-0.1985	-0.1183
SOX17	SOX17	-0.1434	-0.1460	-0.0676	-0.1740	-0.1822	-0.1661	-0.1465	-0.1623	-0.1097
BMP5	BMP5	-0.2202	-0.1663	-0.2637	-0.1688	0.1045	-0.1786	-0.1489	-0.1717	-0.1338
NEUROG1	NEUROG1	-0.1893	-0.1322	-0.0713	-0.1230	-0.2002	-0.1803	-0.1494	-0.1684	-0.1616
ITGB3	CD61/GP3A	-0.2273	-0.1876	-0.2421	-0.1655	-0.0518	-0.0532	-0.1546	-0.1336	-0.1111
KRT17	Keratin 17	-0.2577	-0.2163	-0.0683	-0.1229	-0.1884	-0.0783	-0.1553	-0.2141	-0.1316
FZD3	FZD3	-0.1444	-0.1163	-0.1761	-0.1542	-0.1584	-0.1895	-0.1565	-0.1268	0.2633
CDH1	E-cadherin	-0.1202	-0.2494	-0.2339	-0.1649	0.0122	-0.1867	-0.1571	-0.1935	-0.1703
NOG	Noggin	-0.1964	-0.1958	-0.1124	-0.2235	-0.0450	-0.1714	-0.1574	-0.1941	-0.2165
AFP	AFP	-0.2112	-0.2076	-0.0607	-0.1028	-0.0327	-0.3343	-0.1582	-0.2411	-0.2112
BMP4	BMP4	-0.1620	-0.1814	-0.2201	-0.2273	-0.0566	-0.1089	-0.1594	-0.2011	-0.1735
OLIG2	OLIG2	-0.1949	-0.1606	-0.0769	-0.2375	-0.1802	-0.1088	-0.1598	-0.2328	-0.2595
CNTFR	CNTFRa	0.0134	-0.2693	-0.0583	-0.1533	-0.3107	-0.1927	-0.1618	-0.2540	-0.1063
ITGB4	Integrin b4	-0.3263	-0.1890	-0.0923	-0.2064	-0.0417	-0.1164	-0.1620	-0.2390	-0.2205
WNT2	WNT2	-0.1704	-0.2364	-0.2346	-0.1493	0.0488	-0.2341	-0.1627	-0.2363	-0.1839
ACVR2	ACVR2	-0.1271	-0.2064	-0.1921	-0.1992	-0.1097	-0.1596	-0.1657	-0.1907	0.0249
PUM2	PUM2	-0.2531	-0.2384	-0.0472	-0.1558	-0.1989	-0.1258	-0.1699	-0.1752	-0.1480
DNMT2	DNMT2	-0.2365	-0.1519	-0.2173	-0.0915	-0.1653	-0.1630	-0.1709	-0.2148	-0.1763
DNMT3L	DNMT3L	-0.2456	-0.2617	-0.1686	-0.1856	-0.1201	-0.0551	-0.1728	-0.2698	-0.2890
COL6A2	COL6A2	-0.1184	-0.2052	-0.1702	-0.1249	-0.2955	-0.1335	-0.1746	-0.1438	0.0260
FGF19	FGF19	-0.1752	-0.2252	-0.0479	-0.2436	-0.2343	-0.1260	-0.1754	-0.2064	-0.1147
SIAT8A	SIAT8A	-0.2567	-0.2592	-0.1575	-0.1904	-0.0576	-0.1521	-0.1789	-0.1592	-0.2159
FGF18	FGF18	-0.1435	-0.1538	-0.0740	-0.2650	-0.2298	-0.2084	-0.1791	-0.2233	-0.1757
NES	NES	-0.2733	-0.1820	-0.1096	-0.1362	-0.2503	-0.1306	-0.1803	-0.1831	-0.0948
DNMT1	DNMT1	-0.1276	-0.2340	-0.1807	-0.2451	-0.1716	-0.1396	-0.1831	-0.1885	-0.1417
PLP1	PLP1	-0.1699	-0.2108	-0.1218	-0.1727	-0.1514	-0.2815	-0.1847	-0.1831	-0.1789
MYL4	MYL4	-0.2223	-0.1803	-0.1935	-0.1964	-0.2258	-0.1012	-0.1866	-0.1394	-0.1487
FGF2	FGF2	-0.1848	-0.1646	-0.1884	-0.2337	-0.1788	-0.1783	-0.1881	-0.2460	-0.2918
NKX2B	NKX2B	-0.3250	-0.2256	-0.1068	-0.2220	-0.1397	-0.1107	-0.1883	-0.2480	-0.1034
ITGA7	Integrin a7	-0.1997	-0.2380	-0.1947	-0.1576	-0.1835	-0.1597	-0.1889	-0.2150	-0.2496
GDF5	CDMP-1	-0.1404	-0.2943	-0.1141	-0.2240	-0.1880	-0.1735	-0.1891	-0.2017	-0.2655
LIFR	LIFR	-0.2188	-0.1539	-0.1190	-0.1768	-0.1675	-0.3001	-0.1893	-0.2224	-0.1816
FGFR1	FLG	-0.2144	-0.1505	-0.1813	-0.2817	-0.1312	-0.1861	-0.1909	-0.2007	-0.2435
ACVR1	ALK-2	-0.1517	-0.0142	-0.2005	-0.3559	-0.1937	-0.2297	-0.1910	-0.2191	-0.0874
FGF14	FGF14	-0.2107	-0.2325	-0.1487	-0.2814	-0.1290	-0.1462	-0.1914	-0.2116	-0.2146
FOXM1	MPP2	-0.2554	-0.1956	-0.1726	-0.1350	-0.2194	-0.1784	-0.1927	-0.1653	-0.1743
FGF5	FGF-5	-0.2184	-0.2176	-0.1170	-0.2223	-0.1638	-0.2204	-0.1932	-0.1987	-0.2377
FGF12	FGF12	-0.2560	-0.2515	-0.1330	-0.2342	-0.1027	-0.1827	-0.1934	-0.1331	-0.1668
CDH15	Cadherin 15	-0.1666	-0.1962	-0.3146	-0.2643	-0.0790	-0.1408	-0.1936	-0.1247	0.1224
NCAM2	NCAM2	-0.2582	-0.1990	-0.1872	-0.1363	-0.1731	-0.2160	-0.1950	0.0070	-0.2184
BMPR1B	BMPR1B/ALK-6	-0.0843	-0.2305	-0.0755	-0.2924	-0.2805	-0.2073	-0.1951	-0.2279	0.0425
SOX10	SOX10	-0.2047	-0.2325	-0.1361	-0.1870	-0.3044	-0.1074	-0.1954	-0.2116	-0.2364
OLIG1	OLIG1	-0.1231	-0.1466	-0.2457	-0.2272	-0.2130	-0.2166	-0.1954	-0.2630	-0.2197
FGFR4	FGFR4	-0.1842	-0.2965	-0.1181	-0.2149	-0.2746	-0.0840	-0.1954	-0.1347	-0.1914
FGF10	FGF10	-0.3046	-0.2436	-0.1502	-0.2043	-0.1011	-0.1690	-0.1954	-0.1919	-0.0967
WNT11	WNT11	-0.1909	-0.3516	-0.1140	-0.1502	-0.2043	-0.1625	-0.1956	-0.2191	-0.1441
CD9	CD9	-0.1496	-0.3109	-0.1970	-0.2012	-0.2098	-0.1065	-0.1958	-0.2238	-0.1933
MYH11	MYH11	-0.2542	-0.1885	-0.0796	-0.2464	-0.1521	-0.2604	-0.1969	-0.1717	-0.1434
PTEN	PTEN	-0.2398	-0.1463	-0.1041	-0.2069	-0.3484	-0.1519	-0.1996	-0.1923	-0.2001
POU3F3	POU3F3	-0.2368	-0.1912	-0.2745	-0.2129	-0.1502	-0.1393	-0.2008	-0.2007	-0.1737
GCM2	GCMB	-0.2300	-0.1638	-0.0667	-0.1819	-0.3477	-0.2306	-0.2034	-0.2337	-0.2732
BMP3	BMP3	-0.2594	-0.2178	-0.2042	-0.2702	-0.2081	-0.0610	-0.2035	-0.1888	-0.2174
FGF7	KGF	-0.3071	-0.1975	-0.0708	-0.3689	-0.0876	-0.1925	-0.2041	-0.2299	-0.4409
TGFB2	TGF b2	-0.1253	-0.3919	-0.1522	-0.2567	-0.1312	-0.1740	-0.2052	-0.2528	-0.2192
PUC18	pUC18	-0.1458	-0.3426	-0.0434	-0.3238	-0.1567	-0.2227	-0.2059	-0.1942	-0.1172
FABP6	FABP6	-0.2147	-0.2583	-0.2258	-0.1917	-0.1367	-0.2099	-0.2062	-0.1704	-0.2181
CXCR4	CXCR4	-0.3203	-0.2541	-0.1644	-0.2355	-0.1595	-0.1037	-0.2063	-0.2142	0.0949
FABP4	FABP4	-0.3420	-0.3223	-0.1733	-0.1037	-0.1731	-0.1342	-0.2081	-0.1731	-0.1608
NRG2	Neuregulin2	-0.2349	-0.2477	-0.1250	-0.1937	-0.2233	-0.2306	-0.2092	-0.1926	-0.1019
FLJ21195	Prdc-pending	-0.2593	-0.1953	-0.2878	-0.1541	-0.1741	-0.1858	-0.2094	-0.1764	-0.1736
ITGB6	Integrin b6	-0.2176	-0.2792	-0.3081	-0.1583	-0.1578	-0.1380	-0.2098	-0.2188	-0.1848
KDR	FLK1/VEGFR2	-0.2200	-0.2068	-0.1713	-0.2771	-0.1766	-0.2077	-0.2099	-0.2293	-0.2248

Supplemental table 3.

PDX1	PDX1	-0.3482	-0.1897	-0.1546	-0.1871	-0.2041	-0.1772	-0.2101	-0.2028	-0.1551
RB1	Rb	-0.1905	-0.2608	-0.2122	-0.1974	-0.1884	-0.2148	-0.2107	-0.2367	-0.1922
GDF9	GDF9	-0.1905	-0.2350	-0.2017	-0.1903	-0.1438	-0.3055	-0.2111	-0.3266	-0.2749
PUC18	pUC18	-0.2411	-0.2929	-0.2216	-0.1560	-0.1838	-0.1716	-0.2112	-0.2369	-0.1780
CST3	Cystatin C	-0.2369	-0.2361	-0.2419	-0.0760	-0.1765	-0.3040	-0.2119	-0.3027	-0.1466
DLK1	DLK	-0.2795	-0.1373	-0.1289	-0.3017	-0.2243	-0.1999	-0.2119	-0.1804	-0.1870
POU6F1	POU6F1	-0.0933	-0.2082	-0.1116	-0.2819	-0.3433	-0.2337	-0.2120	-0.2410	-0.1778
FZD2	FZD2	-0.2794	-0.1489	-0.1538	-0.1540	-0.1983	-0.3378	-0.2120	-0.2211	-0.1932
GCG	Glucagon	-0.2560	-0.2677	-0.1311	-0.1364	-0.3271	-0.1577	-0.2127	-0.2133	-0.1889
PECAM1	CD31	-0.3313	-0.1690	-0.0546	-0.2591	-0.1816	-0.2856	-0.2135	-0.2222	-0.2082
GDF11	BMP11/GDF11	-0.2627	-0.1020	-0.2052	-0.2612	-0.2151	-0.2383	-0.2141	-0.2614	-0.1531
GATA4	GATA4	-0.2446	-0.2334	-0.1046	-0.2565	-0.3230	-0.1284	-0.2151	-0.2413	-0.1659
FZD9	FZD9	-0.2074	-0.1572	-0.3525	-0.2030	-0.2115	-0.1599	-0.2153	-0.2074	-0.2118
FGF9	FGF9	-0.2529	-0.2617	-0.1392	-0.2304	-0.2778	-0.1300	-0.2153	-0.2378	-0.2078
NTRK2	Trk B	-0.1062	-0.2977	-0.2185	-0.1411	-0.3827	-0.1482	-0.2157	-0.2272	-0.1604
TNC	TNC	-0.2772	-0.1457	-0.1884	-0.2229	-0.2753	-0.1877	-0.2162	-0.2534	-0.2263
PROML1	PROML1	-0.1320	-0.0653	-0.1837	-0.3202	-0.2389	-0.3632	-0.2172	-0.2579	-0.2735
SOX13	SOX13	-0.1478	-0.1928	-0.1604	-0.2361	-0.3420	-0.2259	-0.2175	-0.4727	-0.2372
PDGFRB	PDGFR	-0.3217	-0.2731	-0.2081	-0.1749	-0.1224	-0.2060	-0.2177	-0.2099	-0.1124
FGF11	FGF11	-0.2190	-0.2276	-0.1265	-0.2418	-0.2677	-0.2247	-0.2179	-0.2339	-0.3401
S100B	S100B	-0.1926	-0.3120	-0.2348	-0.1768	-0.1446	-0.2484	-0.2182	-0.2280	-0.1993
KRT15	KRT15	-0.2522	-0.2313	-0.0531	-0.2923	-0.2933	-0.1915	-0.2190	-0.2045	-0.1994
GJB1	GJB1	-0.2156	-0.2966	-0.2157	-0.2175	-0.2067	-0.1670	-0.2199	-0.3185	-0.3895
FLJ10314	NODAL	-0.1813	-0.2358	-0.1844	-0.2704	-0.2955	-0.1548	-0.2204	-0.1973	-0.2327
DNMT3A	DNMT3A	-0.1889	-0.2095	-0.3041	-0.1528	-0.2807	-0.1870	-0.2205	-0.2058	-0.1545
NUMB	NUMB	-0.1517	-0.1732	-0.2321	-0.2193	-0.2692	-0.2810	-0.2211	-0.1501	-0.2261
SOX4	SOX4	-0.2570	-0.1986	-0.1795	-0.2335	-0.2112	-0.2481	-0.2213	-0.1708	-0.2214
FOXG1A	HBF-2	-0.1961	-0.2163	-0.3241	-0.2885	-0.1200	-0.1879	-0.2221	-0.2429	-0.1722
NKX2-5	NKX2E	-0.3245	-0.2159	-0.2295	-0.2414	-0.2212	-0.1007	-0.2222	-0.2785	-0.1978
GJB3	GJB3	-0.2272	-0.2802	-0.2565	-0.2210	-0.1743	-0.1748	-0.2224	-0.2469	-0.2062
BMP2	BMP2	-0.1923	-0.3562	-0.1512	-0.1961	-0.1931	-0.2594	-0.2247	-0.2475	-0.2208
SLC1A2	glutamate transporter2	-0.2552	-0.2779	-0.1248	-0.2229	-0.2394	-0.2323	-0.2254	-0.1702	-0.1997
IGF1R	IGF-1R	-0.1558	-0.1171	-0.1698	-0.2440	-0.3148	-0.3546	-0.2260	-0.0975	-0.2084
POU3F2	POU3F2	-0.2545	-0.2638	-0.2571	-0.2038	-0.1544	-0.2256	-0.2265	-0.2806	-0.3145
IGF2	IGF-II	-0.1767	-0.2688	-0.1770	-0.2782	-0.1708	-0.2920	-0.2273	0.0248	0.1235
BMPR-II	BMPR-II	-0.2351	-0.2215	-0.1898	-0.1260	-0.3406	-0.2509	-0.2273	-0.1657	-0.1725
EGF	EGF	-0.2028	-0.2643	-0.2708	-0.1586	-0.3679	-0.1012	-0.2276	-0.2499	-0.1709
FGF4	HST	-0.2274	-0.2731	-0.2015	-0.2637	-0.2261	-0.1741	-0.2276	-0.1978	-0.1503
CER1	Cerberus	-0.2728	-0.2372	-0.2414	-0.2235	-0.2007	-0.1946	-0.2284	-0.2006	-0.1689
ICAM5	ICAM-5	-0.2314	-0.3106	-0.2773	-0.1608	-0.2437	-0.1475	-0.2286	-0.2008	-0.1996
NEFL	NEFL	-0.2750	-0.1833	-0.1962	-0.1992	-0.2687	-0.2503	-0.2288	-0.1974	-0.2042
SOX15	SOX15	-0.2651	-0.2667	-0.0744	-0.2595	-0.2832	-0.2246	-0.2289	-0.2099	-0.1551
WNT3	WNT3	-0.4329	-0.1719	-0.2951	-0.2332	-0.0248	-0.2195	-0.2296	-0.3085	-0.2566
NGFB	NGF	-0.0654	-0.4103	-0.2002	-0.1706	-0.3038	-0.2348	-0.2308	-0.2268	-0.1984
CD44	CD44	-0.2743	-0.2356	-0.1970	-0.1474	-0.3224	-0.2163	-0.2322	-0.2818	-0.2346
FGF3	INT2	-0.3681	-0.2555	-0.2806	-0.1985	-0.1397	-0.1520	-0.2324	-0.1686	-0.1488
SOX6	SOX6	-0.3854	-0.2896	-0.0925	-0.1704	-0.2814	-0.1754	-0.2325	-0.1798	-0.3052
PUC18	pUC18	-0.1738	-0.3048	-0.2050	-0.2010	-0.2404	-0.2733	-0.2331	-0.2400	-0.1998
BMP7	BMP7	-0.3407	-0.3091	-0.0644	-0.3945	-0.0784	-0.2135	-0.2334	-0.3290	-0.2034
FGF21	FGF21	-0.3635	-0.2637	-0.0870	-0.2629	-0.2531	-0.1819	-0.2354	-0.2636	-0.2893
GDF2	BMP9	-0.3860	-0.2023	-0.1034	-0.2553	-0.2275	-0.2432	-0.2363	-0.2855	-0.1959
SOX1	SOX1	-0.1846	-0.4129	-0.1388	-0.3509	-0.1580	-0.1731	-0.2364	-0.2878	-0.2479
EGFR	EGFR	-0.2712	-0.1311	-0.2718	-0.2083	-0.1882	-0.3529	-0.2372	-0.2895	-0.2298
CCNG2	Cyclin G2	-0.2379	-0.2464	-0.2310	-0.1923	-0.2528	-0.2703	-0.2384	-0.2024	-0.2609
NCAM1	NCAM	-0.2836	-0.2013	-0.1014	-0.2838	-0.2858	-0.2768	-0.2388	-0.2121	-0.2269
SOX5	SOX5	-0.3009	-0.3127	-0.1577	-0.2257	-0.2077	-0.2433	-0.2413	-0.2067	-0.1296
TP53	p53	-0.2228	-0.2507	-0.1759	-0.2932	-0.3188	-0.1881	-0.2416	-0.1552	-0.2197
POU5F1	POU5F1	-0.3502	-0.2369	-0.1508	-0.3074	-0.3008	-0.1052	-0.2419	-0.1240	-0.1571
ITGA6	Integrin a6	-0.3476	-0.3553	-0.1545	-0.3217	-0.0578	-0.2227	-0.2433	-0.0122	-0.1233
GJA7	GJA7	-0.2610	-0.2459	-0.2349	-0.2107	-0.3142	-0.1935	-0.2433	-0.2749	-0.2181
WNT5B	WNT5B	-0.2395	-0.3711	-0.2079	-0.3359	-0.1617	-0.1450	-0.2435	-0.2108	-0.1407
PROX1	PROX1	-0.1908	-0.2672	-0.2455	-0.2252	-0.1815	-0.3548	-0.2442	-0.2458	-0.2494
IGF1	IGF-1	-0.3368	-0.2953	-0.0472	-0.1968	-0.3522	-0.2370	-0.2442	-0.2463	-0.1833
CDKN2D	p19-INK4D	-0.3853	-0.2483	-0.2995	-0.1656	-0.0940	-0.2797	-0.2454	-0.1469	-0.1462
FZD8	FZD8	-0.3320	-0.2934	-0.2925	-0.2258	-0.2032	-0.1281	-0.2458	-0.1804	-0.1904
NTF3	NT-3	-0.1231	-0.2893	-0.2234	-0.2384	-0.4729	-0.1374	-0.2474	-0.2154	-0.1283
NGFR	NGFR	-0.1731	-0.2716	-0.2929	-0.2802	-0.2744	-0.2036	-0.2493	-0.4197	-0.2479
SNAI1	SNAI1	-0.1431	-0.1339	-0.4715	-0.1441	-0.1976	-0.4100	-0.2500	-0.1288	-0.3645
GDF3	GDF3	-0.2902	-0.2012	-0.1509	-0.1685	-0.0855	-0.6068	-0.2505	-0.2641	-0.1616
ITGA2B	Integrin alpha 2b	-0.3414	-0.2423	-0.1497	-0.3166	-0.2281	-0.2336	-0.2520	-0.2696	-0.1922
FGFR3	FGFR3	-0.2387	-0.2838	-0.2796	-0.2126	-0.2987	-0.2027	-0.2527	-0.2549	0.0261
CDKN2A	p16INK4	-0.3024	-0.3044	-0.2106	-0.1995	-0.2279	-0.2731	-0.2530	-0.2097	-0.2591
FGF16	FGF16	-0.2101	-0.2751	-0.2331	-0.3082	-0.2768	-0.2167	-0.2533	-0.2018	-0.0417
INSRR	INSRR	-0.3204	-0.3318	-0.1488	-0.2623	-0.2287	-0.2324	-0.2541	-0.2553	-0.2431
BDNF	BDNF	-0.1604	-0.1715	-0.1627	-0.2440	-0.3628	-0.4415	-0.2571	-0.2217	-0.3212

Supplemental table 3.

GATA2	GATA2	-0.1986	-0.3445	-0.0819	-0.2513	-0.3965	-0.2879	-0.2601	-0.3029	-0.2665
FGF17	FGF17	-0.2316	-0.1889	-0.3485	-0.2784	-0.2497	-0.2637	-0.2601	-0.1963	-0.1706
BMP15	GDF9B	-0.2812	-0.3201	-0.2469	-0.1841	-0.1648	-0.3652	-0.2604	-0.2407	-0.3220
CDKN1A	P21/Waf1/CIP1	-0.3594	-0.2773	-0.2479	-0.1881	-0.2345	-0.2630	-0.2617	-0.2344	-0.2489
ACVRL1	ACVRL1/ALK-1	-0.2815	-0.3074	-0.3141	-0.2017	-0.2277	-0.2389	-0.2619	-0.1575	-0.2208
ITGA8	Integrin a8	-0.2049	-0.2442	-0.2259	-0.3064	-0.3615	-0.2349	-0.2630	-0.2021	0.1841
ITGAL	LFA1a /CD11A	-0.2689	-0.3817	-0.2908	-0.2465	-0.1516	-0.2493	-0.2648	-0.2662	-0.1829
CTNNAL1	Catenin alpha-like 1	-0.3110	-0.3921	-0.1346	-0.3513	-0.2192	-0.1887	-0.2661	-0.2575	-0.3038
MAP1B	MAP1B	-0.1719	-0.3685	-0.1732	-0.2046	-0.4230	-0.2819	-0.2705	-0.2410	-0.3774
FABP7	FABP7	-0.3090	-0.2863	-0.1093	-0.2473	-0.4202	-0.2517	-0.2706	-0.1616	-0.2132
FGF23	FGF23	-0.2839	-0.2695	-0.1616	-0.2756	-0.3265	-0.3088	-0.2710	-0.3506	-0.2676
ISL1	ISL1	-0.2881	-0.2017	-0.2872	-0.2806	-0.1121	-0.4581	-0.2713	-0.2621	-0.2010
WNT7A	WNT7A	-0.2333	-0.1986	-0.3698	-0.2548	-0.3537	-0.2233	-0.2723	-0.2962	-0.2992
ITGAM	Integrin aM	-0.2622	-0.4006	-0.1825	-0.1733	-0.3278	-0.2987	-0.2742	-0.3429	-0.3693
RBL1	p107	-0.2971	-0.1934	-0.3549	-0.2140	-0.2153	-0.3712	-0.2743	-0.5963	-0.4735
WNT6	WNT6	-0.1698	-0.3194	-0.2582	-0.2607	-0.4388	-0.1990	-0.2743	-0.2512	-0.1678
UTF1	UTF1	-0.5598	-0.3152	-0.3035	0.0962	-0.5059	-0.1222	-0.2851	-0.3480	0.3440
GFAP	GFAP	-0.2191	-0.3042	-0.3967	-0.0843	-0.3859	-0.3255	-0.2859	-0.5439	-0.4968
ITGAE	ITGAE	-0.3261	-0.3335	-0.1245	-0.3398	-0.4011	-0.1909	-0.2860	-0.2155	-0.2107
NPPA	ANP/ANF	-0.1320	-0.2340	-0.2692	-0.2376	-0.4061	-0.4567	-0.2893	-0.2979	-0.3173
FGF1	FGF1	-0.4174	-0.3572	-0.1826	-0.2634	-0.3457	-0.1825	-0.2915	-0.2195	-0.1863
TGFBR2	TGFbR2	-0.2597	-0.2506	-0.2045	-0.3133	-0.5324	-0.2014	-0.2937	-0.2190	-0.2289
BMP8	BMP 8	-0.3658	-0.3463	-0.0956	-0.2777	-0.2897	-0.4047	-0.2966	-0.3205	-0.3285
TERF1	TERF1	-0.3865	-0.3086	-0.2067	-0.3585	-0.1778	-0.3486	-0.2978	-0.3284	-0.2080
GJB5	GJB5	-0.3469	-0.2768	-0.3277	-0.2648	-0.2464	-0.3322	-0.2991	-0.3340	-0.3654
VIM	VIM	-0.3827	-0.3356	-0.1429	-0.4058	-0.2561	-0.2963	-0.3032	-0.4359	-0.2548
MGC21116	GJB4	-0.1600	-0.2752	-0.1995	-0.2629	-0.2221	-0.7222	-0.3070	-0.3712	-0.1989
PDGFA	PDGF a	-0.1136	-0.3387	-0.6084	-0.3017	-0.2708	-0.2673	-0.3168	-0.3101	-0.3182
ABCG2	BCRP	-0.2147	-0.2985	-0.3003	-0.3254	-0.3836	-0.4076	-0.3217	-0.3130	-0.7684
BMPR1A	ALK-3	-0.4016	-0.2771	-0.2862	-0.4528	-0.2297	-0.3274	-0.3291	-0.2752	-0.1483
INHBB	INHBB	-0.3144	-0.4367	-0.3347	-0.3019	-0.2026	-0.3956	-0.3310	-0.4740	-0.4716
CTNNB1	b Catenin	-0.3499	-0.4259	-0.1967	-0.2607	-0.3771	-0.3791	-0.3316	-0.4213	-0.2472
GDF8	GDF8	-0.3382	-0.3928	-0.2438	-0.2942	-0.3790	-0.4010	-0.3415	-0.4582	-0.4484
PTCH	PTCH	-0.2898	-0.3293	-0.2305	-0.3071	-0.1400	-0.7676	-0.3441	-0.5228	-0.5179
IL6R	IL-6 Ra	-0.2994	-0.3380	-0.4484	-0.2404	-0.3136	-0.4382	-0.3463	-0.3087	-0.5270
SYT1	SYT1	-0.4643	-0.2874	-0.0692	-0.2379	-0.3460	-0.6800	-0.3475	-0.4100	-0.5288
WNT7B	WNT7B	-0.3385	-0.3286	-0.2726	-0.3856	-0.2748	-0.5187	-0.3531	-0.8137	-0.7306
PUC18	pUC18	-0.2619	-0.3208	-0.4078	-0.2304	-0.6793	-0.2343	-0.3557	-0.4180	-0.8962
ITGB2	LFA-1/CD18	-0.4188	-0.4833	-0.2734	-0.3547	-0.3731	-0.2793	-0.3638	-0.4184	-0.3628
SOX18	SOX18	-0.3318	-0.2586	-0.6260	-0.1719	-0.5030	-0.3107	-0.3670	-0.3541	-0.5128
CTNNA1	Catenin a	-0.3574	-0.2717	-0.4451	-0.1541	-0.2931	-0.7493	-0.3784	-0.4682	-0.3050
TEP1	TEP1	-0.3840	-0.3147	-0.0690	-0.4318	-0.2793	-0.8421	-0.3868	-0.4825	-0.4481
WNT8A	WNT8A	-0.3579	-0.4550	-0.1689	-0.3839	-0.5010	-0.5186	-0.3976	-0.9794	-0.6457
INA	INA	-0.3881	-0.5032	-0.5345	-0.1639	-0.4770	-0.3660	-0.4054	-0.5185	-0.5075
ACTC	ACTC	-0.2953	-0.3988	-0.2198	-0.4819	-0.3808	-0.8842	-0.4435	-0.4652	-0.6332
ZFP42	ZFP42	-0.3827	-0.3662	-0.3340	-0.3420	-0.6292	-0.7062	-0.4601	-0.8541	-0.8020
BMP6	BMP 6	-0.4790	-0.4547	-0.4536	-0.4849	-0.4354	-0.6568	-0.4941	-1.1735	-1.0088
ITGAX	Integrin aX	-0.4127	-0.5828	-0.5610	-0.3775	-0.5578	-0.4854	-0.4962	-0.4469	-0.8764
CDH3	Cadherin 3	-0.8455	-0.6416	-0.1561	-0.7613	-0.3337	-0.2578	-0.4993	-0.6513	-0.6188
Blank		-0.8508	-0.4619	-0.5173	-0.9810	-0.9781	-1.2660	-0.8425	-1.1104	-0.9774

Supplemental table 3.

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PART IV

Dose-dependent reduction in apomorphine-evoked rotations in hemi-parkinsonian rats by human umbilical cord matrix stem cells.

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder of central nervous system, where stem cells are deemed to have a potential therapeutic benefit by regenerating or protecting the degenerating neurons. Previously, Human umbilical cord matrix stem (hUCMS) cells, which are a type of mesenchymal stem cells, were shown to have a therapeutic benefit in PD model rats. Here, we investigated the dose-dependent effect of hUCMS cells in PD model rats. PD model rats were created by administering 6-hydroxydopamine (6-OHDA) stereotaxically into the left medial forebrain bundle. Four weeks after the 6-OHDA lesion, rats received about 2500 and 25000 hUCMS cells (or sham transplants). The rats did not receive immunosuppressive therapy. Apomorphine-induced rotatory behavior was used to analyze the motor deficits of the PD-model rats. When data was pooled from the previous study with 1000 hUCMS cell transplant, there was a significant dose-dependent decrease in apomorphine-induced rotations overtime up to 10 weeks after transplantation. A few graft cells were recovered at 1-week post-transplantation, but none were recovered at 6-weeks or 10-weeks. In a pilot experiment with hUCMS cell transplantation in immunosuppressed rats, the graft was recovered at 1-week post-transplantation. Quantitative assessment of the TH-positive neurons in substantia nigra (SN) and ventral tegmental area (VTA) revealed that there is no significant difference in the number of dopaminergic neurons on the lesioned side in the rats that received hUCMS cell transplant compared to the control rats. A novel quantitative measure has been introduced to evaluate the transplant

efficacy (Transplant or T-value), where multiple parameters are taken into consideration. The T-value correlates well with the behavioral recovery in the various groups. These results show that it is likely that the hUCM cells may play a role in promoting improvement of motor deficits in PD-model rats, but the mechanism is currently unknown.

Keywords: umbilical cord matrix, stem cells, xenotransplantation, Parkinson's disease, 6-hydroxydopamine, apomorphine, tyrosine hydroxylase,

Introduction:

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease, and is the most common neurodegenerative movement disorder (Dawson and Dawson, 2003). It affects 1% of the U.S. population older than 65 and an estimated 60,000 new cases are being diagnosed annually. There is no cure for the disease, but symptomatic treatment using L-DOPA or carbidopa alleviate some of the symptoms early in the disease (Arenas, 2002). However, as the disease progresses treatment effectiveness decreases and drug-induced dyskinesias appear (Obeso et al., 2000b;Obeso et al., 2000c). Another approach often combined with L-DOPA and carbidopa is deep brain stimulation of the globus pallidus and the subthalamic nuclei (Arenas, 2002;Obeso et al., 2000a). Thus, the study of pathologic mechanisms and therapeutic alternatives of PD utilizing animal models is very critical. There are several rodent models of PD (Beal, 2001); 6-hydroxydpamine (6-OHDA) model being one of the most commonly used models. A chronic 6-OHDA model with progressive degeneration of the DA neurons is described by Sauer and Oertel (Sauer and Oertel, 1994) that may more closely mimic the disease than the acute models.

The therapy of acute and chronic neurological disorders would be significantly enhanced with stem cells endowed with the ability to form several kinds of neurons or glia that can be used to replace the damaged neural cells (Bjorklund and Lindvall, 2000;Bjorklund et al., 2000). Neural stem cell populations have been identified as ependymal cells (Johansson et al., 1999b;Johansson et al.,

1999a), subventricular zone astrocytes (Doetsch et al., 1999), and cells in the subgranular zone of the dentate gyrus (Gage et al., 1998). Thus, harvesting neural stem cells from the human patient cannot be done easily because the location and distribution of the neural stem cells makes it difficult to harvest a substantial population of these cells for *in vitro* expansion/manipulation and subsequent autologous transplantation. Moreover, the role of endogenous neural stem cells in disease is not clear. Therefore, to treat PD, a motor disease characterized by loss of neural catecholaminergic input to the striatum, several different therapeutic approaches have ensued. The most radical approach is the transplantation of human or porcine fetal neural tissue into human brains (Bjorklund and Lindvall, 2000;Deacon et al., 1997). Embryonic stem (ES) cells and the dopaminergic neurons derived from ES cells were shown to be effective in treating animal models of PD (Bjorklund et al., 2002;Kim et al., 2002). Graft versus host disease (GVHD) and host immune rejection are serious problems associated with cell therapy including xenotransplants (Borlongan et al., 1996;Duan et al., 1996;Duan et al., 1995). In addition to cell-replacement, regenerative/restorative therapy has gained importance as an effective therapeutic strategy in managing neurodegenerative diseases, where rescue or restoration of the degenerating neurons is deemed to occur through trophic factors, pharmaceutical agents, immunomodulation etc. (Ourednik et al., 2002;Beal, 2003). Moreover, glial derived neurotrophic factor (GDNF) has been shown to rescue degenerating DA neurons *in vitro* (Ding et al., 2004), and *in vivo* in PD animal models (Bjorklund et al., 1997;Kirik et al., 2000a). Also,

immunomodulation (Aharoni et al., 2005;Stangel, 2004) and immunoisolation (Emerich and Salzberg, 2001) techniques have been shown to have therapeutic benefit in neurological diseases.

Umbilical cord matrix (UCM) or Wharton's jelly, the gelatinous connective tissue found in the umbilical cord, is a source of primitive stem cells. In culture, these cells express stem cell characteristics (Mitchell et al., 2003;Weiss et al., 2005). Our previous work revealed that pUCMS cells express stem cell markers and can be grown *in vitro* for more than 100 population doublings and differentiate into neuron-like cells *in vitro* and *in vivo* (Mitchell et al., 2003;Weiss et al., 2003). Recently, Human UCMS cells were characterized and their surface antigen and gene profile closely resembles to that of mesenchymal stem cells (Weiss et al., 2005). Also, a subset of hUCM cells respond to the differentiation signals *in vitro* and exhibit neuronal characteristics (Mitchell et al., 2003;Weiss et al., 2005). In addition, when transplanted at a low-density, hUCMS cells provide therapeutic benefit in PD model rats owing to the restoration of degenerating DA neurons in SN and VTA (Weiss et al., 2005).

Assessing the therapeutic value of cell transplantation is becoming critical to determine the optimal cell type and intervention (Lindvall et al., 2004). A universal metric for assessing the transplant may include components of the behavior/physiological and morphological outcomes and the host immune response and long term engraftment. Here, we introduce such a metric, referred

to as “Transplant or T-value”, which evaluates the efficacy of the transplanted cells by considering multiple parameters of the graft (survival, proliferation, differentiation and tumorigenecity), and host (behavior, health status and immune response.)

As a follow-up to our previous study (Weiss et al., 2005), the PD model rats received a medium-density (approximately 2500 hUCMS cells) or high-density (approximately 25,000 hUCMS cells) transplant. When the rotatory behavioral data was pooled from the previous study, there was a significant dose-dependent reduction in the apomorphine-induced rotations in the PD model animals that received hUCMS cell transplant compared to the control animals (PBS infusion). However, the transplanted hUCMS cells were not recovered either at 6 weeks or 10 weeks after transplantation. We hypothesize that the transplanted hUCMS cells have a dose-dependent therapeutic benefit by restoring, but not replacing, the host dopaminergic neurons in PD model rats, resulting in recovery in the motor deficit (apomorphine-induced rotatory behavior).

Materials and Methods:

Cell culture and counting

Human UCMS cells were generated and expanded as described previously (Weiss et al., 2005). Briefly, Human UCM cells were harvested from the human umbilical cords by using enzymes, such as Hyaluronidase 1mg/ml (Sigma H-2126) and Collagenase 300units/ml (Sigma C-6885). The hUCM cells were cultured and maintained as described previously (Medicetty et al., 2004;Weiss et al., 2005) in a defined media containing 56% low glucose DMEM (Invitrogen 11885-092), 37% MCDB 201 (sigma M6770), 1% 100x Insulin-Transferrin-Selenium (Invitrogen 51500056), 1% 100x Albumax1 (Invitrogen 11020021), 0.001% 10^{-4} M Dexamethasone (Sigma D4902), 1% 10^{-2} M Ascorbic acid-2 phosphate (sigma A8960), 1% Antibiotic/Antimycotic (Invitrogen 15240-062), 2% FCS (Hyclone-heat inactivated), 0.001% Epidermal growth factor (R&D 236-EG-200), 0.2% Platelet-derived growth factor (R&D 520-BB-050). Cells from the same cord and passage (P4) were used for transplantation in all the animals. Before transplantation, the cells were lifted by a trypsin solution (0.05%) and counted by a hemocytometer, and were adjusted to a final concentration of approximately 2500 and 25,000 cells per microliter. The cell concentration was confirmed before and after the injection to ensure that the correct numbers of cells are delivered.

6-hydroxydopamine (6-OHDA) lesion and behavioral assesment

PD model rats were created by stereotaxic injection of 6-hydroxydopamine (6-OHDA) as previously described (Weiss et al., 2005;Ungerstedt and Arbuthnott, 1970). Anesthetized female Sprague-Dawley rats received a 4.0 µl injection of 32 mM 6-OHDA HCl (Sigma) dissolved in 0.02% ascorbate at 1 µl/min into the left medial forebrain bundle (Bregma -2.8; lateral 2.0; ventral 8.4mm) or an injection of vehicle (sterile PBS) four weeks prior to transplantation. Following surgery, rats were monitored for behavioral changes, weight loss and overall health. Four weeks following lesion surgery the animals received the human UCM cell transplant or sham transplant (sterile saline). The rats were placed in an opaque testing chamber and allowed to acclimate for 5 minutes prior to assessing the number of rotations in a 30 minute period after apomorphine injection (alternate weeks, 0.1 mg/kg, sc). The lesioned rats that did not exhibit at least 200 contralateral rotations/30 minutes after apomorphine treatment were excluded from the experiment. Further, apomorphine-induced rotations were measured at alternate weeks till the end of the experimental period. Based on the difference in the number of apomorphine-induced rotations at the end of survival period and the baseline rotations (number of rotations at the time of transplantation), the animals that received hUCMS cell transplant are designated as either 1. Responder – decrease and 2. Non-responder – no change or increase in the number of rotations compared to the baseline (see supplementary table 4.1).

Immunesuppression

The rats were treated by using cyclosporin A (CsA, 10mg/kg, ip) in Cremophor - ethanol vehicle (1 ml/kg, ip) daily, starting 2 days prior to transplantation of hUCMS cells. In addition, the rats received 20 mg/kg methylprednisolone (Solu-Medrol; Pharmacia-Upjohn) for the first four days following surgery. These methods have been shown to effectively protect pig neural grafts from immune rejection in the rat brain (Borlongan et al., 1996;Wennberg et al., 2001)

Transplantation of hUCMS cells

Human UCM cells were transplanted into anesthetized rats (2% halothane in oxygen) via stereotaxic injection into the left striatum (Bregma +0.5, Lateral 3.4, Ventral 5.0 mm from the surface of the brain). Four weeks after 6-OHDA injection, approximately 2500 and 25,000 hUCM cells in 1ul of the sterile saline were injected over 5 min. The control group received sham transplantation (PBS). At 6 and 10 weeks post-transplantation, rats were randomly selected from various groups, anesthetized and sacrificed by transcardial perfusion with heparinized isotonic saline rinse followed by 10% buffered neutral formalin. The brains were removed, postfixed, and cryoprotected in 20% sucrose overnight. Frozen sections of the brains were cut coronally at 40 μm and the sections were collected into six sets of adjacent sections, each set consisting of every sixth serial section.

Tissue processing and Immunocytochemical processing (IC)

Immunocytochemical (IC) detection of a single antigen was performed on one set of sections as previously described (Weiss et al., 2003) and the adjacent sets of sections were held in reserve in a cryoprotectant solution (Watson, Jr. et al., 1986). The free-floating tissue sections were stained with primary antibodies for anti-human nuclear antigen (mouse host, 1:30, Chemicon), anti-human mitochondria (mouse host, 1:100, Chemicon) anti-TH (rabbit host, 1:2000, East Acres Biologicals), anti-rat CD-4 (mouse host, 1:500, Serotec), anti-rat CD-8 (mouse host, 1:500, Serotec), anti-rat CD-11b (mouse host, 1:250, serotec), anti-rat CD-161(mouse host, 1:250, Serotec). The antigens were visualized either with diaminobenzidine (DAB) and hydrogen peroxide using a commercially available ABC kit (Vectastain) or with immunofluorescence. For immunofluorescence localization, either anti-mouse alexa488 (Molecular probes, 1:200) or 7-amino-4-methylcoumarine-3-acetic acid (AMCA) - Avidin D (Vector Laboratories, 1:200) was used with the biotinylated secondary antibody. The IC-stained sections were mounted on subbed microscope slides, air-dried, and rinsed with distilled water. To detect the immunofluorescence, the sections were observed using epifluorescence illumination with the appropriate filter combinations on a Leica DMRD microscope after clearing and coverslipping with glycerol containing N-propyl gallate (3 parts 2% N-propyl gallate in 0.1M Tris buffer, pH 9.0 and 7 parts glycerol).

Quantification of TH-positive cells in SN and VTA

The TH-positive DA neurons in substantia nigra (SN) and ventral tegmental area (VTA) were quantified by using a design-based unbiased stereological method by using a morphometry/image analysis system (Bioquant Nova Prime, R&M Biometrics). One set of brain sections of all the experimental animals were stained with anti-TH antibody and visualized with DAB. In each section the region of interest is outlined (SN and VTA), and the number of TH-positive cells per sq. mm. of that region were selected and semi-automatically counted. All the sections in the particular set, containing SN and VTA were screened and TH-postive cells were counted, and the average number of TH-positive DA neurons per sq. mm. was calculated for each animal

Statistical analysis

Recovery of function (apomorphine-induced rotations) were evaluated over time (repeated measures) at alternate weeks till the end of the experiment and pooled across groups to compare treatments. All tissue manipulations were conducted in large batches to avoid batch to batch differences in tissue IC-staining. Tissue processing and data collection were conducted in an experimenter-blind fashion. After measurements and counting, the survival group status was decoded prior to statistical analysis. In general, analysis of variance (ANOVA) was used to evaluate group differences. ANOVA was followed by post hoc testing using

Scheffe=s test. Significance was set at $p<0.05$. The mean plus or minus one standard error are presented on graphs.

Transplant value (T-Value) of the graft:

The formula used for calculating the transplant (T) value and explanation are:
T value = 2F + G + H, where F = functional efficacy; G = graft outcome; H = host tolerance.

To calculate the T-value for animals from all the groups (low, medium and high-density graft) of both trials, the required parameters (see below) were collected for each animal. Every term in the equation for F, G and H was expressed as a score of 1-10 assigned in the following way. For each parameter, the range (minimum and maximum value) was divided into 10 equal sets, and range assignments (1-10) were made and represented as a score for that particular parameter. The individual parameter score is then put into the formula. Each term was expressed so that the proportionality is correct as shown in the formula. Further, the T-value is mapped against the behavioral recovery to test the correlation.

Based on the type of parameter, desirable or undesirable, we assigned the best score ‘10’ to the highest group (maximum value of the range) or lowest group (minimum value of the range) respectively. For example, supplementary table 4.2 shows the scoring for the % change in weight, a desirable parameter that

indicates the health status of the animal. F (functional efficacy) = % baseline rotation score ('10' = lowest group) + TH cells in SN ('10' = highest group) + mortality ('10' = lowest group) + weight loss from transplant to sac ('10' = lowest group) + ipsilateral rotations after apomorphine ('10' = lowest group). G (graft outcome) = % donor cells apoptotic ('10' = lowest group) + incidences of tumor ('10' = lowest group) + % donor cells expressing neuronal markers ('10' = highest group) + % donor cells expressing glial markers ('10' = highest group). H (host tolerance) = number of infiltrating CD8 cells ('10' = lowest group).

Results:

Behavioral assessment of the PD model rats

Previously, we have confirmed the effects of 6-OHDA in rat brain by immunostaining for TH, which validates this PD model (Weiss et al., 2005). The rotatory behavior of the PD model rats was assessed by counting the apomorphine-induced rotations for 30 minutes at alternate weeks till the end of the experimental period. Half the experimental animals were sacrificed 6-weeks following transplantation and the remaining were sacrificed at the end of experimental period i.e. 10 weeks post-transplantation. In the previous trial, we have reported the detailed rotatory behavior analysis of the PD model rats that were transplanted with 1000 hUCMS cells (Weiss et al., 2005). Here, owing to the fact that the controls in both the trials were statistically not different (data not shown), we pooled the data from the low-density transplant with the data from the medium-density and high-density transplant to study the dose-dependent effect of hUCMS cells. The PD model rats that received the hUCMS cell transplant, except for the high-density transplant group, showed a significant dose-dependent decrease in the number of rotations compared to the sham transplanted (PBS) animals (see Fig. 4.1). In the first trial (1000 cells), 5 of the 8 animals responded to the transplant i.e. decreased their apomorphine-induced rotation behavior. In the second trial, 6 of 13 animals treated with 2500 cells and 6 of 12 animals treated with 25,000 cells responded (see methods section for description of a 'responder'). Thus, approximately 50% of the animals respond to hUCMS cell treatment. Although half of the rats in the high-density transplant

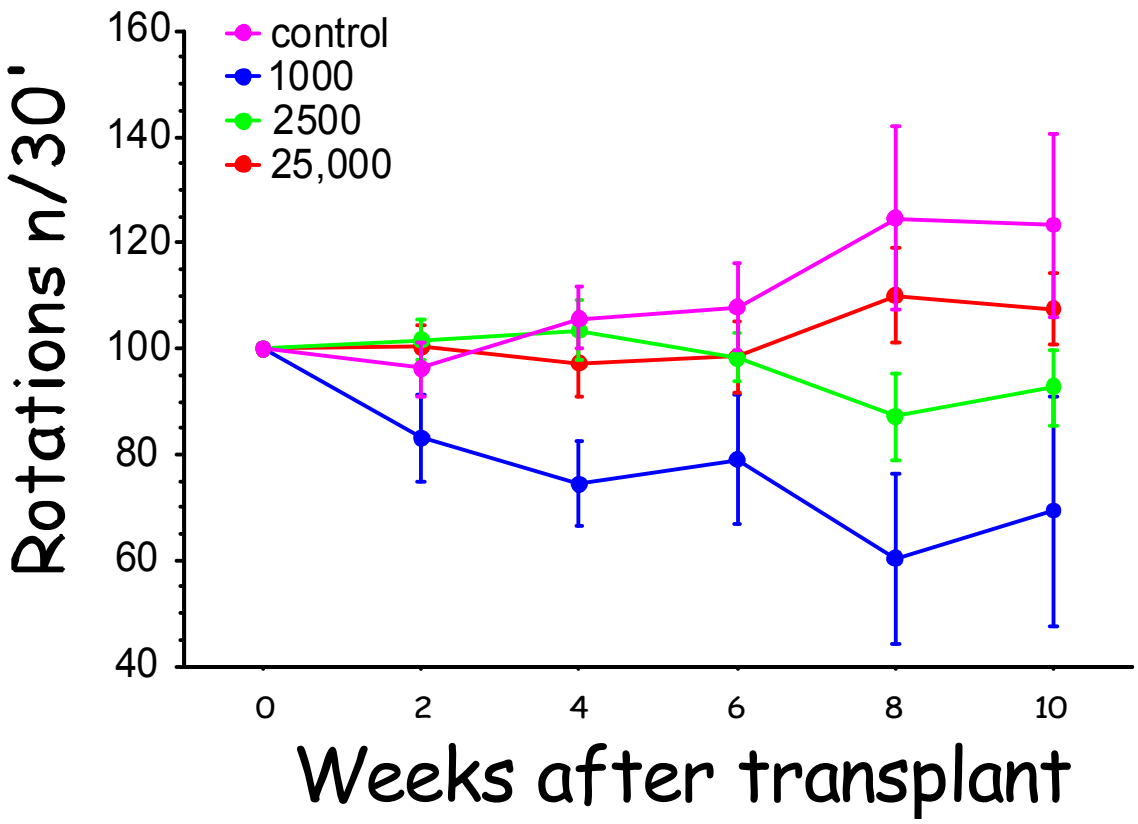

Figure 4.1: Effect of human umbilical cord matrix stem (hUCMS) cell transplant on apomorphine-induced rotation behavior. The data from first trial is pooled with data from second trial. In the first trial, 5 of the 8 animals responded to the transplant, e.g., decreased their apomorphine-induced rotation behavior. In the second trial, 6 of 13 animals treated with 2500 cells and 6 of 12 animals treated with 25000 cells responded. Thus, approximately 50% of the animals respond to UCM treatment.

group responded to hUCMS cell transplantation (see supplementary table 4.1), as a group they are not statistically different from the control group.

Recovery of the graft cells

The transplanted hUCMS cells were recovered by staining the brain sections with anti-human nuclear (AHN) antigen or antibody to human mitochondria. Very few graft (hUCM) cells were recovered at 1 week following transplantation in non-immunosuppressed PD model rats (Fig. 4.2A shows the nuclei of the transplanted hUCM cells stained with AHN antigen and visualized with alexa 488). However, the 6 and 10-week survival animals that received 2500 and 25000 hUCM cell-transplant did not show any positive staining for AHN antibody. In addition, hUCMS cells (high-density, 25000) that were transplanted into immunosuppressed rats were recovered at 1-week post-transplantion (Fig. 4.2B shows anti-human mitochondrial staining visualized with alexa 488) and few transplanted cells diffused from injection site into the brain parenchyma (data not shown).

Assessment of immune-cell infiltration

Brain sections from 1, 6 and 10-week animals with 2500 and 25000 cell-graft were stained with rat anti-CD8 antibody (recognizes T-cytotoxic cells) and visualized by AMCA. There were no rat CD-8 positive cells at the transplantation site in any of the groups, which is similar to what we have noticed earlier with 1000-cell transplant (Weiss et al., 2005). However, there were rat CD11b-positive

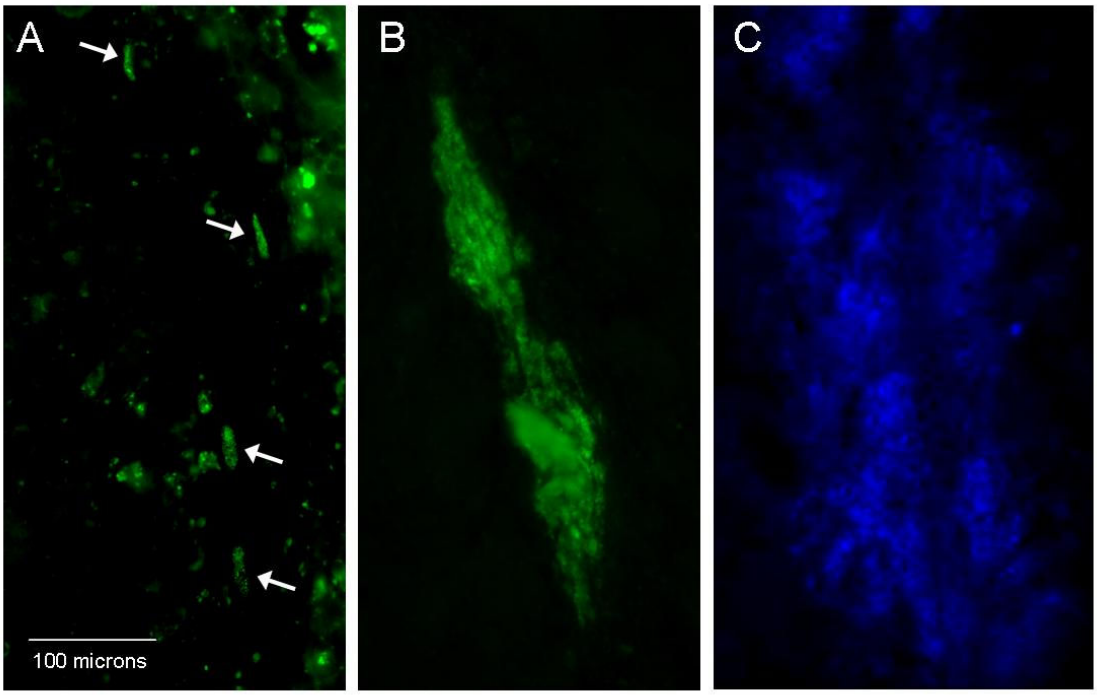

Figure 4.2: (A-B) Recovery of graft cells. Transplanted hUCMS cells were recovered at 1-week following transplantation in non-immunesuppressed rats by immunostaining for anti-human nuclear antigen (arrows in A) and in immunosuppressed rats by immunostaining for anti-human mitochondria (B) localized with Alexa 488. Activated microglia (identified by CD-11b staining) are noticed at the graft site (C), localized by AMCA.

(recognizes activated microglia) cells at the transplant site in the 1-week survival animals (See Fig. 4.2C). There is no staining for rat CD-4 (recognizes T-helper cells) or rat CD-161 (recognizes natural-killer cells) at the transplant site in the 1-week survival animals.

TH- staining and Quantification of DA neurons in SN and VTA

One set of brain sections of all the 6 and 10-week experimental animals were stained with anti-TH antibody and visualized with DAB. Figure 4.3A-F shows the TH-staining in SN and VTA from randomly selected representative animals from various groups and survival periods. As expected from the type of the lesion, a robust TH-staining (large number of DA neurons) on the non-lesion side (right side) and a weak TH-staining (very few DA neurons) is observed in all the groups (control, medium-density and high-density transplant) at 6 weeks or 10 weeks following transplantation. Further, the TH-positive cells in SN and VTA of all the experimental animals were quantified by semi-automatic counts in Bioquant. Statistical analysis of TH-positive cells per sq. mm. in the different groups revealed that there is no significant difference between the various groups either at 6 or 10 weeks post-transplantation (See Figure 4.4).

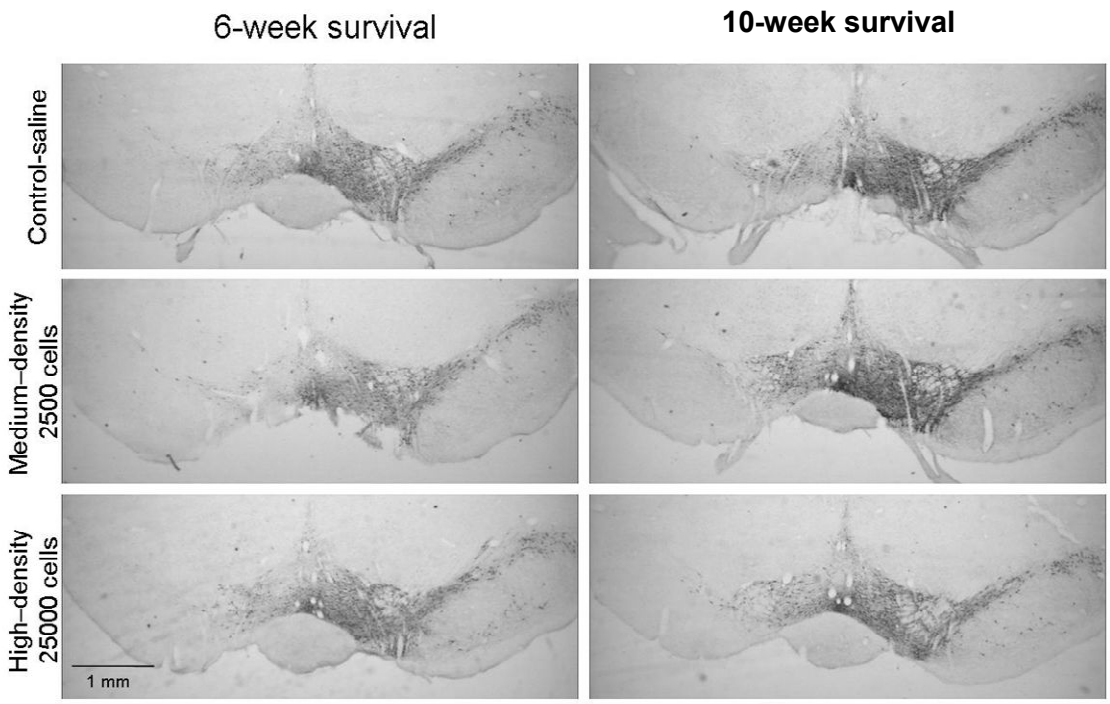

Figure 4.3: Tyrosine hydroxylase (TH) staining in PD model rats with and without hUCMS cell transplant. One set of brain sections from all the experimental animals were stained for TH, a catecholaminergic marker. (A-F) shows sections from representative animals from various groups that were selected randomly and matched for similar morphology. Top panel are TH-stained midbrain sections of the control (sham-transplant) group at 6-week (A) and 10-week (B) post-transplantation. Middle panel comprises TH-stained sections from the medium-density (2500 hUCMS cells) group at 6-week (C) and 10-week (D) post-transplantation. The bottom panel consists of TH-stained sections from the high-density (25,000 hUCMS cells) group at 6-week (E) and 10-week (F) post-transplantation.

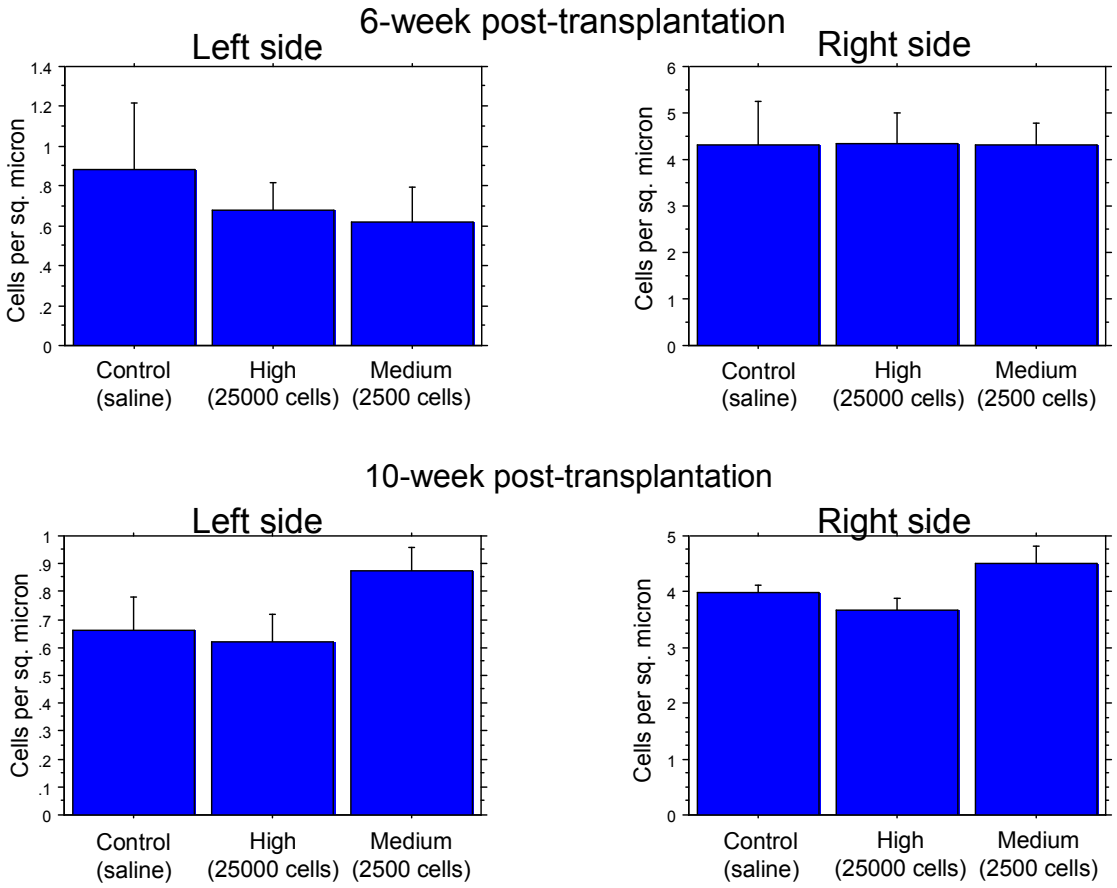

Figure 4.4: Quantification of TH-positive DA neurons in SN and VTA. The TH-positive cells were counted semi-automatically by a design-based stereological method and analyzed through Statview. There is no significant difference in the number TH-positive cells in the ventral midbrain of the control (sham transplant), high-density or medium-density hUCMS cell-transplant groups either at 6 weeks or 10 weeks after transplantation. ($p<0.05$)

T-value

The scoring system for calculating T-value is discussed in detail in methods, and supplementary table 4.2 shows detailed scoring table for one of the desirable parameters, health status, implied by % change in weight over time in the low-density (1000 hUCMS cell) transplant group. All the other parameters were also assigned a score (1-10) in the similar manner. As the graft cells were not recovered, the G-graft outcome component included only 'tumour incidence' parameter. T-values were calculated for all the animals from both trials with all the groups (control, low-density, medium-density and high-density transplant) and a treatment group mean was calculated at each survival period. The final T score for each transplant condition is the average of those of the survival periods. Figure 4.5 demonstrates the validity of the T-value, where it shows a positive correlation between T-value and behavioral improvement.

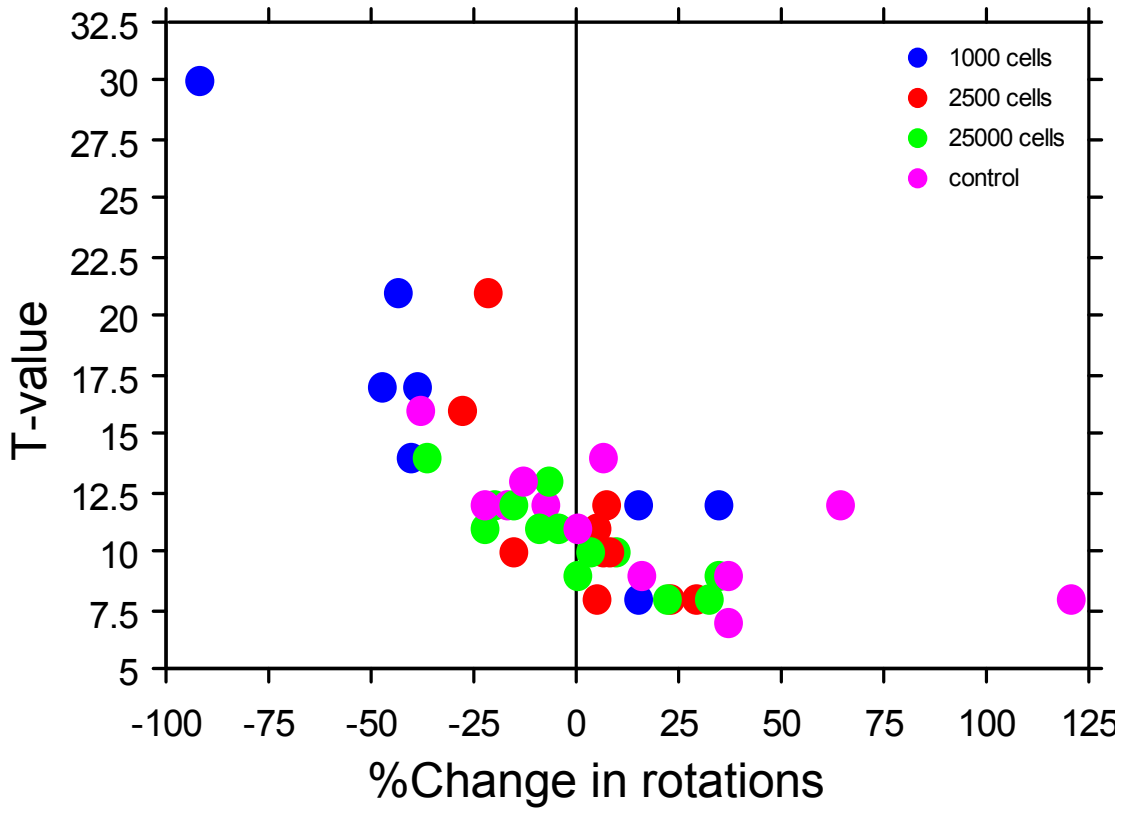

Figure 4.5: T- value. Data from all the animals in both trials was used to generate T - values. This pilot data reveals that the T-value has merit. Animals that displayed superior improvement behaviorally (indicated here by the change in their rotations at sacrifice on x-axis) tended to have a higher T-value (shown on the y - axis).

Discussion

Several rodent models have been used to study the pathology and therapeutic intervention in PD (Beal, 2001), of which the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyrodine) model and 6-OHDA model are most commonly used. The 6-OHDA rodent model is well-known for its unilateral lesions that create a definite behavioral phenotype that is easily quantifiable (Beal, 2001). In addition, a chronic 6-OHDA model has been described (Sauer and Oertel, 1994), which shows a progressive loss of DA neurons in the ventral midbrain. The neurotoxin, 6-OHDA, when injected into medial forebrain bundle causes destruction of the dopaminergic neurons in the ipsilateral SN and VTA leading to PD phenotype. Previously, we have utilized the 6-OHDA model to asses the therapeutic potential of low-density hUCMS cells (Weiss et al., 2005). The 6-OHDA lesion was assessed immunocytochemically by staining the brain sections with an antibody against tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis (Kim et al., 2002). Unilateral staining of TH in the ventral midbrain DA neurons and striatum on the contralateral side of the lesion showed the evidence of the toxic effect of 6-OHDA on the dopaminergic system (Weiss et al., 2005). The degeneration of DA neurons is largely restricted to SN and VTA. The rats were screened based on the apomorphine-induced rotatory behavior for transplantation of hUCMS cells. Apomorphine is a dopamine agonist, which stimulates dopamine receptors, activates the receptors on both sides, but greater activation on the lesion side because of denervation supersensitivity, leading to rotations on contralateral side (Kirik et al., 2000a;Nishimura et al., 2003).

Animal ID	Transplant	Survival	% Rotations Pre-transplant	% Rotations 6 week	% Rotations 10 week	Response
SC101	Medium-density	6 week	100	129	-	NR
SC104	Medium-density	6 week	100	72	-	R
SC108	Medium-density	6 week	100	123	-	NR
SC113	Medium-density	6 week	100	105	-	NR
SC115	Medium-density	6 week	100	106	-	NR
SC116	Medium-density	6 week	100	95	-	R
SC125	Medium-density	6 week	100	84	-	R
SC92	Medium-density	10 week	100	-	105	NR
SC98	Medium-density	10 week	100	-	108	NR
SC102	Medium-density	10 week	100	-	68	R
SC110	Medium-density	10 week	100	-	107	NR
SC114	Medium-density	10 week	100	-	90	R
SC119	Medium-density	10 week	100	-	78	R
SC94	High-density	6 week	100	63	-	R
SC135	High-density	6 week	100	80	-	R
SC139	High-density	6 week	100	77	-	R
SC144	High-density	6 week	100	95	-	R
SC149	High-density	6 week	100	109	-	NR
SC118	High-density	10 week	100	-	84	R
SC126	High-density	10 week	100	-	100	NR
SC132	High-density	10 week	100	-	132	NR
SC134	High-density	10 week	100	-	103	NR
SC137	High-density	10 week	100	-	93	R
SC138	High-density	10 week	100	-	134	NR
SC142	High-density	10 week	100	-	122	NR
SC146	High-density	10 week	100	-	91	R
SC93	control	6 week	100	83	-	C
SC127	control	6 week	100	77	-	C
SC88	control	10 week	100	-	164	C
SC91	control	10 week	100	-	100	C
SC117	control	10 week	100	-	137	C
SC129	control	10 week	100	-	116	C
SC133	control	10 week	100	-	62	C
SC140	control	10 week	100	-	87	C

Supplementary table 4.1: Summary of apomorphine-induced rotations in PD model rats. The number of apomorphine-induced rotations before transplantation is taken as pre-transplant% (baseline – 100%) to compare the changes in the rotatory behavior. The response parameter (R or NR) is only assigned to the PD-model rats that received hUCMS cell graft, but not sham graft (C). R (Responder) - decrease in %rotations compared to baseline (pre-transplant). NR (Non-responder) - no change or increase in %rotations compared to baseline (pre-transplant). C (Control) - animals that did not receive hUCMS cell graft.

In the earlier trial, we observed a significant behavioral improvement with a low-density (approximately 1000) hUCMS cell transplant into the striatum of non-immune suppressed PD rats, which correlated with increased number of TH-positive DA neurons in the SN and VTA on the lesioned side (Weiss et al., 2005). As the controls from the earlier trial were statistically not different from the controls in the current trial, we pooled the behavioral data from the two trials to evaluate the effect of graft density on the apomorphine-induced rotations. When the rotatory behavioral data was pooled from the first trial, we observed a dose-dependent decrease in apomorphine-induced rotations in the PD model rats. In the first trial, 5 of the 8 animals responded to the transplant, i.e. decreased their apomorphine-induced rotation behavior. In the second trial, 6 of 13 animals treated with 2500 cells and 6 of 12 animals treated with 25000 cells responded (see supplementary table 4.1). Thus, approximately 50% of the animals respond to UCM treatment. Although half of the rats with high-density transplant showed decrease in apomorphine-induced rotations, as a group they were not significantly different from the control group. In addition, some rats in the control group show spontaneous improvement in rotatory behavior (supplementary table 4.1). However, the intensity of response (% decrease in the number of rotations), but not the number of responders, makes the low and medium-density transplant groups significantly different from the control group. Moreover, we observed the other desirable parameters, such as health status (represented by the increase in body weight), to be lower in the control group compared to other groups (see supplementary table 4.2). Thus, T-value, which considers multiple

host and graft parameters, may be a better method to evaluate these PD model rats. In addition, the TH-positive DA neurons in the SN and VTA on the lesion side of the medium-density transplant group did not correlate with the behavioral improvement, which is different from what we noticed with the low-density transplant (Weiss et al., 2005). Taken together, this data suggests that the low-density graft may be more beneficial than the medium and high-density grafts.

One of the major issues of xenotransplantation is graft vs host disease (GVDH) and immune rejection (Larsson and Widner, 2000;Larsson et al., 2000;Larsson et al., 2001). Here, none of the animals received immune suppression therapy because, the results from our preliminary work, where we transplanted pUCM cells at various densities, showed that the transplanted cells were not rejected (Weiss et al., 2003). Moreover, when we transplanted 150 pUCM cells in 6-OHDA lesioned rats, the cells survived and proliferated upto 8 weeks after transplantation (Medicetty et al., 2004); based on which we have transplanted 1000 hUCMS in the first trial, followed by 2500 and 25000 hUCMS cells. However, we recovered only a few graft cells in PD model rats at 1-week post-transplantation, but none at 6-weeks or 10-weeks. The fact that we were able to recover a large number of graft cells in the immunosuppressed rats at 1-week post-transplantation suggests that the immune system may play a role in clearing the graft cells in non-immunesuppressed rats. Although the CD-11b positive cells (activated microglia) noticed around the graft at 1-week post-transplantation in non-immunesuppressed animals may add some credibility to the above

Animal No	Transplant	Survival	Wt. at transplant	Wt. at sacrifice	% change in Wt.	Wt. score for T-value
SC 45	low	6 week	245	265	8.2	5
SC 46	low	6 week	260	291	11.9	8
SC 55	low	6 week	266	276	3.8	2
SC 59	low	6 week	250	270	8.0	5
SC 56	control	6 week	261	265	1.5	1
SC 48	control	6 week	255	270	5.9	4
SC 47	low	12 week	251	290	15.5	10
SC 57	low	12 week	253	290	14.6	10
SC 60	low	12 week	260	290	11.5	8
SC 62	low	12 week	288	315	9.4	6
SC 61	control	12 week	253	275	8.7	6
SC 58	control	12 week	252	280	11.1	7
					Wt. Range 15.5-1.5	14
					10 groups	1.4% per group
					10	14.1
					9	12.7
					8	11.3
					7	9.9
					6	8.5
					5	7.1
					4	5.7
					3	4.3
					2	2.9
					1	1.5

Supplementary table 4.2: Scoring method for health status (% change in body weight), which was used to calculate T-value along with other parameters. The above table shows the body weight scores for all the animals in the low-density (1000 hUCMS cell) transplant group. Body weight at transplantation is taken as the baseline to assess the % change in body weight at the end of the survival period. The range is calculated based on the maximum and minimum % change in body weight and further divided into 10 groups with representative scores of 1-10. The animals were assigned a score based on the group they were in.

assumption, there were no CD4, CD8 or CD 161 cells identified at the graft site. In addition, the absence of CD8 cells at 6 or 10 weeks post-transplantation makes it pre-mature to put the blame entirely on the immune system. It is possible that the host immune cells may have cleared the graft cells and left the area by 6-weeks, as previous work with fetal pig mesencephalic tissue grafted into the rat brain indicates that the grafted tissue is rejected in about 4-6 weeks (Larsson et al., 2001). The other speculation is that the graft was not able to support itself and the cells did not survive for long time.

The behavioral recovery of the PD model animals could be due to rescue of the degenerating DA neurons of SN and VTA, which could be mediated by various trophic factors (Bjorklund et al., 1997;Kirik et al., 2000a;Kirik et al., 2000b;Yoshimoto et al., 1995;Chen et al., 1995;Fawcett et al., 1995;Haque et al., 1995). We have evidence that hUCMS cells produce potent trophic factors such as GDNF, VEGF, FGF20 and CNTF (Weiss et al., 2005). Previously, we reported that 40-80% of hUCM cells (depending on the isolate) produce GDNF (glial cell line-derived neurotrophic factor) (Weiss et al., 2005), which is a potent survival factor for dopaminergic neurons (Ding et al., 2004;Arenas et al., 1995;Bjorklund et al., 2000;Helt et al., 2001;Bjorklund et al., 1997). It has been shown that stem cells may promote neuroprotection by immunomodulation (Pluchino et al., 2005b;Pluchino et al., 2005a), which could be another cause for the behavioral improvement in this PD model rats. Recently, Ryan et al. reported that mesenchymal stem cells may evade the allogenic host immune response by,

1. hypoimmunogenecity, 2. preventing T-cell response by dendritic cell modulation and 3. inducing a local immunosuppressive milieu (Ryan et al., 2005). Preliminary data from our lab shows that hUCMS cells express HLA-G (manuscript in preparation), which is an MHC-like protein present at the feto-maternal interface and is shown to have immunosuppressive capabilities (Rouas-Freiss et al., 2000;Rouas-Freiss et al., 1999). In addition, hUCMS cells seem to dampen the proliferative potential of mitogen-stimulated splenocytes (manuscript in preparation). We hope to elaborate on the immunosuppressive abilities of hUCMS cells with ongoing work in our lab.

It is critical to have a universal metric to assess and optimize the cell transplants, where multiple parameters of both the host and the graft are taken into consideration (Lindvall et al., 2004). Here, we introduced a new universal metric, Transplant or T-value, to asses the effect of hUCMS cell transplants on PD model rats. In both the trials, as we could not recover the graft cells at 6, 10 or 12 weeks post-transplantation, the graft outcome 'G' had only the 'tumor incidence' component. Here, the T-value is shown to be a useful metric by its positive correlation with the behavioral recovery. The T-value can be used to compare between cell-type or cell-number and other experimental variables, such as immune suppression. Theoretically, the T value could be adapted for any type of transplantable cell or disease model. In future studies, we intend to use the T-value to optimize hUCMS cell transplantation in PD rats, and to investigate possible mechanisms whereby hUCMS cells mediate their effects.

Transplantation of hUCM cells showed a dose-dependent behavioral recovery in the PD model rats. Based on our work in progress (data not shown), we suggest two likely mechanisms for the behavioral improvement and increased survival of DA neurons in the SN and VTA: 1. Trophic rescue via factors such as GDNF or 2. Decreased neuroinflammation due to immune suppression by hUCM cells. We intend to utilize an *in vitro* PD model (6-OHDA treated PC-12 cells) (Ha et al., 2003;Ryu et al., 2002;Tarabin and Schwaninger, 2004) free of immune cells to elucidate possible trophic effects of hUCMS cells, and *in vitro* immune function assays (Djouad et al., 2003;Le et al., 2004;Pittenger et al., 1999) to determine whether hUCM cells are immunesuppressive. Our preliminary *in vitro* work reveals that hUCMS cells are immunesuppressive (manuscript in preparation). Given the fact that a large number of graft cells were recovered at 1-week post-transplantation in immunesuppressed rats, we intend to compare the effect of hUCMS cells in immunesuppressed and non-immunesuppressed parkinsonian rats. In conclusion, the hUMCS cells, a type of mesenchymal stem cells, may be therapeutically useful in treating central nervous system disorders such as PD.

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PART V

Major conclusions and discussion

Stem cells are not only drawing the attention of scientists, ethicists, policy makers and general public, but still maintain their place as one of the most promising yet controversial scientific discoveries of the 20th century. Although there is some evidence of how these undifferentiated stem cells work, a more sophisticated research is on its way to authenticate and substantiate the applicability of stem cells for therapeutic purposes. Various stem cells were shown to have therapeutic potential in a wide range of diseases, including neurodegenerative diseases such as Parkinson's disease (PD). To date, ESCs and NSCs have been used for cell-therapeutic purposes in animal models of PD, mostly to attempt to replace the damaged dopaminergic (DA) neurons. Although there is some promising work demonstrating the efficacy of ESCs and NSCs in PD model animals (Bjorklund et al., 2002;Kim et al., 2002;Ourednik et al., 2002;Pluchino et al., 2005), other associated limitations, such as pre- and post-transplantation complications has lead researchers in this area to think about alternate sources of stem cells. In addition, the relatively new neural regeneration/rescue strategies are exerting fundamental changes in the concepts of therapeutics for neurodegenerative diseases. Post-natal and adult stem cells that bypass a number of limitations of ESCs and NSCs have been generating interest in the past few years. While mesenchymal stem cells (MSCs) (Pittenger et al., 1999;Prockop et al., 2001;Woodbury et al., 2000)and multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002;Reyes and Verfaillie, 2001;Schwartz et al., 2002) from bone marrow have created genuine interest in adult stem cells,

other sources of stem cells are constantly making news. Here I describe a post-natal or adult stem cell, derived from umbilical cord matrix (UCM), which is an inexhaustible source of mesenchymal-like stem cells that may have therapeutic importance in PD.

Previously, it has been shown that pig UCMS cells express various stem cell characteristics and can be differentiated into neuron-like cells *in vitro* and *in vivo* (Mitchell et al., 2003;Weiss et al., 2003). When transplanted into a normal rat brain, pUCMS cells expressed neuronal markers, such as NF-70 (Weiss et al., 2003), a pig specific neurofilament protein. Here, the safety and proliferative potential of pUCMS cells was evaluated in a 6-OHDA lesioned rat brain (Medicetty et al., 2004). We have noticed that pUCMS cells engrafted and proliferated up to 8 weeks post-transplantation, where we observed a 7-8 fold increase in the number of graft cells. In addition, graft cells expressing TH increased overtime from 1% at 2 weeks to 6% at 8 weeks following transplantation. Moreover, there was no significant gross or histological evidence of immune rejection. Although xenotransplantation could potentially provide an unlimited supply of transplantable tissues and organs for humans, the important risk factors that keep this technique out of the clinic are immune rejection, graft versus host disease (GVHD), cross-species transmission of undetected or unidentified animal infectious agents, and uncertain efficacy/viability. It is known that replacement heart valves from pigs are used for

therapeutic purposes (Chen et al., 2003;Oxenham et al., 2003). However, these therapeutic products have been chemically treated and are not living tissues, which distinguishes them from the viable organs or cells used in xenotransplantation. As pUCMS cells were able to engraft, proliferate and differentiate in a rat brain without eliciting a frank immune response, they may be a potential xenogenic source of stem cells for therapeutic application in PD, with still having to address the safety concerns of xenotransplantation.

Human UCM is a non-controversial and inexhaustible source of post-natal or adult stem cells. The UCM may contain 1. Incompletely migrated EGCs, 2. HSCs dispersed in the surrounding connective tissue or 3. Mesenchymal-like connective tissue cells in the umbilical cord matrix that resembles embryonic mesenchyme. The surface antigen profile and gene-expression profile of human UCMS cells closely resemble to that of mesenchymal stem cells (Weiss et al., 2005). The fact that hUCMS cells do not express the classic HSC markers, CD34 and CD45, tells that they may not be related to HSCs. Although hUCMS cells express some primitive stem cell markers expressed by EGCs (Turnpenny et al., 2005), the morphology in culture and phenotype of the hUCMS cells do not resemble that of EGCs. In addition, at this time it is unclear if hUMCS cells are pluripotent as EGCs (Pan et al., 2005;Park et al., 2004;Turnpenny et al., 2005). Recently, it has been shown that fetal MSCs have higher proliferative capacity and less lineage commitment than adult MSCs (Gotherstrom et al., 2005). It is possible that the hUCMS are similar to that of fetal MSCs rather than adult

MSCs, as suggested by the source and gene expression, for example, preliminary data in our lab shows that hUCMS cells express HLA-G (data not shown), which is expressed by fetal MSCs, but not adult MSCs (Gotherstrom et al., 2005).

One of the major concerns of stem cells in culture is the culture-induced genetic changes in the cells, which may be a big hurdle to pursue translational research and clinical trials. It has been reported that 5 of the 11 human ESC lines in United States are genetically unstable (Maitra et al., 2005). In this context, the hUCMS may have a major advantage over other stem cells as hUCMS cells can be harvested in relatively large numbers compared to other stem cells, which minimizes the culture-induced genetic changes that can occur in stem cells. Based on the length of the cord, 3 – 5 million cells can be extracted from each umbilical cord (Weiss et al., 2005). To minimize the effect of the culture-induced changes in these experiments, we used the cells within 10 passages after extraction.

Here, we noticed a dose-dependent behavioral improvement in PD model rats following hUCMS cell transplantation. The low-density graft (1000 hUCMS cells) produced the best improvement, where more than 50% of the animals showed 50-60% reduction and one animal showed 90% reduction in apomorphine-induced rotatory behavior (Weiss et al., 2005). Also, we observed a significant decrease in rotatory behavior in the animals that received medium-density graft

(2500 hUCMS cells). Although some of the animals that received high-density graft (25,000 hUCMS cells) showed improvement in the rotatory behavior, as a group they were not significantly different from the control group that received vehicle (saline). The non-PD model rats (animals that did not receive 6-OHDA) with or without low-density hUCMS cell transplants were not affected by apomorphine i.e. they did not show any apomorphine-induced rotatory behavior. The latter finding adds to the safety of hUCMS cells as transplantable cells as they do not seem to produce any physiological alterations (as indicated by apomorphine-induced rotations) in normal rats.

However, we were able to recover hUCMS cells 2-days and 1-week post-transplantation, but not at 6, 10 or 12 weeks, in non-immunesuppressed PD model rats. This finding is completely in contrast to what we observed in non-immunesuppressed rats that received pUCMS cell grafts. As mentioned earlier, 150 pUCMS cells survived and proliferated in 6-OHDA lesioned rat brain (Medicetty et al., 2004). This may be due to 1. Non-primate cells including pig cells possess an enzyme □-1, 3-galactosyl transferase (Alisky, 2004) that may help in evading the hyperacute rejection when transplanted into rats, increasing the chances of survival and proliferation, 2. Difference in the cell-phenotype owing to changes in culture media; pUCMS cells were grown in complete medium (see part II, methods section) (Medicetty et al., 2004;Weiss et al., 2003) where as hUCMS cells were expanded in defined medium (see part III, methods section) (Weiss et al., 2005). Optimizing the culture conditions is a major

challenge in stem cell research. Although, we had no problems expanding pUCMS cells in complete medium, the hUCMS cells did not seem to thrive well in complete medium. Later, we started growing hUCMS cells in defined media (used for expanding MAPCs) (Jiang et al., 2002;Reyes and Verfaillie, 2001), and noticed that the cells were phenotypically stable up to 10 passages after extraction (Weiss et al., 2005).

Previously, Bjorklund et al. showed that murine ESCs can differentiate into functional dopaminergic neurons in PD model rats; an important finding in this study is that low-density grafts were better than high-density grafts (Bjorklund et al., 2002). Although this study was very promising, an important limitation in this highly cited publication is that, of the 25 animals treated, grafts did not survive in six, and five had grafts that developed into tumors or teratomas due to uncontrolled growth, and only the remaining 14 rats had cell grafts that survived and became dopaminergic. In the above mentioned study, although the rats received immune suppression, 20% of the animals had tumors or teratomas, 24% of the animals showed no graft survival and 56% of the animals responded to the graft. Although we did not see a dramatic behavioral recovery (as seen with ESCs) (Bjorklund et al., 2002;Kim et al., 2002) in our experiments with hUCMS cells, we have noticed that approximately 50% of the animals respond to the graft. Moreover, there are no tumors or teratomas observed in any of the transplanted animals, which is an important parameter to address the safety

concerns. In addition, preliminary work in our lab suggests that hUCMS cells do not form tumors or teratomas in immunodeficient (SCID) mice (data not shown).

Ron McKay's team at the National Institute of Neurological Disorders and Stroke (NINDS) showed that ESCs, which were genetically engineered and pre-differentiated into dopaminergic neurons *in vitro*, were functional and useful in rescuing PD model rats (Kim et al., 2002). Although, this group prevented tumorigenesis by pre-differentiating ESCs, the transgenic techniques have a long way to go before they can be approved for clinical trials in humans. Also, there are other technical complications associated with transplanting pre-differentiated cells; for example, extensive network formation by the neurites extending from the differentiating cells may be subjected to mechanical damage during transplantation leading to cell death and in turn affecting the graft survival. Both pig and human UCMS cells have shown to be able to differentiate into neuron-like cells in culture. However, we did not have an opportunity to transplant the pre-differentiated UCMS cells to compare the efficacy to that undifferentiated cells. Recently, Fu et al. have reported that pre-differentiated human umbilical cord mesenchymal cells may be useful in halting the progression of disease in PD model rats (Fu et al., 2005). However they have noticed that PD model rats transplanted with undifferentiated human umbilical cord mesenchymal cells deteriorated overtime similar to the controls that received PBS (Fu et al., 2005), which is in contrast to the behavioral improvement that we noticed after

transplantation of 1000 and 2500 undifferentiated hUCMS cells. The fact that we did not see any behavioral improvement in the PD model rats that received 25000 cells may explain the finding reported by Fu et al., where they have transplanted $1x10^5$ undifferentiated cells. In future, we intend to conduct studies to look at the effect of pre-differentiation of UCMS cells in engraftment of the cells and promoting therapeutic benefit to PD models rats.

Here, the hUCMS cells, when transplanted into PD model rats, showed improvement in the apomorphine-induced rotatory behavior. However, we could not recover the graft cells at 6, 10 or 12 weeks post-transplantation. In this context, we believe that hUCMS cells may be more useful to regenerate/rescue than they are to replace damaged DA neurons in PD. We hypothesize that this behavioral improvement may be due to the trophic factors released by hUCMS cells. Recently, it has been shown that umbilical cord blood cells (Borlongan et al., 2004;Newman et al., 2004) and bone marrow stromal cells (Chen et al., 2001a;Chen et al., 2001b;Li et al., 2000) have neuroprotective effect that is mediated by trophic factors. We have noticed that a majority of hUCMS cells *in vitro* express GDNF (Weiss et al., 2005), which is known to be a potent neurotrophic factor for DA cells. Previous studies have shown that GDNF can rescue DA cells in culture as well as in PD animal models (Ding et al., 2004;Arenas et al., 1995;Bjorklund et al., 1997;Kirik et al., 2000). Currently, we are investigating this hypothesis by using an *in vitro* PD model, 6-OHDA treated

PC12 cells (Ha et al., 2003;Ryu et al., 2002). In addition, the gene array shows that hUCMS cells make transcripts of other trophic factors such as, vascular endothelial growth factor (VEGF), fibroblast growth factor – 20 (FGF20) and ciliary neurotrophic factor (Weiss et al., 2005), which were previously shown to be neuroprotective (Greenberg and Jin, 2004a;Greenberg and Jin, 2004b;van der Walt et al., 2004;Cameron et al., 1998). Also, we observed that the behavioral recovery in the PD model rats that received low-density hUCMS cell grafts correlates with the TH-positive DA cells in SN and VTA in the respective animals at 12 weeks following transplantation (Weiss et al., 2005). Although we noticed a significant improvement in the rotatory behavior, we did not see a correlation with the TH-positive cells in the low-density 6-week survival and the medium-density grafts. This may be attributed to the multiple host and graft factors affecting the efficacy of the graft. With higher density graft, there may be increased host-reaction, which may in turn decrease the overall efficacy of the graft, suggesting the importance of the graft and host interaction in determining the efficacy of the graft.

Although interaction between various parameters was shown to be an important indication of the graft efficacy in PD patients, a more comprehensive analysis of the patient and optimization of the graft is suggested to be essential for future PD studies (Lindvall and Bjorklund, 2004). Here, we introduced a novel universal metric (Transplant or T – value) by including the multiple host and graft

parameters (see part IV, methods section), which can be used to asses the graft efficacy. The parameters that are included in T-value, such as behavioral improvement (Dunnett et al., 1987;Nishimura et al., 2003;Olsson et al., 1995), host DA cells (Ourednik et al., 2002), graft outcome (Bjorklund et al., 2002;Kim et al., 2002) etc., have been considered separately by various researchers, but here we wanted to look at all the parameters simultaneously and generate a comprehensive measure of the improvement in the animals and efficacy of the graft. We observed a positive correlation between T-value and behavioral recovery in all the experimental groups of PD model rats, which further corroborates the use of T-value to assess the graft efficacy. In addition, this T-value can be used to, 1. Analyze both replacement and rescue type of stem cell transplants, 2. Compare between different types of stem cell transplants and 3. Assess the efficacy of the cell grafts in other neural and non-neural disease conditions.

Immune cells were implicated to play a critical role in various neurodegenerative disorders including PD (Block and Hong, 2005;Wu et al., 2005;Zhang et al., 2005). Thus, the immune modulatory capabilities of some stem cells may be useful to promote therapeutic benefit in PD. Previous studies indicate that mesenchymal stem cells have immunosuppressive properties (Le et al., 2004;Djouad et al., 2003). Recently, it has been reported that mesenchymal stem cells may evade the allogenic host immune response by their

hypoimmunogenic properties, ability to prevent T-cell response by dendritic cell modulation, and by inducing a local immunosuppressive milieu (Ryan et al., 2005). As we did not notice any immune cells at the transplant site in the PD model rats that received UCMS transplant (Medicetty et al., 2004;Weiss et al., 2005), it is possible that the graft cells may have evaded the host immune response by some immune modulatory mechanism. Moreover, preliminary work in our lab suggests that hUCMS cells may have immunosuppressive properties (manuscript in preparation). Also, it is known that cells at the feto-maternal interface in humans express HLA-G that has been shown to play a role in suppressing immune cells (Carosella et al., 2003;Rouas-Freiss et al., 2003;Rouas-Freiss et al., 2000;Rouas-Freiss et al., 1999). Preliminary studies in our lab suggest that hUCMS cells *in vitro* make transcripts of HLA-G (data not shown). Ongoing studies in our lab would confirm and explain the immunosuppressive capabilities of hUCMS cells.

I believe that replacement therapy by stem cells in PD is still in its initial stages and has a long way to go for clinical trials in human patients. The prospects of using stem cells for replacement therapy in PD are complicated by the tasks that a donor replacement cell should complete to integrate into the host system i.e., a donor replacement cell should not only engraft in the host brain, but also should 1. Evade the host immune response, 2. Differentiate and exhibit the required neural phenotype, 3. Extend neurites and make appropriate connections (form

synapses) with the other resident neurons and glia, 4. Produce appropriate neurotransmitters and conduct action potentials and 5. Survive and function for a long period. Considering the above mentioned daunting tasks for a replacement cell, there is a better chance for stem cells being used to rescue, rather than to replace the damaged DA cells. The rescue phenomenon is comparatively simple and is based on revitalizing the existing, but damaged host neural circuitry. Moreover, it is possible that the trophic and immune modulatory factors that are useful to rescue the damaged DA cells may cross the blood-brain barrier and thus may raise questions about the necessity of a brain transplant to treat PD. Based on the fact that approximately 90% of the PD patients are in the 5^{th} and 6^{th} decades of their life, avoiding a brain transplant would add an enormous benefit for the normal health of the patient. Although it is yet to be proven, it would be ideal to have a stem cell that can be injected through intravenous route and can produce trophic and immune modulatory factors that can cross the blood-brain barrier and rescue the damaged DA cells.

The question remains whether any of these stem cells work in the PD patients; can the animal model results be directly related to a human patient? Recent trials showed that patients with PD did not benefit from receiving fetal neural tissue transplants; indeed, some patients experienced jerky abnormal involuntary movements, known as dyskinesias (Check, 2003). Moreover, it has been shown that L-DOPA, which is used for symptomatic treatment in PD, induces

dyskinesias that are a major concern in managing PD patients (Obeso et al., 2000b;Obeso et al., 2000a). Although the primary cause of dyskinesia remains to be established, studies in animal models are beginning to unravel the cellular and molecular details of this movement disorder. Previous studies have shown that drug-induced dyskinesia is associated with changes in gene expression in DA projection areas in the brain (Cenci et al., 1998) and depends on the topography of DA denervation with in the projection areas (Winkler et al., 2002). These drug and graft induced dyskinesias may pose a significant concern by affecting the behavioral analysis, particularly the drug-induced rotatory behavior, in the PD animal model studies. We speculate, based on the ongoing studies in our lab, that the drug-induced rotatory behavior may be affected by dyskinesias (data not shown), which may lead to ambiguity in interpretation of the data. It is possible that the control animals may show a decrease in the drug-induced rotations due to dyskinesias. However, a dyskinesia scoring system has been recently introduced that evaluates various abnormal involuntary movements, thus providing a tool to assess the reliability of these animal models (Winkler et al., 2002). In future, we intend to follow-up the dyskinetic animals, if there are any, by using the dyskinesia scoring system and if necessary, modify the behavioral tests accordingly; as there are other valid tests to evaluate PD model animals with out using any drugs (Olsson et al., 1995;Dunnett et al., 1987).

In conclusion, the UCM is a source of post-natal or adult stem cells. Pig UCMS cells engraft and proliferate following transplantation into a lesioned-rat brain, where a subset of the graft cells express neuronal markers (Medicetty et al., 2004). Human UCMS cells are non-controversial and can be extracted in large numbers, and phenotypically resemble mesenchymal stem cells (Weiss et al., 2005). When transplanted in PD model rats, hUCMS cells show a dose-dependent improvement in the rotatory behavior, and thus may have therapeutic applications in PD. Further investigation is underway to unveil the mechanism by which hUCMS cells produce this improvement in PD model rats.

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