University of Louisville

ThinkIR: The University of Louisville's Institutional Repository

Electronic Theses and Dissertations

12-2011

Human olfactory epithelial-derived progenitors : a potential source for cell therapy for Parkinson's disease.

Meng Wang
University of Louisville

Follow this and additional works at: <https://ir.library.louisville.edu/etd>

Recommended Citation

Wang, Meng, "Human olfactory epithelial-derived progenitors : a potential source for cell therapy for Parkinson's disease." (2011). *Electronic Theses and Dissertations*. Paper 1513.
<https://doi.org/10.18297/etd/1513>

This Doctoral Dissertation is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.

**HUMAN OLFACTORY EPITHELIAL-DERIVED PROGENITORS:
A POTENTIAL SOURCE FOR CELL THERAPY FOR PARKINSON'S DISEASE**

By

Meng Wang

M.D., Tianjin Medical University, Tianjin, China, 2005

M.S., University of Louisville, Kentucky, US, 2007

A Dissertation

**Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of**

Doctor of Philosophy

**Department of Anatomical Sciences and Neurobiology
University of Louisville
Louisville, Kentucky**

December, 2011

HUMAN OLFACTORY EPITHELIAL-DERIVED PROGENITORS:
A POTENTIAL SOURCE FOR CELL THERAPY FOR PARKINSON'S DISEASE

By

Meng Wang

M.D., Tianjin Medical University, Tianjin, China, 2005

M.S., University of Louisville, Kentucky, US, 2007

A Dissertation Approved on

December, 16th 2011

by the Following Committee:

Dissertation Director, Fred J Roisen, Ph.D.

Nigel Cooper, Ph.D.

Kathleen Klueber, Ph.D.

Irene Litvan, M.D.

Mengsheng Qiu, Ph.D.

Welby Winstead, M.D.

DEDICATION

This dissertation is dedicated to my parents

Mr. Heping Wang

and

Mrs. Haiying Wang

who opened my eyes to this world, and have given me encouragement and invaluable educational opportunities.

To my dear grandparents

Mr. Ruicai Wang

and

Mrs. Xiuzheng Li

who gave me unconditional support and helped me overcome the difficulties in my life.

To my dear Huihang and Lucas

who love and understand me with their lives.

ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Fred Roisen, for his guidance, education, and patience. I would also like to thank the other committee members, Drs. Nigel Cooper, Kathleen Klueber, Mengsheng Qiu, Irene Litvan and Welby Winstead, for their invaluable discussion and comments. Many thanks are to the faculty and students of the Department of Anatomical Sciences and Neurobiology and all my colleagues who have given me helpful suggestions and assistance. Dr. Chengliang Lu, Dr. Yu Lin, Jodi Hallgren, Patrick Baumann, Deborah Black and Holly Waechter have given me great help in experiment design and operation. Dr. Ian Fentie and Mrs. Chris Ekstrom have been experts on reviewing the manuscripts and my thesis. I also thank Mrs. Chris Ekstrom for her help with the photographic images. I would like to express my special thanks to my family, my aunts, uncles and dear cousin, for their love, support and understanding.

Dishman Family Foundation and RhinoCyte Inc. are acknowledged for their financial support to my research project. The Integrated Programs in Biomedical Sciences (IPIBS) are also acknowledged for my initial graduate fellowship.

ABSTRACT

HUMAN OLFACTORY EPITHELIAL-DERIVED PROGENITORS: A POTENTIAL SOURCE FOR CELL THERAPY FOR PARKINSON'S DISEASE

Meng Wang

December 16th, 2011

Human adult olfactory epithelium contains neural progenitors (hONPs) which replace damaged cellular components throughout life. Methods to isolate and expand the hONPs have been developed in our laboratory. In response to morphogens, the hONPs differentiate along several neural lineages. This study optimized conditions for the differentiation of hONPs towards dopaminergic neurons. The hONPs were treated with Sonic Hedgehog, in the presence or absence of Retinoic acid and/or forskolin. Transcription factors (Nurr1, Pitx3 and Lmx1a) that promote embryonic mouse or chicken dopaminergic development were employed to determine if they would modulate lineage restriction of these adult human progenitors. Transcription factor expression and tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, were detected in the transfected cells after 4-month selection with G418, indicating transfected hONPs were stably restricted towards a dopaminergic lineage. Furthermore, enzyme immunoassay was employed to detect the synthesis and release of dopamine. The most efficient dopamine transfection paradigm was determined. Equivalent levels of several neurotrophic factors were detected in both the pre- and post-transfected hONPs which

have potential roles in the maintenance, survival and proliferation of dopaminergic neurons.

This study engrafted cells modified by the most efficient transfection paradigm for dopamine formation into a unilateral neurotoxin, 6-hydroxydopamine (6-OHDA)-induced Parkinsonian rat model. Thirty-five percent of the animals engrafted with hONPs had improved behavioral recovery as demonstrated by the amphetamine induced rotation test as well as a corner preference and cylinder paw preference, over a period of more than 24 weeks. No difference was observed between the pre- and post-transfected groups indicating that the host environment facilitated dopaminergic differentiation *in situ*. Human fibroblasts did not diminish the Parkinsonian rotational deficits at any point during the study. The engrafted hONP population remained intact and TH positive for a minimum of six months *in vivo*. Higher dopamine levels were detected in the striatum of behaviorally recovered animals than in equivalent regions of their non-recovered counterparts. Throughout these experiments, no evidence of tumorigenicity was observed. These studies support our hypothesis that human adult olfactory epithelial-derived progenitors represent a unique autologous cell type for a cell-based strategy for the treatment of Parkinson's disease.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
LIST OF TABLES AND FIGURES.....	vii
CHAPTER	
I. GENERAL INTRODUCTION	1
A. Cell Replacement Therapy.....	4
B. Stem Cells.....	5
C. Human Adult Olfactory Epithelial	
Derived Neural Progenitors.....	8
II. LINEAGE RESTRICTION OF ADULT HUMAN	
OLFACTORY EPITHELIAL DERIVED PROGENITORS TO	
DOPAMINERGIC NEURONS	10
A. Introduction	11
B. Material and Methods	14
1. Cell Culture.....	14
2. Construction of expression plasmids.....	15
3. Transfection and selection.....	16
4. Treatment with Morphogens.....	16
5. Immunocytochemistry.....	17

6. Western Blot Analysis	18
7. Dopamine assay	19
8. Neurotrophin Assay	19
C. Results	20
1. Card Transfection of Olfactory-derived progenitors (hONPs) to achieve dopaminergic lineage restriction	20
2. Transfected hONPs remain restricted to dopaminergic lineage after removal from cryostorage	27
3. Lineage restricted hONPs produced and released dopamine	30
4. The effect of morphogens on tyrosine hydroxylase (TH) expression, dopamine formation and release	33
5. Stably transfected and pre-transfected hONPs produce neurotrophins (BDNF, CNTF and NT-3) at equivalent levels	39
D. Discussion	42
1. Pitx3 and nurr1 induce the DA neuron maturation synergistically	43
2. Treatment of hONPs with morphogens enhances intracellular and extracellular dopamine levels	46
3. Lineage restricted hONPs retain their capability to produce neurotrophic factors	48
III. PILOT STUDIES FOR ANIMAL MODELS OF PD	51
A. Selection of injection sites	53
B. Determination of Cell Number for the Transplantation	57
C. Complimentary Behavior Tests	63

1. Adjusting stepping test	63
2. Rope test	63
IV. TRANSPLANTATION OF HONPS IN A RAT MODLE OF PD	65
A. Introduction	66
B. Material and Methods	67
1. Cell Preparation	67
2. Animal Model and Cell Transplantation	68
2.1. Establishment of Rotational Parkinsonian Rat Model	68
2.2. Assignment of Experimental Groups	72
2.3. Cell Transplantation	72
3. Behavioral Analysis	73
3.1. Amphetamine-induced Rotation Test	73
3.2 Complementary Behavioral Tests	73
4. Immunohistochemistry	74
5. Dopamine assay	74
C. Results	75
1. The Effect of cell engraftment on behavioral activity	75
2. Transplanted hONPs Promote TH Expression in the Toxin-injured Sites	77
3. HONPs remain intact and TH positive for a minimum of 6 months after transplantation	87
4. Analysis of the dopamine levels	94
D. Discussion	97

V. SUMMARY AND FUTURE DIRECTIONS	105
REFERENCES	115
CURRICULUM VITAE	136

LIST OF FIGURES

FIGURE	PAGE
Figure 1. Chemical formula and biosynthesis for dopamine	3
Figure 2. Construction of Expression Plasmid.....	15
Figure 3. Immunocytochemical analysis of pre- and post-transfected hONPs.....	22
Figure 4. Western Blot of pre- and post-transfected hONPs.....	25
Figure 5. Immunocytochemistry and western blot analysis of hONPs recovered from cryostorage	28
Figure 6. Intracellular and extracellular dopamine analysis of pre- and post-transfected hONPs in vitro.....	31
Figure 7. HONPs treated in DFBNM with Shh.....	34
Figure 8. HONPs cultured in DFBNM supplemented with Shh, in the presence or absence of retinoic acid (RA) and forskolin (FN)	37
Figure 9. Neurotrophin levels in pre- and post-transfected hONPs.....	40
Figure 10. Injection points of 6-OHDA and for transplantation of pre- and post- transfected hONPs, human fibroblast and medium.....	58
Figure 11. Experimental equipment set-up and animal mounting.....	70

Figure 12. Animal rotation test analysis·····	78
Figure 13. Corner preference test and cylinder test analysis·····	80
Figure 14. TH staining of neural toxin lesioned animal brain sections·····	83
Figure 15. TH staining of hONP, human fibroblast or medium implanted animal brain sections·····	85
Figure 16. Immunohistochemistry analysis of pre- and post-transfected hONPs twenty-four weeks after transplantation·····	88
Figure 17. Pre- and post-transfected hONPs migrate after transfection·····	90
Figure 18. Dopamine analysis in animal brains·····	95

LIST OF TABLES

TABLE

Table 1. Qualifying model rate on different toxin injection sites.....	55
Table 2. Cell Transplantation and Rotation Analysis.....	60
Table 3. Cell counting analysis in the brain of the behavioral recovered or non-recovered animals which received pre-and post-transfected hONPs.....	92

CHAPTER I

GENERAL INTRODUCTION

Parkinson's disease (PD) remains one of the leading causes of chronic degenerative neurological disability, which affects more than 6,000,000 people world-wide, with approximately 60,000 new cases diagnosed each year in the United States (National Parkinson Foundation, 2010). The incidence rises with age, being approximately 1:1000 overall and 1% of the population over the age of 60 and 4% in those over 80 years. Unfortunately, the mortality rate of PD has increased steadily in recent years (de Lau and Breteler, 2006; Anderson and Caldwell, 2007) as the population longevity has increased (Savica et al., 2010). One of the early stage symptoms is the loss of smell (Zucco et al., 1991; Zucco et al., 2001) and the later symptoms are movement-related, including shaking, rigidity, slowness of movement and difficulty with walking and gait (Inoue et al., 2007; Garcia-Ruiz, 2011). At more advanced stages of the disease, problems with dementia may commonly arise (Klassen et al., 2011; Parekh, 2011). The cause of PD remains unknown and it is characterized by the extensive loss of dopaminergic (DA) neurons in the substantia nigra (SN) in the midbrain (Hornykiewicz, 1973b).

Dopamine is a neurotransmitter that can function as a hormone which mainly inhibits the release of prolactin from the anterior lobe of the pituitary (Benes, 2001). Dopamine is synthesized in body first by the hydroxylation of the amino acid L-tyrosine to L-DOPA via the enzyme tyrosine hydroxylase (TH), which has been widely used as a marker for dopaminergic neurons (Sauer et al., 1993; Takeuchi et al., 2005; Redmond et al., 2007). L-dopa is then decarboxylized by aromatic L-amino acid decarboxylase to the final product, dopamine (Figure1) (Barger and Dale, 1910). Dopaminergic neurons are primarily found in the substantia nigra (SN) and the ventral tegmental area (Wise, 2004)

and they have many important roles in the brain, including voluntary movements, behavior and motivation (Vadasz et al., 1992b, 1992a; Stoessl, 1996; Da Cunha et al., 2009).

Figure 1. Chemical formula and biosynthesis for dopamine.

Currently the principle treatment for PD is oral L-3,4-dihydroxyphenylalanine (L-dopa) (Bidet-Ildei et al., 2011), which is the precursor of dopamine that can pass the blood-brain-barrier (Hornykiewicz, 1973a). L-dopa promotes symptomatic relief. Patients who took L-dopa improved in their motor function, activities of daily living and quality of life of the patients (Evans et al., 1980; Kalinderi et al., 2011). However, with time the drug becomes less effective for two reasons: 1. During the progression of the disease the neurons become less sensitive to the drug (Callaway, 2011) and 2. L-dopa does not prevent or rescue the DA neurons from degeneration (Lang and Lozano, 1998; Sheng et al., 2010). Furthermore, dyskinesia was eventually developed after several years of treatment with L-dopa (Friedman, 1985; Wedekind, 2005). Dyskinesia is characterized by twisting, jerking or dance-like movement, mostly of the arms and/or face. This symptom results from changes in striatal signaling after long-term pulsatile dopaminergic stimulation (Stacy and Galbreath, 2008). Clearly, an alternative treatment that can provide long-term recovery with no side-effects is needed.

A. Cell Replacement Therapy

The basic concept of cell replacement therapy is to restore function lost as a result of the disease in the central nervous system (CNS) by replacing degenerating or lost cells with viable functional cells. Recent research has attempted to find cell populations that can be used to replace lost or degenerating dopaminergic neurons (Anderson and Caldwell, 2007; Parish et al., 2008). Studies have employed neural cell grafts obtained from the fetal ventral mesencephalic (VM) dopaminergic neurons, and were able to successfully relieve the Parkinsonian symptoms following the embryonic tissue transplantation (Lindvall et al., 1988; Madrazo et al., 1988; Lindvall et al., 1992;

Freeman et al., 1995; Borlongan, 2000; Ganser et al., 2010). However, this treatment frequently resulted in significant dyskinesia (Freed et al., 2001; Olanow et al., 2003; Barker and Kuan, 2010; Lane et al., 2010). Even when positive clinical improvements were achieved in the absence of dyskinesia, the amount of tissue required for each PD patient necessitated a minimum of 4-5 fetal brains (Mendez et al., 2005). This requirement increased the possibility of viral or bacterial infection and the lack of donor source has significantly limited the utility of this approach. In addition the number of surviving neurons was highly limited as the majority of the engrafted cells died in the initial days following transplantation (Borlongan, 2000; Barker and Kuan, 2010; Ganser et al., 2010). The limited supply of fetal VM cells coupled with their poor graft viability severely limited the therapeutic utility of this population for the treatment of PD. Therefore, an alternate expandable source of dopamine cells has become a major research focus (Daadi, 2002; Doss et al., 2004; Lindvall et al., 2004; Xiong et al., 2011).

B. Stem Cells

Stem cells are undifferentiated cells with an unlimited capacity for self-renewal and the potential for lineage restriction (maturation) into one or more specific cell types, depending on their origin and the micro-environmental signals that they receive (Lindvall et al., 2004; Hwang et al., 2010). These characteristics make stem cells an attractive target population for PD cell replacement therapy (Snyder and Olanow, 2005; Sonntag et al., 2005; Kim, 2011; Tonnesen et al., 2011). Recent studies also suggest that the engraftment of stem cells or progenitors can up-regulate or enhance existing endogenous progenitor populations (Redmond et al., 2007; Abdel-Salam, 2011; Ruff et al., 2011).

Stem cells can be divided into three major groups: embryonic stem cells, induced pluripotent somatic cells and adult human stem cells. Embryonic stem cells (ESC) originate from the inner cell mass of blastocysts (Thomson et al., 1998). Mouse and porcine ESCs have been employed in cell replacement strategies for the treatment of PD animal models and behavioral improvements have been observed (Lonardo et al., 2010; Yang et al., 2010). However, a source of human cells is essential for clinical trials. Human embryonic stem cells were first isolated in 1998 from totipotent cells of the early mammalian embryo (Thomson et al., 1998). This was a breakthrough in the stem cell research field for these cells maintained their capacity for stable developmental restriction to form derivatives of all three embryonic germ layers even after prolonged culture (Thomson and Marshall, 1998). Dopaminergic lineage restricted ESCs were transplanted into different kinds of PD models, such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned non-human primate model (Bjugstad et al., 2008), or the 6-hydroxydopamine lesioned rodent model (Park et al., 2003; Brederlau et al., 2006; Geeta et al., 2008). Parkinsonian deficits were greatly relieved in the models that received ESC engraftment. However, unfortunately 50% of these animals were found to develop tumors within 6-8 weeks after cell transplantation (Arnhold et al., 2004; Hedlund et al., 2007). Furthermore, ethical concerns in using human embryos have limited the use of this cell source in clinical studies.

Human induced pluripotent stem cells (iPSC) may also represent a promising resource according to recent studies (Hargus et al., 2010; Chang et al., 2011; Soldner et al., 2011). They can be isolated from a variety of tissues, such as human fibroblasts (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), human or rat primordial germ

cells (Shamblott et al., 1998; Okita et al., 2007; Hamanaka et al., 2011), or mammalian embryos (Martin, 1981; Nichols et al., 1998). These cells can be reprogrammed to an “embryonic-like” state by transfer of nuclear contents into oocytes or by fusion with ES cells after being obtained from adult human somatic cells (Takahashi and Yamanaka, 2006). Human iPSCs are similar to hESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity (Takahashi et al., 2007). Therefore, human embryonic stem cells (hESCs) (Brederlau et al., 2006) and human induced pluripotent stem cells (iPSC) are both promising resources according to recent studies, with behavioral improvement in animal models of PD (Hargus et al., 2010; Chang et al., 2011; Soldner et al., 2011). However, like the ESC transplantation studies, in many cases animals received iPSCs, tumors containing a variety of tissues from all three germ layers formed within 9 weeks following the stem cell engraftment (Takahashi and Yamanaka, 2006; Chang et al., 2011). This result severely limits the utility of these two cell sources.

Collectively the studies illustrated above provide “proof of concept” that cell replacement therapy is a viable and hopeful therapeutic strategy for the treatment of individuals with PD. What is needed is a cell source to replace lost or damaged DA neurons which is stable, will not be tumorigenic, avoids the need for immunosuppressive agents, and eliminates the search for available donors as well as the ethical concerns associated with embryonic tissues.

C. Human Adult Olfactory Epithelial Derived Neural Progenitors

The olfactory epithelium is the only tissue in adult human that contains neural progenitors, which undergo neurogenesis throughout life without trauma (Sosnowski et

al., 1995; Calof et al., 1998; Zhang et al., 2000). Therefore, it is considered a unique source for the development of adult human stem cells. Human olfactory epithelium-derived progenitors (hONP) have been identified and successfully isolated in our laboratory (Roisen et al., 2001). To date more than 150 patient-specific cell lines of human olfactory neural progenitors (hONPs) have been established from primary cultures of human adult olfactory epithelium isolated from cadavers (Roisen et al., 2001) and patients undergoing endoscopic sinus surgery (Winstead et al., 2005). This tissue is a unique source for neural progenitors that can be harvested by minimally invasive endoscopic nasal surgery without a craniotomy (Winstead et al., 2005). This population is an autologous cell source which provides total histocompatibility and thus eliminates the need for immunosuppressive therapy as well as the ethical concerns and long waiting lists for available matched tissue.

Previously our laboratory has shown that the hONPs have the potential to differentiate along several neural lineages following exposure to environmental signals *in vitro* (Marshall et al., 2005; Zhang et al., 2006). Therefore, the present study was designed to determine: 1. If hONPs could be lineage restricted towards dopaminergic neurons with a proper local microenvironment; 2. To optimize the methodology for hONPs differentiation to dopaminergic neurons; 3. To determine if hONPs will function in a unilateral 6-hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease (Wright et al., 2009).

In this *dissertation*, the second chapter will describe how the hONPs can be lineage restricted to dopaminergic neurons, with the method of genetic manipulation and morphogenic treatment. It will also be shown that the transfection did not alter the

capability of the hONPs to produce neurotrophic factors, which are essential in the development of dopaminergic neurons. The most efficient transfection paradigm will be discussed and further transplanted into an animal model of PD. Chapter III will address the pilot studies for the following *in vivo* studies: the ideal animal model and the proper engrafting cell number/concentration and site. In chapter IV, the *in vivo* studies utilizing transfected and non-transfected hONPs implanted into the striatum, as well as a population of human fibroblasts and the medium as controls will be described. The observed behavioral recovery following engraftment of transfected and non-transfected hONP will be presented. Since equivalent results were obtained with both the pre- and post-transfected hONPs, these studies suggest that the hONPs without genetic manipulation have a high potential in the treatment of PD models. It was also demonstrated that the dopamine levels in animal brains were related to their behavioral improvement. In the final chapters, evidence will be presented which support our hypothesis that hONPs may represent an ideal autologous population for cell therapy for Parkinson's disease. The future studies are discussed which aim to identify mechanism of hONPs action on behavioral improvement in the PD models and to increase the recovery rate.

CHAPTER II

**LINEAGE RESTRICTION OF ADULT HUMAN OLFACTORY
EPITHELIAL DERIVED PROGENITORS TO DOPAMINERGIC
NEURONS**

Meng Wang, Chenliang Lu, Hong Li, Mengsheng Qiu, Welby, Winstead
and Fred J. Roisen

Published in Stem Cell Discovery, 2011, Vol.1 No.3 PP.29-43.

A. Introduction

As it has been introduced in Chapter I, this study is aiming to find an ideal source for cell-mediated treatment for Parkinson's disease due to their unlimited capacity for self-renewal and the potential for lineage restriction (maturation) into one or more specific cell types, depending on their origin and the micro-environmental signals that they receive (Lindvall et al., 2004; Hwang et al., 2010). Stem cells are considered an attractive target population for PD cell replacement therapy (Snyder and Olanow, 2005; Sonntag et al., 2005; Kim, 2011; Tonnesen et al., 2011). Human embryonic stem cells (hESCs), lineage-restricted towards dopaminergic neurons when transplanted into a rodent model of PD, provide a significant relief of symptoms (Schulz et al., 2004). However, with time, animals engrafted with hESCs have frequently developed teratomas (Brederlau et al., 2006). Human induced pluripotent stem cells (iPSC) are promising resource according to recent studies (Hargus et al., 2010; Chang et al., 2011; Soldner et al., 2011). These cells can be obtained from adult human somatic cells and reprogrammed to an "embryonic-like" state by transfer of nuclear contents into oocytes or by fusion with ES cells (Takahashi and Yamanaka, 2006). Human iPSCs are similar to hESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity (Takahashi et al., 2007). Transplantation of iPSCs has been shown to diminish some of the Parkinsonian behavioral deficits in several rodent models of PD; unfortunately in many cases similar to their embryonic counterparts the engrafted population formed, tumors containing a variety of tissues from all three germ layers (Takahashi and Yamanaka, 2006; Chang et al., 2011). Collectively the studies illustrated above provide "proof of concept" that cell replacement therapy is a

viable and hopeful therapeutic strategy for the treatment of individuals with PD. What is needed is a cell source to replace lost or damaged DA neurons which is stable, will not be tumorigenic, avoids the need for immunosuppressive agents, and eliminates the search for available donors as well as the ethical concerns associated with embryonic tissues.

The olfactory epithelium (OE) is a unique source for neural progenitors (multipotent neural stem cells) that can be harvested by minimally invasive endoscopic nasal surgery without a craniotomy (Winstead et al., 2005a). Furthermore, since no demonstrable olfactory deficits result from OE biopsy, the tissue can be used to generate an autologous progenitor population from patients with PD (Winstead et al., 2005a). An autologous cell source provides total histocompatibility and thus eliminates the need for immunosuppressive therapy as well as long waiting lists for available matched tissue. Previously our laboratory developed methods for the isolation and culture of a neurosphere forming population of neural progenitors (Roisen et al., 2001a). To date more than 150-patient-specific cell lines of human olfactory neural progenitors (hONPs) have been established from primary cultures of human adult olfactory epithelium isolated from cadavers (Roisen et al., 2001a) and patients undergoing endoscopic sinus surgery (Winstead et al., 2005a). Our studies have shown that the hONPs have the potential to differentiate along several neural lineages following exposure to environmental signals *in vitro* (Marshall et al., 2005; Zhang et al., 2006a). Therefore, with a proper local microenvironment, the hONPs have the potential to mature to dopaminergic neurons (Zhang et al., 2006a).

Molecular techniques were applied for the transfection of Nurr1 (Perlmann and Wallen-Mackenzie, 2004b; Kim, 2011), Pitx3 (Maxwell et al., 2005; Courtois et al.,

2010) and *Lmx1a*, transcription factors which promote dopaminergic differentiation. It has been reported that gene *pitx3* and *nurr1* are essential for the survival and differentiation of DA neurons in the striatum (Perlmann and Wallen-Mackenzie, 2004a; Maxwell et al., 2005; Haubenberger et al., 2011). Both transcription factors function as dopaminergic promoters in chick, mouse, or human embryonic cells (Saucedo-Cardenas et al., 1997; Hwang et al., 2003a; Courtois et al., 2010; Katunar et al., 2010), and we first demonstrated that they participate in dopamine production in adult human olfactory-derived progenitors (Soldner et al., 2011). Furthermore, the genes *pitx3* and *nurr1* were found to induce TH expression synergistically (Martinat et al., 2006; Soldner et al., 2011). In these studies the hONPs were genetically modified by transfecting gene *pitx3* and *nurr1*, after which they were tyrosine hydroxylase (TH) positive even after four months of selection. The transfection effects of different paradigms were evaluated and compared.

Several studies have shown that neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), etc. are important for the survival and function of dopaminergic neurons in CNS (Karamohamed et al., 2005; Singh et al., 2006; Li and Ding, 2010; Edalat et al., 2011; Pessach and Notarangelo, 2011). Recent studies also indicate that the neurotrophins have the potential to optimize the local micro-environment of the damaged area, and thereby induce endogenous stem cells to replace or rescue degenerating neurons (Lindvall and Kokaia, 2010; Kassis et al., 2011). HONPs derived from adult human olfactory epithelium have been shown to produce and release neurotrophins (Zhang et al., 2000; Zhang et al., 2003; Marshall et al., 2006), which could further support their use in a cell-

based therapy for PD. Therefore, this study also evaluated the ability of pre and post transfected hONPs to synthesize key neurotrophins.

The 1st objective of this study was to determine if hONPs could be lineage restricted towards dopaminergic neurons and if so to optimize the methodology, followed by determining and evaluating the function of IPN transfected and pre-transfected hONPs in a unilateral 6-hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease (Wright et al., 2009).

B. Material and Methods

1. Cell culture

The two patient-specific olfactory progenitor lines used in this study were obtained from adult olfactory epithelium harvested from a 42-year-old female patient and a 20-year-old male via endoscopic biopsy (Roisen et al., 2001b). The tissues were cultured to allow the emergence and harvest of hONPs as previously described (Zhang et al., 2004; Winstead et al., 2005b). The hONPs were thawed from frozen stock that was maintained in liquid nitrogen and cultured in minimal essential medium (MEM) with 10% heat inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY) (10%OE) for one week. The hONPs were adapted to serum-free growth media via serial dilution of serum every day for 4 days until the cells were finally cultured in DFBNM (DMEM/F12 supplemented with 1% B27 and 0.5% N2 and 100 µg/ml gentamycin (GIBCO, Grand Island, NY) (Zhang et al., 2004). Parallel independent experiments were performed on hONP lines from the two different patient lines. Since equivalent results were achieved, data from only one line has been presented.

2. Construction of expression plasmids (Figure 2)

The mouse *nurr1* cDNA was cloned into the pLNCX2 expression vector (Clontech) between *Cla*I. Similarly, the rat *pitx3* and mouse *lmx1a* cDNA were inserted into pLNCX2 vector between *Cla*I. For the *nurr1* and *pitx3* co-expression vector, *nurr1* cDNA was cloned into pIRES (Clontech) between *Xba*I and *Sal*I, and *pitx3* was inserted between *Eco*RI. The pLNCX2 and pIRES expression vectors served as controls. All expression vectors were verified by extensive DNA sequencing.

Figure 2. Construction of Expression Plasmid

All plasmid constructs were introduced into the hONPs by liposomal transfection. The cells were plated on glass coverslips in six-well plates (5×10^4 cells per 35-mm well) in DFBNM without antibiotics 1 day before transfection. HONPs were transfected with each plasmid (4 μ g/well) for 24 hours according to the manufacture's protocol (Lipofectamine 2000, Invitrogen, Carlsbad, California). One day after transfection, the cells were fed with 10% FBS in MEM and selected with G418 (400 μ g/ml; Invitrogen, Carlsbad, California). The selection pressure was kept for up to 4 months to insure a purified stably transfected cell population. Immunocytochemistry and Western blot analysis were applied to detect several dopaminergic neuronal markers. After a four-month selection, the transfected hONPs were frozen in liquid nitrogen for additional four-six months of storage. After removal from cryostorage and several days' recovery in MEM with 10% FBS at 37°C, the dopaminergic lineage restriction was probed with immunocytochemistry and Western blot analysis.

4. Treatment with Morphogens

The hONPs were treated with Shh, in the presence or absence of RA (1 μ M) and/or FN (5 μ M) (Zhang et al., 2004). Highly purified Shh (kindly provided under a Material Transfer Agreement with Curis and Wyeth, Inc.) was applied to hONPs and compared to a commercially available control sample obtained from Sigma to determine the extent to which purification of Shh can affect the expression of tyrosine hydroxylase (TH). The hONPs were plated on glass coverslips in six-well plates (5×10^4 cells/35mm well) in DFBNM and treated with medium containing various concentrations and combinations of RA, FN, and Shh for 7 days (CO₂ atmosphere at 5% and temperature of 37°C). Treatment with Shh included several concentrations; 0.25mg/ml (Shh0.25), 0.1mg/ml (Shh0.1),

0.05mg/ml (Shh0.05), 0.025mg/ml (Shh0.025) in the presence or absence of retinoic acid 1 μ M RA (RA1) and/or forskolin, 5 μ M FN (FN5). After treatment, the TH expression was determined at 1-7 days *in vitro* by immunocytochemical analysis. Once the optimized environment for inducing dopaminergic neurons was determined, the medium containing the optimized combination was applied to stably transfected hONPs to further improve the yield of these neurons.

5. Immunocytochemistry

The hONPs (5×10^4 cells/well) were plated on 35 mm round glass coverslips in six-well plates (Becton, Dickinson and Co.) and incubated at 37 °C in 5% CO₂/95% air for 24 hours and treated with RA, FN, and Shh or transfected and selected for different periods of time prior to fixation for immunofluorescence. 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1,000, 2 mg/ml, Molecular Probes, Eugene, OR) was applied in culture for 30 minutes at 37 °C for vital nuclear staining. The coverslips were rinsed with cytoskeletal buffer (CB) twice and fixed in 3% paraformaldehyde in CB (10 minutes). 0.2% Triton X-100 (10 minutes, Sigma) in tris buffered saline (TBS) was applied and cells were incubated (1 hour) in 3% bovine serum albumin (BSA) in TBS. Primary antibodies were applied overnight (4 °C). After 30 minutes washing (10 minutes each, 3 times) in TBS, the cells were incubated with secondary antibodies: Texas-red conjugated goat anti-rabbit immunoglobulin G (IgG) , Texas-red-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-mouse IgG and/or Cy2-conjugated goat anti-rabbit IgG (all diluted 1:600, Cy2, Jackson Immunology Research Laboratories; Texas red, Molecular Probes). The coverslips were rinsed in TBS for 30 minutes (10 minutes each, 3 times) and mounted on slides. The slides were examined with confocal microscopy. All

experiments were repeated a minimum of two times to ensure the specificity of staining; only one set of data has been presented since similar results were obtained.

6. Western Blot Analysis

Western blot analysis was used to further examine and confirm the immunofluorescence studies. Proteins from hONPs transfected with control vectors, as well as hONPs transfected with the vectors plus each combination of transcription factors (pLNCX2-pitx3, pLNCX2-nurr1, pLNCX2-lmx1a, pIRES-pitx3-nurr1), cultured in DFBNM, selected in all groups were collected in cell lysis buffer (Sigma, St. Louis, MO). After 15 minutes of incubation on ice, samples were centrifuged for 30 minutes (4°C) and the protein concentration of each supernatant was determined. The protein samples (20µg/well) were electrophoresed on 10% SDS-polyacrylamide gels along with standardized-molecular-size marker proteins in an adjacent lane and transferred from gel to nitrocellulose paper. Nonspecific binding was blocked (1 hour) with 5% nonfat dry milk in TBS-Tween (TBST) buffer. Blots were incubated (4°C overnight) in primary antibodies (anti-TH, MAB; anti-actin, MAB). Blots were washed three times for 10 minutes in TBST, after which they were incubated (1 hour, room temperature) monoclonal horseradish peroxidase-labeled anti-mouse IgG (1:2,000). ECL Western blotting detection reagents (Amersham Biosciences) were used to identify bound antibodies. Densitometry of the protein bands was carried out on a high performance chemiluminescence film (Amersham Biosciences). Data was analyzed using the Image-J software programs supplied by the NIH official website (<http://rsb.info.nih.gov/ij/>).

7. Dopamine assay

Stably transfected hONPs were plated into flasks (25cm², Corning) at 10⁵ per flask before they were adapted to the absence of serum via serial dilution of serum every day for 4 days until the cells were finally cultured in DFBNM, which was collected daily after the serum was totally eliminated from the medium. The DFBNM collected from each restricted NSFC line was then concentrated to 1/50 volume respectively by centrifugal filters (Amicon Ultra-15, Millipore). The differentiated hONPs were then collected and lysed (lysis buffer, Sigma). Dopamine expression was analyzed quantitatively in the concentrated medium as well as in the cell lysates with a dopamine enzyme immunoassay kit (Dopamine EIA, Immuno Biological Laboratories, Inc.), according to the manufacture's protocol.

8. Neurotrophin Assay

Pre- and post-transfected hONPs were plated into flasks (25cm², Corning) at 5×10⁵ per flask and cultured in 10% OE media for two days before they were adapted to the absence of serum via serial dilution of serum every day until they were finally cultured in DFBNM. The differentiated hONPs were then collected and lysed (lysis buffer, Sigma). Neurotrophins were detected in the extracted protein with different enzyme-linked immunosorbent assay (ELISA) kits (BDNF, Chemicon; CNTF, Quantikine; NT-3, Chemicon) respectively, according to the manufacture's protocol. The ELISA absorbance (OD) was obtained with a microplate spectrophotometer (Spectramax Plus), and the results were plotted and calculated with the compatible software (Softmax Pro).

C. Results

Cryopreserved vials of the two representative hONP lines were obtained from storage and grown for 1-2 weeks prior to the start of these experiments to insure equivalent passage (4-8) and sufficient cell numbers for the following studies.

1. Transfection of Olfactory-derived progenitors (hONPs) to achieve dopaminergic lineage restriction.

1.1 HONPs were obtained from previously frozen stock with low passage number (4-8) and maintained in MEM10 medium during their recovery period. These mitotically active cells divided every 18 – 20 hour which typically required passage three times per week as previously described. The heterogeneous nature of the hONP population prior to transfection was determined by immunocytochemistry. No reactivity was observed for pitx3, nurr1, lmx1a with pre-transfected hONPs and only a few (5-10%) of them were positive for the dopamine precursor, TH, when treated conditionally (Zhang et al., 2006b). Low passages (Passage 4-8) of hONPs from 2 different patient-specific cell lines were employed in parallel transfection experiments. To examine the phenotypic expression of hONPs after transfection and selection, representative cultures as well as their respective pre-transfection controls were evaluated. Non-transfected hONPs or those transfected with lipofectamine alone died within 1 week after selection with 400µg/ml G418. In contrast, 30% of the transfected cells (both with the concerned genes and the control vectors) survived under the selection pressure. Transfection with control vectors, single genes, or pitx3-nurr1 combined resulted in no morphologic changes compared to the typical pretreated hONPs. However, the transfected hONPs divided more slowly, with a new doubling time of three to four days, which required a feeding schedule of only

twice a week and necessitated passage every 9-10 days. Immunofluorescent analysis of the transfected populations demonstrated that hONPs were stably transfected and TH expressed.

1.2 Human olfactory derived hONPs were transfected by pIRES-pitx3-nurr1 to restrict them towards DA neurons. The vector alone was employed as a control. To obtain a purified population of restricted cells the transfected population was maintained in G418 for selection. Although only several weeks of selection produced relatively pure populations, an interval of four months was employed to insure stability and purity. HONPs remained TH positive after transfection of pIRES-pitx3-Nurr1, whereas the transfection of control vectors exhibited no phenotypic changes, demonstrating that hONPs can be restricted towards dopaminergic neurons. (Figure3)

1.3. HONPs were transfected with pLNCX2-nurr1, pLNCX2-pitx3, pLNCX2-lmx1a or the vector alone as a control. The transfected cells were exposed to G418 for selection for periods up to 4 months. HONPs were TH positive after transfection of pLNCX2-nurr1 and pLNCX2-pitx3, whereas the transfection of control vectors resulted in no phenotypic changes. Therefore pLNCX2-nurr1 or pLNCX2-pitx3 can be employed to lineage restrict the hONPs towards dopaminergic neurons. In contrast, the hONPs transfected with pLNCX2-lmx1a remained unreactive for TH, although positive of myc, which demonstrated the successful incorporation of the plasmid. (Figure 3)

Figure 3. Immunocytochemical analysis. HONPs transfected with pIRES-Pitx3- Nurr1, pLNCX2- Pitx3 or pLNCX2-Nurr1 were tyrosine hydroxylase (TH) positive after 4 months selection with G418 (c, d, f, g), while the lines transfected with pIRES or pLNCX2 were TH negative (b, e). HONPs transfected with pLNCX2-Lmx1a were Myc positive, demonstrating that the plasmid was transfected into the nucleus (h).

1.4. Western Blot analysis was employed to confirm quantitatively the immunocytochemical studies of the transfected NSFC populations. The following transfected lines were analyzed for TH expression: hONPs transfected with pIRES-pitx3-nurr1, pLNCX2-nurr1, pLNCX2-pitx3 and pLNCX2-lmx1a all of which were TH positive, which indicated their potential to release dopamine. In contrast, the hONP populations' transfected with the control vectors (pIRES and pLNCX2) did not exhibit TH expression. β -actin, a protein that is widely expressed in all mammalian and avian cells was used as a reference protein for the comparison of TH expression by the various lines. Image-J was applied for the data analysis. Each curve from B to M in Figure 2 illustrates the density of bands evident on the western gel (Figure4 A), and the area that each curve circles was measured. The bars in picture N represent the ratio of TH expression and ACTIN expression in the cell line. HONPs transfected with pIRES-pitx3-nurr1 exhibited the highest ratio for the TH and ACTIN expression, while the cells transfected with the control vector (pLNCX2 or pIRES) had the least TH staining (Figure 4 B-N). These results demonstrate that individual transcription factors have unique abilities in promoting the dopaminergic restriction of hONPs.

Figure 4 A. Western blot analysis. B-G. Scanning densitometry demonstrates ACTIN-expression in a hONP line of pLNCX2, pLNCX2-pitx3, pLNCX2-nurr1, pIRES and pIRES-pitx3-nurr1 respectively. H-M. Densitometry of TH-expression as shown in A. N. Histogram demonstrating the ratio of TH/ Actin produced by each population.

2. Transfected hONPs remain restricted to dopaminergic lineage after removal from cryostorage.

After 4 month-selection, the dopaminergic lineage restricted cells were cryopreserved in liquid nitrogen for additional 4-6 months. Following their removal from cryostorage and several days' recovery in MEM10 at 37°C, all but one of the transfected hONP populations survived under the selection pressure of 400µg/ml G418, demonstrating that these cells were stably transfected and retained their potential for long term storage and clinical application. Immunocytochemistry and Western blot analysis was applied to these previously stored populations to examine their TH expression. The hONPs transfected with pLNCX2-pitx3, pLNCX2-nurr1 and pIRES-pitx3-nurr1 remained healthy and TH positive under the pressure of selection, while the pLNCX2-lmx1a transfected line did not (Figure 5).

Figure 5. Immunocytochemistry (A-G) and western blot analysis (I) demonstrating that hONPs transfected with pLNCX2-pitx3, pLNCX2-nurr1 and pIRES-pitx3-nurr1 remain healthy and TH positive following removal from cryostorage under selection pressure (D, F, G). In contrast, the Lncx2-lmx1a transfected line no longer expressed TH (H).

3. Lineage restricted hONPs produced and released dopamine.

After removal from the cryostorage, dopamine production was detected in the hONP lines which were stably transfected with concerned genes, while the cells transfected with control vectors and the non-transfected hONPs didn't produce dopamine. The dopamine level of each sample was then divided by the concentration of protein in each specific hONP line to calculate the efficiency of dopamine production. Among all the 4 gene transfected lines, hONPs transfected with pIRES-pitx3-nurr1 exhibited the most efficient dopamine formation (Figure 6). Spent medium was collected 4 days after culturing the lineage restricted hONPs. This medium was then concentrated to 1/50 volume respectively, and dopamine E.I.A. was applied to detect the dopamine release (extracellular levels). Data was calculated in the same manner as the intracellular dopamine analysis. Lower levels of dopamine were detected in the concentrated media compared to the corresponding analysis of the cell lysis. The greatest level of dopamine release was detected in pIRES-pitx3-nurr1 transfected hONPs compared to the other restricted cell lines (Figure 6).

Intracellular Dopamine Assay

Extracellular Dopamine Assay

Figure 6. Histograms demonstrating the ratio of dopamine formation (pg/100µl) to total protein concentration (mg/ml) of cells transfected with pIRES-pitx3-nurr1, pLNCX2-pitx3, pLNCX2-nurr1, pLNCX2-lmx1a, pIRES, pLNCX2 and non-transfected hONPs. HONPs transfected with pIRES-pitx3-nurr1 exhibited the highest levels of intracellular and extracellular dopamine production. Dopamine production and release were enhanced in hONPs treated with the morphogens.

4. The effect of morphogens on tyrosine hydroxylase (TH) expression, dopamine formation and release.

4.1. HONPs were cultured in DFBNM along with RA (1 μ M), FN (5 μ M) and either of two different sources (purities) of Shh for four days. Both Shh treatments resulted in greater expression than in those cultured solely in DFBNM. TH expression was greater in the cells that were treated with highly purified Shh than the commercial product obtained from SIGMA when applied for same period of time. (Figure 7).

Figure 7. HONPs treated in DFBNM with a highly purified Shh(C) exhibited greater reactivity to tyrosine hydroxylase (TH) than those treated with commercially available Shh (B) for 3 days in the presence of RA and FN.

4.2. HONPs treated with RA1FN5 and highly purified Shh expressed seemingly more intensive TH reactivity in the positive cells (Figure 7 A). Therefore, the concentration of Shh was reduced to determine the lowest concentration of Shh that could drive the hONPs towards dopaminergic neurons. In contrast to the response when a high level of Shh was applied, the reduction of the Shh to 0.025mg/ml applied with RA (1 μ M) & FN (5 μ M) did not produce an immediate response. The hONPs became TH positive only after 18 hours of treatment with highly purified Shh; however, they were healthy and maintained TH expression for longer periods. The application of RA and FN promoted an even greater expression of TH (Figure 8 A). Therefore, the optimal conditions for restricting the hONP lineage to dopaminergic neurons (under these defined conditions) was determined to be DFBNM supplemented with RA1FN5Shh0.025 (Figure 8 B).

Figure 8. A. HONPs cultured in DFBNM supplemented with 0.025mg/ml of Shh, in the presence or absence of retinoic acid (RA)(1 μ M) and forskolin (FN)(5 μ m) for days indicated. B. HONPs were tyrosine hydroxylase (TH) positive following 7 days treatment with RA1FN5Shh.

4.3 Stably transfected hONPs were treated with a cocktail of RA1FN5Shh0.025 to determine if a combination of genetic modification and morphogenic treatment would increase intracellular and intercellular dopamine levels. Spent medium was collected 4 days after morphogenic treatment and concentrated to a 1/50 volume. The treated lineage restricted hONPs were also collected. Dopamine E.I.A. was applied to both cell lysis sample and concentrated medium. Dopamine formation efficiency was calculated as previously described. HONPs transfected with pIRES-pitx3-nurr1 were the most efficient population with respect to dopamine formation and release after morphogenic treatment (Figure 6 A & B). Compared to intracellular and extracellular dopamine levels of the lineage restricted hONPs in the absence of morphogens, dopaminergic expression was greatly enhanced in the stably transfected hONPs in the presence of the combination of Shh, RA and FN (Figure 6 A & B). These studies suggest that morphogenic treatment can play an important role in dopamine formation and release by the lineage restricted hONPs.

5. Stably transfected and pre-transfected hONPs produce neurotrophins (BDNF, CNTF and NT-3) at equivalent levels

The non- (pre)-transfected hONPs were found to produce neurotrophic factors such as BDNF (56.09 ± 10.24 pg/ml), CNTF (18.72 ± 1.43 pg/ml) and NT-3 (24.87 ± 6.53 pg/ml). The stably transfected lines were examined to determine if lineage restriction to dopaminergic neurons alters the synthetic capacity and activity of these neurotrophins; no significant differences in intracellular neurotrophin (BDNF, CNTF, NT-3) levels between transfected and non-transfected hONP lines were observed ($P > 0.01$), indicating that transfection did not alter neurotrophin synthesis. (Figure 9)

Figure 9. Histogram demonstrating the neurotrophin levels in hONPs (pg/ml) transfected with pIRES-pitx3-nurr1, pLNCX2-pitx3, pLNCX2-nurr1, pLNCX2-lmx1a, pIRES, pLNCX2 and non-transfected NSFCs. Lineage restriction did not alter neurotrophin production.

D. Discussion

Parkinson's disease, as a neuronal degenerative disease, is characterized by loss of specific dopaminergic neurons in substantia nigra (Hornykiewicz, 1973). Although a variety of pharmacological agents have been employed in the treatment of PD their effects are transient. "Proof of concept studies" with embryonic adrenal medulla cells (Fitzpatrick et al., 2009) although ending in failure demonstrated the potential of a stem cell based therapy. Recently substantial effort has been devoted to the search for a suitable cell source for a cell replacement strategy for the treatment of PD. Many studies have focused on the use of embryonic stem cells; studies utilizing embryonic cells derived from mice or porcine were found to be functional in relieving PD like symptoms in PD animal models (Yang et al., 2010; Tonnesen et al., 2011), and positive results obtained from human oriented ES cells further advance the use and promise of stem cells as a potential source for cell therapy for PD (Tatard et al., 2007; Arenas, 2010; Blandini et al., 2010). However, these studies were all generally hampered by the significant side effects due to the transplantation of ES cells, such as dyskinesias and/or the formation of teratomas (Brederlau et al., 2006; Arenas, 2010; Brundin et al., 2010). Unfortunately, low cell viability following transplantation, tissue compatibility, a limited of source and ethical concerns further diminish the therapeutic utility of ES cells. In contrast, the use of adult human olfactory epithelium derived progenitors, as a unique autologous cell resource, which can be obtained with minimally invasive surgery can avoid these negative factors and also eliminate the need for immunosuppression. The studies described in this manuscript demonstrate that hONPs can be stably lineage restricted under an optimized paradigm, so that they produce and release dopamine, which makes

them potential candidates for cell-based therapy for PD. Additionally, the genetic modification didn't alter the capability of hONPs to produce and release key neurotrophic factors, which have the potential to support neuronal survival, as well as rescue degenerating neurons. These factors can also provide permissive micro-environments that may induce endogenous stem cell generation and differentiation. (Torp et al., 2006; Hess and Borlongan, 2008; Bao et al., 2011).

In the present study, several factors have been observed to optimize the environment for hONPs and facilitate their differentiation to dopaminergic neurons, including genetic modification and morphogenic treatments, along with another emphasis, the capability of hONPs to produce neurotrophic factors, which will all be discussed individually below.

1. Pitx3 and nurr1 induce the DA neuron maturation synergistically.

The pitx3 gene belongs to the Pitx family of transcription factor genes and has been shown to be required for the expression of TH, the precursor of dopamine, both *in vitro* and in mice from E11.5 (Maxwell et al., 2005). It has been reported that pitx3 is crucial to the formation of SN and the specification and/or the survival of the subpopulation of the DA neurons in striatum (Cazorla et al., 2000; Haubenberger et al., 2011; Reddy et al., 2011). The earlier studies suggest that pitx3 increased TH promoter induction in mouse and rat cell lines, but not in human cell lines (Cazorla et al., 2000; Lebel et al., 2001). However, human embryonic stem cells were employed in experiments to demonstrate the regulation of TH expression by pitx3 (Hwang et al., 2003b; Martinat et al., 2006; Liu et al., 2009). These studies suggested that pitx3 is a key transcriptional regulator of genes required specifically for the mesencephalic dopaminergic (mesDA) phenotype (Smidt et al., 1997; Liu et al., 2009) and for TH expression (Maxwell et al., 2005; Reddy et al.,

2011). Nurr1 is a member of the nuclear receptor super family of transcription factors that is expressed in both developing and mature dopaminergic neurons in the central nervous system in mice (Saucedo-Cardenas et al., 1997). Previous studies have shown that nurr1 is essential to both survival and differentiation of the ventral mesencephalic dopaminergic precursor neurons (Smits et al., 2003; Kim, 2011). Nurr1 has also been reported to be essential in the expression of TH, which is required for DA synthesis; and for vesicular monoamine transporter 2 (VMAT2), which is related to DA storage; and dopamine transporter (DAT), which is crucial for DA re-uptake (Smits et al., 2003). In addition, a recent study has shown that Nurr1 plays a previously unexpected role in protecting TH positive neurons from neurotoxicity (Winner, 2008). Furthermore, nurr1 is the only known transcription factor that is associated with the dopaminergic neurotransmitter identity in mesDA neurons (Saucedo-Cardenas et al., 1997). Therefore, both pitx3 and nurr1 have been shown to be crucial to the formation of SN and the specification and/or the survival of the DA neurons in midbrain in rodents (Perlmann and Wallen-Mackenzie, 2004b; Simeone, 2005; Krasnova et al., 2011). The results obtained in the present study indicate that overexpression of pitx3 and/or nurr1 promotes the expression of DA neuron marker, TH in human adult olfactory epithelial-derived progenitors *in vitro*. HONP lines that were stably transfected with pitx3 and/or nurr1 and selected for 4 months, remained healthy and TH positive following 6 months cryostorage in liquid nitrogen. Furthermore, the direct detection of dopamine production was also evaluated. Lysates of pitx3 or nurr1 transfected hONPs were dopaminergic as determined by dopamine E.I.A. These results suggest that the transcription factors, pitx3 and nurr1, not only function as a dopaminergic promoters in chick, mouse, or human embryonic

cells (Saucedo-Cardenas et al., 1997; Hwang et al., 2003b; Courtois et al., 2010; Katunar et al., 2010), but also participate in dopamine production in adult human olfactory-derived progenitors. Based on previous studies which focused on the regulatory function of *pitx3* and *nurr1* in dopaminergic neuron promotion (Smidt et al., 1997; Cazorla et al., 2000; Hwang et al., 2003b; Smits et al., 2003; Simeone, 2005; Vazin et al., 2009) and the studies described in this manuscript, we hypothesized that *pitx3* and *nurr1* may collaborate to induce a higher efficiency of dopamine production in midbrain DA neuron maturation. Previously a synergistic effect between *pitx3* and *nurr1* on TH expression has been reported, which appeared to be species dependent occurring in human but not in embryonic murine stem cells (Lebel et al., 2001; Martinat et al., 2006; Messmer et al., 2007). The current studies demonstrate that the simultaneous transfection of *pitx3* and *nurr1* into the hONPs produced higher levels of TH expression and dopamine production than transfection of either of the individual genes. We evaluated the effect of transfection on the level of the precursor (TH) and final intracellular and extracellular dopamine levels to confirm and compare the efficiency of the different transfected hONP lines. Therefore, our data, in combination with published reports in rodents (Zetterstrom et al., 1997; Nunes et al., 2003) and with human embryonic stem cells (Martinat et al., 2006; Jacobs et al., 2009), indicate that *pitx3* and *nurr1* cooperatively induce the maturation of DA neurons. We extend the previous studies to show the feasibility of genetic modification of adult human olfactory-derived progenitors to promote the generation of DA neurons. These studies strongly suggest that the co-expression of *pitx3* and *nurr1* will enhance significantly the lineage restriction of adult human progenitors toward

dopaminergic neurons which can be employed in cell-replacement paradigms for the treatment of PD.

2. Treatment of hONPs with morphogens enhances intracellular and extracellular dopamine levels.

Human adult derived progenitors have the potential to differentiate along several neural lineages in response to morphogenic signals *in vitro* (Zhang et al., 2005). For example, 11.6 ($\pm$ 1.5) % of hONPs expressed TH following a 7 day treatment of RA1FN5Shh (1 μ M RA, 5 μ M FN and 15nM Shh), indicating that a dopaminergic lineage can be driven by exposure to these morphogens (Zhang et al., 2006b). Sonic hedgehog (Shh), (RA) and Forskolin (FN) have all been shown to be crucial developmental factors that regulate neuronal specification and differentiation (Roisen et al., 1972a; Roisen et al., 1972b; Ericson et al., 1997; Novitsch et al., 2003; Kurauchi et al., 2011; Trzaska and Rameshwar, 2011). Shh has been shown to be required for the generation of ventral midbrain motor neurons (Wichterle et al., 2002; Ko et al., 2009) as well as dopaminergic neurons in rodents (Arenas, 2010; Blandini et al., 2010; Krasnova et al., 2011) and chick embryos (Brundin et al., 2010). This study suggests that Shh increases the expression of TH and that the purity of Shh is an important determinant of TH expression. RA regulates neuronal differentiation in embryonic stem cells (Bibel et al., 2004; Moliner et al., 2008) and adult human neuronal progenitors (Hsieh et al., 2004; Cooper et al., 2010). RA has several pathways through which it can effect cellular differentiation (Canon et al., 2004; Fathi et al., 2010). FN is an adenylyl cyclase activator that increases intercellular levels of cAMP that can stimulate axonal elongation (Roisen et al., 1972a; Roisen et al., 1972b) and induce embryonic rat/mouse motor neuron survival (Hanson et al., 1998; Kobayashi

et al., 2011). Following the treatment of RA and FN, the progenitor nature of hONPs is diminished, as characterized by a loss of nestin expression, and the presence of more mature neuronal markers. In this study, a combination of highly purified Shh, RA and FN was applied to the lineage restricted hONPs. The intracellular level of dopamine was demonstrated to be significantly increased by this treatment. This result confirms and extends the published data by showing that these morphogens can increase TH expression by progenitors obtained from adult humans (Zhang et al., 2006b). Furthermore, following a 4 day treatment of RA1FN5Shh, the dopamine level of the spent conditioned medium was significantly enhanced, indicating that the morphogens promoted the release of dopamine, which is important for future studies transplanting lineage restricted hONPs into PD animal models. Among all 4 lineage restricted hONP lines, those cells transfected with pIRES-pitx3-nurr1 produced and released the highest levels of dopamine in the presence of Shh, RA and FN. This result is consistent with the analysis of the lineage restricted cells in the absence of treatment with the morphogens. This data further supports the conclusion that hONPs transfected with pIRES-pitx3-nurr1 are the most efficient line in dopamine production studies to date, and therefore are likely candidates for engraftment into an animal model of PD. Shh is secreted by the notochord and floor plate at early stage of development (Echelard et al., 1993), RA is detectable in the midbrain of chick and mice embryos (Maden, 2002), and FN is highly concentrated in the rat substantia nigra (Gehlert, 1986). The local distribution of these morphogens *in situ* should influence the engrafted hONPs and may further support their survival and dopamine release following transplantation. The higher level of dopamine released following Shh, RA and FN treatment suggests their potential utility for cell-replacement

therapy for PD. Previous studies on the non-human primate PD models, demonstrated that the transplanted responsive human embryonic progenitor cells were still capable of differentiation to DA phenotype within the micro-environment around the lesioned adult host SN, an unexpected finding was that the engraftment also up-regulated an endogenous progenitor population (Redmond et al., 2007). The results of our studies utilizing a paradigm that combines transfection and morphogen induced lineage modulation highlight the potential therapeutic utility of olfactory epithelial-derived neural progenitors as an autologous cell source for cell-based replacement strategies for patients with Parkinson's disease.

3. Lineage restricted hONPs retain their capability to produce neurotrophic factors

It's been reported that neurotrophins such as BDNF, CNTF and NT-3 are crucial in the recovery of primate and rodent models of Parkinson's disease (Redmond et al., 2007; Yoneyama et al., 2011). BDNF is a member of the neurotrophin family which support the maturation and survival of dopaminergic neurons in substantia nigra (Singh et al., 2006; Maia et al., 2011). In the presence of BDNF, more TH positive cells can be found in cultures of ventral mesencephalic tissue than in the absence of the neurotrophin (Braun et al., 2011; Maia et al., 2011). NT-3 belongs to the same family of neurotrophins as BDNF, and has been shown to play a protective role in the degeneration of adult central noradrenergic neurons *in vivo* (Sadan et al., 2009; He et al., 2010). CNTF has been reported to rescue the degenerating striatal neurons in primate and rodent models (Fu et al., 2010; Edalat et al., 2011). Furthermore, the absence of CNTF leads to the apoptosis of motor neurons in adult mice (von Bohlen und Halbach and Unsicker, 2009; Li and Ding, 2010). Collectively these studies strongly suggest an important role for these

neurotrophins in future therapeutic strategies for neurodegenerative diseases, including PD, Alzheimer's disease and Huntington disease. Therefore, a cell population that can produce neurotrophins could be an ideal source for cell therapy for these diseases. They can provide protective micro-environments *in vivo* and prevent, rescue and or replace neuronal degeneration. The pre-transfected hONPs were found to produce several neurotrophins including BDNF and NT-3 when in a serum enriched medium (Marshall et al., 2006). The stably transfected lines were examined to determine if lineage restriction to dopaminergic neurons or absence of serum alters the synthesis of these neurotrophins since they play a role in neuronal survival, differentiation and maturation. As shown in the results, the transfection of hONPs did not alter trophin production. The post-transfected hONPs produce BDNF, NT-3 and CNTF at equivalent levels with the pre-transfected progenitors. Therefore, genetically modified hONPs can not only serve as replacements of the dead or dysfunctional dopaminergic neurons but also can provide protective micro-environments to help rescue dying or damaged neurons from further degeneration and to enhance the endogenous progenitor populations. The stably lineage restricted hONPs are unique populations with high potential for cell transplantation for animal models of Parkinson's disease.

The long term goal of this study is to develop restricted hONP lines that will have therapeutic utility in cell replacement strategies for patients with PD. The *in vivo* viability and stability are important variables, especially considering the likelihood that with time the engrafted population may die and require replacement. Therefore, experiments were undertaken to determine the stability and viability of frozen stocks of transfected cells. HONPs survived under the pressure of selection after removal from cryostorage and

retained their ability to express TH, as well as produce and release dopamine and neurotrophins, which further demonstrates the unique potential of these progenitors to perhaps serve as an autologous cell source for cell-based strategies for the long-term treatment of Parkinson's disease.

CHAPTER III

PILOT STUDIES FOR THE EVALUATION OF THE *IN VIVO* EFFECTS OF HONPS IN A PD ANIMAL MODEL

Chapter II identified the most efficient dopamine-producing paradigm. This Chapter described pilot studies that were undertaken to evaluate an animal model of PD in which the *in vitro* results could be validated *in vivo*. Currently, there are two widely used animal models of PD: the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced model (MPTP) (Chang et al., 2011; Yasuda et al., 2011) and unilateral 6-hydroxydopamine (6-OHDA) lesioned model of Parkinson's disease (Cai et al., 2010). Studies with MPTP involve mainly although not exclusively on primates (Itakura et al., 1988; Burov Iu et al., 1995; Oiwa et al., 2006). In contrast, the 6-OHDA model has been more popular in the rodents (Matsukawa et al., 2007; Cai et al., 2010). Both models have been well established and work equivalently in developing animal models of PD. The principal advantage of using 6-OHDA for these studies is that this neural toxin is very sensitive to dopamine agonists (Reyhani-Rad S., 2011). Since we had already determined the most efficient dopamine-producing paradigm (Chapter II), we designed the studies to evaluate the action of engrafted transfected hONPs on the 6-OHDA model of PD, along with the cellular and medium controls. Initially, a toxin injection equivalent to a total of 20 µg (Azzouz et al., 2004; Massie et al., 2010; Danielyan et al., 2011) was employed. At this level most of the animals started to exhibit Parkinsonian deficits within 3 weeks following the injection; however, some of the animals behaviorally recovered with no treatment as time progressed. This could be because not all of the dopaminergic neurons degenerated or were severely damaged in response to the toxic injection, and spontaneous recovery occurred. Therefore, in later experiments an increased toxin dosage was used to make sure that no spontaneous recovery would occur. The total free base 6-OHDA hydrochloride (Sigma H116) was increased to 28 µg (dissolved to the concentration of

3.5µg/µl, with a total of 8µl, at 4 individual injection sites). As anticipated, no spontaneous behavioral improvement was observed up to 27 weeks after the toxin injection.

A. Selection of injection sites

The next step was to determine which location would be the most ideal area for the neurotoxin injection, among the three most widely used sites: the Medium Forebrain Bundle (MFB) (Zhang et al., 2007), Substantia Nigra (SN) (Dymecki and Freed, 1989), or the striatum (Danielyan et al., 2011).

One hundred and thirty-eight female Sprague Dawley rats (Charles River, Wilmington, MA, USA; 200-250g) were maintained under a 12-h light/dark cycle with constant temperature and humidity. Food and water was available ad libitum. Twenty-four hours prior to surgery, animals were weighed and assigned to individual groups (Striatum, MFB, SN) with ID numbers.

Prior to surgery the animals are anesthetized using ketamine (Hospire Inc. Lakeforest, IL 60045) / xylazine (Ben Venue Lab. Bedford, OH 44146) 37.7mg/5mg/kg, (0.1ml/100g, IP). After anesthesia and 30 minutes before lesion, Desipramine (Sigma D3900; 25mg/kg, IM) was given to protect noradrenergic neurons from 6-OHDA toxicity, and Pargyline (Sigma P8013; 50 mg/kg, IM) to inhibit endogenous monoamine oxidase. A prophylactic dose of general antibiotics, penicillin (Butler, Dublin, OH 43017; 100,000 units/kg, IM) was given to prevent infection. Twenty-eight micrograms of free base 6-OHDA hydrochloride (Sigma H116) was injected into the animal brains according to the three groups. Injection sites were located from the Bregma:

Striatum: AP: -1.3 mm, L: 2.6 mm, D: 5 mm;

AP: -0.4 mm, L: 3.0 mm, D: 5 mm;

AP: 0.4 mm, L: 4.2 mm, D: 5 mm;

AP: 1.3 mm, L: 4.5 mm, D: 5 mm.

Medium Forebrain Bundle: AP: -4.4 mm, L: 1.2 mm; D: 7.8mm

Substantia Nigra: AP: -5.0 mm, L: 2.0 mm; D: 7.8 mm

Five milliliters of 0.9% saline was given to counteract any fluid/blood loss. Penicillin (Butler, Dublin, OH 43017; 100,000 units/kg, IM) and Buprenorphine (Bedford Lab, Bedford, OH 44146; 0.02 mg/kg, IM) were administered for post-operation care for an additional 2 days after surgery.

Three weeks after toxin injection, all animals were examined by using a rotation test that is widely employed as a standard for evaluation of Parkinson's disease models (Nikkhah et al., 1993; Lei et al., 2011). In the rotation test, amphetamine as a stimulant was injected intraperitoneally, and rotation numbers were evaluated 15 minutes after the injection. Three 15 minute segments were evaluated for a total of 45 minutes. Among these three groups, the SN-injured model was eliminated because lower percentage of animals were considered "qualified" models (over 6 turns in rotation test (described in Chapter IV)) from this group, compared to the animals having received their toxic injection in either the MFB or the striatum. The striatum was eventually selected as our 6-OHDA injection site because multiple points of injections lead to more complete neural degeneration. The striatum is a larger area, compared to the MFB, which will allow for a more accurate placement of multiple injections.

Table 1. Qualifying Model Rate

6-OHDA Lesion Site	Qualifying Model Rate (>6 turns/min in Rotation Test)	
	20µg 6-OHDA	28µg 6-OHDA
MFB	9/24 (37.5%)	21/28 (75%)
SN	1/6 (16.67%)	6/14 (42.85%)
Striatum	21/38 (55.26%)	20/28 (71.42%)

Table 1. Three weeks after the 6-OHDA injection, the animals received intraperitoneal amphetamine injection at 0.35mg/100g body weight for rotation test. The qualified rats (more than 6 turns/minute) will be utilized for the cell transplantation and control animals., Three different toxin injection sites (MFB, SN, and Striatum) were tried initially with the total of 20µg 6-OHDA. Only up to 55% of the animals (Striatum group) exhibited qualifying rotation numbers. When the 6-OHDA was increased to 28µg, more than 70% of the animals achieved qualification (more than 6 turns / minute) in two regions, MFB and striatum, while only 43% reached this level of deficit in the SN group. Therefore the SN as a neurotoxin injection site for establishing the animal PD model was not utilized..

B. Determination of Cell Number for the Transplantation

Pilot studies were initiated to determine the optimal number of hONPs for injection to achieve the maximum recovery rate. PIRE5-Pitx3-Nurr1 transfected hONPs, which previously (Chapter II) had been shown to be the optimized dopamine formation paradigm, were transplanted to the qualified PD models, at their striatum: AP: -0.8 mm, L: 2.8 mm, D: 5 mm; AP: 0 mm, L: 3.6 mm, D: 5 mm; AP: 0.8 mm, L: 4.4 mm, D: 5 mm (Figure 9). Four different numbers of cells (5k, 10k, 15k, 50k) in six microliters DFBNM were injected into the rats, which were then maintained for 16 weeks. One third of the 15k hONP-injected animals recovered in the behavioral tests, which was the highest level of recovery, compared to the other concentrations (20% for 5k, 0 for 10k and 18% for 50k hONP transplanted animals). Therefore, it was determined that the striatum was the optimal site of toxin injection for zero spontaneous recovery over time, and the most ideal transplanted cell number for enhanced recovery was determined to be 15k.

Figure 9. The four 6-OHDA toxin injection points were distributed both anterior and posterior to the bregma, at: AP:+1.3mm, ML:+2.6mm, DV:+5.0mm; AP:+0.4mm, ML:+3.0mm, DV:+5.0mm; AP: -0.4mm, ML:+4.2mm, DV:+5.0mm; AP: -1.3mm, ML:+4.5mm, DV:+5.0mm. The three hONPs transplantation points were located in between the toxic injury sites, at: AP:+0.8mm, ML:+2.8mm, DV:+5.0mm; AP:+0.0mm, ML:+3.0mm, DV:+5.0mm; AP:-0.8mm, ML:+4.4mm, DV:+5.0mm

Table 2. Cell Transplantation and Rotation Analysis

6-OHDA Injection Site	Injected Cell Number	Rotation Numbers		Recovery Rate
		3w after toxin Injection	16w after Cell Engraftment	
MFB	5k	904	865	1 out of 5 20%
	5k	873	1204	
	5k	1733	464	
	5k	726	1024	
	5k	752	824	
	10k	1006	1568	0
	10k	1391	1470	
	10k	1175	408	
	10k	1251	1487	
	10k	382	1344	
	15k	864	720	2 out of 4 50%
	15k	1378	548	
	15k	1518	716	
	15k	897	647	
	50k	502	1303	1 out of 5 20%
	50k	714	1107	
	50k	1491	1632	
	50k	689	1367	
	50k	897	455	
	Medium Control	1172	719	0
	Medium Control	654	454	
	Medium Control	1317	880	
	Medium Control	965	968	
	Medium Control	1385	1182	
	Medium Control	583	1047	
	Medium Control	604	1088	

Striatum	15k	1058	1374	2 out of 7 28.57%
	15k	13842	2383	
	15k	1235	500	
	15k	1500	399	
	15k	1279	1619	
	15k	466	1133	
	15k	1039	612	
	50k	721	1647	1 out of 6 16.67%
	50k	600	1282	
	50k	1563	1953	
	50k	938	1144	
	50k	747	348	
	50k	1469	1766	
	Medium Control	639	1052	0
	Medium Control	760	1500	
	Medium Control	621	1493	
	Medium Control	1834	1531	
	Medium Control	512	977	
	Medium Control	741	1432	
	Medium Control	1060	682	

Table 2. HONPs were transplanted into the qualified PD models. Different cell concentrations were engrafted. A concentration of 15k hONPs produced the most recovery as determined by the rotation test, compared to the other doses evaluated. Therefore 15k hONPs was used for the studies described in Chapter IV.. Red text indicates the reduced rotation numbers after cell transplantation.

B. Complimentary Behavior Tests

Most standard evaluation assays for 6-OHDA PD models rely primarily on the rotation test. To supplement this evaluation we explored the utility of several complimentary behavior tests including corner preference test, cylinder vertical climbing test, stepping test and rope test, The corner preference test and cylinder vertical climbing test will be further described and discussed in Chapter IV since they were adapted for the in vivo studies.

1. Adjusting Stepping Test

Adjusting stepping test has been employed by some research groups to evaluate the Parkinsonian symptoms in rat models (Olsson et al., 1995; Kim et al., 2002). The hind-limbs and one of the fore-limbs of animals were held by the investigator, with the other forelimb exposed to a 90 cm rough wood board. The animal was moved slowly sideways (5 seconds for 90 cm), twice in the forehand direction. The number of adjusting steps was counted for both paws, and analyzed by comparing the left versus right limb usage along within the distance along the board. A baseline study was performed and the normal rats used both forelimbs equivalently when side-walking on the board, before the rats received toxic injuries in their brains. This test was eliminated because this model seemed less sensitive to this specific test than the other methods. The animals which showed behavioral differences in other tests after toxin or cell injection did not exhibit an obvious contrast in adjusting stepping test.

2. Rope Test

The animals were allowed to hang on a rope (diameter 3cm) through the use of their fore-limbs and the time that they were able to remain hanging was recorded with the

protection of a large basin underneath the animals. Most normal rats were able to hang on with their fore-limbs for a relatively long time (over 1 minute). In contrast, the toxin injured animals were too weak to support their body weight and fell into the protecting basin within a few seconds after they were placed on the rope. However, several weeks after this test was initiated, some animals started to learn how to pull themselves up and they would stand on the rope instead of hanging with two limbs. This capability varied between animals and was not related to the treatment group which made it difficult to count or calculate the hanging time. Therefore, the rope test was eliminated from the experiment as well because of the lack of consistent response within a given treatment group.

Based on the pilot studies described above, we determined that the neural toxin, 6-OHDA, would be injected at four points in the animal striatum, and 15k IPN transfected hONPs would be transplanted thereafter. Besides the main standard for PD models, amphetamine-induced rotation test, complimentary corner preference and a cylinder vertical climbing test would be employed and further discussed in Chapter IV.

CHAPTER IV

TRANSPLANTATION OF hONPs IN A RAT MODEL OF PD

A. Introduction

Parkinson's disease (PD) is a major worldwide neurodegenerative disease whose incidence has been steadily rising as the population longevity increases (Savica et al., 2010). PD is characterized by the extensive loss of functional dopaminergic (DA) neurons in substantia nigra (SN) within the midbrain (Hornykiewicz, 1973). This study is to determine the therapeutic utility of hONPs in cell-based treatment for PD. In this study, the hONPs were genetically modified by transfecting gene *pitx3* and *nurr1*, after which they were tyrosine hydroxylase (TH) positive even after four months of selection. The pIRES-*pitx3*-*nurr1* (IPN) transfected hONPs can be cryo-stored in liquid nitrogen for a minimum of six months, and retain their ability to produce and release dopamine, and therefore have the distinct advantage of serving as stable resource for cell therapy for Parkinson's disease (Soldner et al.). Furthermore, the pre-transfected and post-transfected hONPs have equivalent capacity to produce neurotrophins including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), neurotrophin-3 (NT-3), etc., all of which are important for the survival and function of dopaminergic neurons in the CNS (Soldner et al.) (Singh et al., 2006; Pessach and Notarangelo, 2011). Recent studies also indicate that the neurotrophins have the potential to optimize the local microenvironment of the damaged area, and thereby induce endogenous stem cells to replace or rescue degenerating neurons (Lindvall and Kokaia, 2010; Kassis et al., 2011). Therefore, genetically modified, as well as the non-transfected hONPs have a dual potential to serve as replacements for dead or dysfunctional dopaminergic neurons and also provide protective permissive microenvironments which can rescue dying or

damaged neurons from further degeneration while simultaneously having the potential to activate endogenous progenitors.

The object of the following study was to determine and evaluate the function of IPN transfected and pre-transfected hONPs in a unilateral 6-hydroxydopamine (6-OHDA) lesioned rat model of Parkinson's disease (Wright et al., 2009). This model was established in 1970 (Ungerstedt and Arbuthnott, 1970) and is widely used in the studies related to Parkinson's disease (Hargus et al., 2010; Rauch et al., 2010; Danielyan et al., 2011). The human fibroblasts served as cellular controls, while the culture media alone was employed as a vehicle control. In the pilot studies, two models with two different toxin injection sites: the medium forebrain bundle (MFB) (Zhang et al., 2007) and the striatum (Danielyan et al., 2011) were evaluated. Both models performed equivalently; the striatum was selected as our injection site for the data shown in this manuscript. Different cell numbers/ and vehicle volumes were transplanted into the animal models in the initial studies and only the optimized concentration was applied in the experiments discussed in this manuscript.

B. MATERIAL AND METHODS

1. Cell Preparations

pIRES-Pitx3-Nurr1 transfected and pre-transfected hONPs from the same patient-specific cell line were thawed from frozen stock that was maintained in liquid nitrogen and cultured in minimal essential medium (MEM) with 10% heat inactivated fetal bovine serum (FBS, GIBCO, Grand Island, NY) (10%OE) for one week, as described and developed previously (Roisen et al., 2001; Wang, 2011). Human skin fibroblasts (ACTT crl-1836) were cultured under the same conditions. The hONPs were adapted to serum-

free growth media via serial dilution of serum every day for 3 days until the cells were finally cultured in DFBNM (DMEM/F12 supplemented with 1% B27 and 0.5% N2 and 100 µg/ml gentamycin (GIBCO, Grand Island, NY)) on the day of engraftment. Cells were detached and suspended in DFBNM and viability of the cells was analyzed using 0.4% trypan blue stain (GIBCO, 15250) according to manufacturer's protocol. Only healthy living cells were counted and a concentration of 2500 cells/µl was prepared and kept on ice.

2. Animal Model and Cell Transplantation

All animal care and surgical interventions were undertaken in strict accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals, and with the approval of the University's Institutional Animal Care and Use Committee and Institutional Biosafety Committee. The harvest of hONP was approved by the University Institutional Review Board – IRB 521.01. An informed consent form was approved by the IRB.

2.1. Establishment of Rotational Parkinsonian Rat Model

Female Sprague Dawley rats (Charles River, Wilmington, MA, USA; 200-250g) were maintained under a 12-h light/dark cycle with constant temperature and humidity. Food and water was available ad libitum. Twenty-four hours prior to surgery, animals were weighed and assigned ID numbers.

Prior to surgery the animals were anesthetized using ketamine (Hospire Inc. Lakeforest, IL 60045)/ xylazine (Ben Venue Lab. Bedford, OH 44146) 37.7mg/5mg/kg, (0.1ml/100g, IP). After anesthesia and 30 minutes before lesion, Desipramine (Sigma D3900; 25mg/kg, IM) was given to protect noradrenergic neurons from 6-OHDA

toxicity, and Pargyline (Sigma P8013; 50 mg/kg, IM) to inhibit endogenous monoamine oxidase. A prophylactic dose of general antibiotics, penicillin (Butler, Dublin, OH 43017; 100,000 units/kg, IM) and was given to prevent infection.

Animal hair was shaved and the skin prepared w/betadine solution (Purdue Products L.P. Stamford, CT 06901) at the surgical site. Animals were mounted in stereotaxic apparatus, and the scalp was opened. A small burr hole was drilled into the skull with a dental drill. For striatum injection, 28 µg of free base 6-OHDA hydrochloride (Sigma H116) was dissolved immediately before use, in 8 µl sterile saline containing 0.01% ascorbic acid and injected into the right striatum at the coordinates given by the brain atlas of Paxinos and Watson (Paxinos and Watson, 1996). Injection sites were located from the bregma: AP: -1.3 mm, L: 2.6 mm, D: 5 mm; AP: -0.4 mm, L: 3.0 mm, D: 5 mm; AP: 0.4 mm, L: 4.2 mm, D: 5 mm; AP: 1.3 mm, L: 4.5 mm, D: 5 mm. Two µl of 6-OHDA solution was dispensed into each point with G#31 needle at a rate of 2 µl /minute. The needle was left in place for an additional 2 minutes to prevent backflow and then slowly removed. The burr hole was filled with a piece of gel foam and the scalp was closed. Five mL of 0.9% saline was given to counteract any fluid/blood loss. Penicillin (Butler, Dublin, OH 43017; 100,000 units/kg, IM) and Buprenorphine (Bedford Lab, Bedford, OH 44146; 0.02 mg/kg, IM) were administered for post-operative care for an additional 2 days post-surgery.

Figure 11. Experimental equipment set-up and animal mounting. The surgery was performed under the scope and the animal was mounted on the stereotaxic apparatus. The 6-OHDA solution was dispensed with G#31 needle in a 10µl syringe.

2.2. Assignment of Experimental Groups

The rotation test (described below) was the standard behavioral test used to evaluate the performance of the rat models. Grouping of the qualified rats (≥ 6 rotations/min on average, based on a total of 542 ± 270 turns for 45 minutes) was performed before cell engraftment. Animals were distributed to one of four groups with equivalent rotation numbers. Three groups received unilateral (right, same side with 6-OHDA diffusion) cell transplantation (IPN (N=39), pre-transfected hONPs (N=30), Human Fibroblast (N=7) and one group received a vehicle injection (N =30). A separate group was designed as a sham group, which was not administered anything throughout 24 weeks post toxin injury (N=14).

2.3. Cell Transplantation

The rats were anesthetized, prepared and mounted in the same way as they were for the surgery for toxin administration. A small burr hole was drilled into the skull with a dental drill. Fifteen thousand cells in a total volume of 6 μ l were implanted into the striatum of the animal in three specific locations (AP: -0.8 mm, L: 2.8 mm, D: 5 mm; AP: 0 mm, L: 3.6 mm, D: 5 mm; AP: 0.8 mm, L: 4.4 mm, D: 5 mm). The injection in each point was administered for 1 minute and the syringe was allowed to remain in place for an additional 2 minutes after which it was withdrawn slowly to prevent reflux of the solution. At the surgery site the skin was sutured with 5-0 silk stitches and 5ml 0.9% saline was given intradermally. Penicillin (Butler, Dublin, OH 43017; 100,000 units/kg, IM) and Buprenorphine (Bedford Lab, Bedford, OH 44146; 0.02 mg/kg, IM) were administered for post-operation care for an additional 2 days after surgery. Cyclosporine (Bedford Lab, Bedford, OH 44146) was injected intramuscularly at the dosage of

10mg/kg body weight every other day for at least 10 weeks starting from the day of transplantation. All cellular control and non-engrafted animals received the same dosage of cyclosporine at the same frequency.

3. Behavioral Analysis

3.1 Amphetamine-induced Rotation Test:

Three weeks after the initial 6-OHDA injections, the rats were stimulated with amphetamine (3.0 mg/kg, IP, 3.0mg/ml, 0.1ml/100g.rat; Sigma A5880). A determination of the number of rotations began 15minutes after drug injection to allow for drug diffusion and continued for 45 minutes (recorded in a 3 × 15 minute pattern) afterwards. On average, only the rats that rotated 6 or more turns per minute (for a total of 270 rotations in 45 minutes) were utilized for the remainder of the experiment. Those rats were assigned randomly into controls or cell engrafted groups as described above. After cell engraftment, the rotation test was preformed once every two weeks starting from the 3rd week, until 6 months.

3.2 Complementary Behavioral Tests:

Corner Test: Rats were placed in a right-angle corner of a box with the forelimbs raised off the floor of the box. The direction that the rat turned to leave the corner was recorded for 8 consecutive times. The number of left turns out of 8 trials were summarized and averaged in each group.

Limb-use Asymmetry (Cylinder) Test: Rats were placed in a clear glass cylinder (30 cm tall by 22cm diameter). The number of wall contacts, made by their forelimbs (left, right or both), was recorded for 3 minutes. The ratio of left/right usage was calculated and averaged in each group.

These complementary behavioral tests were performed once before the neurotoxin injection for baseline and were continued once every week starting from the 2nd week post treatment until 6 months after cell transplantation or medium administration.

4. Immunohistochemistry

Animals were deeply anaesthetized with ketamine/xylazine administered intraperitoneally and perfused transcardially with a phosphate buffered saline (PBS) wash followed by a 4% buffered paraformaldehyde (Sigma, P-6148) fixative. Brains were removed and cryoprotected overnight in a 4% buffered paraformaldehyde fixative followed by 20% sucrose (Sigma, S9378) at 4°C. The striatum of the animal was located and dissected with a rat brain cutting block (slicer) and mounted in frozen Optimal Cutting Temperature compound (Sakura, Torrance, CA). Coronal sections (12 micrometers) were cut on a sliding microtome (Leica, CM3050S). One out of six sections were collected and stored serially at -80°C if not stained immediately. Sections were reacted with an antibody against tyrosine hydroxylase (TH, Monoclonal, Sigma) using the ABC method (Elite, PK-6100), with DAB (Vector, SK-4100) staining, passed through an alcohol/xylene series and coverslipped with Permount (Fisher Scientific, SP15-100). Some sections were employed for the immunofluorescence staining for double labeling of TH expression and anti-human localization.

5. Dopamine assay

The rats were terminated with an over dose of ketamine/xylazine 4 months after cell or medium injection, and brains were removed immediately. The striatum of each rat brain was dissected with a rat brain slicer and lysed in RIPA buffer (Sigma, R0278) with a tissue grinder (Kimble Chase, 101020), in the presence of protease inhibitor (Sigma,

P8340). Total protein of each sample was collected and dopamine production was analyzed quantitatively with a dopamine enzyme immunoassay kit (Dopamine EIA, Immuno Biological Laboratories, Inc.), according to the manufacture's protocol.

RESULTS

1. The Effect of cell engraftment on behavioral activity

Animals receiving hONPs exhibited reduced Parkinson-like behavioral deficits following the engraftment of IPN-transfected and the matched pre-transfected cells into the striatum of animals treated for over 3 weeks with 6-OHDA. In contrast, animals transplanted with the medium vehicle or human fibroblasts remained unchanged from neurotoxin-treated controls. Twenty-four weeks after engraftment and 27 weeks after the 6-OHDA lesion, approximately 36% (14 out of 39) of the engrafted animals in IPN-transfected group exhibited improved behavioral recovery in a rotation test. The transfection of gene *Pitx3* and *Nurr1* initiated the dopaminergic differentiation of the hONPs and resulted in positive improvements which could be detected by the 6th week post cell transplantation. On the other hand, 33% (10 out of 30) of the pre-transfected hONPs, which were originally employed as a cellular control group, also had a reduced rotation activity although the improvement required a longer post-engraftment period. The IPN transfected animals initially exhibited improvement in week six post engraftment, while the pre-transfected animals did not show any signs of behavioral improvement until 12 weeks after transplantation. However, at the conclusion of these experiments (27 weeks) no significant difference in level or degree of recovery was detected between pre-transfected and post-transfected groups ($P>0.05$). Furthermore the improved rotation levels were reduced to 43% of the initial levels in both the pre-

transfected and post-transfected improved animals. Human fibroblasts were employed as a cellular control to evaluate further the specific role of the hONPs in cell replacement therapy for the 6-OHDA Parkinson model. Animals that received the fibroblasts had no improvement in behavioral activity for the duration of this experiment which was 18 weeks post-engraftment. The recovery rate of the fibroblast engrafted animals was significantly different from the IPN-transfected or pre-transfected hONP group ($P<0.05$); there was no difference between the media control or fibroblast engrafted animals ($P>0.05$). The medium controls never exhibited improved rotational deficits throughout the 24 weeks following their treatment. In contrast, a significant difference was observed between the hONPs (pre-and/or post-transfected) animals and the medium only control and/or fibroblast engrafted groups ($P<0.01$) (Figure 12 A, B).

Furthermore, the corner preference tests and cylinder vertical climbing tests were performed for comparison with the rotational results. Baseline studies were performed before the animals received their toxin lesion. The normal animals tended to turn left and right equivalently in the corner test, while the injured animals, those receiving the unilateral 6-OHDA turned toward the toxin injected side (right side in this study) much more frequently than to the left. In contrast, the corner preference test demonstrated that those animals that exhibited obvious reduction in the rotation test had reduced preference and turned to both the left and right when faced into a corner. However, animals that had no rotational improvement similarly had an overwhelmingly corner preference to the right. In contrast, the fibroblast engrafted and the medium only control animals continued their right preference, turning to toward the right 7 times more than left (Figure

13 A). In the cylinder vertical climbing test normal animals used their right and left front paws equally against the glass wall as they reached for the top of the cylinder, while the toxin injured animals had a strong preference to use their right more than their left front paws. In this study, the animals that exhibited improved rotational deficits also had improvements in cylinder vertical climbing test. Their left/right paw usage increased from 0.1 to 0.4 over 24 weeks following engraftment, which was three times greater than the human fibroblast transplanted or medium only treated controls (Figure 13 B).

Figure 12. Thirty-five percent of the animals engrafted with transfected hONPs and thirty-three percent of the animals engrafted with pre-transfected hONPs (non-transfected) exhibited reduced rotation under the same level of amphetamine stimulation. In contrast, animals in the control groups that received only the medium or human fibroblasts had no reduction in rotational activity (A). A significant difference was observed between the hONP engrafted (transfected or non-transfected) and medium control group ($P < 0.01$). In contrast, there was no significant difference between the transfected and non-transfected engrafted groups, nor was there a difference between the fibroblast and the medium only control groups ($P > 0.05$) (B). Error bars indicate standard deviation.

Figure 13. The corner preference test (A) and the cylinder vertical climbing test (B) showed consistent results with the amphetamine induced rotation test. The animals which reduced in rotation numbers also exhibited behavioral improvements in corner and cylinder tests. In the corner test, the left turn numbers tended to be 50%, meaning equivalent left versus right preference when randomly facing a right corner in the pre- and post-transfected hONP injected animals. In contrast, in the medium and the fibroblast controlling group, the animals will mostly turn to the toxin injected side (right side in this study). In the cylinder vertical climbing test, the hONP transplanted animals use their left paws three times more than the medium or fibroblast implanted animals. Error bars indicate standard deviation.

2. Transplanted hONPs Promote TH Expression in the Toxin-injured Sites

Unilateral treatment of the rat striatum with the neurotoxin 6-OHDA destroyed all TH positive cells after three weeks as demonstrated by the lack of immunoreactivity in the area of treatment. Furthermore, no detectable spontaneous recovery of TH positive cells occurred throughout 24 weeks post-neurotoxin injection nor was TH positive immunoreactivity observed in the toxin treated regions of the sham operated controls.(Sham group, Figure 14 A-D) nor was it detected in the human fibroblast or medium only injected control groups (Figure 15 C, D). The left sides of the brains, which did not receive the toxin injury, expressed TH in the striatum, while the right side striatum did not demonstrate any TH regeneration over 27 weeks after the toxin injection, nor did it restore any in the cellular or medium controls. In contrast, both the pre- and post-transfected hONP engrafted animals, which improved in the behavior tests, exhibited greater TH expression in sections of their striatum, compared to the controls (Figure 15 A, B).

Figure 14. Dopaminergic neurons degenerated in the 6-OHDA lesioned side (right) of the animals throughout the time line, and did not recover spontaneously (A-D). TH expression started to be dismissed since the 3rd week after 6-OHDA injection, and there is no spontaneous restoration of TH up to 24 weeks after toxic injury.

Figure 15. More dopaminergic neurons were found in the lesioned sites of animals engrafted with transfected and non-transfected hONPs (A, B), while the medium or human fibroblast injected controls had a significant deficit of dopaminergic neurons (C, D).

3. HONPs remain intact and TH positive for a minimum of 6 months after transplantation

Twenty-four weeks after engraftment, dopaminergic neurons were detected in the toxin-injured striatum of the hONP transplanted animals (Figure 16 A, B). The engrafted hONP population remained intact and TH positive for a minimum of six months in vivo. These cells strongly resembled multipolar neurons which were characterized by long processes that frequently passed out of the plane of focus. (Figure 16 C, F). Furthermore, TH positive processes were found well beyond the injection sites (Figure 17 A, B). Cell bodies and processes that expressed TH were detected 800 μ m away from the initial implantation area. As stated above, about 36% of the animals, that received hONP injection, recovered in both post-transfected and pre-transfected groups. A quantitative study was performed and significant differences in TH positive cell numbers were detected between transfected recovered (T-Recov) and transfected non-recovered (T-NoRecov) animals ($P<0.05$) and between pre-transfected recovered (PreT-Recov) and the pre-transfected non-recovered (NoPreT-NoReov) animals ($P<0.05$). No significant differences between the recovered transfected and recovered pre-transfected animals were observed ($P<0.05$). Similarly no differences were found between the non-recovered animals of these two groups ($P>0.05$) (Table 1). No TH positive cells were observed in the toxin-injured striatum of the human fibroblast, the medium only injected, or the sham controls. Human fibroblast cells were not detectable 4 months after transplantation.

Figure 16. IPN-Transfected hONPs (A,-C) and the pre-transfected hONPs (D-F) were intact and TH positive 24 weeks after the cell engraftment. High magnification confocal microscopy reveals that both the transfected and non-transfected hONPs had TH positive processes (C, F).

Figure 17. IPN-Transfected hONPs (A) and the pre-transfected hONPs (B) were observed intact and TH positive 24 weeks after the cell engraftment, 800 μ m beyond the initial injection sites.

Table 3. P values of the comparison of the cell numbers in animal striatum

P Value	TRecov	TNoRecov	PreTRecov	PreTNoRecov
TRecov		0.008	0.63	
TNoRecov	0.08			0.11
PreTRecov	0.63			0.009
PreTNoRecov		0.11	0.009	

Table 3. There was no significant difference in cell numbers between the recovered transfected and the non-transfected hONP engrafted animals ($P>0.1$) or between the non-recovered transfected and non-transfected hONP transplanted animals, ($P>0.1$), However, there was a significant difference between the behaviorally improved and the nonrecovered animals ($P<0.05$). Definition of abbreviations TRecov: Transfected Recovered; TNoRecov: Transfected Non-recovered; PreTRecov: Pre-transfected Recovered; PreTNoRecov: Pre-transfected Non-recovered.

4. Analysis of the dopamine levels.

Dopamine enzyme immunoassay was employed 4 months after toxin injury to detect the dopamine level in the striatum sections of half brains. The left half brains that never received 6-OHDA injuries contained 1.44 ± 0.68 dopamine (pg)/total protein (ig) (D/TP), which is fourteen times greater than the toxin injured and medium only injected brains (0.11 ± 0.04 D/TP). The recovered animals from pre-transfected and post-transfected groups had D/PT levels of 0.27 ± 0.07 (pg/ig) and 0.41 ± 0.11 (pg/ig) respectively. The D/PT levels of pre-transfected non-recovered animals averaged at 0.11 ± 0.03 (pg/ig), while the post-transfected non-recovered averaged level was 0.13 ± 0.03 (pg/ig). The behaviorally improved animals exhibited higher levels of dopamine compared to the non-recovered or the medium only controls. The dopamine level in the left side brains, which never received toxin injection, was significantly higher than all the right sides. There was no significant difference (NS) between the transfected recovered and non-transfected recovered, or the transfected non-recovered and non-transfected non-recovered animals. However, the dopamine levels in the treated brains of recovered and non-recovered animals were statistically different from each other (Figure 18).

Dopamine Analysis in Animal Brains

Figure 18. The left side of the brain that never received the toxin injection retained the highest dopamine level and was significantly different from all right side brain levels. Significant difference was observed between the recovered and non-recovered the animals ($P < 0.05$, **). However, There was no significant difference ($P > 0.1$, NS) between transfected recovered and non-transfected recovered, or transfected non-recovered and non-transfected non-recovered animals.

C. DISCUSSION

Currently, the etiology and cure for Parkinson's disease remain unknown (Hornykiewicz, 1973). Although the disease is characterized by the degeneration of dopaminergic neurons in a region of the midbrain known as the substantia nigra, the underlying mechanisms have been elusive. Pharmacological agents, although widely used, only transiently relieve symptoms, losing their effectiveness with prolonged use (Lloyd and Hornykiewicz, 1973; Sharpe et al., 1973). A considerable level of proof of concept research aimed at developing a cell replacement therapy is ongoing (Anderson and Caldwell, 2007; Parish et al., 2008). Early studies included the transplantation of embryonic tissues such as mesencephalic tissue (Piccini et al., 2000; Freed et al., 2001), fetal nigral cells (Nikkhah et al., 1994; Kordower et al., 1998), or ventral midbrains (Mendez et al., 2005). With the relief of Parkinsonian symptoms, these trials raised several concerns: 1. The lack of donor tissue; each patient receiving this surgery required 3-5 fetal brains (Freed et al., 2001), which increased the risk of bacterial or viral infection. 2. Even with well-experienced surgical team, the outcome varied with each individual. 3. Dyskinesia eventually occurred in a significant proportion of patients (Barker and Kuan, 2010; Lane et al., 2010). Therefore, an alternate expandable source for dopaminergic cells has been a major research focus (Daadi, 2002; Lindvall et al., 2004). Stem cells represent a potential population for cell-replacement treatment of Parkinson's disease due to their capacity for self-renewal and ability to differentiate into other cell types (Lindvall et al., 2004). Human embryonic stem cells were one of the first stem cell populations employed in a PD model, and significant decrease in rotation tests from pre-transplantation levels following cell transplantation have been reported (Bjorklund et al.,

2002; Correia et al., 2005; Brederlau et al., 2006). A large number of studies focused on iPSCs, which were derived from human fibroblasts (Takahashi and Yamanaka, 2006; Takahashi et al., 2007), human or rat primordial germ cells (Shamblott et al., 1998; Okita et al., 2007; Hamanaka et al., 2011), or mammalian embryos (Martin, 1981; Nichols et al., 1998). These cells promoted behavioral recovery when transplanted into Parkinsonian animal models (Hargus et al., 2010). However, like embryonic stem cells, in most cases (50% or more) they generated teratomas within 6 weeks (Arnhold et al., 2004; Hedlund et al., 2007; Chang et al., 2011).

The long-term goal of the present study was to find a stable, non-tumorigenic cell source that could be used in a cell-based therapy for Parkinson's disease. Our lab developed methods to isolate and expand neural progenitors from human adult olfactory epithelium (31). The epithelial tissue was obtained via endoscopic biopsy from the olfactory region of the nasal cavity without invasive surgery or significant injury to the donor. The cells were then cultured for 8-12 weeks until the progenitors (hONPs) were obtained as previously described (Kordower et al., 1998; Winstead et al., 2005). The use of hONPs that can be obtained from the patient and then returned to the patient would eliminate ethical concerns as well as the need for immunosuppressive agents since they would be autologous. Previous studies demonstrated that hONPs can differentiate into neurons in response to their local environment (Zhang et al., 2005; Zhang et al., 2006).

The hONPs were lineage restricted to dopaminergic neurons by transfection with the *Nurr1* and *Pitx3* genes, which worked synergistically in this process (Wang, 2011). Furthermore, hONPs produce a variety of neurotrophic factors *in vitro* (Marshall et al., 2005) and *in vivo* following their engraftment (Lu et al., 2011). Recent studies have

shown that hONPs can act as biological mini pumps releasing neurotrophic factors that create a permissive environment for regeneration (Lu et al., 2011). Furthermore, several of the released neurotrophins including BDNF CNTF and NT-3 have been shown to be crucial in the recovery of primate and rodent models of Parkinson's disease (Redmond et al., 2007; Yoneyama et al., 2011). The pre-transfected hONPs were found to produce many of these essential neurotrophins including BDNF and NT-3 even when in a serum enriched medium (Marshall et al., 2005). In this study transfection of hONPs did not alter neurotrophin production. The post-transfected hONPs produce BDNF, NT-3 and CNTF at levels equivalent to the pre-transfected progenitors. Therefore, genetically modified hONPs can not only serve as replacements for the dead, dying or dysfunctional dopaminergic neurons but they also have the potential to provide a protective premissive microenvironment to help rescue dying or damaged neurons from further degeneration and to enhance the endogenous progenitor populations. Transfected and pre-transfected populations support a dual mechanism of synthesis and release of both dopamine and neurotrophins which collectively have the capacity to enhance the deteriorating, non-permissive environment created by the 6-OHDA administration in the Parkinsonian rat model. In contrast, the fibroblast engrafted cellular controls and those animals that received medium only as a vehicle control showed no improvement which reflected the non-permissive environment created by the neurotoxin. The amphetamine-induced rotation test was applied as the main standard. This procedure has been widely used to evaluate this Parkinsonian model (Nikkhah et al., 1993; Lei et al., 2011). However, a corner preference and vertical climbing assessment were used to further support the results of the rotational studies. Once the rotation numbers decrease consistent with the

literature improvement in Parkinsonian deficits were noted (Anderson and Caldwell, 2007; Vidailhet, 2011). Furthermore, when the rotations were reduced to half of the starting level, the rat was operationally considered partially recovered. We predicted that animals that received IPN-transfected cells would recover behaviorally, while the non-transfected hONPs implanted animals would not recover, or at best would have less recovery compared to the transfected group, because the non-transfected hONPs produced less dopamine than the transfected hONPs *in vitro*. However, as described above, 35% of the animals that received IPN-transfected cells behaviorally recovered according to the rotation test. Surprisingly, 33% of the animals engrafted with non-transfected hONPs exhibited reduced rotation numbers equivalent to those engrafted with the IPN-transfected cells. There was no significant difference in the final level of recovery between the two groups. The difference between the two groups was that the non-transfected cell-injected animals exhibited a rotational reduction 6 weeks later than the IPN-transfected progenitor-implanted rats on average. This might reflect the time required for the non-transfected hONPs to differentiate into dopaminergic neurons and or to affect the local micro-environment and thereby stimulate autologous stem cell populations to form dopaminergic neurons. It has been reported that the trophic factors act neuroprotectively in Parkinson's disease models (Torp et al., 2006; Kong et al., 2008). We therefore hypothesized that the neurotrophins produced by transfected and non-transfected hONPs would play an essential role in recovery from Parkinson's disease. Thus neurotrophic molecules could have a dual role; "cellular protection" in addition to cellular replacement for the dopaminergic cells.

Behavioral recovery has also been reported in Primate Parkinson's models in response to the human neural stem cell transplantation; these animals exhibited behavioral improvement during a 60 day period after stem cell transplantation.(Redmond et al., 2007; Redmond et al., 2010). The authors of these studies suggested that the Parkinsonian primate CNS may benefit from replacement of degenerating DA neurons by differentiated human stem cells, and/or the trophic, protective, and guidance effects of stem cell-derived progeny which is consistent with our results. Other studies employing human embryonic (Redmond et al., 2010) or adult (Bjugstad et al., 2008) stem cells in the 6-OHDA rat model also reported some behavioral recovery of the Parkinsonian deficits following cell transplantation. These authors suggested that it was likely that the grafted human stem cells may be protective against the toxicity from the 6-OHDA in rats' striatum. Collectively these support our hypothesis that the behavioral recovery in the hONP transfected animals may be the result of two separate but complimentary actions: the trophin-enriched protective microenvironment and or the replacement dopaminergic neurons.

A series of control groups were included to demonstrate that the behavioral recovery was specifically due to the engrafted pre- and post-transfected hONPs rather than the toxin-lesioned environment alone, with the medium or with the injection of a non hONP cell types. A sham group was included with the same dosage and location of 6-OHDA injection, and evaluated for the entire 27 weeks. Immunohistochemistry was employed to determine if the DA neurons would be restored in the sham, medium, or cellular control groups. No TH expression was detected in any of the control groups, 24 weeks after cell/medium engraftment, which was 27 weeks after the toxin injection, indicating that

the microenvironment of the lesioned sites can only support DA neuron development induced by the hONPs. Therefore, the hONPs represent a unique cell type for cell-mediated therapy of Parkinson's disease, with the ability to differentiate into dopaminergic neurons and to stimulate the microenvironment for host stem cell activation *in situ*.

Immunohistochemistry demonstrated that both the transfected and non-transfected hONPs survived and remained TH positive for the duration of the experiment (a minimum of 24 weeks after transplantation, indicating that these cells have the long-term potential to provide dopamine and neurotrophin rich environments. In contrast, the human fibroblasts were not detected 24 weeks after the engraftment which further demonstrates the specific stability and utility of the hONP population. Furthermore, the TH positive hONPs were observed as far as 800 μm from their engraftment site, demonstrating that hONPs can migrate in the local environment of the 6-OHDA lesioned striatum which is essential to cell integration. Similar results were reported by other groups using human embryonic stem cells and iPSCs (Svendsen et al., 1997; Bjugstad et al., 2008; Zhu et al., 2009; Wolff et al., 2011), which survived and promoted improvement in the behavior tests in the animals with Parkinsonian symptoms. Bjugstad et al. reported that the neural stem cells isolated from the human fetal telencephalon migrated along the nigrostriatal pathway 4 months after transplantation in an adult monkey in an alternate, 1-methyl 4-phenyl 1,2,3,6-tetrahydro pyridine (MPTP) model of Parkinson's disease. More cells were detected along the pathway 3 months post engraftment, demonstrating that after transplantation, the hNSCs did not remain entirely in the injection site, but migrated along certain pathways (Bjugstad et al., 2008). Studies

with iPSCs engraftment in a MPTP lesioned mouse model demonstrate that these cells also migrate from the site of transplantation in the striatum, to the site of lesion, localizing in the substantia nigra (Wolff et al., 2011). Collectively these studies are in agreement with the present study and demonstrate significant cell migration after transplantation, although different species and models were employed.

It has been reported that the PD models which were transplanted with ESCs or iPSCs eventually developed teratoma and died within 6 weeks for ESCs (Freed et al., 2001; Arnhold et al., 2004; Hedlund et al., 2007), and 7-9 weeks for iPSCs (Kong et al., 2008; Hwang et al., 2010) after transplantation. In the present study with hONPs, no tumor formation was detected 24 weeks after cell transplantation, indicating that the hONPs represent a more stable, lineage committed population and thus are more suited for cell replacement-therapy for Parkinson's disease. Furthermore, the hONPs can be cryostored in liquid nitrogen without loss of viability for future engraftment should serial treatments become necessary (Wang, 2011).

Dopamine EIA was applied to detect the dopamine level in animal brains. It has been shown that the brains of Parkinsonian models have decreased dopamine levels. (Santaniello et al., 2010). In the hONPS engrafted brains the dopamine levels in the behaviorally recovered animals were higher than in animals without behavioral improvement suggesting that the hONPs were functional and that the recovery was in part the result of the increased dopamine levels. Other groups also reported that intracranial transplantation with iPSCS resulted in a significant improvement of striatal concentrations of dopamine in the behaviorally recovered MPTP mouse model of PD as measured by high-performance liquid chromatography (HPLC) (Wolff et al., 2011)

which further supported the likelihood that the observed behavioral improvement was the result of the increased dopamine as well as neurotrophin support provided by the engrafted hONPS.

CHAPTER V

GENERAL DISCUSSION, SUMMARY AND FUTURE DIRECTIONS

Parkinson's disease (PD) is one of the leading neurodegenerative disorders in the world (Anderson and Caldwell, 2007); as the population longevity increases the incidence will further expand. (Savica et al., 2010). It is believed that the cause of PD is related to the loss of dopaminergic neurons in Substantia Nigra (SN), a small area in the midbrain (Hornykiewicz, 1973b). This degeneration results in less dopamine, which functions as a neurotransmitter, in the nigrostriatal pathways (Hornykiewicz, 1973a). Lower levels of dopamine result in a variety of movement disorders (Herrero et al., 2011). Dopamine, when given as a medication, is not able to pass the blood brain barrier. Therefore the oral L-dopa, a precursor in dopamine synthesis, can pass the blood-brain-barrier and has served as the principle and traditional treatment for PD (Bidet-Ildei et al., 2011). However, L-dopa does not provide long-term relief because: 1. The patient become less sensitive to the medicine over time (Callaway, 2011); 2. The L-dopa does not prevent degeneration of the dopaminergic neurons (Lang and Lozano, 1998; Sheng et al., 2010); 3. It usually leads to severe dyskinesia after several years of use (Friedman, 1985; Wedekind, 2005). Therefore, an alternative therapy is definitely needed.

Cell replacement therapy has become a major focus with promise for a future treatment of patients with Parkinson's disease. The concept of this strategy is to replace the degenerated or unhealthy neurons (dopaminergic neurons) with the new functional cells. Therefore, many researchers are involved in the search for an ideal cell source for transplantation into PD models (Lindvall et al., 1990; Olanow et al., 2001). Early studies that employed neural cell grafts obtained from the human fetal ventral mesencephalic (VM) dopaminergic neurons, successfully relieved the Parkinsonian symptoms following transplantation (Lindvall et al., 1988; Madrazo et al., 1988; Lindvall et al., 1992;

Borlongan, 2000; Ganser et al., 2010). However, treatment of a single patient with this procedure required 4-5 fetal brains, because of the low neuronal viability of these fetal cells once they were transplanted (Mendez et al., 2005). Furthermore, this treatment required immunosuppressive therapy because of the numerous donor tissues (Lindvall et al., 1990). In comparison even significantly lower numbers (15 K vs 100K) of transplanted hONPs can be detected 24 weeks after engraftment; the cells remain TH positive indicating their high viability and health *in vivo*. More importantly, the hONPs are unique having an autologous source (Roisen et al., 2001), which means that the PD patient can be both the donor and the recipient. This tissue source also eliminates the ethical concerns associated with the use of fetal tissue, the long waiting list for a matched donor and the need for immunosuppressive procedures. The hONPs are stably isolated progenitors and can be stored in liquid nitrogen for years, or cultured *in vitro* for over 200 passages (2 years) no matter the gender or age of the patient. The telomerase activity of hONP cultures remained relatively constant over an *in vitro* period of 6 weeks to 2 years which corresponded to several hundred passages ($P > 0.05$). The hONPs exhibit a relatively consistent level of metabolic activity as well; no differences ($P > 0.05$) in ornithine decarboxylase activity were found irrespective of the donor age, sex or the time in culture (Marshall et al., 2005). The high stability of these cells makes them an ideal source for experimental and clinical trials since they can be obtained from any patient.

The hONPs can be lineage restricted to dopaminergic neurons by genetic modification with gene Pitx3 and Nurr1, as well as the treatment with a combination of morphogens (RA, FN and Shh). When transfected with double genes Pitx3 and Nurr1, which have been shown to be essential in survival and development of dopaminergic

neurons in embryonic mouse/chicken (Saucedo-Cardenas et al., 1997; Cazorla et al., 2000; Haubenberger et al., 2011; Reddy et al., 2011), higher TH expression and increased dopamine production were observed compared to the non-transfected or the results obtained with the single gene transfected paradigm. This observation is consistent with reports from other groups that report a synergistic effect between Pitx3 and Nurr1 on TH expression in embryonic human or embryonic murine stem cells (Martinat et al., 2006). However, this is controversial in human or murine embryonic stem cells with some other groups claiming the opposite conclusion (Messmer et al., 2007). Our results suggested a synergetic effect of Pitx3 and Nurr1 in development of dopaminergic neurons in adult human stem cells. The transfected hONPs were cultured under the pressure of G418 selection for an extensive 4 months after the transfection to ensure the pure transfected populations were obtained. After the initial four month-selection, the transfected hONPs were stored in liquid nitrogen for an additional four to six months after which they were found to remain TH positive and to produce dopamine when removed from the cryostorage. This result suggested that the hONPs were stably transfected and continuously remained the dopaminergic in nature. This level of stability will allow the option of multiple injections over time in future studies should they become necessary when hONPs are employed in clinical trials. Furthermore, dopamine production by hONPs has been shown to increase when treated with a combination of morphogens (Zhang et al., 2006; Wang, 2011). Sonic hedgehog (Shh), (RA) and Forskolin (FN) have all been shown to be crucial developmental factors that regulate neuronal specification and differentiation (Roisen et al., 1972a; Roisen et al., 1972b; Ericson et al., 1997; Novitch et al., 2003; Kurauchi et al., 2011; Trzaska and Rameshwar, 2011). A

combination of 0.025 mg/ml highly purified Shh applied with RA (1 μ M) and FN (5 μ M) increased the TH expression and dopamine production maximally indicating that the morphogens promoted the release of dopamine, which is important to note when planning future transplantation studies into PD models with morphogen treated and lineage restricted hONPs.

Some neurotrophic factors, such as BDNF, CNTF and NT-3, have been shown to be essential to the behavioral recovery in the primate and rodent models of Parkinson's disease (Redmond et al., 2007; Yoneyama et al., 2011). Therefore, a cell population that can produce these neurotrophins could be an important source for cell therapy for this disease. They can provide protective micro-environments *in vivo* and, rescue and/or provide a population to replace lost or degenerating neurons. Pre-transfected hONPs were found to produce several neurotrophins including BDNF and NT-3 when in a serum enriched medium (Marshall et al., 2006). In a previous study we demonstrated that transfection did not alter the capability of hONPs to produce these neurotrophins (Wang, 2011). Therefore, the Pitx3 and Nurr1 transfected hONPs can not only serve as replacements of the dead or dysfunctional dopaminergic neurons but also can provide protective micro-environments that may help rescue dying or damaged neurons from further degeneration and to enhance the endogenous progenitor populations. The multiphasic action of transfected hONPs could allow them to function as an ideal source for cell therapy for the treatment of PD.

There are as many research groups using stem cells as there are different transplantation sources, including embryonic stem cells (ESCs) (Lonardo et al., 2010; Yang et al., 2010) and induced pluripotent stem cells (IPSCs) (Hargus et al., 2010; Chang

et al., 2011). Stem cells are an attractive potential source because of their unlimited capacity for self-renewal and the potential for lineage restriction (maturation) into one or more specific cell types, in response to their origin and the micro-environmental signals that they receive (Lindvall et al., 2004; Hwang et al., 2010). Positive behavioral improvements with PD animal models have been achieved with transplantation of both ESCs and iPSCs, however, more than half of the animals that received either the ESCs or iPSCs eventually developed teratomas within 9 weeks post transplantation (Arnhold et al., 2004; Takahashi and Yamanaka, 2006; Hedlund et al., 2007). In our study, the animals were maintained as long as 24 weeks after the cell engraftment and no tumorigenesis was observed. It has been reported that the risk of tumor formation can be reduced by pre-differentiation of the stem cells *in vitro*, before the transplantation (Brederlau et al., 2006; Li et al., 2008). However, it has been demonstrated that pre-differentiated stem cells are more difficult to adapt to the host environment, and will therefore more likely to be rejected by the host (Dressel, 2011). This conflict highlights a bottle neck in stem cell transplantation strategy. In the present study, hONPs were shown to be unique because the undifferentiated pre-transfected cells were as effective as their lineage restricted counterparts, indicating that no genetic manipulation was needed for hONPs to serve as an effective source for a cell based therapy for PD. As previously discussed several additional benefits are also gained through the use of hONPs including the elimination of the ethical and practical concerns due to transfection of animal genes into human cells. Furthermore, the number of cells needed when either ESCs or iPSCs sources were used in PD animal models (100,000-160,000) (Arnhold et al., 2004; Brederlau et al., 2006; Cai et al., 2010; Hargus et al., 2010) was eight times greater than the number of hONPs

(15,000) required to achieve a positive behavioral recovery, which further supports the unique advantage of hONPs over other cell types and opens the possibility of multiple injections over time should they become necessary.

Immunohistochemical localization of both the transfected and non-transfected hONPs demonstrated intact and TH positive cells in the behaviorally improved animals 24 weeks after the transplantation. Significant fewer cells were observed in the non-recovered animals in both groups compared to the animals with improved behavioral tests. In the future it will be essential to define the time course of the degeneration of engrafted hONPs so that it can be minimized or followed with a series of serial injections. One way that this could be determined would employ the unilateral 6-OHDA lesioned rat model. HONPs could be implanted into the striatum 3 weeks after the toxin injection. The animals could be terminated at various intervals (3 wk, 5 wk, 7 wk and 9 wk) after engraftment. Once the animals were perfused, the immunohistochemistry could be employed to detect the number and locations of the transplanted cells to accurately track cell migration and degeneration. This study would provide data showing the cell fate: the location of the hONPs at a specific time. It is reported that the hESCs will migrate along the striatal-nigral pathway after transplantation (Burnstein et al., 2004; Mukhida et al., 2008). Therefore, the hypothesis is that the engrafted hONPs may also migrate along specific pathways. By determining the location at each time point, the migration would be demonstrated and perhaps hONPs undergo a specific degeneration when they reach a specific site. Therefore additional cell engraftment at the site of degeneration could be aimed to overcome this limitation and thereby increase the recovery rate among the animals.

In this study, EIA was applied to detect the dopamine levels in animal brains. In the hONPS engrafted brains the dopamine levels in the behaviorally recovered animals were higher than in animals without behavioral improvement suggesting that the hONPs were functional and that the recovery was in part the result of the increased dopamine levels. Similarly, it has been reported that intracranial transplantation with iPSCs resulted in a significant improvement of striatal concentrations of dopamine in the behaviorally recovered MPTP mouse model of PD as measured by high-performance liquid chromatography (HPLC) (Wolff et al., 2011). That study used 10^5 adult human endometrial derived stem cells (HEDSC) engrafted 5 days after the MPTP lesion in the mouse model. The study demonstrated mean DA concentrations were significantly higher in MPTP lesioned mice after HEDSC compared to MPTP lesioned mice treated with sham phosphate buffered saline (PBS) transplant. This further supports the likelihood that the observed behavioral improvement was the result of the increased dopamine as well as neurotrophin support provided by the engrafted hONPs. To evaluate this further in a future study, another control group could be included: the dopamine transporter (DAT) can be blocked by an inhibitor, such as 4-Hydroxy-1-methyl-4-(4-methylphenyl)-3-piperidyl 4-Methylphenyl Ketone, or clinically used drugs, such as benztropine and mazindol (Zahniser et al., 1999; Wang et al., 2000). If none of the animals recover in the following behavioral tests after cell transplantation, it would support the conclusion that behavioral improvement is directly related to the dopamine level in animal brains; if some, but fewer animals recover from the Parkinsonian deficits, it could mean or indicate that the neurotrophic factors produced by the hONPs provide a microenvironment that can partially protect the dopaminergic neurons from degeneration

or stimulate an endogenous stem cell formation; and if the same recovery rate is observed, which is unlikely, it would suggest that the behavioral improvement is not related to the dopamine levels in striatum. On the other hand, it has been reported that neurotrophins are crucial in the behavioral recovery of PD models (Singh et al., 2006). Therefore it is possible that 6-OHDA lesioned animals could be treated solely with the trophic factors like BDNF (Sadan et al., 2009; Ahlskog, 2011), GDNF (Georgievska et al., 2002; Azzouz et al., 2004), or CNTF (von Bohlen und Halbach and Unsicker, 2009) with some success. In which case, it would also be important to determine the optimized combination of the trophic factors that could be an efficient supplemental treatment aiding behavioral recovery in the PD models.

In conclusion, human adult olfactory epithelial-derived neural progenitors have a great potential as a population for cell-based therapy for Parkinson's disease because of their capacity to survive, produce dopamine and provide neurotrophic support while simultaneously not becoming tumorigenic in the toxin-lesioned environment of the striatum as well as the neurotoxic environment of the Parkinsonian brain. Furthermore, hONPs have a significant advantage since they can be harvested from the patient's olfactory epithelium without highly invasive surgical procedures and thus represent an autologous cell source where the patient is both the donor and the recipient. This benefit would eliminate the need for waiting lists for histocompatible tissues as well as the use of immunosuppressive agents typically applied following cell engraftment. Finally these studies demonstrate that genetic engineering (transfection) is not required for an effective dopaminergic formation but that the microenvironment of the substantia nigra can modulate hONPs to become functional, stable, dopamine releasing cells that can offer

long-term survival following their engraftment without tumor formation. Future studies are needed to determine if the benefits of hONPs described in this thesis will also apply in the clinic.

REFERENCES

- Abdel-Salam OM. 2011. Stem Cell Therapy for Alzheimer's Disease. *CNS Neurol Disord Drug Targets*.
- Ahlskog JE. 2011. Does vigorous exercise have a neuroprotective effect in Parkinson disease? *Neurology* 77:288-294.
- Anderson L, Caldwell MA. 2007a. Human neural progenitor cell transplants into the subthalamic nucleus lead to functional recovery in a rat model of Parkinson's disease. *Neurobiol Dis* 27:133-140.
- Arenas E. 2010. Towards stem cell replacement therapies for Parkinson's disease. *Biochem Biophys Res Commun* 396:152-156.
- Arnhold S, Klein H, Semkova I, Addicks K, Schraermeyer U. 2004. Neurally selected embryonic stem cells induce tumor formation after long-term survival following engraftment into the subretinal space. *Investigative ophthalmology & visual science* 45:4251-4255.
- Azzouz M, Ralph S, Wong LF, Day D, Askham Z, Barber RD, Mitrophanous KA, Kingsman SM, Mazarakis ND. 2004. Neuroprotection in a rat Parkinson model by GDNF gene therapy using EIAV vector. *Neuroreport* 15:985-990.
- Bao X, Wei J, Feng M, Lu S, Li G, Dou W, Ma W, Ma S, An Y, Qin C, Zhao RC, Wang R. 2011. Transplantation of human bone marrow-derived mesenchymal stem cells promotes behavioral recovery and endogenous neurogenesis after cerebral ischemia in rats. *Brain Res* 1367:103-113.
- Barger G, Dale HH. 1910. Chemical structure and sympathomimetic action of amines. *The Journal of physiology* 41:19-59.
- Barker RA, Kuan WL. 2010. Graft-induced dyskinesias in Parkinson's disease: what is it all about? *Cell Stem Cell* 7:148-149.

- Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde YA. 2004. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. *Nature neuroscience* 7:1003-1009.
- Bidet-Ildei C, Pollak P, Kandel S, Fraix V, Orliaguet JP. 2011a. Handwriting in patients with Parkinson disease: Effect of l-dopa and stimulation of the sub-thalamic nucleus on motor anticipation. *Hum Mov Sci* 30:783-791.
- Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci U S A* 99:2344-2349.
- Bjugstad KB, Teng YD, Redmond DE, Jr., Elsworth JD, Roth RH, Cornelius SK, Snyder EY, Sladek JR, Jr. 2008. Human neural stem cells migrate along the nigrostriatal pathway in a primate model of Parkinson's disease. *Exp Neurol* 211:362-369.
- Blandini F, Cova L, Armentero MT, Zennaro E, Levandis G, Bossolasco P, Calzarossa C, Mellone M, Giuseppe B, Delilieri GL, Polli E, Nappi G, Silani V. 2010. Transplantation of undifferentiated human mesenchymal stem cells protects against 6-hydroxydopamine neurotoxicity in the rat. *Cell Transplant* 19:203-217.
- Borlongan CV. 2000. Transplantation therapy for Parkinson's disease. *Expert Opin Investig Drugs* 9:2319-2330.
- Braun AA, Herring NR, Schaefer TL, Hemmerle AM, Dickerson JW, Seroogy KB, Vorhees CV, Williams MT. 2011. Neurotoxic (+)-methamphetamine treatment in rats increases brain-derived neurotrophic factor and tropomyosin receptor kinase B expression in multiple brain regions. *Neuroscience*.
- Brederlau A, Correia AS, Anisimov SV, Elmi M, Paul G, Roybon L, Morizane A, Bergquist F, Riebe I, Nannmark U, Carta M, Hanse E, Takahashi J, Sasai Y, Funa K, Brundin P, Eriksson PS, Li JY. 2006. Transplantation of human embryonic stem cell-derived cells to a rat model of Parkinson's disease: effect of in vitro differentiation on graft survival and teratoma formation. *Stem Cells* 24:1433-1440.
- Brundin P, Barker RA, Parmar M. 2010. Neural grafting in Parkinson's disease Problems and possibilities. *Prog Brain Res* 184:265-294.

- Burnstein RM, Foltynie T, He X, Menon DK, Svendsen CN, Caldwell MA. 2004. Differentiation and migration of long term expanded human neural progenitors in a partial lesion model of Parkinson's disease. *The international journal of biochemistry & cell biology* 36:702-713.
- Burov Iu V, Shul'govskii VV, Petrov GV, Tereshchenko LV, Iudin AV. 1995. [Operant behavioral disorders in monkeys with an MPTP-induced Parkinson-like syndrome]. *Fiziologicheskii zhurnal imeni IM Sechenova / Rossiiskaia akademiia nauk* 81:113-116.
- Cai J, Yang M, Poremsky E, Kidd S, Schneider JS, Iacovitti L. 2010. Dopaminergic neurons derived from human induced pluripotent stem cells survive and integrate into 6-OHDA-lesioned rats. *Stem Cells Dev* 19:1017-1023.
- Callaway E. 2011a. Gene therapy offers hope for Parkinson's disease. In: *Nature News*.
- Calof AL, Mumm JS, Rim PC, Shou J. 1998. The neuronal stem cell of the olfactory epithelium. *J Neurobiol* 36:190-205.
- Canon E, Cosgaya JM, Scsucova S, Aranda A. 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. *Mol Biol Cell* 15:5583-5592.
- Cazorla P, Smidt MP, O'Malley KL, Burbach JP. 2000. A response element for the homeodomain transcription factor Ptx3 in the tyrosine hydroxylase gene promoter. *J Neurochem* 74:1829-1837.
- Chang YL, Chen SJ, Kao CL, Hung SC, Ding DC, Yu CC, Chen YJ, Ku HH, Lin CP, Lee KH, Chen YC, Wang JJ, Hsu CC, Chen LK, Li HY, Chiou SH. 2011. Docosahexaenoic Acid Promotes Dopaminergic Differentiation in Induced Pluripotent Stem Cells and Inhibits Teratoma Formation in Rats with Parkinson-like Pathology. *Cell Transplant*.
- Cooper O, Hargus G, Deleidi M, Blak A, Osborn T, Marlow E, Lee K, Levy A, Perez-Torres E, Yow A, Isacson O. 2010. Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol Cell Neurosci* 45:258-266.
- Correia AS, Anisimov SV, Li JY, Brundin P. 2005. Stem cell-based therapy for Parkinson's disease. *Ann Med* 37:487-498.

- Courtois ET, Castillo CG, Seiz EG, Ramos M, Bueno C, Liste I, Martinez-Serrano A. 2010. In vitro and in vivo enhanced generation of human A9 dopamine neurons from neural stem cells by Bcl-XL. *J Biol Chem* 285:9881-9897.
- Da Cunha C, Wietzikoski EC, Bortolanza M, Dombrowski PA, dos Santos LM, Boschen SL, Miyoshi E, Vital MA, Boerngen-Lacerda R, Andreatini R. 2009. Non-motor function of the midbrain dopaminergic neurons. *Journal of neural transmission Supplementum*:147-160.
- Daadi MM. 2002a. Activation and differentiation of endogenous neural stem cell progeny in the rat Parkinson animal model. *Methods Mol Biol* 198:265-271.
- Daadi MM. 2002b. Activation and differentiation of endogenous neural stem cell progeny in the rat Parkinson animal model. *Methods in molecular biology* 198:265-271.
- Danielyan L, Schafer R, von Ameln-Mayerhofer A, Bernhard F, Verleysdonk S, Buadze M, Lourhmati A, Klopfer T, Schaumann F, Schmid B, Koehle C, Proksch B, Weissert R, Reichardt HM, van den Brandt J, Buniatian GH, Schwab M, Gleiter CH, Frey WH, 2nd. 2011. Therapeutic efficacy of intranasally delivered mesenchymal stem cells in a rat model of Parkinson disease. *Rejuvenation Res* 14:3-16.
- de Lau LM, Breteler MM. 2006. Epidemiology of Parkinson's disease. *Lancet Neurol* 5:525-535.
- Doss MX, Koehler CI, Gissel C, Hescheler J, Sachinidis A. 2004. Embryonic stem cells: a promising tool for cell replacement therapy. *Journal of cellular and molecular medicine* 8:465-473.
- Dressel R. 2011. Effects of histocompatibility and host immune responses on the tumorigenicity of pluripotent stem cells. *Seminars in immunopathology* 33:573-591.
- Dymecki J, Freed WJ. 1989. [Results of cross-species transplantation of substantia nigra to the lateral ventricle of the brain of rats with experimentally-induced Parkinson disease]. *Neuropatologia polska* 27:557-571.

- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417-1430.
- Edalat H, Hajebrahimi Z, Movahedin M, Tavallaei M, Amiri S, Mowla SJ. 2011. p75NTR suppression in rat bone marrow stromal stem cells significantly reduced their rate of apoptosis during neural differentiation. *Neurosci Lett*.
- Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J. 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* 90:169-180.
- Evans MA, Triggs EJ, Broe GA, Saines N. 1980. Systemic activity of orally administered L-dopa in the elderly Parkinson patient. *European journal of clinical pharmacology* 17:215-221.
- Fathi F, Altiraihi T, Mowla SJ, Movahedin M. 2010. Transplantation of retinoic acid treated murine embryonic stem cells & behavioural deficit in Parkinsonian rats. *Indian J Med Res* 131:536-544.
- Fitzpatrick KM, Raschke J, Emborg ME. 2009. Cell-based therapies for Parkinson's disease: past, present, and future. *Antioxid Redox Signal* 11:2189-2208.
- Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S. 2001a. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med* 344:710-719.
- Freeman TB, Olanow CW, Hauser RA, Nauert GM, Smith DA, Borlongan CV, Sanberg PR, Holt DA, Kordower JH, Vingerhoets FJ, et al. 1995. Bilateral fetal nigral transplantation into the postcommissural putamen in Parkinson's disease. *Annals of neurology* 38:379-388.
- Friedman A. 1985. [Dyskinesia as a complication of the treatment of Parkinson disease with L-dopa--clinical observations]. *Neurol Neurochir Pol* 19:291-294.
- Fu Q, Song XF, Liao GL, Deng CL, Cui L. 2010. Myoblasts differentiated from adipose-derived stem cells to treat stress urinary incontinence. *Urology* 75:718-723.

- Ganser C, Papazoglou A, Just L, Nikkhah G. 2010. Neuroprotective effects of erythropoietin on 6-hydroxydopamine-treated ventral mesencephalic dopamine-rich cultures. *Exp Cell Res* 316:737-746.
- Garcia-Ruiz PJ. 2011. Gait disturbances in Parkinson disease. Did freezing of gait exist before levodopa? Historical review. *J Neurol Sci* 307:15-17.
- Geeta R, Ramnath RL, Rao HS, Chandra V. 2008. One year survival and significant reversal of motor deficits in parkinsonian rats transplanted with hESC derived dopaminergic neurons. *Biochemical and biophysical research communications* 373:258-264.
- Gehlert DR. 1986. Regional modulation of [3H]forskolin binding in the rat brain by guanylyl-5'-imidodiphosphate and sodium fluoride: comparison with the distribution of guanine nucleotide binding sites. *The Journal of pharmacology and experimental therapeutics* 239:952-958.
- Georgievska B, Kirik D, Rosenblad C, Lundberg C, Bjorklund A. 2002. Neuroprotection in the rat Parkinson model by intrastriatal GDNF gene transfer using a lentiviral vector. *Neuroreport* 13:75-82.
- Hamanaka S, Yamaguchi T, Kobayashi T, Kato-Itoh M, Yamazaki S, Sato H, Umino A, Wakiyama Y, Arai M, Sanbo M, Hirabayashi M, Nakauchi H. 2011. Generation of germline-competent rat induced pluripotent stem cells. *PLoS One* 6:e22008.
- Hanson MG, Jr., Shen S, Wiemelt AP, McMorris FA, Barres BA. 1998. Cyclic AMP elevation is sufficient to promote the survival of spinal motor neurons in vitro. *J Neurosci* 18:7361-7371.
- Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, Yow A, Soldner F, Hockemeyer D, Hallett PJ, Osborn T, Jaenisch R, Isacson O. 2010. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. *Proc Natl Acad Sci U S A* 107:15921-15926.
- Haubenberger D, Reinthaler E, Mueller JC, Pirker W, Katzenschlager R, Froehlich R, Bruecke T, Daniel G, Auff E, Zimprich A. 2011. Association of transcription factor polymorphisms PITX3 and EN1 with Parkinson's disease. *Neurobiol Aging* 32:302-307.

- He BC, Chen L, Zuo GW, Zhang W, Bi Y, Huang J, Wang Y, Jiang W, Luo Q, Shi Q, Zhang BQ, Liu B, Lei X, Luo J, Luo X, Wagner ER, Kim SH, He CJ, Hu Y, Shen J, Zhou Q, Rastegar F, Deng ZL, Luu HH, He TC, Haydon RC. 2010. Synergistic antitumor effect of the activated PPARgamma and retinoid receptors on human osteosarcoma. *Clin Cancer Res* 16:2235-2245.
- Hedlund E, Pruszek J, Ferree A, Vinuela A, Hong S, Isacson O, Kim KS. 2007. Selection of embryonic stem cell-derived enhanced green fluorescent protein-positive dopamine neurons using the tyrosine hydroxylase promoter is confounded by reporter gene expression in immature cell populations. *Stem Cells* 25:1126-1135.
- Herrero MT, Pagonabarraga J, Linazasoro G. 2011. Neuroprotective role of dopamine agonists: evidence from animal models and clinical studies. *Neurologist* 17:S54-66.
- Hess DC, Borlongan CV. 2008. Stem cells and neurological diseases. *Cell Prolif* 41 Suppl 1:94-114.
- Hornykiewicz O. 1973a. Dopamine in the basal ganglia. Its role and therapeutic implications (including the clinical use of L-DOPA). *Br Med Bull* 29:172-178.
- Hornykiewicz O. 1973b. Parkinson's disease: from brain homogenate to treatment. *Fed Proc* 32:183-190.
- Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH. 2004. Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America* 101:16659-16664.
- Hwang DY, Ardayfio P, Kang UJ, Semina EV, Kim KS. 2003b. Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. *Brain Res Mol Brain Res* 114:123-131.
- Hwang DY, Kim DS, Kim DW. 2010. Human ES and iPS cells as cell sources for the treatment of Parkinson's disease: current state and problems. *J Cell Biochem* 109:292-301.
- Inoue M, Mima T, Kojima Y, Satoi H, Makino F, Kanda M, Shibasaki H. 2007. [A case presenting with both features of essential tremor and Parkinson tremor]. *Rinsho shinkeigaku = Clinical neurology* 47:413-418.

- Itakura T, Kamei I, Nakai K, Nakai M, Naka Y, Nakakita K, Imai H, Komai N. 1988. [Transplantation of the superior cervical ganglion into the brain--a new experimental therapy of Parkinson disease]. *No to shinkei* = Brain and nerve 40:285-290.
- Jacobs FM, van Erp S, van der Linden AJ, von Oerthel L, Burbach JP, Smidt MP. 2009. Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. *Development* 136:531-540.
- Kalinderi K, Fidani L, Katsarou Z, Bostantjopoulou S. 2011. Pharmacological treatment and the prospect of pharmacogenetics in Parkinson's disease. *International journal of clinical practice* 65:1289-1294.
- Karamohamed S, Latourelle JC, Racette BA, Perlmutter JS, Wooten GF, Lew M, Klein C, Shill H, Golbe LI, Mark MH, Guttman M, Nicholson G, Wilk JB, Saint-Hilaire M, DeStefano AL, Prakash R, Tobin S, Williamson J, Suchowersky O, Labell N, Growdon BN, Singer C, Watts R, Goldwurm S, Pezzoli G, Baker KB, Giroux ML, Pramstaller PP, Burn DJ, Chinnery P, Sherman S, Vieregge P, Litvan I, Gusella JF, Myers RH, Parsian A. 2005. BDNF genetic variants are associated with onset age of familial Parkinson disease: GenePD Study. *Neurology* 65:1823-1825.
- Kassis I, Vaknin-Dembinsky A, Karussis D. 2011. Bone marrow mesenchymal stem cells: agents of immunomodulation and neuroprotection. *Curr Stem Cell Res Ther* 6:63-68.
- Katunar MR, Saez T, Brusco A, Antonelli MC. 2010. Ontogenetic expression of dopamine-related transcription factors and tyrosine hydroxylase in prenatally stressed rats. *Neurotox Res* 18:69-81.
- Kim HJ. 2011. Stem cell potential in Parkinson's disease and molecular factors for the generation of dopamine neurons. *Biochim Biophys Acta* 1812:1-11.
- Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sanchez-Pernaute R, Bankiewicz K, McKay R. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* 418:50-56.
- Klassen BT, Hentz JG, Shill HA, Driver-Dunckley E, Evidente VG, Sabbagh MN, Adler CH, Caviness JN. 2011. Quantitative EEG as a predictive biomarker for Parkinson disease dementia. *Neurology* 77:118-124.

- Ko JY, Lee HS, Park CH, Koh HC, Lee YS, Lee SH. 2009. Conditions for tumor-free and dopamine neuron-enriched grafts after transplanting human ES cell-derived neural precursor cells. *Mol Ther* 17:1761-1770.
- Kobayashi K, Umeda-Yano S, Yamamori H, Takeda M, Suzuki H, Hashimoto R. 2011. Correlated alterations in serotonergic and dopaminergic modulations at the hippocampal mossy fiber synapse in mice lacking dysbindin. *PLoS One* 6:e18113.
- Kong XY, Cai Z, Pan L, Zhang L, Shu J, Dong YL, Yang N, Li Q, Huang XJ, Zuo PP. 2008. Transplantation of human amniotic cells exerts neuroprotection in MPTP-induced Parkinson disease mice. *Brain Res* 1205:108-115.
- Kordower JH, Freeman TB, Chen EY, Mufson EJ, Sanberg PR, Hauser RA, Snow B, Olanow CW. 1998. Fetal nigral grafts survive and mediate clinical benefit in a patient with Parkinson's disease. *Mov Disord* 13:383-393.
- Krasnova IN, Ladenheim B, Hodges AB, Volkow ND, Cadet JL. 2011. Chronic methamphetamine administration causes differential regulation of transcription factors in the rat midbrain. *PLoS One* 6:e19179.
- Kurauchi Y, Hisatsune A, Isohama Y, Sawa T, Akaike T, Shudo K, Katsuki H. 2011. Midbrain dopaminergic neurons utilize nitric oxide/cyclic GMP signaling to recruit ERK that links retinoic acid receptor stimulation to up-regulation of BDNF. *Journal of neurochemistry* 116:323-333.
- Lane EL, Bjorklund A, Dunnett SB, Winkler C. 2010. Neural grafting in Parkinson's disease unraveling the mechanisms underlying graft-induced dyskinesia. *Prog Brain Res* 184:295-309.
- Lang AE, Lozano AM. 1998b. Parkinson's disease. First of two parts. *N Engl J Med* 339:1044-1053.
- Lebel M, Gauthier Y, Moreau A, Drouin J. 2001. Pitx3 activates mouse tyrosine hydroxylase promoter via a high-affinity binding site. *J Neurochem* 77:558-567.
- Lei Z, Jiang Y, Li T, Zhu J, Zeng S. 2011. Signaling of glial cell line-derived neurotrophic factor and its receptor GFR α 1 induce Nurr1 and Pitx3 to promote survival of grafted midbrain-derived neural stem cells in a rat model of Parkinson disease. *Journal of neuropathology and experimental neurology* 70:736-747.

- Li JY, Christophersen NS, Hall V, Soulet D, Brundin P. 2008. Critical issues of clinical human embryonic stem cell therapy for brain repair. *Trends Neurosci* 31:146-153.
- Li W, Ding S. 2010. Small molecules that modulate embryonic stem cell fate and somatic cell reprogramming. *Trends Pharmacol Sci* 31:36-45.
- Lindvall O, Brundin P, Widner H, Rehnström S, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, Marsden CD, et al. 1990. Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. *Science* 247:574-577.
- Lindvall O, Kokaia Z. 2010. Stem cells in human neurodegenerative disorders--time for clinical translation? *J Clin Invest* 120:29-40.
- Lindvall O, Kokaia Z, Martinez-Serrano A. 2004a. Stem cell therapy for human neurodegenerative disorders-how to make it work. *Nat Med* 10 Suppl:S42-50.
- Lindvall O, Rehnström S, Gustavii B, Brundin P, Astedt B, Widner H, Lindholm T, Björklund A, Leenders KL, Rothwell JC, et al. 1988. Fetal dopamine-rich mesencephalic grafts in Parkinson's disease. *Lancet* 2:1483-1484.
- Lindvall O, Widner H, Rehnström S, Brundin P, Odin P, Gustavii B, Frackowiak R, Leenders KL, Sawle G, Rothwell JC, et al. 1992. Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants. *Annals of neurology* 31:155-165.
- Liu S, Tian Z, Yin F, Zhao Q, Fan M. 2009. Generation of dopaminergic neurons from human fetal mesencephalic progenitors after co-culture with striatal-conditioned media and exposure to lowered oxygen. *Brain Res Bull* 80:62-68.
- Lloyd KG, Hornykiewicz O. 1973. L-glutamic acid decarboxylase in Parkinson's disease: effect of L-dopa therapy. *Nature* 243:521-523.
- Lonardo E, Parish CL, Ponticelli S, Marasco D, Ribeiro D, Ruvo M, De Falco S, Arenas E, Minchiotti G. 2010. A small synthetic cGMP blocking Peptide improves neural induction, dopaminergic differentiation, and functional integration of mouse embryonic stem cells in a rat model of Parkinson's disease. *Stem Cells* 28:1326-1337.

- Lu C, Hallgren J, Lin Y, Black D, Ekstrom L, Fentie IH, Brock G, Song Y, Winstead W, Roisen FJ. 2011. Human olfactory-derived neural progenitors diminish locomotory deficits following spinal cord contusion injury. *J Neurodegeneration and Regeneration* 4:1-18.
- Maden M. 2002. Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci* 3:843-853.
- Madrazo I, Leon V, Torres C, Aguilera MC, Varela G, Alvarez F, Fraga A, Drucker-Colin R, Ostrosky F, Skurovich M, et al. 1988. Transplantation of fetal substantia nigra and adrenal medulla to the caudate nucleus in two patients with Parkinson's disease. *N Engl J Med* 318:51.
- Maia J, Santos T, Aday S, Agasse F, Cortes L, Malva JO, Bernardino L, Ferreira L. 2011. Controlling the neuronal differentiation of stem cells by the intracellular delivery of retinoic acid-loaded nanoparticles. *ACS Nano* 5:97-106.
- Marshall CT, Guo Z, Lu C, Klueber KM, Khalyfa A, Cooper NG, Roisen FJ. 2005. Human adult olfactory neuroepithelial derived progenitors retain telomerase activity and lack apoptotic activity. *Brain Res* 1045:45-56.
- Marshall CT, Lu C, Winstead W, Zhang X, Xiao M, Harding G, Klueber KM, Roisen FJ. 2006. The therapeutic potential of human olfactory-derived stem cells. *Histol Histopathol* 21:633-643.
- Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78:7634-7638.
- Martinat C, Bacci JJ, Leete T, Kim J, Vanti WB, Newman AH, Cha JH, Gether U, Wang H, Abeliovich A. 2006. Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. *Proc Natl Acad Sci U S A* 103:2874-2879.
- Massie A, Schallier A, Vermoesen K, Arckens L, Michotte Y. 2010. Biphasic and bilateral changes in striatal VGLUT1 and 2 protein expression in hemi-Parkinson rats. *Neurochem Int* 57:111-118.

- Matsukawa N, Maki M, Yasuhara T, Hara K, Yu G, Xu L, Kim KM, Morgan JC, Sethi KD, Borlongan CV. 2007. Overexpression of D2/D3 receptors increases efficacy of ropinirole in chronically 6-OHDA-lesioned Parkinsonian rats. *Brain Res* 1160:113-123.
- Maxwell SL, Ho HY, Kuehner E, Zhao S, Li M. 2005. Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Dev Biol* 282:467-479.
- Mendez I, Sanchez-Pernaute R, Cooper O, Vinuela A, Ferrari D, Bjorklund L, Dagher A, Isacson O. 2005a. Cell type analysis of functional fetal dopamine cell suspension transplants in the striatum and substantia nigra of patients with Parkinson's disease. *Brain : a journal of neurology* 128:1498-1510.
- Messmer K, Remington MP, Skidmore F, Fishman PS. 2007b. Induction of tyrosine hydroxylase expression by the transcription factor Pitx3. *Int J Dev Neurosci* 25:29-37.
- Moliner A, Enfors P, Ibanez CF, Andang M. 2008. Mouse embryonic stem cell-derived spheres with distinct neurogenic potentials. *Stem cells and development* 17:233-244.
- Mukhida K, Baghbaderani BA, Hong M, Lewington M, Phillips T, McLeod M, Sen A, Behie LA, Mendez I. 2008. Survival, differentiation, and migration of bioreactor-expanded human neural precursor cells in a model of Parkinson disease in rats. *Neurosurg Focus* 24:E8.
- NationalParkinsonFounfdation. 2010. <http://www.parkinson.org>. In: National Parkinson Foundation.
- Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95:379-391.
- Nikkhah G, Cunningham MG, Jodicke A, Knappe U, Bjorklund A. 1994. Improved graft survival and striatal reinnervation by microtransplantation of fetal nigral cell suspensions in the rat Parkinson model. *Brain Res* 633:133-143.

- Nikkhah G, Duan WM, Knappe U, Jodicke A, Bjorklund A. 1993. Restoration of complex sensorimotor behavior and skilled forelimb use by a modified nigral cell suspension transplantation approach in the rat Parkinson model. *Neuroscience* 56:33-43.
- Novitsch BG, Wichterle H, Jessell TM, Sockanathan S. 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* 40:81-95.
- Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP. 2003. Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proceedings of the National Academy of Sciences of the United States of America* 100:4245-4250.
- Oiwa Y, Nakai K, Itakura T. 2006. Histological effects of intraputaminial infusion of glial cell line-derived neurotrophic factor in Parkinson disease model macaque monkeys. *Neurologia medico-chirurgica* 46:267-275; discussion 275-266.
- Okita K, Ichisaka T, Yamanaka S. 2007. Generation of germline-competent induced pluripotent stem cells. *Nature* 448:313-317.
- Olanow CW, Freeman T, Kordower J. 2001. Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med* 345:146; author reply 147.
- Olanow CW, Goetz CG, Kordower JH, Stoessl AJ, Sossi V, Brin MF, Shannon KM, Nauert GM, Perl DP, Godbold J, Freeman TB. 2003. A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Annals of neurology* 54:403-414.
- Olsson M, Nikkhah G, Bentlage C, Bjorklund A. 1995. Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *J Neurosci* 15:3863-3875.
- Parekh V. 2011. Parkinson disease: sniffing out dementia. *Nat Rev Neurol* 7:358.
- Parish CL, Castelo-Branco G, Rawal N, Tonnesen J, Sorensen AT, Salto C, Kokaia M, Lindvall O, Arenas E. 2008. Wnt5a-treated midbrain neural stem cells improve dopamine cell replacement therapy in parkinsonian mice. *J Clin Invest* 118:149-160.

- Park S, Kim EY, Ghil GS, Joo WS, Wang KC, Kim YS, Lee YJ, Lim J. 2003. Genetically modified human embryonic stem cells relieve symptomatic motor behavior in a rat model of Parkinson's disease. *Neurosci Lett* 353:91-94.
- Perlmann T, Wallen-Mackenzie A. 2004a. Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. *Cell Tissue Res* 318:45-52.
- Pessach IM, Notarangelo LD. 2011a. Gene therapy for primary immunodeficiencies: Looking ahead, toward gene correction. *J Allergy Clin Immunol*.
- Pessach IM, Notarangelo LD. 2011b. Gene therapy for primary immunodeficiencies: Looking ahead, toward gene correction. *J Allergy Clin Immunol* 127:1344-1350.
- Piccini P, Lindvall O, Bjorklund A, Brundin P, Hagell P, Ceravolo R, Oertel W, Quinn N, Samuel M, Rehnström S, Widner H, Brooks DJ. 2000. Delayed recovery of movement-related cortical function in Parkinson's disease after striatal dopaminergic grafts. *Annals of neurology* 48:689-695.
- Rauch F, Schwabe K, Krauss JK. 2010. Effect of deep brain stimulation in the pedunculopontine nucleus on motor function in the rat 6-hydroxydopamine Parkinson model. *Behav Brain Res* 210:46-53.
- Reddy SD, Rayala SK, Ohshiro K, Pakala SB, Kobori N, Dash P, Yun S, Qin J, O'Malley BW, Kumar R. 2011. Multiple coregulatory control of tyrosine hydroxylase gene transcription. *Proceedings of the National Academy of Sciences of the United States of America* 108:4200-4205.
- Redmond DE, Jr., Bjugstad KB, Teng YD, Ourednik V, Ourednik J, Wakeman DR, Parsons XH, Gonzalez R, Blanchard BC, Kim SU, Gu Z, Lipton SA, Markakis EA, Roth RH, Elsworth JD, Sladek JR, Jr., Sidman RL, Snyder EY. 2007. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proceedings of the National Academy of Sciences of the United States of America* 104:12175-12180.
- Redmond DE, Jr., Weiss S, Elsworth JD, Roth RH, Wakeman DR, Bjugstad KB, Collier TJ, Blanchard BC, Teng YD, Snyder EY, Sladek JR, Jr. 2010. Cellular repair in the parkinsonian nonhuman primate brain. *Rejuvenation Res* 13:188-194.

- Reyhani-Rad S, AMN, Javad Mahmoudi, Morteza Samini. 2011. Intraventricular Pallidum Injection of 6-OH Dopamine Induces Motor Deficit in Rat, a Novel Experimental Model for Catalepsy. *Australian Journal of Basic and Applied Sciences* 5:109-112.
- Roisen FJ, Klueber KM, Lu CL, Hatcher LM, Dozier A, Shields CB, Maguire S. 2001a. Adult human olfactory stem cells. *Brain Res* 890:11-22.
- Roisen FJ, Murphy RA, Braden WG. 1972a. Dibutyryl cyclic adenosine monophosphate stimulation of colcemid-inhibited axonal elongation. *Science* 177:809-811.
- Roisen FJ, Murphy RA, Pichichero ME, Braden WG. 1972b. Cyclic adenosine monophosphate stimulation of axonal elongation. *Science* 175:73-74.
- Ruff CA, Wilcox JT, Fehlings MG. 2011. Cell-based transplantation strategies to promote plasticity following spinal cord injury. *Exp Neurol*.
- Sadan O, Bahat-Stromza M, Barhum Y, Levy YS, Pisnevsky A, Peretz H, Ilan AB, Bulvik S, Shemesh N, Krepel D, Cohen Y, Melamed E, Offen D. 2009. Protective effects of neurotrophic factor-secreting cells in a 6-OHDA rat model of Parkinson disease. *Stem cells and development* 18:1179-1190.
- Santaniello S, Gale JT, Montgomery EB, Sarma SV. 2010. Modeling the motor striatum under Deep Brain Stimulation in normal and MPTP conditions. Conference proceedings : Annual International Conference of the IEEE Engineering in Medicine and Biology Society IEEE Engineering in Medicine and Biology Society Conference 2010:2065-2068.
- Saucedo-Cardenas O, Kardon R, Ediger TR, Lydon JP, Conneely OM. 1997. Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1. *Gene* 187:135-139.
- Sauer H, Fischer W, Nikkhah G, Wiegand SJ, Brundin P, Lindsay RM, Bjorklund A. 1993. Brain-derived neurotrophic factor enhances function rather than survival of intrastriatal dopamine cell-rich grafts. *Brain Res* 626:37-44.
- Savica R, Rocca WA, Ahlskog JE. 2010. When does Parkinson disease start? *Arch Neurol* 67:798-801.

- Schulz TC, Noggle SA, Palmarini GM, Weiler DA, Lyons IG, Pensa KA, Meedeniya AC, Davidson BP, Lambert NA, Condie BG. 2004. Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem Cells* 22:1218-1238.
- Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, Blumenthal PD, Huggins GR, Gearhart JD. 1998. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci U S A* 95:13726-13731.
- Sharpe JA, Rewcastle NB, Lloyd KG, Hornykiewicz O, Hill M, Tasker RR. 1973. Striatonigral degeneration. Response to levodopa therapy with pathological and neurochemical correlation. *J Neurol Sci* 19:275-286.
- Sheng D, Qu D, Kwok KH, Ng SS, Lim AY, Aw SS, Lee CW, Sung WK, Tan EK, Lufkin T, Jesuthasan S, Sinnakaruppan M, Liu J. 2010. Deletion of the WD40 domain of LRRK2 in Zebrafish causes Parkinsonism-like loss of neurons and locomotive defect. *PLoS Genet* 6:e1000914.
- Simeone A. 2005. Genetic control of dopaminergic neuron differentiation. *Trends Neurosci* 28:62-65; discussion 65-66.
- Singh S, Ahmad R, Mathur D, Sagar RK, Krishana B. 2006a. Neuroprotective effect of BDNF in young and aged 6-OHDA treated rat model of Parkinson disease. *Indian J Exp Biol* 44:699-704.
- Singh S, Ahmad R, Mathur D, Sagar RK, Krishana B. 2006b. Neuroprotective effect of BDNF in young and aged 6-OHDA treated rat model of Parkinson disease. *Indian J Exp Biol* 44:699-704.
- Smidt MP, van Schaick HS, Lanctot C, Tremblay JJ, Cox JJ, van der Kleij AA, Wolterink G, Drouin J, Burbach JP. 1997. A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proceedings of the National Academy of Sciences of the United States of America* 94:13305-13310.
- Smits SM, Ponnio T, Conneely OM, Burbach JP, Smidt MP. 2003. Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. *Eur J Neurosci* 18:1731-1738.

- Snyder BJ, Olanow CW. 2005. Stem cell treatment for Parkinson's disease: an update for 2005. *Curr Opin Neurol* 18:376-385.
- Soldner F, Laganieri J, Cheng AW, Hockemeyer D, Gao Q, Alagappan R, Khurana V, Golbe LI, Myers RH, Lindquist S, Zhang L, Guschin D, Fong LK, Vu BJ, Meng X, Urnov FD, Rebar EJ, Gregory PD, Zhang HS, Jaenisch R. 2011. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell* 146:318-331.
- Sonntag KC, Simantov R, Isacson O. 2005. Stem cells may reshape the prospect of Parkinson's disease therapy. *Brain Res Mol Brain Res* 134:34-51.
- Sosnowski JS, Gupta M, Reid KH, Roisen FJ. 1995. Chemical traumatization of adult mouse olfactory epithelium in situ stimulates growth and differentiation of olfactory neurons in vitro. *Brain Res* 702:37-48.
- Stacy M, Galbreath A. 2008. Optimizing long-term therapy for Parkinson disease: levodopa, dopamine agonists, and treatment-associated dyskinesia. *Clin Neuropharmacol* 31:51-56.
- Stoessl AJ. 1996. Which dopamine receptor(s) do we need for motor function? Lessons from gene targeting and translational blockade. *Parkinsonism Relat Disord* 2:167-175.
- Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB. 1997. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp Neurol* 148:135-146.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861-872.
- Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663-676.

- Takeuchi Y, Hashizume C, Chon EM, Momozawa Y, Masuda K, Kikusui T, Mori Y. 2005. Canine tyrosine hydroxylase (TH) gene and dopamine beta -hydroxylase (DBH) gene: their sequences, genetic polymorphisms, and diversities among five different dog breeds. *The Journal of veterinary medical science / the Japanese Society of Veterinary Science* 67:861-867.
- Tatard VM, Sindji L, Branton JG, Aubert-Pouessel A, Colleau J, Benoit JP, Montero-Menei CN. 2007. Pharmacologically active microcarriers releasing glial cell line - derived neurotrophic factor: Survival and differentiation of embryonic dopaminergic neurons after grafting in hemiparkinsonian rats. *Biomaterials* 28:1978-1988.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-1147.
- Thomson JA, Marshall VS. 1998. Primate embryonic stem cells. *Current topics in developmental biology* 38:133-165.
- Tonnesen J, Parish CL, Sorensen AT, Andersson A, Lundberg C, Deisseroth K, Arenas E, Lindvall O, Kokaia M. 2011. Functional integration of grafted neural stem cell-derived dopaminergic neurons monitored by optogenetics in an in vitro Parkinson model. *PLoS One* 6:e17560.
- Torp R, Singh PB, Sorensen DR, Dietrichs E, Hirschberg H. 2006. [Growth factors as neuroprotective treatment in Parkinson disease?]. *Tidsskr Nor Laegeforen* 126:899-901.
- Trzaska KA, Rameshwar P. 2011. Dopaminergic neuronal differentiation protocol for human mesenchymal stem cells. *Methods in molecular biology* 698:295-303.
- Ungerstedt U, Arbuthnott GW. 1970. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. *Brain Res* 24:485-493.
- Vadasz C, Kobor G, Lajtha A. 1992a. Motor activity and the mesotelencephalic dopamine function. I. High-resolution temporal and genetic analysis of open-field behavior. *Behav Brain Res* 48:29-39.

- Vadasz C, Kobor G, Lajtha A. 1992b. Motor activity and the mesotelencephalic dopamine function. II. Multivariate analysis of genetically segregating generations. *Behav Brain Res* 48:41-47.
- Vazin T, Becker KG, Chen J, Spivak CE, Lupica CR, Zhang Y, Worden L, Freed WJ. 2009. A novel combination of factors, termed SPIE, which promotes dopaminergic neuron differentiation from human embryonic stem cells. *PLoS One* 4:e6606.
- Vidailhet M. 2011. Movement disorders in 2010: Parkinson disease-symptoms and treatments. *Nat Rev Neurol* 7:70-72.
- von Bohlen und Halbach O, Unsicker K. 2009b. Neurotrophic support of midbrain dopaminergic neurons. *Adv Exp Med Biol* 651:73-80.
- Wang ML, C; Li, H; Qiu, M; Winstead, I. W; Roisen, F. 2011. Lineage Restriction of Adult Human Olfactory-Derived Progenitors to Dopaminergic Neurons. In: *Stem Cell Discovery*, 10/18/2011 ed. p 29-43.
- Wang S, Sakamuri S, Enyedy IJ, Kozikowski AP, Deschaux O, Bandyopadhyay BC, Tella SR, Zaman WA, Johnson KM. 2000. Discovery of a novel dopamine transporter inhibitor, 4-hydroxy-1-methyl-4-(4-methylphenyl)-3-piperidyl 4-methylphenyl ketone, as a potential cocaine antagonist through 3D-database pharmacophore searching. Molecular modeling, structure-activity relationships, and behavioral pharmacological studies. *Journal of medicinal chemistry* 43:351-360.
- Wedekind S. 2005. [L-dopa effectiveness decreases over time. What helps Parkinson patients stay mobile?]. *MMW Fortschritte der Medizin* 147:10.
- Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385-397.
- Winner B. 2008. The impact of Nurr1 to antagonizes neurotoxicity of activated microglia in Parkinson's disease (PD) model. In: *NEUROSCIENCE 2008. WASHINGTON D. C., USA.*
- Winstead W, Marshall CT, Lu CL, Klueber KM, Roisen FJ. 2005a. Endoscopic biopsy of human olfactory epithelium as a source of progenitor cells. *Am J Rhinol* 19:83-90.

- Winstead W, Marshall CT, Lu CL, Klueber KM, Roisen FJ. 2005b. Endoscopic biopsy of human olfactory epithelium as a source of progenitor cells. *Am J Rhinol* 19:83-90.
- Wise RA. 2004. Dopamine, learning and motivation. *Nature reviews Neuroscience* 5:483-494.
- Wolff EF, Gao XB, Yao KV, Andrews ZB, Du H, Elsworth JD, Taylor HS. 2011. Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. *Journal of cellular and molecular medicine* 15:747-755.
- Wright AK, Garcia-Munoz M, Arbuthnott GW. 2009. Slowly progressive dopamine cell loss--a model on which to test neuroprotective strategies for Parkinson's disease? *Rev Neurosci* 20:85-94.
- Xiong N, Zhang Z, Huang J, Chen C, Jia M, Xiong J, Liu X, Wang F, Cao X, Liang Z, Sun S, Lin Z, Wang T. 2011. VEGF-expressing human umbilical cord mesenchymal stem cells, an improved therapy strategy for Parkinson's disease. *Gene Ther* 18:394-402.
- Yang JR, Liao CH, Pang CY, Huang LL, Lin YT, Chen YL, Shiue YL, Chen LR. 2010a. Directed differentiation into neural lineages and therapeutic potential of porcine embryonic stem cells in rat Parkinson's disease model. *Cell Reprogram* 12:447-461.
- Yasuda T, Hayakawa H, Nihira T, Ren YR, Nakata Y, Nagai M, Hattori N, Miyake K, Takada M, Shimada T, Mizuno Y, Mochizuki H. 2011. Parkin-mediated protection of dopaminergic neurons in a chronic MPTP-minipump mouse model of Parkinson disease. *Journal of neuropathology and experimental neurology* 70:686-697.
- Yoneyama M, Kawada K, Shiba T, Ogita K. 2011b. Endogenous nitric oxide generation linked to ryanodine receptors activates cyclic GMP / protein kinase G pathway for cell proliferation of neural stem/progenitor cells derived from embryonic hippocampus. *Journal of pharmacological sciences* 115:182-195.
- Zahniser NR, Larson GA, Gerhardt GA. 1999. In vivo dopamine clearance rate in rat striatum: regulation by extracellular dopamine concentration and dopamine transporter inhibitors. *The Journal of pharmacology and experimental therapeutics* 289:266-277.

- Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. 1997. Dopamine neuron agenesis in *Nurr1*-deficient mice. *Science* 276:248-250.
- Zhang HL, Wu JJ, Ren HM, Wang J, Su YR, Jiang YP. 2007. Therapeutic effect of microencapsulated porcine retinal pigmented epithelial cells transplantation on rat model of Parkinson's disease. *Neurosci Bull* 23:137-144.
- Zhang J, Geula C, Lu C, Koziel H, Hatcher LM, Roisen FJ. 2003. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. *Exp Neurol* 183:469-481.
- Zhang X, Cai J, Klueber KM, Guo Z, Lu C, Qiu M, Roisen FJ. 2005. Induction of oligodendrocytes from adult human olfactory epithelial-derived progenitors by transcription factors. *Stem Cells* 23:442-453.
- Zhang X, Cai J, Klueber KM, Guo Z, Lu C, Winstead WI, Qiu M, Roisen FJ. 2006a. Role of transcription factors in motoneuron differentiation of adult human olfactory neuroepithelial-derived progenitors. *Stem Cells* 24:434-442.
- Zhang X, Klueber KM, Guo Z, Cai J, Lu C, Winstead WI, Qiu M, Roisen FJ. 2006c. Induction of neuronal differentiation of adult human olfactory neuroepithelial-derived progenitors. *Brain Res* 1073-1074:109-119.
- Zhang X, Klueber KM, Guo Z, Lu C, Roisen FJ. 2004. Adult human olfactory neural progenitors cultured in defined medium. *Exp Neurol* 186:112-123.
- Zhang XD, Guo ZF, Liu N, Roisen FJ. 2000b. [Effects of bFGF and BDNF on the cells of injured adult mouse olfactory epithelium in vitro]. *Sheng Li Xue Bao* 52:193-198.
- Zhu Q, Ma J, Yu L, Yuan C. 2009. Grafted neural stem cells migrate to substantia nigra and improve behavior in Parkinsonian rats. *Neurosci Lett* 462:213-218.
- Zucco G, Zeni MT, Perrone A, Piccolo I. 2001. Olfactory sensitivity in early-stage Parkinson patients affected by more marked unilateral disorder. *Perceptual and motor skills* 92:894-898.
- Zucco GM, Zaglis D, Wambsganss CS. 1991. Olfactory deficits in elderly subjects and Parkinson patients. *Perceptual and motor skills* 73:895-898.

CURRICULUM VITAE

Meng Wang

Education

Ph.D. in Anatomical Sciences & Neurobiology	University of Louisville, USA	2007-2011
M.S. in Anatomical Sciences & Neurobiology	University of Louisville, USA	2005-2007
M.D.	Tianjin Medical University, China	2000-2005

Academic Experience

2005-Present	Department of Anatomical Sciences & Neurobiology, University of Louisville, U.S.A	Graduate Research Assistant
2007-2008	Department of Anatomical Sciences & Neurobiology, University of Louisville, U.S.A	Teaching Assistant Neuroanatomy (ASNB 607)
2003-2005	Second Hospital of Tianjin Medical University, Tianjin, China	Observing Doctor
2002-2004	Parasite Laboratory, College of Basic Medicine, Tianjin Medical University, Tianjin, China	Undergraduate Research Assistant
2000-2005	College of Basic Medicine, Tianjin Medical University, Tianjin, China	Medical Student

Professional Societies

2007- Present Society for Neuroscience, Student Member

Patent (Pending)

Olfactory Epithelial-derived Stem Cells and Methods of Use Therefor

Inventors: Fred Roisen, Ph.D.

Chengliang Lu, M.D.

Meng Wang, M.D., M.S.

Mengsheng Qiu, Ph.D.

Honors and Awards

2010	Michael Tanner Memorial Award for Excellence in Graduate Student Sciences	Louisville, KY
2009	Second Place in Graduate Poster Competition in Society for Neuroscience, Louisville Chapter	Louisville, KY
2005-20 07	IPIBS Fellowship from University of Louisville	Louisville, KY
2004	Outstanding Volunteer of Tianjin Medical University	Tianjin, China
2003	Third Place in the 8 th National Challenge Cup Scientific Paper Competition, as first Author and Presenter	Tianjin, China
2002	First Place in the 7 th Tianjin Challenge Cup Scientific Paper Competition, as first Author and Presenter	Tianjin, China

Community Service

2004-2005	Vice-President of the Student Union	Tianjin Medical University
2002-2004	President of Student Volunteer Association	Tianjin Medical University

Publication

- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Lineage Restriction of Adult Human Olfactory-derived Progenitors to Dopaminergic Neurons. *Stem Cell Discovery*, Vol. 1, No.3, 29-43 (2011)

Manuscripts in Preparation

- **Meng Wang**, Chengliang Lu, Fred Roisen. Lineage Restricted Adult Human Olfactory-derived Progenitors in a Rodent Model of Parkinson's Disease (*In Preparation*)

Conference Abstract and Presentation

- **Meng Wang**, Chenliang Lu, Fred Roisen. Human Adult olfactory epithelial-derived progenitors: A potential therapy for parkinson's disease. *Neuroscience 2011, Washington, D.C., USA, Nov 12-16 2011*
- **Meng Wang**, Chengliang Lu, Fred Roisen. Human Adult Olfactory Epithelial-Derived Progenitors: A Potential Therapy for Parkinson's Disease. *Research! Louisville 2011, Louisville, KY, Oct.10-14 2011*
- **Meng Wang**, Chengliang Lu, Mengsheng Qiu, Welby Winstead, Fred Roisen. Human Adult olfactory epithelial-derived progenitors: A potential therapy for parkinson's disease. *21st Annual Neuroscience Day, Louisville Chapter, Louisville, KY, Apr. 21 2011*
- **Meng Wang**, Chengliang Lu, Mengsheng Qiu, Welby Winstead, Fred Roisen. Human Adult olfactory epithelial-derived progenitors: A potential therapy for parkinson's disease. *Neuroscience 2010, San Diego, CA, USA, Nov.12-16 2010*
- **Meng Wang**, Chengliang Lu, Mengsheng Qiu, Welby Winstead, Fred Roisen. Human Adult Olfactory Epithelial-Derived Progenitors: A Potential Therapy for Parkinson's Disease. *Research! Louisville 2010, Louisville, KY, Oct.11-15 2010*
- **Meng Wang**, Chengliang Lu, Mengsheng Qiu, Welby Winstead, Fred Roisen. Human Adult Olfactory Epithelial-Derived Progenitors: A Potential Therapy for Parkinson's Disease. *20th Annual Neuroscience Day, Louisville Chapter, Louisville, KY, May 6 2010*
- **Meng Wang**, Patric Baumann, Deborah Black, Jodi Hallgren, Yu Lin, Chengliang Lu, Mengsheng Qiu, Holly Waechter, Welby Winstead, Fred Roisen. Human adult olfactory epithelial-derived progenitors: A potential cell-mediated therapy for Parkinson's disease. *Neuroscience 2009, Chicago, IL, USA, Nov. 13-17 2009*
- **Meng Wang**, Patric Baumann, Deborah Black, Jodi Hallgren, Yu Lin, Chengliang Lu, Mengsheng Qiu, Holly Waechter, Welby Winstead, Fred Roisen. Dopaminergic Restriction of Human Adult Olfactory Epithelial-Derived Progenitors: A Potential

Source for Cell –Mediated Therapy for Parkinson’s Disease. ***Research!Louisville 2009, Louisville, KY***

- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Genetic Modification of Adult Human Olfactory-Derived Progenitors towards Dopaminergic Neurons. ***19th Annual Neuroscience Day, Louisville Chapter, Louisville, KY, Apr.16 2009***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Adult human olfactory-derived progenitors can be lineage restricted to dopaminergic neurons. ***2nd Annual Graduate Student Research Symposium, Louisville, KY, Mar. 2009***
- **Meng Wang**, Student Representative of UofL, Graduate Research Day at the Capitol, poster presentation to Kentucky legislation, ***Frankfort, KY, Feb.2, 2009***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Adult human olfactory-derived progenitors can be lineage restricted to dopaminergic neurons. ***Neuroscience 2008, Washington DC, USA, Nov. 15-19 2008***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Adult human olfactory epithelial-derived progenitors: a potential autologous population for cell therapy for Parkinson's disease. ***Research!Louisville 2008, Louisville, KY***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Genetic Modification of Adult Human Olfactory-Derived Progenitors towards Dopaminergic Neurons. ***18th Annual Neuroscience Day, Louisville Chapter, Louisville, KY, Apr.17 2008***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Dopaminergic Differentiation of Adult Human Olfactory-Derived Progenitors. ***1st Annual Graduate Student Research Symposium, Louisville, KY, Mar. 2008***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Lineage restriction of adult human olfactory-derived progenitors to dopaminergic neurons. ***Neuroscience 2007, San Diego, CA, USA, Nov.3-7 2007***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Lineage restriction of adult human olfactory-derived progenitors to dopaminergic neurons. ***Research!Louisville 2007, Louisville, KY, Oct. 16-19 2007***
- **Meng Wang**, Chengliang Lu, Hong Li, Mengsheng Qiu, Welby Winstead, Fred Roisen. Lineage restriction of adult human olfactory-derived progenitors to dopaminergic neurons. ***17th Annual Neuroscience Day, Louisville Chapter, Louisville, KY, Apr.12 2007***