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Introduction

Grafting of primary embryonic neural tissue has proven that cell replacement therapy is a viable approach to the treatment of Parkinson's disease (PD). The number of successfully grafted patients, however, is relatively small: partly due to limited access to suitable donor tissue. Alternative sources of donor tissue are urgently needed for cell transplantation to become a widely available therapy for PD. Human embryonic stem cells (hESCs) have been suggested to be a potential tissue source for cell replacement therapy in PD. They can be differentiated into a variety of cell types including neurons (Stojkovic et al., 2004). Previous studies have shown that mouse ESC can indeed differentiate into dopaminergic (DA) neurons under certain culture conditions (Kawasaki et al., 2000; Lee et al., 2000; Wakayama et al., 2001; Kim et al., 2002; Barberi et al., 2003). Cells derived from mouse ESC can also survive transplantation into rodent brains (Kawasaki et al., 2000; Bjorklund et al., 2002; Kim et al., 2002; Morizane et al., 2002; Nishimura et al., 2003). When non-differentiated mouse ESCs were grafted into the striatum, substantial numbers of mouse ESC differentiated into DA neurons. Many grafted rats, however, developed teratomas (Bjorklund et al., 2002). If mouse ESCs were differentiated into neurons *in vitro* prior to transplantation, much higher numbers of DA neurons were observed after grafting. There was convincing evidence for functional recovery, in terms of improvement of lesion-induced behavioral deficits, without the formation of teratomas (Kawasaki et al., 2000; Kim et al., 2002; Barberi et al., 2003). Similar results have also been reported when using monkey ESCs (Kawasaki et al., 2002; Takagi et al., 2005). Our own results suggest that a risk of teratoma formation inversely correlates with the duration of the *in vitro* differentiation protocol (Brederlau & Correia et al., 2006). While the yield of hESC-derived DA neurons is not a limiting factor of transplantation success anymore, the major problems associated with the actual application of hESC-based therapies for PD are associated with 1) poor survival of mature DA neurons and 2) risk of teratoma and tumor formation in the site of transplantation. The former is caused by the presence of residual undifferentiated/mitotic cells amongst the transplanted cells.

Body

After establishing a laboratory suitable for hESC research, a continuous colony of proliferating hESCs (NIH-approved SA002 (Sahlgrenska 2) hESC line; Heins et al., 2004) was supported, providing a continuous flow of cell material for extensive experimentation. A primary *in vitro* differentiation protocol developed has relied upon epigenetic factors including co-culturing hESCs with stromal-derived cell inducing activity (SDIA)-releasing stromal cells (namely, PA6 cells) and supplementation of growth/differentiation media with basic fibroblast growth factor (bFGF, FGF2). As described in the annual reports, a prolonged *in vitro* differentiation of hESCs exposed to these factors has resulted in the formation of tyrosine hydroxylase-positive (TH⁺) neurons; moreover, dopamine (DA) release was confirmed by High Performance Liquid Chromatography (HPLC) analysis, proving the identity of cells (Fig. 1).

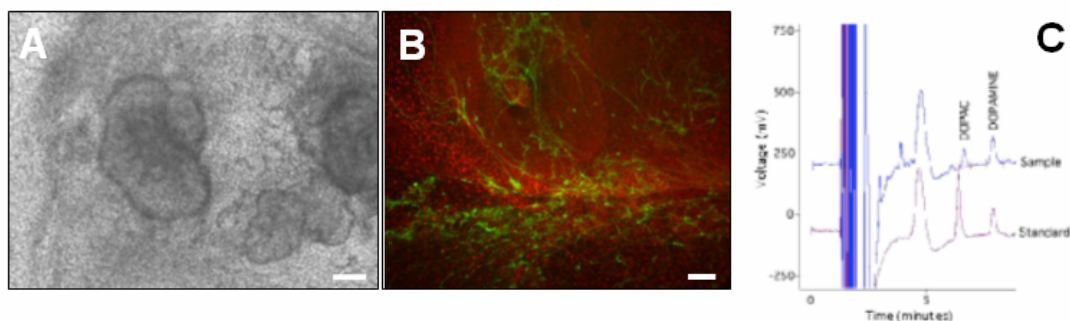


Figure 1. Human Embryonic Stem Cell (hESCs)-derived cells committed towards dopaminergic differentiation pathway by co-culturing with PA6 stromal cells for 16 days. (A) Phase contrast image of structures formed in hESC colony. (B) Immunocytochemical analysis of cell composition: tyrosine hydroxylase (TH), green; human nuclei marker, red. Scale bars = 100 μ m. (C) High Performance Liquid Chromatography (HPLC) chromatogram for dopamine release from hESCs co-cultured with PA6-cells for 20 days (blue line). Pink line represents DA standard.

Following evaluation of the yield of TH⁺ neurons achieved (up to 7.4% in the total number of hESC-derived cells), 100,000 viable cells were transplanted to the striatum of 6-hydroxydopamine (6-OHDA)-lesioned rats, an established animal model of Parkinson's disease. The effectiveness of the lesion was tested by amphetamine-induced rotation prior to the grafting procedure. Animals were kept immunosuppressed, and post-grafting alterations in their behavior were evaluated during 13 weeks using amphetamine-induced rotation test. No statistically significant improvement in the behavior of the grafted rats was detected (though some trend to the improvement was evident in particular groups). However, the experiment has resulted in a major finding: it has proven that the risk of teratoma formation (originated from the residual undifferentiated cells in the mixed cell population) is inversely correlated with the length of *in vitro* differentiation protocol preceding the transplantation procedure. Moreover, the key points associated with (i) 100% rate of teratoma formation in the transplanted animals; (ii) zero risk of teratoma formation and (iii) a transitional stage of the protocol were successfully identified (Brederlau & Correia et al., 2006). It was also concluded that in order to achieve a significant effect upon the behavior of the model animals, DA cell survival in the site of transplantation should be significantly improved. Taken together, we have suggested a concept of 'window of opportunity' characterized by zero risk of teratoma/tumor formation and reasonably high rate of hESC-derived DA neuron survival in the site of transplantation (Fig. 2).

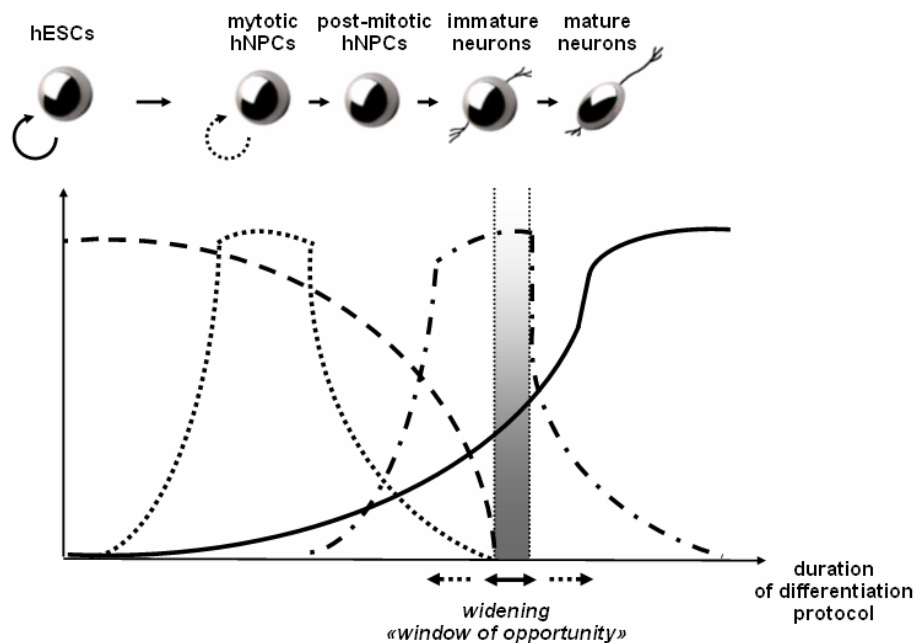


Figure 2. A concept of “window of opportunity” in hESC-based therapy for PD. Dashed line: risk of teratoma formation; Dotted line: risk of non-teratoma tumor formation; Solid line: Yield of hESC-derived DA neurons (as estimated by TH staining); Dash-dotted line: NPC and DA neuron survival rate in transplantation; Shaded area: “window of opportunity” promising clinical benefits.

There is a clear controversy between 1) the ability of hESC-derived DA neurons to withstand handling/transplantation procedures and survive in the site of transplantation and 2) the degree of their differentiation. A straightforward approach taken relied upon increasing the yield of hESC-derived DA neurons by supplementing the *in vitro* differentiation protocol with novel *substantia nigra pars compacta*-related factor (fibroblast growth factor 20; FGF20). Using it in combination with SDIA and basic fibroblast growth factor (bFGF) we were able to attain a balance of differentiation and proliferation processes (Figures 3-4).

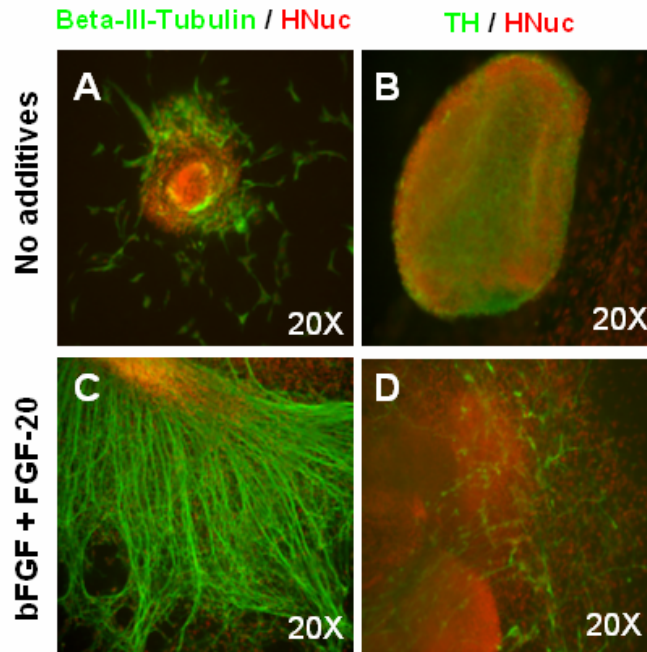


Figure 3. Effect of bFGF/FGF-20 supplementation upon basic in vitro differentiation protocol (co-culturing hESCs with PA6 stromal cells), 3 weeks, immunocytochemical analysis. (A, C) Beta-III-Tubulin (green) / Human Nuclei marker (red); (B, D) Tyrosine hydroxylase (green) / Human Nuclei marker (red). Images were recorded using (20x) objective.

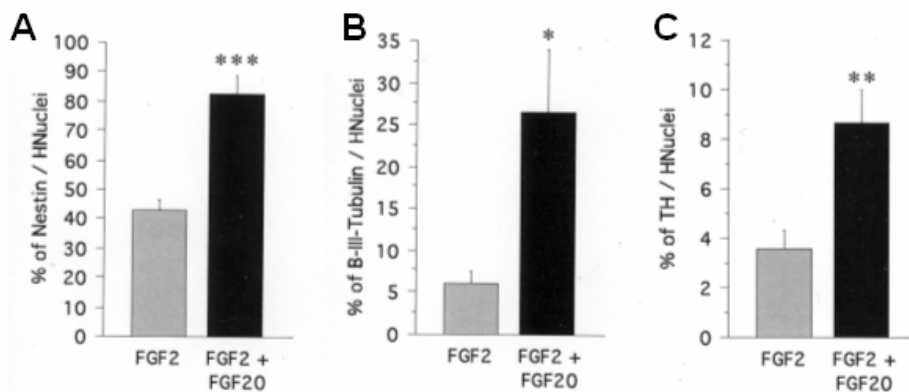


Figure 4. Effect of bFGF/FGF-20 supplementation on the basic in vitro differentiation protocol (co-culturing hESCs with PA6 stromal cells). (A) Number of Nestin+ cells, (B) Beta-III-Tubulin+ cells and (C) Tyrosine hydroxylase+ cells / Human nuclei antigen marker+ cells after 3 weeks of co-culturing. bFGF (4 ng/ml); bFGF (4 ng/ml) + FGF-20 (200 ng/ml). Asterisks denote: (*), $p < 0.05$; (**), $p < 0.01$; (***), $p < 0.001$.

Moreover, an extensive multi-disciplinary approach had allowed us to highlight important biological mechanisms implemented by FGF20. Acting in a dose-dependant manner, it affects not only differentiation, but also proliferation of hESC-derived DA progenitors (Fig. 5). This finding is of great importance in the particular field of ESC/neuronal differentiation research.

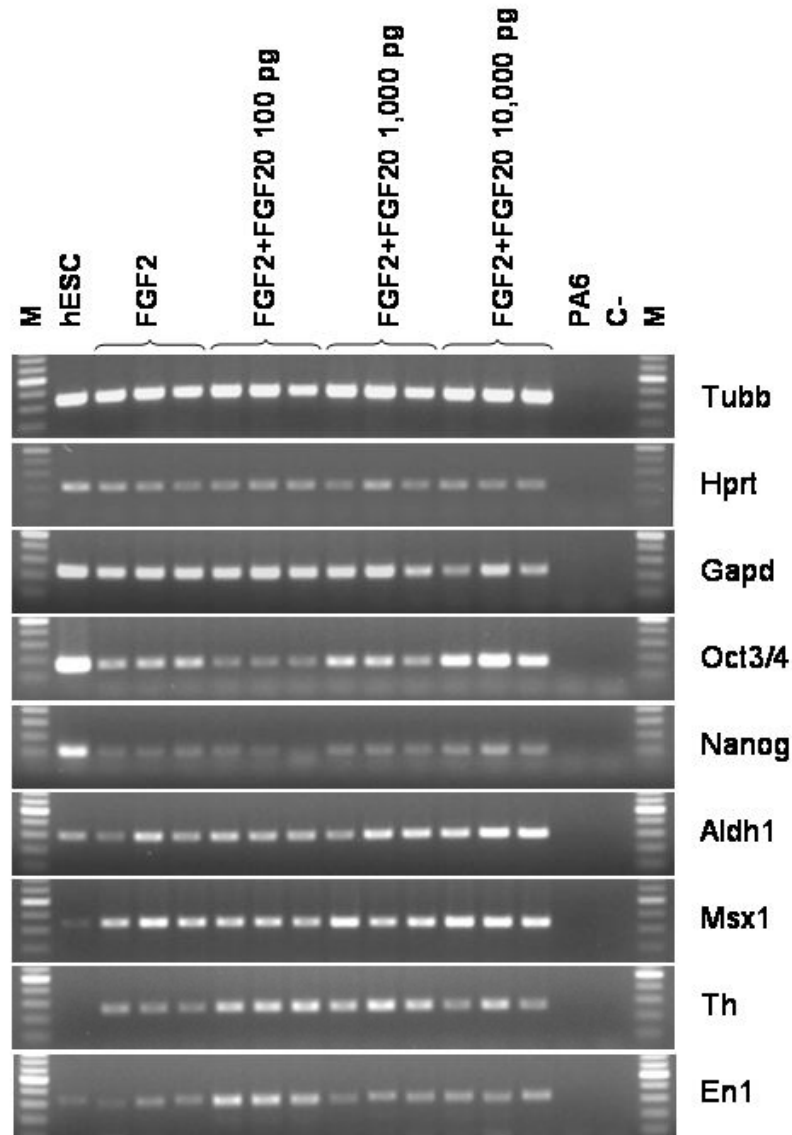


Figure 5. Fibroblast growth factor 20 (FGF20) affects both differentiation and proliferation of hESC-derived DA progenitors in a dose-dependant manner. *Tubb*, *Hprt*, *Gapd* – housekeeping genes; *Oct3/4* and *Nanog* – markers of cell proliferation; *Aldh1*, *Msx1*, *Th* and *En1* – markers of DA lineage commitment.

To further widen the ‘window of opportunity’ by accelerating the differentiation process (and thus eliminating the contaminating fraction of residual undifferentiated hESCs) and to improve cell survival in the site of transplantation radically, we have suggested a novel concept: culturing transplantation-ready cells not in 2-D (cell monolayer) but in 3-D structures. Thus, the major stress to highly sensitive cells caused by enzymatic digestion, mechanical dissociation and prolonged off-incubator handling would be avoided. Additionally, axotomy (i.e., disruption of grown cellular processes) will be prevented. In addition to basic differentiation-promoting factors (including SDIA

and FGF20), a close cell-cell interaction within the individual 3-D structures is considered being a potent differentiation-driving factor itself, accelerating the protocol. Numerous experiments have proved that the selected cell system (the SA002 hESC line) can not withstand the enzymatical/mechanical dissociation essential for the uniform 3-D structure formation. It was therefore concluded that cells should be pre-differentiated/committed toward a dopaminergic lineage prior to dissociation. We were able to derive a 2-stage protocol based on 1) hESC commitment to neuronal/dopaminergic progenitors by 16-17 day co-culturing with SDIA-releasing cells (PA6 cells) and exposure to basic fibroblast growth factor (bFGF/FGF2), 2) dissociation of cells (which are relatively insensitive in the intermediate stage) and 3) formation of 3-D structures induced by constant agitation of the cell suspension inside a CO₂ incubator and terminal differentiation (6-7 days) (Fig. 6).

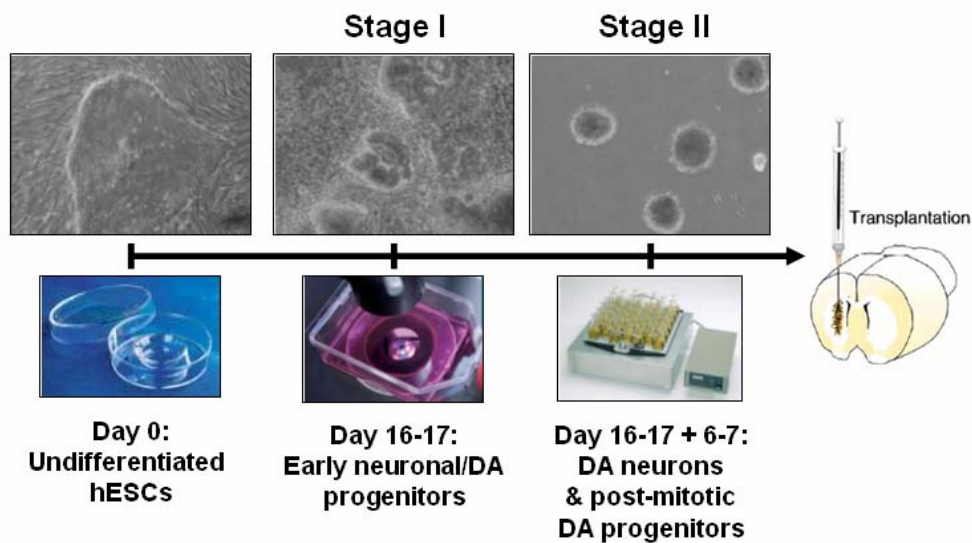


Figure 6. Principle of the 2-stage method suggested: co-exposure of hESCs to stromal-derived cell-inducing activity (SDIA; PA6 cells) and growth factors for 16-17 days (Stage I) is followed by the formation and growth of TU's for 6-7 days, further enriched with DA neurons and post-mitotic DA progenitors (Stage II) suitable for transplantation. Total length of the protocol = 16-17 + 6-7 days.

A shaker/incubator system was established and we were able to adapt the suggested protocol, successfully yielding a population of 3-D structures (that were termed 'Transplantable Units' (TUs). Those TUs are characterized by: 1) having the desired dimensions (the parameter that depends on the pre-settings like the concentration of the cell suspension and incubation length), 2) being relatively uniform and 3) containing a significant number of tyrosine hydroxylase (TH)-positive cells (a feature achieved by the continuous exposure of the forming/formed TUs to differentiation-promoting factors) (Fig. 7-10).

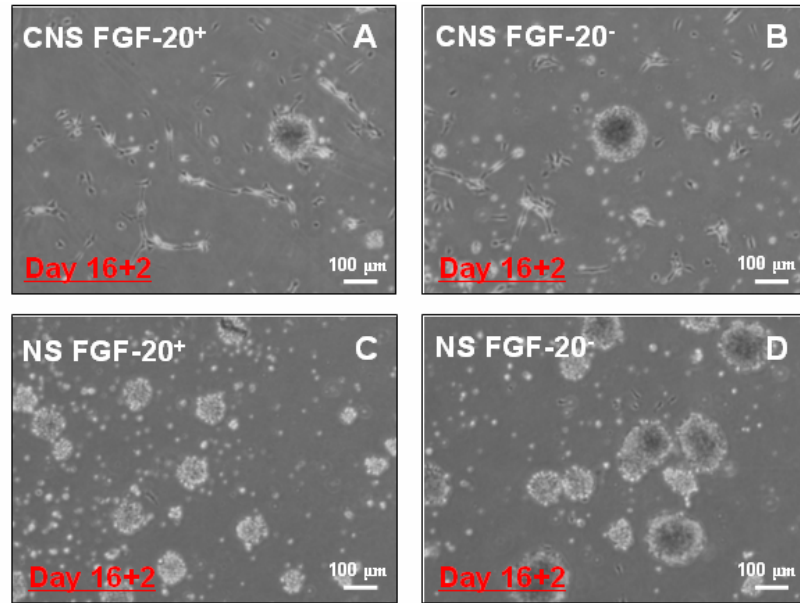


Figure 7. Stage II of differentiation protocol, Day 16+2. After Stage I (hESC/PA6 co-culturing for 16 days), the committed cells are dissociated enzymatically and cultured in Petri dishes on a rotating shaker (60 rpm) with a supplement of PA6-conditioned medium (CNS, A,B) or in serum free (NS, C,D) conditions in presence (A,C) or absence (B,D) of FGF20 (100 pg/ml). Two days rotating culture (Day 16+2) contain heterogeneous aggregates (TU formation) and few cells (contaminating PA6 cells) attached to the bottom of Nunc Petri dishes in all conditions.

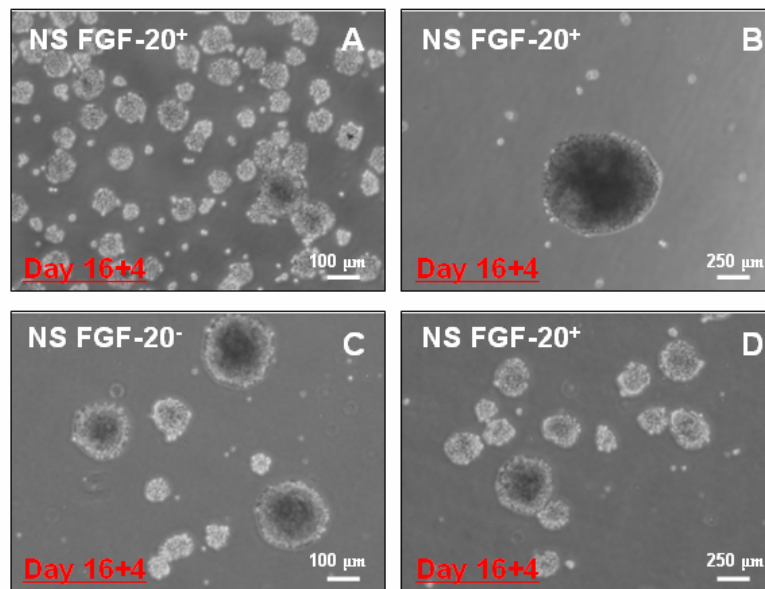


Figure 8. Stage II of differentiation protocol, Day 16+4. During the four days of rotating culture in serum-free (NS) media, highly heterogeneous aggregates are formed in the presence (A,B) and absence (C,D) of FGF20.

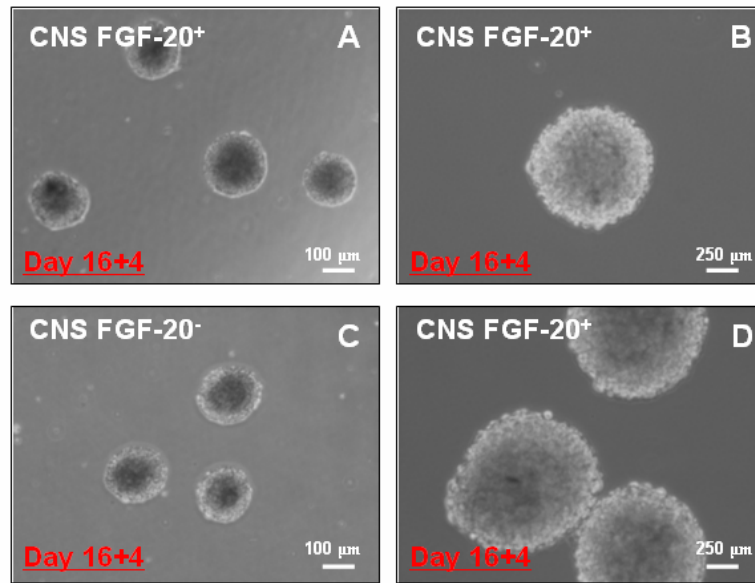


Figure 9. Stage II of differentiation protocol, Day 16+4. During the four days of rotating culture in conditioned media, transplant units (TU's) grow steadily in PA6 cells-conditioned media (CNS) in the presence (A,B) and absence (C,D) of FGF20. Note that re-plating TU's (with ½ media exchange) leads to the purification of rotation culture system of contaminating cells (with PA6 cells being attached to the surface of the first (replaced) dish and dead cells washed away).

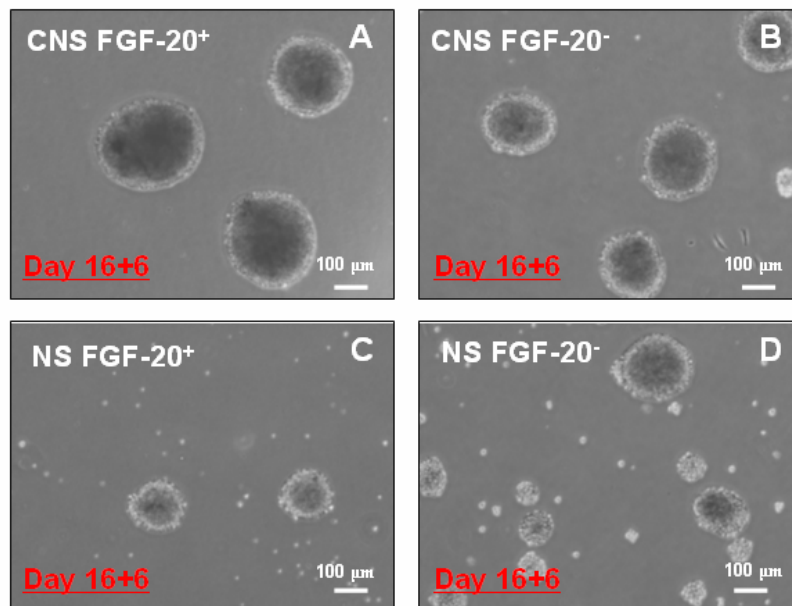


Figure 10. Stage II of the differentiation protocol, Day 16+6. During the six days of rotating culture, more homogeneous TU's are formed in PA6 cell-conditioned media (CNS, A,B); the size of TU's is substantially larger in FGF20-supplemented culture conditions (A). No clear increase of TU size/number of TU's formed in serum-free culture conditions (NS, C,D) over the same time period.

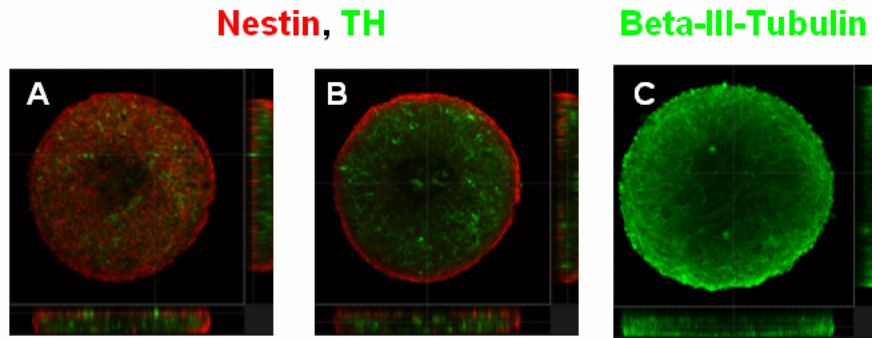


Figure 11. Cellular composition of transplant units (TU's) after 6 days of rotating culture (16+6 days total) supplemented with PA6 cell-conditioned media (CNS). Considerable numbers of cells in TU's express the neural progenitor marker nestin (A,B, red), immature neuronal marker Beta-III-tubulin (C, green) and DA-ergic neuronal marker tyrosine hydroxylase (TH; A,B, red).

After extensive characterization of TUs yielded by different protocols (i.e., with variations in the duration of Stage I and II, differentiation media, additives, etc.; Fig. 11), we have dedicated a significant effort to develop a practical technique for TU transplantation. We were able to effectively deliver the desired number of cells to the rat brain using a microsyringe with a custom-made needle. The latter was loaded with the desired number of TUs (containing an equivalent of ~100,000 cells, as estimated by dissociation and counting cell numbers of representative TUs under the microscope), and an 'air lock' concept was used to prevent untimely release of TUs from the syringe needle (Fig. 12). A set of 6-9 TUs could be efficiently delivered to the site of transplantation in a non-traumatic volume of 3.5-4 μ l of buffer.

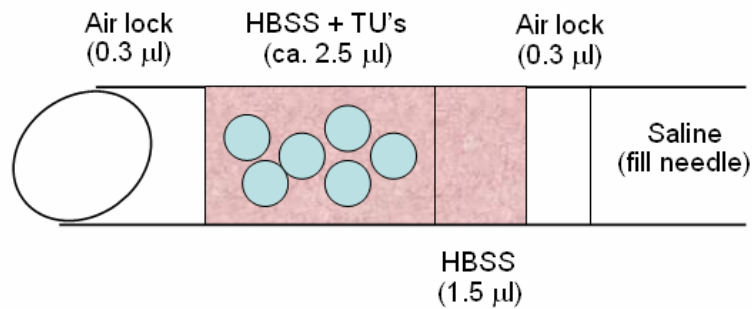


Figure 12. Schematic drawing representing loading the transplant units (TU's; count 5-6) into a needle of a Hamilton syringe (22 gauge, internal diameter = 0.41 mm) for a successful implantation. By using this method, we are able to transplant the required number of TU's and achieve grafts with over 100,000 viable cells in total. Total volume of needle load is relatively small (<5 μ l).

A total of 32 animals (6-OHDA-lesioned hemiparkinsonian rats) were transplanted in three independent sessions. By the latest session, we were fully confident in TU harvest/handling/delivery techniques; moreover, TU handling time was shortened from 3 hrs to 20 min. Extensive characterization of graft sites (following sacrificing transplanted rats 2 days – 8 weeks post-surgery) had demonstrated a notable improvement in cell survival (Figs. 13-14).

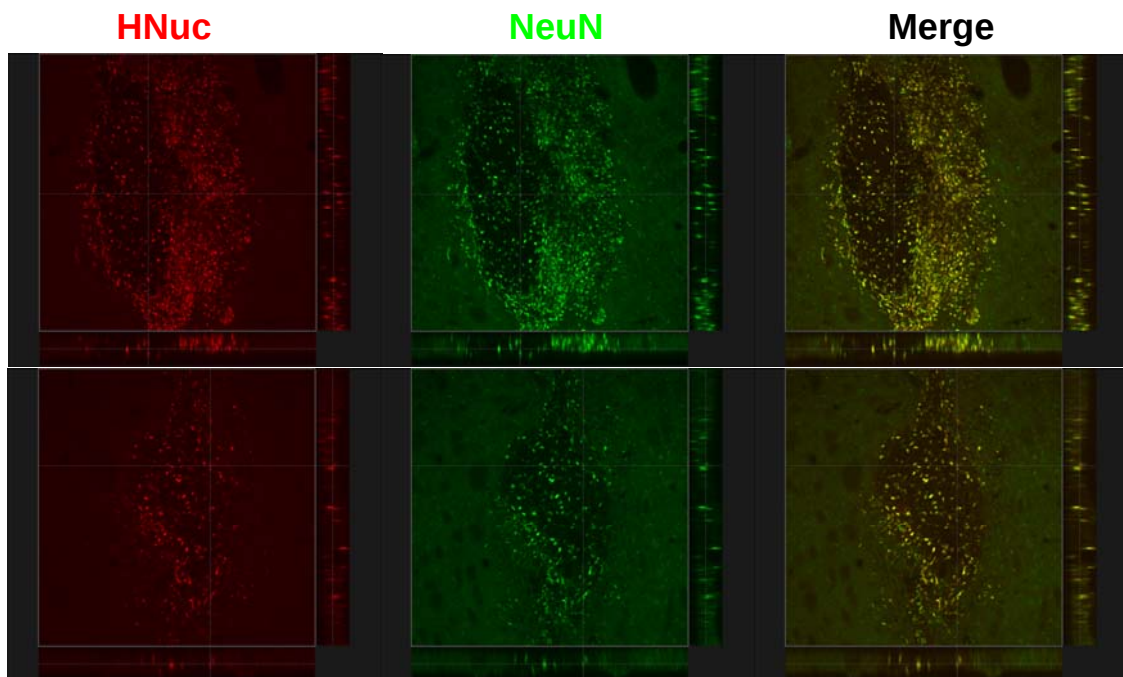


Figure 13. Two weeks following transplantation of 5-6 TU's to the rat striatum, a considerable number of grafted cells appear viable and retains a mature neuronal phenotype. HNuc, Human nuclear antigen marker (red); NeuN, neuron specific protein NeuN, a marker for mature neurons (green).

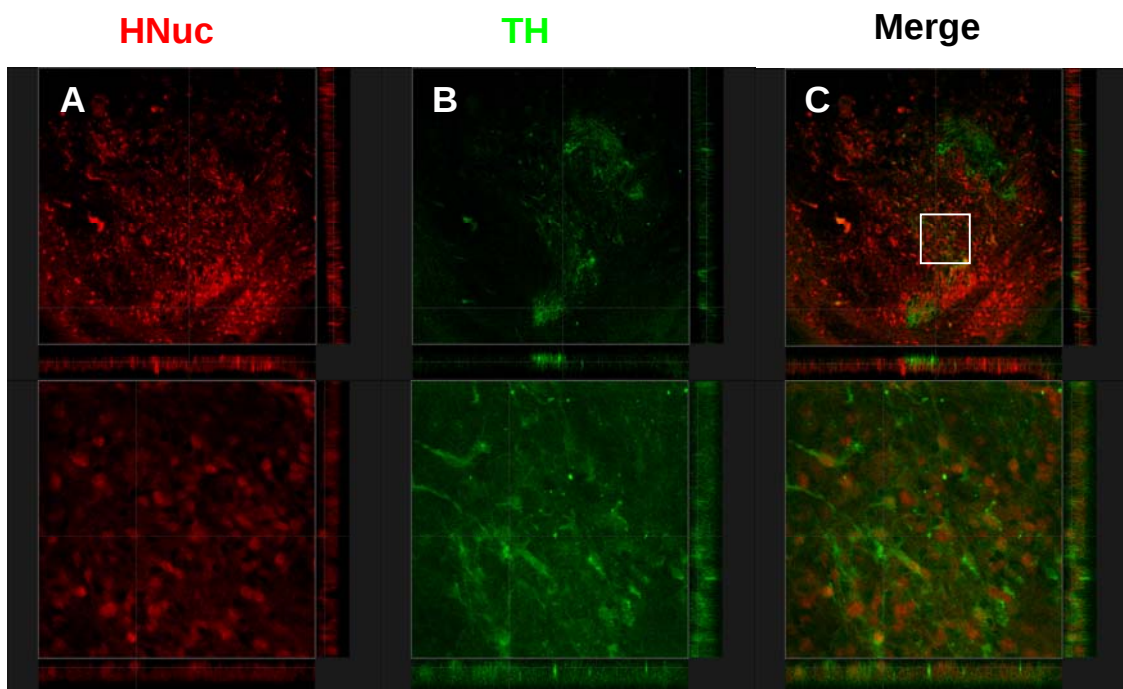


Figure 14. Eight weeks following transplantation of 5-6 TU's to rat striatum, a limited number of grafted TH⁺ cells appear viable and retains a mature neuronal phenotype. HNuc, Human nuclear antigen marker (red); TH, tyrosine hydroxylase (green).

Notably, none of the transplanted rats had developed teratoma. However, 2 of 32 transplanted rats have developed non-teratoma tumors (histologically similar to schwannomas; Fig. 15), indicating the presence of not-totipotent, yet still proliferating cells in the TUs.

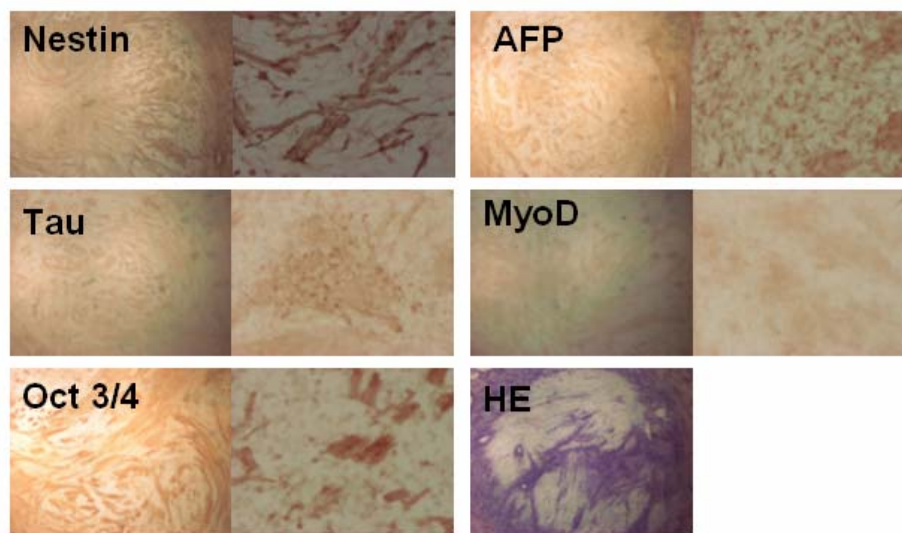


Figure 15. Immunohistological characterization of graft 8 weeks following the transplantation of TU's containing an equivalent of 100,000 cells. Non-teratoma tumor outgrowth is evident, histologically (HE staining) tumor resembles a schwannoma.

A few transplanted animals were lost due to side effects of the surgery procedure itself (wound infection) or the immunosuppression technique. Although we observed a trend of improved animal motor functions (as evaluated by periodic amphetamine-induced rotation), it was not statistically significant. It is therefore clear that despite the major progress achieved, further work is essential to 1) further improve cell survival, 2) purify TU content off mitotic cells and 3) further perfect TU handling/surgical techniques and immunosuppressing regimens. Additionally, the yield of TH-positive cells within TUs is expected to be improved by incorporating some adaptations to both Stage I and Stage II (addition of certain growth factors promoting dopaminergic differentiation). Taken together, after careful re-evaluations of the extensive experimentation performed over the course of the project, an updated protocol of hESCs *in vitro* differentiation was derived, providing an improved yield of TH⁺ neurons. The homogeneous population of TUs yielded from this protocol could be effectively handled and transplanted into the rat model of PD using the technique perfected in the previous transplantation experiments. We believe that improved cell survival will lead to more significant improvements in motor functions of the model animals, while zero risk of teratoma/non-teratoma tumors could be achieved.

Genetic characterization of hESC-derived cells on the various stages of the differentiation protocol is of great importance. Large-scale screening of the genetic profile of these cells, combined with extensive comparative analysis could provide invaluable information regarding the mechanisms of DA differentiation. To address this important goal, we have constructed and tested a unique custom microarray specifically designed for studies in the field of stem cell differentiation into neuronal/DA cells. The platform (NeuroStem Chip) includes over 1300 specific gene targets, related to the (i) “stemness”-related features of hESCs, (ii) processes of differentiation and development, and (iii) specific features of neuronal subpopulations, further supplemented with a vast number of controls (Table 1).

Table 1. Selected categories of NeuroStem chip entries.

Category	Functional role	Examples
I. Stemness	Recognized markers of stemness Candidate markers of stemness Germ cell markers Hematopoietic stem cell markers Mesenchymal stem cell marker	Oct3/4, Nanog, Tdgfl Cpxm1, Hook1, Ddx21 Rif1, Bnc1, Bnc2 Hoxb4, Cdcpl, C1qr1 Bmpr1a, Bmpr2, Cd49a
II. Proliferation	Proliferation markers Neural proliferation markers	Ki67, Pcna, Myc Emx1, Gbx2
III. Development	Differentiation Neuronal differentiation Dopaminergic differentiation Neuronal maturation Neuronal process formation Axonal elongation and branching	Lifr, Ebaf, Lyar Ren, Rai1, Neurod2 Dlx1, Dlx2, Lmx1a Mecp2, Ebf3, Sox4 Hmgb1, Rage Pi3, Map1b, Slit1
IV. Neural markers	Pan-neural markers Markers of dopaminergic neurons Markers of cholinergic neurons Markers of spinal neurons Glial markers Astrocyte markers Oligodendrocyte markers	Gap43, Nfh, Eno2 Th, Aadc, Dat Ngf Hoxc6 S100 β , Cd68 Gfap, Tapa1 Mag, Mobp, Omg
V. Distinct markers	Normal tissues Liver Pancreas Skeletal muscle Cardiac muscle Smooth muscle Endothelial cells Blood cell subtypes Cancer cells Pancreatic cancer	Gata6, G6pd, Fabp1 Tff3, Sst, Pax4 Itga7, Dmd, Tnnt3 Nkx2.5, Anf, Myhca Actg2, Cnn1, Sm22 α Flt1, Vwf, Pecam1 Cd4, Cd8, Cd19 Maspin Kras2

	Colon cancer Breast cancer Prostatic cancer Lung cancer Ovarian cancer Hodgkin's lymphoma	Mina53 Klk7 Hpn, Mat8 Rab5a, Tp63 Mgb2 Ptp4a, Atf5, p21snft
VI. Relevant groups	Apoptosis-related Telomere-related Antioxidants Imprinted genes FZD group WNT group BMP group STAT group FGF group GDF group Caspases group Cyclins group Kruppel-like group	p53, Psip1, Birc2 Tert, Terf2, Rap1 Sod1, Sod2, Gpx1 Tseb3, Gnas1, Grb10 Fzd1, Fzd3, Sfrp1 Wnt1, Wnt7a, Wisp1 Bmp1, Bmp2, Bambi Stat1, Stat3, Pias1 Fgf1, Fgf2, Fgfr4 Gdf2, Gdf3, Gdf9 Casp1, Casp2, Hsp70 Ccna1, Ccnc, Cdk1 Klf2, Klf9, Znfl84

Using this unique platform, we have performed a comprehensive characterization of the initial hESC population (SA02 line) and cells committed towards DA lineage (16 days of co-culturing with PA6 cells) (Anisimov et al., 2007). We have also used this platform to identify key factors responsible for the ability of human foreskin fibroblasts to perform their unique biological function of 'feeder cells', supporting growth and proliferation of hESCs. A limited group of gene targets was identified by straightforward analytical algorithms and was validated by the RT-PCR technique: those related to secretion processes and cell surface membrane. We believe that the most important factors of 'feeder cell' properties were pinpointed. This is important not only for basic stem cell biology, but also for practical application of hESC-based techniques. Transition to 'feeder-free' protocols would be essential in terms of decreasing the complexity of culturing protocols and eliminating the risk of contaminating harvested hESCs or hESC-derived cells with fractions of mouse (and thus xenogenic) or non-hESC human cells.

Key Research accomplishments in the project

1. Refinement of the *in vitro* differentiation protocol resulting in a balance of proliferation and differentiation in the cultures and yielding 3-D structures (Transplantable Units, TUs) suitable for transplantation without further dissociation.
2. Refinement of the technique allowing rapid handling and reliable intracerebral delivery of TUs in model animals; resulting in an improved cell survival.
3. Identification of the *substantia nigra pars compacta*-specific factor Fibroblast growth factor 20 (FGF20) as a factor affecting both differentiation and proliferation of hESC-derived DA progenitors in a dose-dependant manner.

Reportable outcomes

Presentations:

S.V. Anisimov:

S.V. Anisimov. NeuroStem custom microarray: a new tool of experimental stem cell research (invited speaker). Symposium of Lund Strategic Center for Stem Cell Biology and Cell Therapy, Lund University. Lund, Sweden. May 18, 2005.

A.S. Correia, A. Brederlau, S.V. Anisimov, G. Paul, L. Roybon, P. Eriksson, P. Brundin, J.-Y. Li. Developing human embryonic stem cell-based therapy for Parkinson's disease. Meeting of SRCSDARF-JDRF Joint Programme in Stem Cell Research. Sigtuna, Sweden. June 16-17, 2005.

A.S. Correia, A. Brederlau, S.V. Anisimov, G. Paul, L. Roybon, P. Eriksson, P. Brundin, J.-Y. Li. Developing human embryonic stem cell-based therapy for Parkinson's disease. International Society for Stem Cell Research 3rd Annual Meeting. San Francisco, CA, USA. June 23-25, 2005.

S.V. Anisimov. Application of microarray technology to experimental neurology (invited speaker). From Neuron to Neurology and Psychiatry. The Baltic Summer School. University of Copenhagen, The Panum Institute. Copenhagen, Denmark. August 14-26, 2005.

S.V. Anisimov. The search for novel sources of dopamine neurons suitable for transplantation in Parkinson's disease (replacement speaker). 2nd European Science Foundation Functional Genomics Conference "Functional Genomics and Disease". Oslo, Norway. September 6-10, 2005.

S.V. Anisimov. Gene expression in neurodegenerative diseases (invited speaker, session chairman). NSR (Nervous System and Repair) and NCoE (Nordic Center of Excellence) Joint Symposium: Disease models in vivo and in vitro. Lund University, Lund, Sweden. September 21-24, 2005.

S.V. Anisimov. Developing human embryonic stem cell-based system for cell replacement therapy in Parkinson's disease (invited speaker). Swedish Research Council NeuroFortis (Strong Research Environment in Brain Damage and Repair) Annual Meeting. Isaberg, Sweden. January 11-12, 2006.

S.V. Anisimov. Developing human embryonic stem cell-based system for cell replacement therapy in Parkinson's disease (invited speaker). General Biology of stem cell systems. EuroSTELLS Meeting. Venice, Italy. March 19-21, 2006.

P. Brundin, S.V. Anisimov. Possible cell sources for transplantation in stroke: human ES cells and adult neural stem cells (P.Brundin, S.V.Anisimov, invited speakers, joint presentation). Pre-clinical Evaluation of Stem Cell Therapy in Stroke (STEMS) kick-off meeting. Paris, France. December 4, 2006.

Abstracts:

N.S. Christophersen, S.V. Anisimov, B. Juliusson, J. Jørgensen, P. Brundin. Discovering dopaminergic differentiation-associated gene expression profile alterations in an immortalized human mesencephalic cell line using specialized custom microarray.

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Conclusions

Our own results suggest that a risk of teratoma formation inversely correlates with the duration of the *in vitro* differentiation protocol (Brederlau & Correia et al., 2006). While the yield of hESC-derived DA neurons is not a limiting factor of transplantation success anymore, the major problems associated with the actual application of hESC-based therapies for PD are associated with 1) poor survival of mature DA neurons and 2) risk of teratoma and tumor formation in the site of transplantation. The former is caused by the presence of residual undifferentiated/mitotic cells amongst the transplanted cells. We have refined the *in vitro* differentiation protocol resulting in a balance of proliferation and differentiation in the cultures and yielding 3-D structures (Transplantable Units, TUs) suitable for transplantation without further dissociation. We have also refined the technique allowing rapid handling and reliable intracerebral delivery of TUs in model animals; resulting in an improved cell survival. Finally we have identified the *substantia nigra pars compacta*-specific factor Fibroblast growth factor 20 (FGF20) as a factor affecting both differentiation and proliferation of hESC-derived DA progenitors in a dose-dependant manner.

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