Efficient expansion and dopaminergic differentiation of human fetal ventral midbrain neural stem cells by midbrain morphogens

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ABSTRACT:

Human fetal midbrain tissue grafting has provided proof-of-concept for dopamine cell replacement therapy (CRT) in Parkinson's disease (PD). However, limited tissue availability has hindered the development and widespread use of this experimental therapy. Here we present a method for generating large numbers of midbrain dopaminergic (DA) neurons based on expanding and differentiating neural stem/progenitor cells present in the human ventral midbrain (hVM) tissue. Our results show that hVM neurospheres (hVMN) with low cell numbers, unlike their rodent counterparts, expand the total number of cells 3-fold, whilst retaining their capacity to differentiate into midbrain DA neurons. Moreover, Wnt5a promoted DA differentiation of expanded cells resulting in improved morphological maturation, midbrain DA marker expression, DA release and electrophysiological properties. This method results in cell preparations that, after expansion and differentiation, can contain 6-fold more midbrain DA neurons than the starting VM preparation. Thus, our results provide evidence that by improving expansion and differentiation of progenitors present in the hVM it is possible to greatly enrich cell preparations for DA neurons. This method could substantially reduce the amount of human fetal midbrain tissue necessary for CRT in patients with PD, which could have major implications for the widespread adoption of this approach.

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by resting tremor, rigidity and slowness of movement (hypokinesia). The motor features of PD are due in part to the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which project to the striatum and are involved in motor control. One promising therapeutic approach is cell-replacement therapy (CRT), in which DA neurons and/or precursors are grafted into the striatum in order to restore the lost nigrostriatal DA neurotransmission. Several cell types have been considered as potential sources of DA neurons, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells which, due to their capacity to expand and differentiate, can generate large numbers of DA neurons and induce behavioral improvement in animal models of PD (Barberi, et al., 2003, Hargus, et al., 2010, Kawasaki, et al., 2000, Kim, et al., 2002, Kirks et al., 2012; Lee, et al., 2000, Sanchez-Pernaute et al., 2008; Wernig, et al., 2008). However, the lack of purity of the differentiated cultures, the risk of excessive proliferation and teratoma formation, as well as the poor survival of human ES cell–derived DA cells after transplantation in animal models have so far prevented their use in clinical trials (Roy, et al., 2006, Sonntag, et al., 2007). In the past, human fetal ventral midbrain (VM) tissue has been used as a cell source for CRT, as these cells are correctly specified *in vivo*, pose no risk of tumor/teratoma formation and have been used in clinical trials to provide proof-of-principle that DA neurons derived from fetal human VM tissue can survive and offer significant benefits in patients (Kordower, et al., 1995, Mendez, et al., 2005, Piccini, et al., 1999, Politis et al 2010). However, several issues such as tissue availability, quality, standardization and the need for a high number of fetuses (6 to 7) to treat one PD patient make this strategy impractical and unlikely to become a competitive therapeutic option (Bjorklund, et al., 2003, Freed, et al., 2001,

Olanow, et al., 2003, Winkler, et al., 2005). Previous studies have shown that human ventral midbrain-derived cells can be propagated with mitogens (Hovakimyan, et al., 2006, Jin, et al., 2005, Maciaczyk, et al., 2008, Milosevic, et al., 2006, Sanchez-Pernaute, et al., 2001, Storch, et al., 2001, Wang, et al., 2004). In these studies cells were typically expanded in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor 2 (bFGF) (Hovakimyan, et al., 2006, Sanchez-Pernaute, et al., 2001, Storch, et al., 2001) and differentiated by removal of mitogens and addition of neurotrophins such as brain derived neurotrophic factor (BDNF) (Maciaczyk, et al., 2008) and glial cell-line derived neurotrophic factor (GDNF) (Jin, et al., 2005, Storch, et al., 2001), ascorbic acid, cyclic adenosine monophosphate (cAMP) (Sanchez-Pernaute, et al., 2001) or cytokines (Jin, et al., 2005, Storch, et al., 2001). Immortalized human mesencephalic cell lines have also been established (Donato, et al., 2007, Lotharius, et al., 2002, Villa, et al., 2009), however DA neurons could only be generated and maintained after stable overexpression of Bcl-X_L, an anti-apoptotic gene (Courtois, et al., 2010). Surprisingly, none of the studies up to date have examined the use of region-specific developmentally appropriate morphogens for the expansion and differentiation of hVM cells.

Morphogens are secreted factors that form gradients and play a prominent role in development by providing regional and cellular identity to cells in the neural tube. The most important morphogens for midbrain development are Sonic Hedhehog (Shh, a ventralizing signal), Fibroblast growth factor 8 (Fgf8, that provides the midbrain-hindbrain identity), and Wnt5a (which regulates cell orientation and differentiation, reviewed in Inestrosa and Arenas, 2010). Interestingly, we have previously shown that mouse and rat VM neural stem cells can be propagated as neurospheres (VMN) and differentiated in the presence of Shh, FGF8 and Wnt5a. In these conditions, VMNs gave

rise to a large number of functional DA neurons that were able to survive, integrate and induce functional recovery after transplantation in animal models of Parkinson's disease (Parish, et al., 2008). However, much less is known about the capacity of human endogenous VM progenitors to respond to morphogens and give rise to human DA neurons *in vitro*. Here, we examined whether VM morphogens, such as Shh, FGF8 and Wnt5a, can be applied to human VM neural stem cells, in order to improve the yield of DA neurons derived from human fetal midbrain tissue. Our results show that hVMN can be cultured as spheres and that the total number of cells can be expanded 3 fold over a 2-3 week period. Furthermore these VMN-expanded cells maintain the same DA differentiation potential after each passage and addition of Wnt5a increases the number of DA neurons per field by 3.3-fold, compared to controls. These increases together (total cells and TH cell density) resulted in 6-fold increase in the $\%TH^+$ cells/Hoechst and total TH^+ cells obtained from one VM. Moreover, morphogen treatment maintained or increased the expression of typical midbrain markers such as TH, Nurr1, Foxa2, Lmx1a and Lmx1b, compared to non-expanded hVM tissue. In particular, we found that Wnt5a increased the morphological and molecular differentiation of VMN cells into midbrain DA neurons, as well as their functionality, as assessed by DA release and electrophysiological properties. In summary, our results show that morphogen-treated human neural stem/progenitor cells gives rise to a 6-fold increase in the number of DA neurons compared to the starting VM preparation. Hence, the use of developmentally relevant midbrain factors for the expansion and differentiation of hVM cells may significantly reduce the need for multiple donor fetuses for grafting individual PD patients, thereby increasing the feasibility and accessibility of this technology in the future development of CRT for Parkinson's disease.

MATERIALS AND METHODS

hVM tissue

Human fetal ventral mesencephalic tissue was collected and experiments performed at the Centre for Brain Repair (University of Cambridge), Freiburg University Medical Center and Wallenberg Neuroscience Center (Lund University). All fetuses were collected from routine termination of pregnancies under full ethical approval in line with the United Kingdom's Department of Health guidelines and local ethical approval (Local Research Ethics Committee, reference nr. 96/085), the local ethical committee of the University of Freiburg meeting the regulations of the German law (research project nr. 96/03) and the Swedish National Board of Health and Welfare (Socialstyrelsen, Dnr. 23 11667/2008 and Dnr. 23 2981/2009).

hVMN cultures

The VM was dissected from fetuses at post-conception (PC) week 6-11. VM tissues were dissociated in serum-free N2 medium supplemented with BDNF (30 ng/ml, R&D) using collagenase/dispase (700 μg/ml; Roche) with agitation on an orbital mixer incubator (80 rpm, 30 minutes) followed by mechanical dissociation by gently pipetting through 200 μl pipettes or flame-narrowed Pasteur pipettes. For proliferation, 2/3 of the cells from the dissociated tissue were plated at a final density of 150,000 cells/cm^2 on non-coated or non-adherent tissue culture plates in serum-free N2 medium (1:1 mixture of F12 and MEM, 15 mM HEPES buffer, 1 mM glutamine, 6 mg/ml glucose (Sigma-Aldrich), 3 mg/ml Albumax, and N2 supplement, all purchased from Invitrogen) supplemented with Shh (100 ng/ml), FGF8 (100ng/ml), BDNF (30ng/ml) and bFGF

(20ng/ml), all purchased from R&D. These cultures were referred to as passage 0 (P0). For differentiation, 1/3 of the cells were plated onto poly-ornithine (15 μg/ml, Sigma-Aldrich) and laminin-coated 48 well plates (10 μg/ml, Trevigen) and left to differentiate for 4-14 days in N2 medium supplemented with BDNF (20 ng/ml, R&D), GDNF (10 ng/ml, R&D), TGF3β (1 ng/ml, R&D), ascorbic acid (2mM, Sigma-Aldrich) and dibutyryl cAMP (1mM, Sigma-Aldrich) and Wnt5a (100 ng/ml, R&D) or the equivalent volume of 0.1% bovine serum albumin (BSA) as a control for Wnt5a treatment. These cultures were referred to as differentiation 0 (D0). Cells in proliferation grew as spheres and were supplemented with fresh medium every 3 days. After 7 days of proliferation, half of the spheres were passaged using collagenase/dispase and half were plated for differentiation using the same conditions as described above; these cultures were referred to as passage 1 (P1) and differentiation 1 (D1), respectively. After 14 days of proliferation, the remaining spheres (referred to as passage 2, P2) were plated for differentiation (referred to as differentiation 2, D2).

Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde for 20 minutes, washed three times with PBS and pre-incubated for 1 hour in blocking solution (PBS, 0.25% Triton-X 100 and 5% normal goat serum) followed by incubation at 4°C overnight with one or more of the following primary antibodies diluted in blocking solution: rabbit anti-tyrosine hydroxylase (TH, 1:300, PelFreeze), mouse anti-βIII tubulin (Tuj1;1:1000, Promega, Abcam), mouse anti-TH (1:200 Immunostar), Goat anti-Lmx1a (1:200 Santa Cruz), Goat anti-Nurr1 (1:200, R&D). After washing, cultures were incubated for 1 hour at room temperature with the appropriate fluorophore conjugated secondary antibodies

(1:700, AlexaFluor 555, 647 and 488). All cultures were counterstained with Hoechst 33258 for 10 minutes (1:1000, Invitrogen) to enable visualization of all nuclei.

RT-PCR analysis

Cells were lysed and total RNA was extracted using the RNeasy Mini Kit (Qiagen), 1 μg was treated with RQ1 RNase-free DNase (Promega, Madison, WI) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random primers (Invitrogen) (RT^+ reaction). Parallel reactions without the reverse transcriptase enzyme were done as a control (RT^- reaction). PCR conditions and Sybr green real-time quantitative PCR assays were performed as previously described (Rawal, et al., 2006). Expression levels were obtained by normalization with the value of the housekeeping gene encoding 18S rRNA (Ambion, Austin, USA) obtained for every sample in parallel assays. Fold changes were determined using the delta-delta CT method. The primer sequences were as follows:

h18S (forward): GCCTTTGCCATCACTGCCATT,

h18S (reverse): GCCAGTGGTCTTGGTGTGCT;

hSOX2 (forward): GGGAAATGGGAGGGGTGCAAAAGAGG,

hSOX2 (reverse): TTGCGTGAGTGTGGATGGGATTGGTG;

hNESTIN (forward): CAGGAGCGGCTGCGGGCTACTGAAAAGTTCC,

hNESTIN (reverse): CAGGGCTGAGGGGTGGTGCCAAGGAGG;

hGFAP (forward): CTCAAGAGGAACATCGTGGTAAAG,

hGFAP (reverse): TCCTGCTTCGAGTCCTTAATGAC;

hβIII-TUBULIN (forward): GCCTCTTCTCACAAGTACGTGCCTCG,

hβIII-TUBULIN (reverse): GGGGCGAAGCCGGGCATGAACAAGTGCAG;

hLMX1B (forward): GGCACGAGGAGTGTTTGCAGT,

hLMX1B (reverse): GTTTGCAGTACAGTTTCCGATCC;

hLMX1A (forward): CAGGGAAAGGAACTGCTGAG,

hLMX1A (reverse): CTGGTTTTGGAACCACACCT;

hFOXA2 (forward): GACTCCAGCCTCCTCAACTG,

hFOXA2 (reverse): CGAGGAGGACATGAGGTTGT;

hTH (forward): GAGGGGAAGGCCGTGCTAAA,

hTH (reverse): GAGGCGCACGAAGTACTCCA;

hNURR1 (forward): CAGCTCCGATTTCTTAACTCCAG;

hNURR1 (reverse): GGTGAGGTCCATGCTAAACTTGA.

Cell Counts and Statistical Analyses

The relationship between variables (initial number of cells, cell numbers at P2 and embryo age) was determined by linear regression. For TH^+cells/field, TH^+ cells were counted in 8-10 random fields covered by $Tuj1^+$ cells in three 48-well plate wells per condition (differentiation round, D0-D2 and/or Wnt5a treatment). Counts were done using a 25x magnification eye piece with an eye grid and a 20x objective. Each field

was determined by the eye grid, which corresponded to an area of 0,39 mm^2 at 20x magnification. Cell numbers were averaged for each condition and compared using a Student's t-test. The $\%TH^+$ cells/Hoechst was calculated using the number of TH^+ cells/field and the number of $Hoechst^+$ cells/field at D0, counted as described above. Changes in mRNA levels were compared using a one-way ANOVA and a Bonferroni's Multiple Comparison Post Test. Analysis of the effects of time (D0 vs D2) and treatment (control vs Wnt5a) was performed using a two-way ANOVA and Bonferroni's Multiple Comparison Post Test. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. $p<0.05$ was considered as a statistically significant difference (*), $p<0.01$ (**), $p<0.001$ (***). Results in the text and graphs are presented as mean ± standard error of the mean (s.e.m).

DA release measurements by HPLC

DA release was measured in D0 and D2 cultures differentiated for 2 weeks in the absence or presence of Wnt5a. Cells were plated on a 12well plate and incubated in 200µl of N2 medium supplemented with 56mM KCl for 30min. The media were then collected and stabilized with 20µl of 1 M perchloric acid ($HClO_4$) containing 0.05% sodium metabisulphate ($Na_2S_2O_5$) and 0.01% disodium EDTA. Samples were stored at −70°C and on the day of analysis, they were centrifuged at 10000 rpm for 10min and filtered though minispin filters for additional 3min at 10000 rpm before being injected into the HPLC. For each sample, 20µl was injected into the HPLC, which consisted of a LC-20AT pump (Shimadzu), SIL-20A Autosampler (Shimadzu) and C18 reverse phase column (Bio-Rad, Hercules, USA). Detection was via a 3mm VT-03 flow cell with

glassy carbon working electrode (Antec Leyden) and Decade II Electrochemical Detector (Antec Leyden). The mobile phase consisted of 17% v/v methanol in purified deionized water containing 70mM KH2PO4, 0.5mM EDTA and 8.0mM sulfonic acid, pH 3.0 and was run at a flow rate of 0.5ml/min. Peaks were processed by the Azur chromatographic software. Concentrations of DA were calculated for each sample.

Electrophysiological characterization of differentiated VMN cultures

Cells were cultured as described above and allowed to differentiate for 14 days on PDL/laminin coated glass coverslips. Whole cell current-clamp recordings were performed in D2 cultures differentiated for 2 weeks in the absence or presence of Wnt5a (100ng/ml). Recordings were performed using micropipettes that were pulled from flame-polished borosilicate glass to resistances of 4-7 MΩ. The pipette internal solution contained (in mM) 135 K-gluconate, 4 KCl, 2 NaCl, 10 HEPES, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, and 14 phosphocreatine-Tris (pH 7.25, 280 mOsm). Intracellular potentials were not corrected for liquid junction potentials. Recordings were conducted at room temperature. A series of hyperpolarizing and depolarizing step currents were injected to measure the intrinsic properties of each neuron. Synaptic activity (excitatory post-synaptic current; EPSC) was examined at resting membrane potentials following these depolarizing steps. Data was collected using the HEKA Elektronik EPC-10, lowpass filtered at 5 kHz and sampled at 20 kHz. Patchmaster software (HEKA; Lambrecht, Germany) was used for data acquisition, and analysis was performed using IgorPro software (Wavemetrics; Oregon, USA).

RESULTS

hVMN can be expanded in vitro

We have previously shown that mouse VM neural stem cells/progenitors can be propagated and differentiated into DA neurons in the presence of VM morphogens. However, the capacity of rodent VM neural stem cells/progenitors to generate midbrain DA neurons has also been shown to decrease with passaging (Parish, et al., 2008). Moreover, the ability of VM neural stem cells/progenitors to generate increased numbers of DA neurons in response to morphogen exposure during expansion and differentiation has not been examined.

We first set out to examine whether human VM stem/progenitor cells could be propagated in the presence of FGF2 and typical midbrain developmental signals, such as Shh and FGF8. In our protocol, cells obtained from week 6-9 fetuses were induced to proliferate, serially passaged over a two week period, and differentiated following each passage in order to assess their potential to give rise to DA neurons (Figure 1A). One of our first observations was that expansion of cells derived from the VM was very consistent for every tissue (from one well to another), but quite variable from one tissue sample to another (Figure 1B). In particular we found that large pieces of VM tissue gave rise to a large number of cells (>1.3 x 10^6, about 2 million cells) after dissociation of the starting material, but did not propagate and died after Passage 1 (red lines in Figure 1B, C). Tissue preparations containing about 1 million cells (0.7-1.3 x 10^6 cells, grey lines in Figure 1B, C) propagated and reached passage 2 in three out of seven cases, but total cell numbers did not increase compared to the starting preparation (Figure 1C). Finally, in cultures where the dissection of the VM was restrictive and initial cell counts were low (0.2-0.7 x 10^6 cells), all samples expanded and a 3-fold

increase in cell numbers was detected after 2 weeks (Figure 1B,C, black lines, n=6). Linear regression analysis revealed that the number of cells after the second period of proliferation significantly correlated with the initial cell numbers (Figure 1D), but importantly not with the age of the embryo from which the tissue was dissected (Figure 1E). These results show that smaller pieces of tissue with low cell numbers expand better and suggest that restricted dissections of the VM might be advantageous in order to obtain an optimal proliferation of week 6-9 hVM stem/progenitor cells.

Another interesting observation was that the number of cells did not decrease in all of the cultures after one week of proliferation, at passage1 (Figure 1B), as in rodent cultures (Parish et al., 2008). The loss of cells was more apparent in cultures with medium and high initial cell numbers, despite all cell preparations being plated at the same cell density. This may be explained in part by reduced access to nutrients by progenitors in larger pieces of tissue before dissociation, to longer dissociation time and/or to the presence of adjacent cell populations in larger VM preparations, which may either have a deleterious or inhibitory effects on the expansion of VM progenitors. While cultures with high initial number of cells were in continuous decline, or where not able to survive longer than one week in culture, cultures with low initial cell numbers expanded very efficiently after passage 1 (Figure 1B). Thus our findings suggest that a restrictive dissection of the VM, mild tissue dissociation and low initial cell numbers (about 500.000 cells), are preferable for the optimal expansion of human VM tissue from 6-9 weeks old donors.

hVMN retain their DA differentiation potential and capacity to respond to Wnt5a after passaging and expansion.

To determine the DA differentiation potential of the propagated cultures, cells were plated onto poly-ornithine/laminin-coated plates and treated for four days with survival and differentiation-inducing factors, including BDNF, GDNF, TGFβ3, ascorbic acid and dibutyryl cAMP. Primary VM tissue-derived cells readily differentiated and gave rise to cells that were immunoreactive for the pan-neuronal marker Tuj1 and for the rate limiting enzyme in the synthesis of dopamine, tyrosine hydroxylase (TH). Differentiation of the original VM starting material (D0) revealed that 65% of the cells in the culture were $TuJ1^+$ and 6% TH^+ (Figure 2A, Ctrl). TH^+ cells exhibited typical DA morphology with processes and varicose-like structures. We then examined the effects of Wnt5a on DA differentiation. We used a concentration of 100ng/ml of Wnt5a, which we had previously found (Schulte et al, 2005, Bryja et al, 2007) to maximally activate Wnt/PCP signaling while avoiding the toxic effects of Chaps, the detergent and vehicle used to solubilize Wnt5a. However, addition of Wnt5a did not result in any significant increase in the proportion of TH^+ cells nor in the number of cells per field (Figure 2A and 2D). When passage 1 hVMN cells (expanded for 1 week) were differentiated for 4 days (D1), using the factors described above, abundant expression of Tuj1 and a high number of TH^+ neurons was detected (Figure 2B). Most notably, when the number of TH^+ cells/field at D1 was compared to that at D0, a 3-fold increase was detected (Figure 2D), but Wnt5a did not show any significant effect compared to control. Finally, when hVMN cells were passaged twice (expanded for 14 days) and differentiated for 4 days (D2), we found an increase in the number of both $TuJ1^+$ and TH^+ neurons (Figure 2C). This result indicated that cells expanded for 2 weeks retain their capacity to generate neurons and more specifically the capacity to differentiate into DA neurons. At this stage, treatment with Wnt5a significantly increased the number of TH^+ neurons per field 3.3-fold compared to control (from 10% to 33%, Figure 2D).

To further confirm that cells expanded for 14 days are able to give rise to correctly specified VM DA neurons, we analyzed the expression of several midbrain markers in P2 and D2 cultures and compared them to their expression in primary hVM tissue by qPCR (Figure 3). Interestingly, P2 cultures did not show any significant difference in the expression levels of *TH*, *NURR1, FOXA2, LMX1A* and *LMX1B* compared to primary VM tissue, indicating that the expansion of hVM tissue for 2 weeks in the presence of Shh, FGF8 and FGF2 did not significantly alter gene expression (Figure 3 and Supplemental Figure1). However, when P2 cultures were differentiated for 4 days in the presence of survival factors and Wnt5a (D2), the levels of *TH* and *NURR1* increased 70- and 10-fold, respectively, compared to hVM tissue (Figure 3A and B). While the levels of expression of *FOXA2*, *LMX1A* and *LMX1B* showed trends towards an increase, no significant difference was detected between D2 and VM tissue (Figure 3C-E). A significant increase in *TH* and *NURR1* was also detected at D2 compared to P2, indicating that our protocol allowed efficient differentiation of expanded cultures. In sum, our results indicate that expanded hVMN cultures do not lose their midbrain DA identity and that upon differentiation they increase or maintain the expression of midbrain DA phenotypic markers.

Finally, we demonstrated that VM spheres could be passaged and maintained in a proliferative state beyond two weeks. However, we found that expansion for 3 passages (21 days) in the present culture conditions did not further increase, but rather decreased, the number of TH^+ cells in the cultures (data not shown). When P3 spheres were differentiated (D3), they expressed differentiation markers such as *TUJ1* and *GFAP*, and DA-specific markers such as *LMX1B*, *NURR1* and *TH* (Supplemental Figure 2). These results indicate that despite the lower TH^+ cell number, expanded hVMN cultures

do not lose their regional identity or their capacity to differentiate into midbrain DA neurons.

In summary, the data presented here indicates that, in the present culture conditions, the overall number of midbrain DA neurons in hVM cultures propagated as neurospheres does not increase beyond passage 2. Moreover, differentiation at this stage (D2) increased, or at least maintained, the expression of all phenotypic ventral midbrain markers examined, compared to hVM tissue. Thus, our experiments indicate that a high number of midbrain DA neurons can be obtained in 18 days, after expansion of hVM tissue for 2 weeks followed by differentiation for 4 days.

The number of human dopaminergic neurons increases after expansion, passage and differentiation.

Our results indicate that hVM tissue can be expanded and differentiated to increase the yield of midbrain TH^+ DA neurons per human fetal midbrain. This result is in clear contrast with our previous results showing a reduction in the number of TH^+ cells after passaging and differentiation of rodent VM tissue (Parish, et al., 2008). Our present findings indicate that human progenitors, unlike their rodent counterparts, have a greater capacity to expand and differentiate into DA neurons. We therefore examined the extent to which hVM tissue can be expanded and contribute to an increase in the number of TH^+ cells when differentiated in the presence of Wnt5a. We found that compared to cells directly differentiated (D0), the differentiation of hVM cells that underwent two rounds of expansion (D2) increased not only the number of TH^+ cells/field (3-fold in Wnt5a treated cells, Figure 4A,B) but also the percentage of TH^+ cells in the cell preparation (%TH/Hoechst) 6-fold (Figure 4C). These results indicate that it is possible

to increase the total number of TH^+ midbrain DA neurons derived from one human VM tissue preparation through expansion and differentiation and that Wnt5a plays a key role during differentiation.

Extended differentiation in Wnt5a improves morphological differentiation, marker expression and functional properties of midbrain DA neurons derived from hVMN.

We next examined whether the cells produced by the method described above continue developing normally and become functional after differentiation for two weeks. We first examined control- or Wnt5a-treated D0, D1 and D2 cultures for morphological maturation, as assessed by the presence of elaborated dendritic trees and axons. Interestingly, features of morphological maturation improved in cells differentiated after passaging, with D2 cultures being more mature than D1, which in turn were more differentiated than D0. In addition of this, Wnt5a treated cultures contained more TH^+ cells that exhibited even more elaborate morphologies and longer processes (Figure 5A). Combined, thee results indicated that extended cultivation and passaging in the present conditions is not deleterious, but rather beneficial for DA differentiation and survival. In order to verify the maturity of DA neurons produced at D2, we examined the expression of transcription factors critical for midbrain DA neuron development. In the absence of Wnt5a we found immature progenitors ($Lmx1a^+$; $Nurr1^-$; TH^- cells), DA neuroblasts ($Lmx1a^+$; $Nurr1^+$ TH^- cells) and midbrain DA neurons ($Lmx1a^+$; $Nurr1^+$; TH^+ cells). Treatment with Wnt5a decreased the number of immature progenitors and increased the two populations of postmitotic cells, DA neuroblasts ($Lmx1a^+$; $Nurr1^+$ cells) and DA neurons (TH^+; $Nurr1^+$; $Lmx1a^+$ cells) (Figure 5B). Thus, our results indicate that Wnt5a increases the number of DA neurons in the cultures by enhancing

the differentiation of VM DA progenitors into postmitotic neuroblasts and midbrain DA neurons.

In order to assess neuronal functionality, we first examined DA release in D0 and D2 cultures differentiated in the absence or the presence of Wnt5a for two weeks. We first found that DA release was below detection in D0 cultures differentiated without Wnt5a, but it was readily detected in D2 cultures treated with Wnt5a. Although there was a trend for increased DA release with extended cultivation (D0 vs D2 cultures), we only found a significant difference between control and Wnt5a treated cultures, indicating that exposure to Wnt5a improves DA release (Figure 5C). These results also show that extensive culturing does not impair the functionality of DA neurons, as assessed by DA release. We next examined the electrophysiological maturity of neurons in the culture by performing whole-cell patch clamp recordings, targeting cells with a neuronal morphology. Both control and Wnt5a treated neurons exhibited a hyperpolarized resting membrane potential, which was slightly lower and less variable in treated cells (-44.7 ±2.8 mV; n=6) compared to control cells (-40.8 ±6.1 mV; n=5). In both conditions, neurons exhibited spikelets and clear sub-threshold oscillations in response to depolarization (Figure 5D; top trace). Spikelets were larger in amplitude for Wnt5a treated cells compared to control cells (Figure 5E; p=0.084). Additionally, we could observe neurons in Wnt5a treated cultures firing multiple spikes with more mature phenotypes (Fig 5D; lower trace), which was never seen in control conditions. We analyzed the recurrent network activity in the cultures by quantifying the frequency of EPSCs on the recorded cells at resting membrane potential. Interestingly we found a marked increase of excitatory input onto the Wnt5a treated cells (Fig 5F; p=0.004). In sum, these results indicate that Wnt5a treated cultures are more mature and active than control cultures differentiated in the absence of Wnt5a.

In summary, our results show that expansion of human VM tissue for 2 weeks followed by differentiation in the presence or absence of Wnt5a produces cultures containing large numbers of DA neurons. Moreover, we found that Wnt5a treatment during differentiation resulted in cultures exhibiting improved neuronal morphological differentiation (greater maturity of dendritic arbors and axons) with appropriate electrophysiological properties; increased differentiation of progenitors into postmitotic neuroblasts, increased number of midbrain DA neurons and increased DA release.

DISCUSSION

Human fetal ventral midbrain (hVM) is a well known but a limited source of DA neurons for CRT in Parkinson's disease. We now show that is possible to expand hVM progenitors as neurospheres (hVMN) for several passages and that an optimal 3-fold expansion of hVMN cells can be achieved after 2 passages and culture for 2 weeks in proliferating and VM patterning conditions. We also found that the combination of 2 weeks of expansion and 4 day differentiation of hVMN in the presence of Wnt5a leads to a 3.3-fold increase in DA cell density (TH^+ cells/area), compared to unexpanded cells differentiated for 4 days without Wnt5a. Consistent with an increase in TH cell density (x3.3) and the total number of cells (x3), we found that expansion and differentiation of 1 human VM produced a 6-fold increase in the total number of TH^+ cells, compared to cells directly differentiated for 4 days in the absence of Wnt5a. This result is clearly different from that previously obtained with rodent VMN, which did not expand (Parish et al., 2009). The different behavior of hVMN compared to rodent VMN might reflect species differences either in the intrinsic properties of VM progenitors or in their extrinsic factor requirements. We conclude that hVMN, unlike rodent VMN cultures,

can significantly expand and increase the total number of TH^+ cells obtained from 1 VM, and as such is an attractive source of TH^+ cells.

Our results show that the use of appropriate midbrain-specific cell extrinsic factors, including morphogens, mitogens and growth factors that control midbrain DA progenitor identity, expansion and differentiation, improves the efficiency of the generation of midbrain DA neurons derived from hVM tissue. Our study differs from previous reports in that we expanded DA progenitors in the presence of a combination of factors used in midbrain specification and DA differentiation of embryonic stem cells (Shh, FGF8 and FGF2), instead of FGF2 and EGF, the classical forebrain neurosphere culture conditions. We hypothesize that by providing region-specific instructive signals to proliferating VM progenitors, these cells retain their capacity to continue generating midbrain DA neurons. Finally, our study differs from previous studies in the use of Wnt5a for the efficient differentiation of human DA precursors into TH^+ midbrain DA neurons. Interestingly, clear effects of Wnt5a were only detected upon differentiation after the second passage, when cell preparations were more homogeneous. Our results also indicate that cell preparations differentiated after two passages yield the greatest number of TH^+ cells that can be obtained from one VM tissue. This increase in the number of midbrain DA neurons per hVM is of such a magnitude that it could allow for a significant reduction in the amount of donor VM tissue required to treat PD patients if this was to taken forward to the clinic. Moreover, since extended differentiation of such cultures for two weeks in the presence of Wnt5a resulted in cell reparations that acquired remarkable morphological maturity, expression of specific midbrain markers, improved electrophysiological properties and enhanced DA release, our results suggest that these cells could be good candidates for transplantation. The number of DA neurons necessary to elicit a functional recovery in PD patients has been estimated to be

around 100,000 cells (Kordower et al., 1995). We hereby show that our method increases an initial population of about 30,000 TH^+ cells (6% TH^+ cells of 500,000 cells in a human VM) by 6-fold, a number 1.8 times greater than the number of DA neurons present in the human adult substantia nigra (100,000 cells in the SNpc, Damier et al, 1999). Thus, our procedure might allow for the complete grafting of one side of the PD patient's brain using tissue from only one single donor. Clearly, several variables need to be defined and further refinements are needed before cells obtained by this method can be used clinically in such a capacity. We know, for instance, that Wnt5a promotes DA neuritogenesis (Blakely et al., 2011, current observations), which may compromise the transplantability of the DA neurons. Future studies should thus focus on identifying how long Wnt5a exposure should last (to avoid excessive outgrowth) as well as on testing the capacity of hVMN derived-DA neurons to survive and functionally engraft in animal models of PD.

In conclusion, hVM tissue grown in the presence of developmentally relevant factors, such as Shh, FGF8 and Wnt5a, offers an efficient *in vitro* DA cell preparation protocol that may find an application in future CRT for PD patients.

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FIGURE LEGENDS

Figure 1: ***hVMN expansion in vitro.*** (A) Experimental design. Human VM fetal tissue was dissociated into a single-cell suspension, referred to as starting material, and subsequently cultured to promote proliferation (P0) or differentiation (D0). After one week, expanding cultures formed spheres. Half the VMN were passaged (P1) and the remainder plated for differentiation (D1). P1 cells were induced to proliferate for a further 7 days (P2) and plated for final differentiation (D2). Proliferation was performed in the presence of Shh, FGF8, FGF2 and BDNF, and differentiation in the presence of ascorbic acid, BDNF, GDNF, TGFβ, db-cAMP and Wnt5a. (B) Graph representing the number of cells in the starting material and at passage 1 and 2. Each line depicts an independent VM culture. Expansion of large pieces of hVM issue, which contained a high initial number of cells (red lines), and some of the preparations with medium cell numbers (grey lines) yielded no surviving cells at passage 2 (no value represented at P2). Surprisingly, restricted dissections gave rise to starting preparations with low cell numbers (approximately 200,000 to 700,000 cells, black lines) that expanded remarkably well and lead to a 3 fold increase in cell numbers after the second period of proliferation (day 14, 1,500.000 cells). (C) Individual cultures were grouped according to the yield after tissue dissociation into low (around 500,000 cells, black lines), medium (around 1,000.000 cells, grey lines) or high (around 2,000.000 cells, red lines). In preparations with low cells numbers there was a 3-fold increase in cell numbers after 2 weeks in proliferation conditions, whilst medium and high preparations did not expand well. **Low**: N= 6, SM- 0.462 ± 0.070; P1- 1.023 ± 0.2830; P2- 1.429 ± 0.156. **Medium**: N = 7, SM- 1.024 ± 0.055; P1- 0.670 ± 0.295; P2- 0.301± 0.208. **High**: N = 2, SM- 2.115 ± 0.115; P1- 0.1725 ± 0.072; P2- 0). (C) Linear regression analysis confirmed that the expansion of cells derived from the VM was highly dependent on the

numbers of cells after dissociation (r^2 = 0.4127, N = 15, p = 0.0098**), but not on the age of the embryo (D) (r^2 = 0.020, N = 15, p = 0.770).

Figure 2: ***Dopaminergic differentiation potential of hVMN following serial passaging.*** (A) A large number of Tuj1 and TH^+ cells were found at D0. Wnt5a treatment did not promote a significant increase in the numbers of TH^+ cells counted per field (TH/Field- **Ctrl:** 10.23 ± 3.276, **Wnt5a:** 10.64 ± 1.869, N=9). (B) After 7 days of proliferation, differentiated neurospheres contained a high number of Tuj1 and TH^+ cells, but did not appear to be significantly influenced by the presence of Wnt5a (**Ctrl:** 21.78± 13.77, **Wnt5a:** 31.45 ± 18.43 N=4). (C) After 2 weeks of expansion, high numbers of $TuJ1^+$ and TH^+ neurons were still observed. (D) At this stage, Wnt5a treatment significantly increased the number of TH^+ cells per field (**Ctrl:** 17.03 ± 7.715; **Wnt5a:** 33.40 ± 8.664, N= 6, p= 0.0108* paired t-test). Scale bars = 50 μm for left and middle column micrographs; 25 μm for pictures in right hand column.

Figure 3: ***Figure 3: Expression of VM DA markers in expanding and differentiating human ventral midbrain cultures.*** (A-E) qPCR analysis revealed that the hVM derived cells retained or increased the expression of VM-specific markers after passaging (P2) and differentiation (D2), compared to starting material. Statistical analysis was performed using a one-way ANOVA and Bonferroni's post hoc test. *p<0.05, **p<0.01; n=3-4 independent cultures per condition**.**

Figure 4: ***Propagation and differentiation of human VM progenitors expands the number of midbrain TH$^+$ DA neurons 6-fold.*** (A) The number of TH$^+$ cells in the starting cell preparation, differentiated in the presence of Wnt5a (D0), was compared with cells propagated for two weeks and differentiated in the presence of Wnt5a. (B) A 3-fold increase was detected in the number of TH$^+$ cells/field (**D0:** 10.64 ± 1.869 N=9; **D2:** 33.67 ± 7.328 N=7, p= 0.0042**, unpaired t-test). Scale bars = 50 μm for 20x pictures, 25 μm for 20x pictures. (C) The percentage of TH$^+$ cells out of the total (% TH$^+$/Hoechst$^+$ cells) increased by 6-fold when control D0 and Wnt5a D2 were compared (* p<0.05 by two-way ANOVA and Tukey's post hoc test), indicating that our expansion and differentiation protocol effectively increases the number of TH$^+$ cells that can be obtained from 1 human VM tissue.

Figure 5: ***Extended differentiation and Wnt5a treatment improve morphological differentiation, marker expression and the functional properties of hVMN-derived DA neurons.*** (A) Morphological differentiation improved with passaging as assessed by elaborated morphologies and longer processes in D2 and D1 cultures when compared to D0. Differentiation was further increased by Wnt5a treatment. (B) D2 cultures contained DA immature DA progenitors (Lmx1a$^+$; Nurr1$^-$; TH$^-$ cells), DA neuroblasts (Lmx1a$^+$; Nurr1$^+$; TH$^-$ cells) and DA neurons (Lmx1a$^+$; Nurr1$^+$; TH$^+$ cells). Wnt5a treatment enhanced differentiation by increasing the number of DA neuroblasts and DA neurons and decreasing the number of immature DA progenitors. (C) Extensive culturing does not impair the functionality of the DA neurons, as assessed by DA release, which was further enhanced by Wnt5a treatment (Ctrl vs Wnt5a: U=4.00, p=0.0373*, Mann Whitney U test, one-tailed). (D) D2 neurons exhibit

electrophysiological properties of mature neurons such as a hyperpolarized resting membrane potential, spikelets and clear sub-threshold oscillations in response to depolarization. Wnt5a-treated cultures were more mature and active, as assessed by (E) larger spikelet amplitudes and (F) increased frequency of EPSCs ($p=0.004^{**}$).

Figure 1

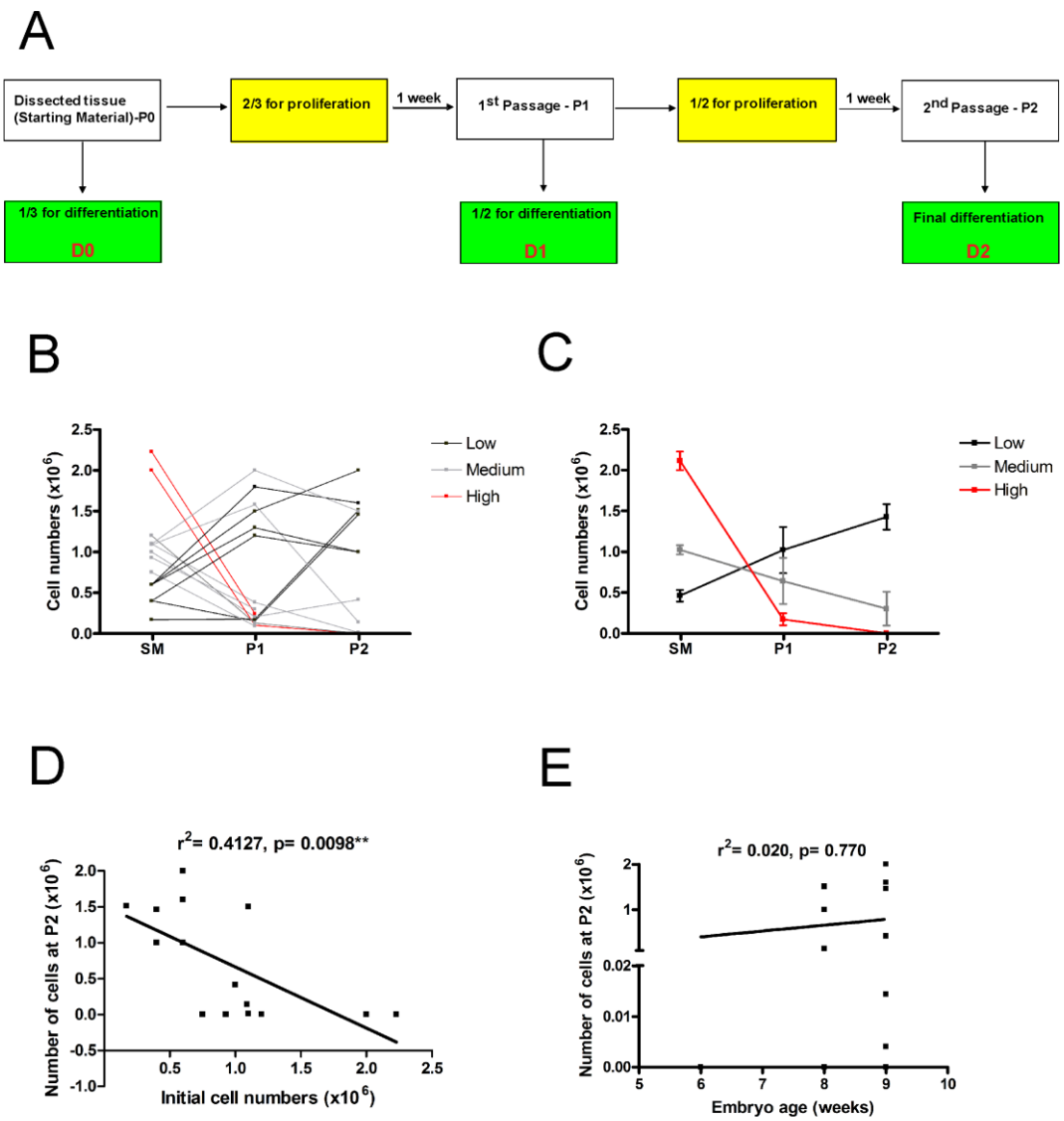


Figure 2

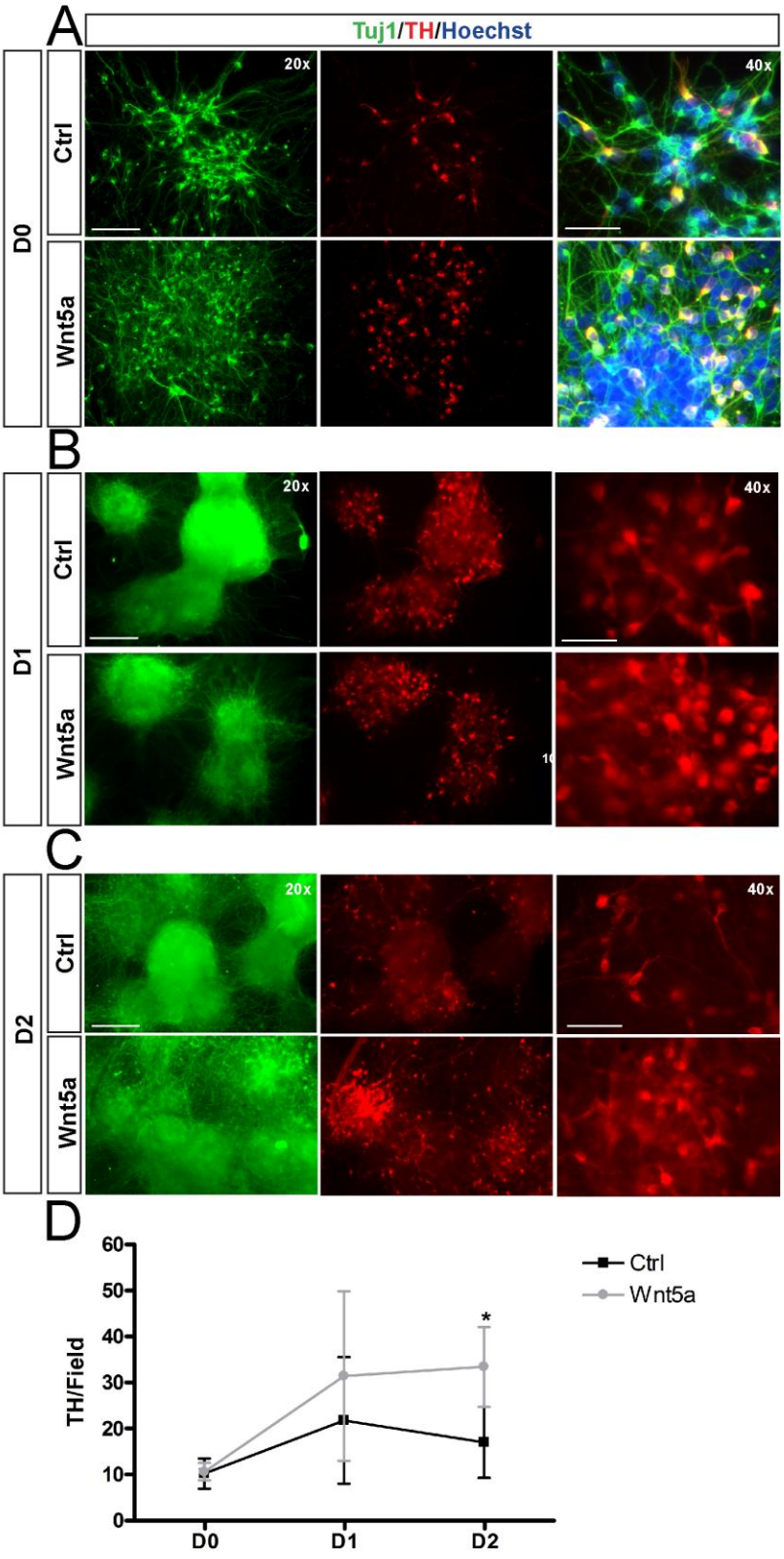


Figure 3

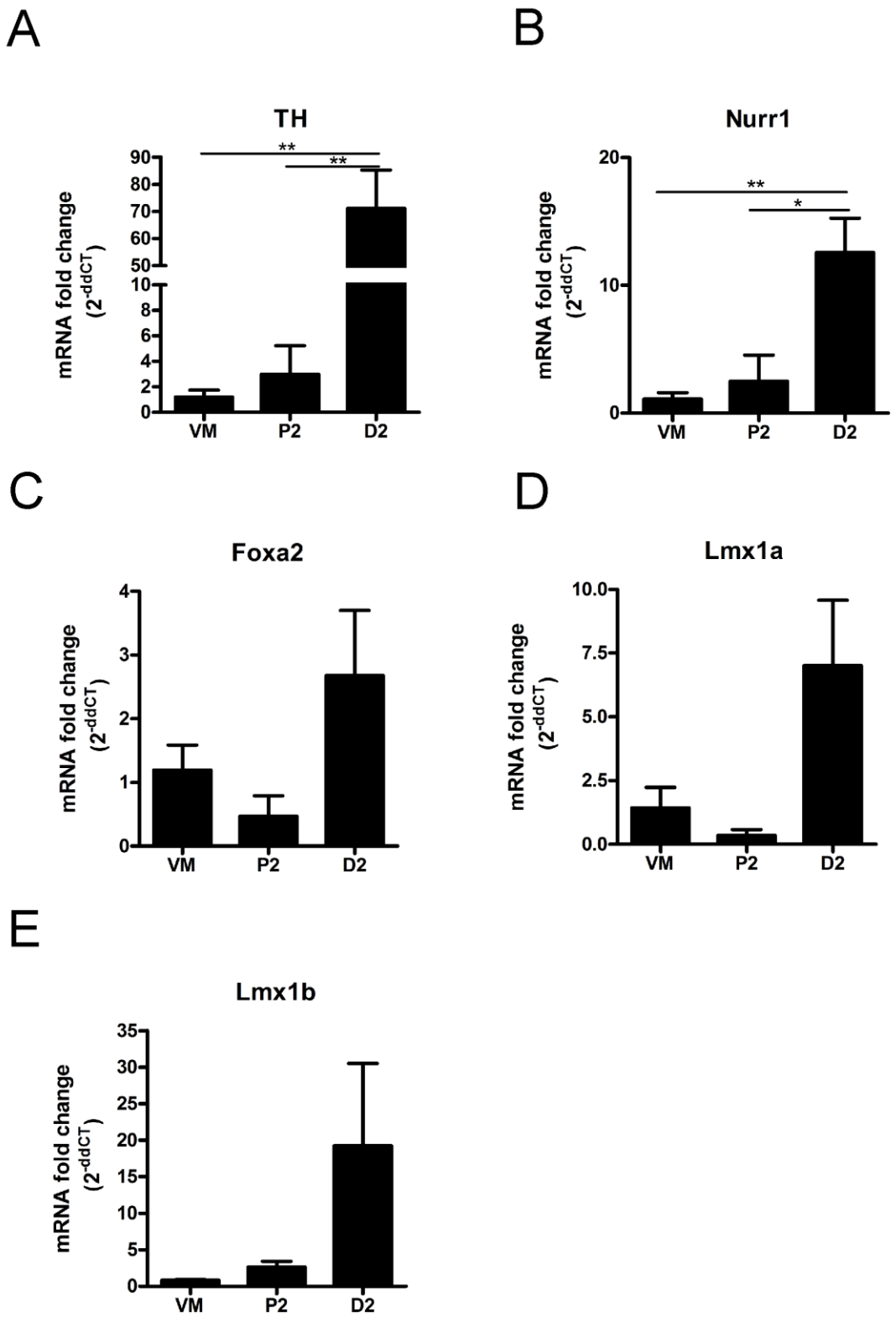


Figure 4

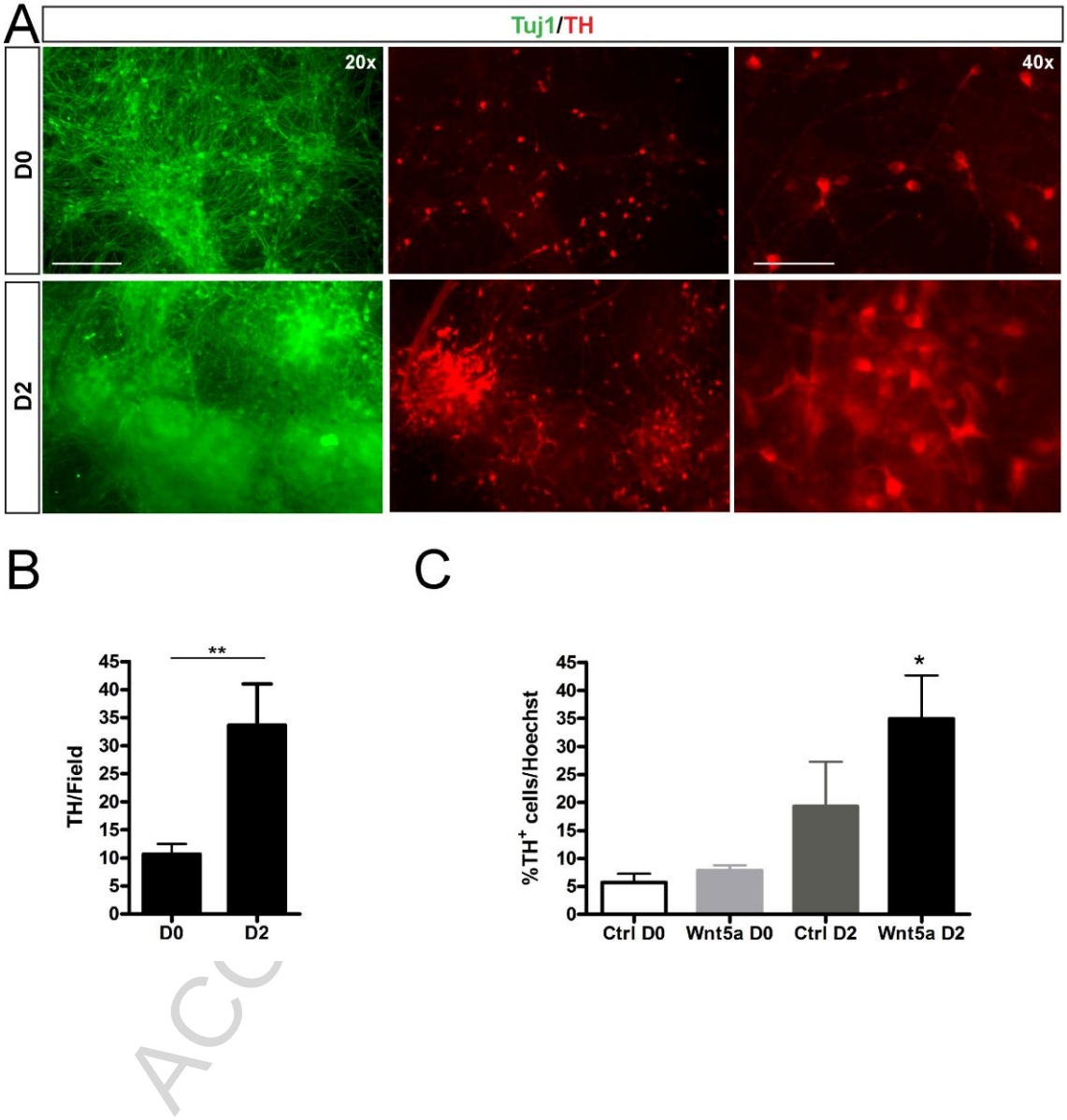
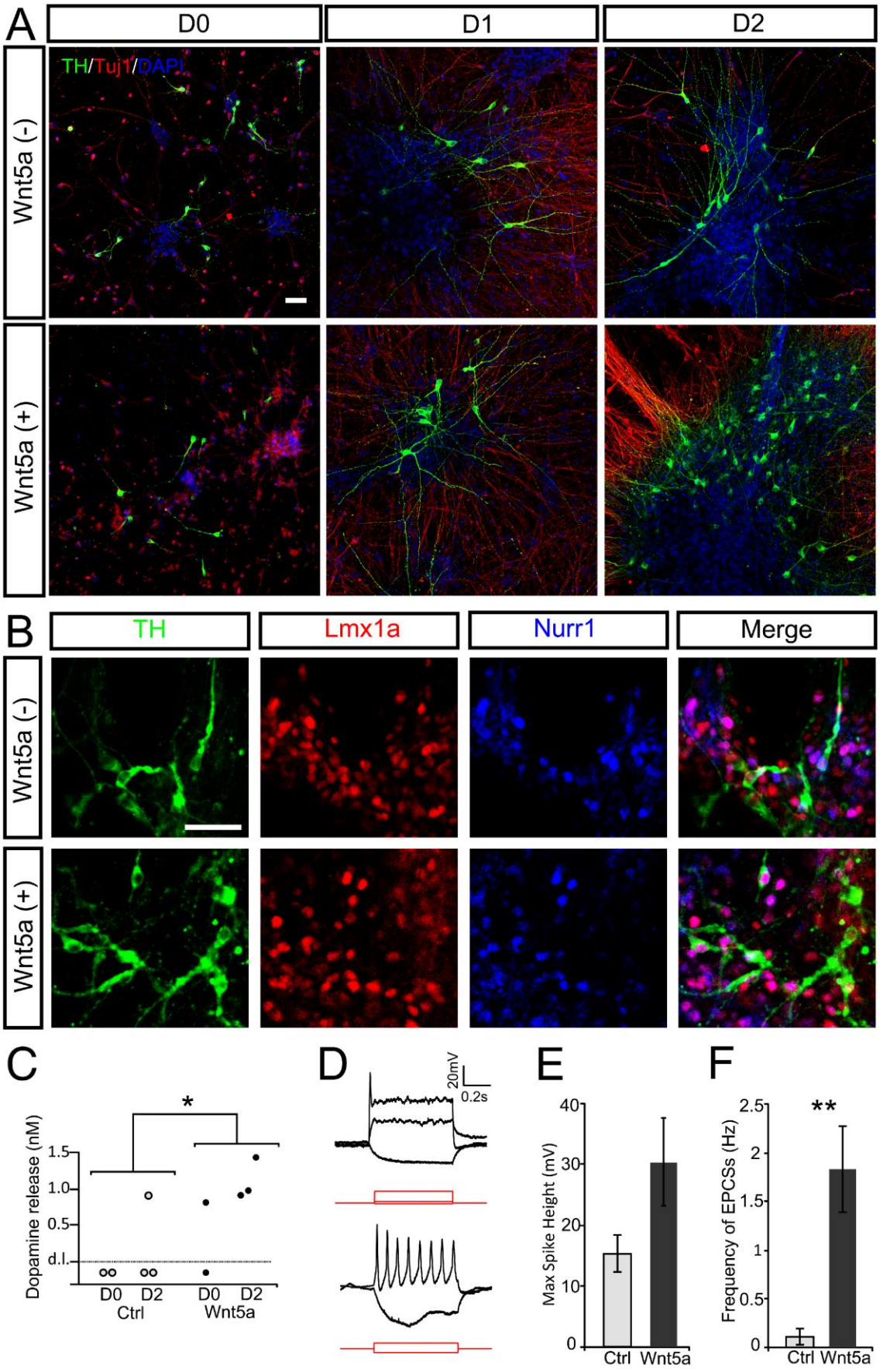


Figure 5



HIGHLIGHTS

- We present a method to increase the yield of human midbrain dopaminergic neurons
- Human ventral midbrain neural stem/progenitor cells can be expanded 3 fold
- Expanded cells retain their capacity to differentiate into dopaminergic neurons
- Wnt5a enhances the differentiation of human ventral midbrain progenitors into mature dopaminergic neurons
- After expansion and differentiation, cell preparations can contain 6-fold more midbrain dopaminergic neurons than the starting ventral midbrain preparation

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