Human midbrain precursors activate the expected developmental genetic program and differentiate long-term to functional A9 dopamine neurons \textit{in vitro}. 
Enhancement by Bcl-X\textsubscript{L}

Emma G. Seiz, Milagros Ramos-Gómez, Elise T. Courtois, Jan Tønnesen, Merab Kokaia, Isabel Liste Noya, Alberto Martínez-Serrano

\textbf{ABSTRACT}

Understanding the molecular programs of the generation of human dopaminergic neurons (DAN) from their ventral mesencephalic (VM) precursors is of key importance for basic studies, progress in cell therapy, drug screening and pharmacology in the context of Parkinson's disease. The nature of human DAN precursors in \textit{vitro} is poorly understood, their properties unstable, and their availability highly limited. Here we present positive evidence that human VM precursors retaining their genuine properties and long-term capacity to generate A9 type \textit{Substantia nigra} human DAN (hVM1 model cell line) can be propagated in culture. During a one month differentiation, these cells activate all key genes needed to progress from pro-neural and pro-dopaminergic precursors to mature and functional DAN. For the first time, we demonstrate that gene cascades are correctly activated during differentiation, resulting in the generation of mature DAN. These DAN have morphological and functional properties indistinguishable from those generated by VM primary neuronal cultures. In addition, we have found that the forced expression of Bcl-X\textsubscript{L} induces an increase in the expression of key developmental genes (MSX1,}

Abbreviations: A9-DAN, (dopaminergic neuron from the A9 group); 6-OHDA, (6-hydroxydopamine); DA, (dopamine); DAN, (dopaminergic neuron); FB, (forebrain); FP, (floor plate); hESC, (human Embryonic stem cells); hNSC, (human neural stem cells); hVM, (human ventral mesencephalon); SNpc, (\textit{substantia nigra pars compacta}); TH, (tyrosine hydroxylase); VM, (ventral mesencephalon); VM DAN, (dopaminergic neuron from ventral mesencephalon); VM hNSC, (human neural stem cells from ventral mesencephalon)


Introduction

Dopaminergic neurons (DAn) of the ventral mesencephalon (VM) play essential roles in reward-based behaviors and control of voluntary movement, and the progressive loss of DAn from the A9 group (A9-DAn) of the Substantia nigra pars compacta (SNpc) causes motor impairments characteristic of Parkinson's disease (PD)[1,2]. Much effort has been done to identify the precursors of VM DAn and to understand the genetic cues and cascades controlling the development of VM DAn in experimental animals and humans. Such knowledge has the potential to improve the capacity to generate or engineer correctly differentiated human A9-DAn in vitro from their stem/precursor cells, and use them for basic studies, pharmacological drug screening and the development of cell replacement therapies [3-8].

VM DAn originate from precursors residing in the mesencephalic floor plate (FP), both in rodents and humans, which show features of radial glia [9-12], reviewed by [6,13-24]. In spite of the importance of identifying a well-characterized and reliable source of human A9-DAn, the present situation is that all neural stem/precursor cultures explored so far present numerous limitations. In addition, there is a large controversy on the actual capacity of various cultures derived from the fetal human VM to generate A9-DAn, in a stable manner [8,25-29]. In parallel to the work with non-immortal precursor cells, several groups, including ours, have recently developed immortalized cell lines of human neural stem/precursor cells (hNSCs), some of them derived from the fetal human VM (MESCl.10, NGC-407, ReNcell VM, vm c-mycER™ and hVMl) [30-34]. In these model cell lines, the non-transforming, immortalizing gene, v-myc overcomes the problem of limited expansion. The knowledge on the properties of the precursors being expanded in these cell lines, the preservation of their regional specification, and their capacity to activate correct genetic developmental programs, is very limited and uncertain, so far. In the present work, and using the hVMl cell line as a model system, we have mainly aimed at defining in detail the molecular properties of, and the developmental steps taken by VM hNSCs when differentiating to A9-DAn (one month, long-term differentiation). This constitutes an essential step to fully understand the nature of the cells, and the means to proliferate them and preserve their neurogenic capacity.

In addition, we have investigated the potential effects that the forced expression of Bcl-XL may have on the properties of hVMl cells and on their differentiation. The capacity of hNSCs and of human embryonic stem cells (hESCs) to generate DAn displaying the correct A9 phenotype is enhanced by Bcl-XL [35-37]. Although a survival effect was attributed to Bcl-XL in both systems, the enhanced expression of transcription factors related to DAn development has been investigated in the present work, to better understand Bcl-XL actions in VM hNSCs (hVMl cells). Such knowledge will increase our understanding of the instability of VM precursor cells' properties in vitro, expand on the non-antiapoptotic functions of Bcl-XL and help to replace its use with other interventions that would not pose safety hurdles, since blocking apoptosis could drive the cells closer to uncontrolled growth.

Materials and methods

Cell culture

VM hNSCs isolation, immortalization, culture, Bcl-XL forced expression and ethics permissions were previously described [34,36]. Briefly, the hVMl cell line was generated from VM tissue of a 10 weeks post-conception old human fetus by v-myc immortalization [34]. Low passage number hVMl cells were further modified to over-express Bcl-XL [36]. The polyclonal cell lines naïve hVMl and the ones with the forced expression of Bcl-XL are referred here as control or Bcl-XL hVMl cells. Detailed cell culture methods are provided under Supplementary information. Experiments were carried out using hVMl cells at passage 9 to 12, and Bcl-XL cells at passages 25-30, in order to work with cells with similar neurogenic potential (see the Results section). Mouse primary neuron cultures were obtained from VM of E12-E14 mice, following procedure detailed in [38].

RNA extraction and Q-RT-PCR

Total RNA was isolated from proliferating or differentiated cells using high pure RNA isolation kit (Roche, Basel, Switzerland). The cDNA was synthesized from 1 μg of RNA using the high capacity cDNA Achieved kit (applied biosystems, Carlsbad, CA). Relative quantification of RNA expression was performed by TaqMan real time PCR using commercial probes described in Supplementary information, and was performed using the ABI PRISM 7900 HT Sequence Detection System as detailed in [36]. Calibrator samples to study the temporal expression profile of each gene in each cell line were day 0 (d0) samples. To study differences in the expression of a given gene between control and Bcl-XL cells, hVMl at d0 was taken as the calibrator sample and results are shown as the ratio hVMl Bcl-XL/hVMl at d0 (d0) samples. To study differences in the expression of a given gene between control and Bcl-XL cells, hVMl at d0 was taken as the calibrator sample and results are shown as the ratio hVMl Bcl-XL/hVMl. The relative expression of a gene with respect to that of TH was defined as (gene of interest/TH RQ). All Q-RT-PCR data were obtained from biological triplicates, each run three times (technical triplicates).

Immunocytochemistry (ICC) and western blotting (WB)

Samples were processed following standard protocols [34,36] and using antibodies described in Supplementary information.

Morphometric analysis

Neurite’s morphology of TH+ cells in cultures differentiated for 12-30 day was analyzed as described [39]. A minimum of 50 TH+
cells was photographed in randomly chosen fields for each sample and analyzed using NeuroN/Idage (NIH, USA). The cell and processes were semi-automatically traced and assigned the status of primary, secondary, tertiary or quaternary neurites.

Electrophysiology

Functional properties of differentiated hVM1 Bcl-X

Functional properties of differentiated hVM1 Bcl-X<sub>L</sub> cells were evaluated by the whole-cell patch-clamp technique as described previously [40]. In short, cover slips with attached differentiated cells were transferred to a recording chamber continuously perfused at a rate of 3 ml/min, at room temperature, with carbogenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) artificial cerebrospinal fluid (aCSF). Resting membrane potential (RMP) was measured in current-clamp mode at 0 pA. Input resistance (Rs) was assessed from 5 mV square voltage pulses at RMP. For current/voltage (I/V) relationship curves, we measured the membrane potential, while injecting 500 ms positive or negative currents stepwise. In some experiments, tetrodotoxin (TTX, 2 μM) was added to the perfusion medium to confirm that action potentials were sodium channel-dependent. All data are presented as mean±standard error of the mean (SEM). Analyses for temporal changes were done by linear regression tests. Level of significance was set at p<0.05.

Activity assays of dopamine transporter (DAT)

Dopamine reuptake

Thirty-days differentiated control or Bcl-X<sub>L</sub> hVM1 cells and mouse VM primary neuronal cultures (E12, C57/BL6 mice, maintained for 1 week) were used to measure dopamine transport. Nomifensine (NMF, 10 μM, Sigma), a DAT inhibitor, was used to block unspecific transport. Cells (plus/minus NMF) were incubated with 50 nM [3H]-DA (di-hydroxy-phenylethylamine 3.4–(ring-2,5,6-3H), 30–60 Ci/mmol, PerkinElmer, Waltham, MS) for 30 min at 37°C. The reaction was stopped by removing the medium and washing the samples twice with PBS. Cells were lysed with 0.5 M NaOH and 0.2 ml scintillation cocktail was added per well.

Live cells imaging with 4-di-2-ASP

ASP<sup>+</sup> (4-(diethylamino) stilil)-N-methylpyrimidine iodide) is a fluorescent analog of l-methyl-4-phenyl pyrimidine, a well-studied neurotoxin transported by monoamine transporters. NMF was used to prevent binding to DAT in control samples. Cells were incubated with 0.5 μM ASP<sup>+</sup> (Invitrogen, Carlsbad, CA) for 15 min at 37°C, and extensively washed with modified Tyrodes’ solution. Trypan Blue (30 μM, Sigma) was added to the medium to reduce autofluorescence. Live cell images were collected using a confocal LSM510 inverted microscope coupled to an Axiovert200M (Zeiss) optical microscope. Fluorescence images (488 nm excitation, 620 nm emission) were mounted off-line onto the corresponding bright field images.

Expression of proneural genes at early differentiation times

In FB hNSCs mitogen withdrawal triggers differentiation, and thus the percentage of nestin and Ki-67 positive cells decreases to about 20% by d7 [42]. In hVM1 cells, differentiation is accompanied by the disappearance of nestin<sup>+</sup> cells (NSCs), and the exit of the majority of the cells in culture from the cell cycle (becoming Ki-67 negative) (Supplementary information Fig. 2). In hVM1 cells, differentiation was increased in Bcl-X<sub>L</sub> cells in comparison to control ones by 30% at d7 and 100% (2-fold) at d30 of differentiation (Fig. 1C). The cholinergic (ChAT<sup>+</sup>) phenotype was not detected throughout the experiment and serotonergic (5-HT<sup>+</sup>) neurons were only occasionally found (less than 0.01%). As expected for VM precursors, the cultures generated 4.05±0.56 GABA<sup>+</sup> and 1.94±0.26 Glutamate<sup>+</sup> neurons.

Results

Forebrain and VM hNSCs preserve their different regional information

Preservation of the regional properties in long term-cultured hNSCs is not granted. Therefore, the expression levels of two very early neuroectoderm markers (PAX6, EN1) and of SNpc genes (TH, DAT, GIRQ2) were studied in FB and VM hNSCs (human neural stem cells) (Fig. 1A). PAX6, a determinant of neuroectoderm fate, was expressed at higher levels in FB than in VM cells. EN1, a transcription factor characteristic of the midbrain neurepithelium, was expressed at two orders of magnitude higher levels in hVM1 than in hNS1 cells. In parallel, TH and DAT expression was substantially higher in hVM1 cells, and, most importantly, GIRQ2 expression, typical of A9 DA neurons, was differentially expressed at much higher levels in VM than in FB cells. hVM1 cells also expressed OTX2 and SOX2 (Supplementary information Fig. 1).

We have reported that DA<sub>N</sub> generation from VM hNSCs decreases with passaging, and that neurogenesis is enhanced and stabilized after forced expression of Bcl-X<sub>L</sub> [35,36,41,42]. Since in the present study we wanted to work with hNSCs producing neurons (β-III-tubulin<sup>+</sup>) at the same rate, to focus on the dopaminergic phenotype, we chose to work with different passage numbers of control and Bcl-X<sub>L</sub> hVM1 cells (9–12 and 25–30, respectively), generating in each case approximately 20% neurons (β-III-tub<sup>+</sup>, Fig. 1B and C). In the same cultures, the rate of TH<sup>+</sup> neuron generation was increased in Bcl-X<sub>L</sub> cells in comparison to control ones by 30% at d7 and 100% (2-fold) at d30 of differentiation (Fig. 1C). The cholinergic (ChAT<sup>+</sup>) phenotype was not detected throughout the experiment and serotonergic (5-HT<sup>+</sup>) neurons were only occasionally found (less than 0.01%). As expected for VM precursors, the cultures generated 4.05±0.56 GABA<sup>+</sup> and 1.94±0.26 Glutamate<sup>+</sup> neurons.

Expression of proneural genes at early differentiation times

In FB hNSCs mitogen withdrawal triggers differentiation, and thus the percentage of nestin and Ki-67 positive cells decreases to about 20% by d7 [42]. In hVM1 cells, differentiation is accompanied by the disappearance of nestin<sup>+</sup> cells (NSCs), and the exit of the majority of the cells in culture from the cell cycle (becoming Ki-67 negative) (Supplementary information Fig. 2). Bcl-X<sub>L</sub> did not induce any significant change at any time point or marker studied, but for a trend for a faster disappearance of nestin<sup>+</sup> cells.

Simultaneously, during the first week of differentiation, the human homologues of three proneural genes known to have
Expression of pro-dopaminergic transcription factor genes at early differentiation times

EN1, LMX1B, NURR1 and PITX3 are needed for the correct development of VM A9-DAn [6,17], and were studied in parallel to TH (tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis; serving here as a reporter of the presence of DAn).

A) Temporal expression (data from each cell type compared to levels at d0): All studied genes, but PITX3, showed a similar time course of expression between the control and Bcl-XL cells (Fig. 3A). EN1 expression remained nearly constant during the one week of differentiation, as expected, whereas LMX1B, NURR1 and TH progressively increased their expression levels in both cell types, consistent with the earlier expression of proneural genes (Fig. 2). Note that the expression levels of LMX1B, NURR1 and PITX3 by d4 are only different from those at d0 in the Bcl-Xl cells. The case of PITX3 was interesting since the control cells expressed it, but did not up-regulate the gene, even by d7 (and even later, see below). This aspect could be important to explain a lower yield of DAn in the control cultures, when compared to Bcl-XI ones.

B) Net Bcl-Xl effects on gene expression levels: When the expression data were compared at each day being Bcl-Xl the only variable (Fig. 3B) we found a number of interesting
differences between control and Bcl-XL cells. First, as expected from the increased number of TH+ neurons in Bcl-XL cells at d7 (Fig. 1C), we observed a ~4-fold increase in TH mRNA levels induced by Bcl-XL. This was accompanied and possibly explained by the increased levels of all other genes in Bcl-XL cells: EN1 (~1.5-fold by d0), LMX1B (~5-fold by d7), NURR1 (~2-fold by d7) and particularly PITX3 (~10-fold enhancement by d4, just the time when its expression is

needed, to cooperate in the generation of DAn). Note that by d4 of differentiation TH+ neurons are starting to appear in the cultures (~1–2% in the case of control hVM1 cells [34], Fig. 1C).

DAα generated from hVM1 hNSCs produce and release DA after depolarization [34–36], both in the presence and absence of elevated Bcl-XL levels. For a TH+ neuron to synthesize DA, at least two other enzymes are needed, aromatic aminoacid de-carboxylase (AADC), and GTP-Cyclohydrolase I (GTPCH, needed for the synthesis of the TH cofactor tetra-hydro-biopterin (BH4)). As shown in Supplementary information Fig. 3 both genes are expressed in control and Bcl-XL cells. Their expression levels increased during the first week of differentiation in control hVM1 cells, whereas AADC increased and GTPCH remained nearly constant for Bcl-XL cells (Supplementary information Fig. 3A). When the expression data for these two genes were referred to those of TH (Supplementary information Fig. 3B) we found that their expression levels were significantly higher in Bcl-XL cells than compared to control hVM1 ones, suggestive of an increased dopaminergic phenotype of the latter (for a given TH level). The expression of Dopamine-ß-hydroxylase (DBH), which converts DA further into noradrenaline and adrenaline, present in TH+ adrenergic neurons, was not detected by Q-PCR even after 40 cycles of amplification.

**Long term differentiation and expression of maturation, function-related genes**

As shown in Fig. 1, the number of neurons and TH+ neurons does not increase with differentiation time beyond d12 of differentiation. To

---

**Fig. 2** - Expression of proneuronal genes during early differentiation times. (A) Temporal gene expression profile of MSX1 in each cell line determined by Q-RT-PCR (see legend of Supp. Info. Fig. 1 for detailed calculations). Results are mean ± SEM (n=3) (*p<0.05, **p<0.001 vs d0; t-test). (B) Bcl-XL effects on relative MSX1 mRNA levels expressed as ratio hVM1 Bcl-XL/hVM1 at each studied day (see legend of Supp. Info. Fig. 1 for detailed calculations). Results are mean ± SEM (n=3) (*p<0.05, indicates significant differences to 1 (equal values); one sample t-test).

---

**Fig. 3** - Expression of pro-dopaminergic genes during early differentiation times. (A) Relative mRNA temporal expression profile of EN1, LMX1b, NURR1, PITX3 and TH during one week of differentiation. Results are mean ± SEM (n=3) (***p<0.01, ****p<0.001 vs hVM1 d0; $\overline{p}$<0.01, $\overline{p}$<0.001 vs hVM1 Bcl-XL d0; +p<0.05, ++p<0.001 d4 vs d7; t-test). (B) Bcl-XL effects on relative mRNA levels of the studied genes expressed as ratio hVM1 Bcl-XL/hVM1 at day 0, 4 and 7 of differentiation. Results are mean ± SEM (n=3) (*p<0.05, **p<0.01, indicate that the mean differ significantly from 1 (equal values, 95% confidence interval); one sample t-test).
cultures, an enhancement of TH protein or cells in the case of Bcl-X
L TH+ neurons (Fig. 1C). For DAT, VMAT2, DRD2 and GIRK2, the only increased 2-fold (Fig. 1C).

This points to a long differentiation time required by TH+ neurons to become functionally active (as proposed in [26,40,48]). In the case of Bcl-XL, cells, the time course of expression was notoriously different, since DAT, VMAT and DRD2 expression peaked at d12 instead of d30 (Fig. 4B). This might be suggestive of the DAn maturing faster in the presence of elevated levels of Bcl-XL.

(A) Temporal profile: TH mRNA levels peaked at d12 in control cells, decreasing afterwards (Fig. 4Ai), in parallel to TH+ neurons (Fig. 1C); opposite, the four function-related genes continued increasing their expression during the month studied (Fig. 4B). This points to a long differentiation time required by TH+ neurons to become functionally active (as proposed in [26,40,48]). In the case of Bcl-XL, cells, the time course of expression was notoriously different, since DAT, VMAT and DRD2 expression peaked at d12 instead of d30 (Fig. 4B). This might be suggestive of the DAn maturing faster in the presence of elevated levels of Bcl-XL.

(B) Net Bcl-XL effects on gene expression: TH expression was enhanced 2-fold in Bcl-XL cells by d30 (Fig. 4Ai), in parallel to TH+ neurons (Fig. 1C). For DAT, VMAT2, DRD2 and GIRK2, the ratio of expression in the presence or absence of increased Bcl-XL levels (data treated as in Fig. 2B and Fig. 3B, ratio Bcl-XL to control cells) did not reveal any relevant changes (results not shown). When expression data of the four genes in each cell line were normalized to TH levels (an indication of their DA phenotype), we found that Bcl-XL did not make the neurons more mature per unit of TH expression (Fig. 4C), but prevents the decrease in expression of these genes observed in control cells by d30. Similar results were obtained after WB or ICC determinations shown in Fig. 4D and E, showing that by d30 there is more TH protein or cells in the case of Bcl-XL cultures, an enhancement that is accompanied by the increased presence of the other four proteins.

Long-term expression of transcription factors required for function and survival

Recent studies have proposed a maintenance and survival role for some of the factors studied here (EN1, LMX1B, NURR1 and PITX3), in addition to their known developmental roles [49–55] (reviewed in [6,15,48]). We therefore analyzed their expression in 30d-differentiated control and Bcl-XL hVM1 cells. When referring expression data to the level at d0, in control cells, NURR1 was the only gene expressed long term at slightly higher levels than in non-differentiated cells, whilst EN1 and LMX1B expression returned to basal levels (Fig. 5A) (PITX3 never got activated, see Fig. 3). In sharp contrast, the forced expression of Bcl-XL yields cultures where most genes were expressed long-term at higher levels than in dividing cells (10–40 fold enhancement) (EN1 is slightly below levels seen at d0, 0.86-fold) (Fig. 5A). The net fold enhancement of transcription levels induced by Bcl-XL (Fig. 5B) was 20–50 folds for LMX1B, NURR1 and PITX3, and 1.42-fold for EN1. This enhancement cannot be attributed solely to a survival action of Bcl-XL since TH+ neurons only increased 2-fold (Fig. 1C).

Morphological and functional long-term maturation of the DAn

A detailed morphometric account of the shape and arborization of the TH+ neurons after differentiation on hVM1 cells, and of Bcl-XL effects on these parameters, has not been previously undertaken. To this end, we differentiated the cultures for one month (plus/minus forced expression of Bcl-XL), and studied their evolution. To challenge the human system, we used as a control the best possible counterpart, a primary neuronal culture derived from fetal mouse VM. The results in terms of soma size, and the shape, number and length of neurites present in the TH+ neurons are summarized in Fig. 6. Similarly to primary neurons, in control and Bcl-XL hVM1 cells we found mid size neurons with ovoid, polygonal or fusiform morphology, typical of A9-DAn. Neurons were bi- or multi-polar, and the mean soma area was not different between primary neurons and those derived from control or Bcl-XL hVM1 cells (Fig. 6A and B). Details of all parameters studied for each cell type, times of differentiation and neurite order are provided in Supplementary information Fig. 4. The number of each type of neurite (Fig. 6C) and their average length (Fig. 6D) at d30 of differentiation, overall show that the neurite number seem to be lower in the order primary culture>hVM1>hVM1-Bcl-XL, whereas neurite length gets compensated showing values that increase in the order hVM1-Bcl-XL>hVM1> primary cultures. One may then conclude that the degree of arborization (Fig. 6E) is rather similar between primary and hVM1 derived TH+ neurons, the most noticeable difference being a reduction at d30 to approximately one half in Bcl-XL derived neurons.

Control hVM1 cells differentiate into functional neurons, according to calcium handling and electrophysiological parameters [40]. Since electrophysiological properties of SNpc are well established, here we only studied differentiated cultures of Bcl-XL hVM1 cells. Twenty-six of forty-nine cells that were analyzed electrophysiologically displayed early neuronal properties, including broad, relatively immature action potentials that where blocked by the sodium channel blocker TTX, and rectification of membrane currents (Fig. 7A and B). Electrophysiological evaluation, at d4 to d13 of differentiation, did not reveal any changes over time in input resistance (Ri) or resting membrane potential (RMP), though some tendency for an increase of RMP was apparent (Fig. 7C and D). The overall RMP average for the whole population of neurons over all time points was –48.2 mV and the Ri was 1404±188 MΩ. The remaining population of the recorded cells (23 cells) displayed passive membrane properties and a lower Ri at 111±12.6 MΩ and was considered as of a glial identity (Fig. 7E). The differences in membrane resistance and lack of presence of membrane rectifying properties allowed for very clear discrimination between neuronal and non-neuronal cell populations (Fig. 7F).

In summary, both control [40] and Bcl-XL hVM1 cells (present work) differentiate in vitro exhibiting electrophysiological properties indicative of both neuronal and glial phenotypes. Electrophysiological neuronal characteristics of both cell lines indicate that DAn neurons that are part of the neuronal population show immature action potential, relatively depolarized resting membrane potential, and properties typical of A9 DAn like high input resistance (2000 MΩ), and broad, low amplitude action potentials. Slow electrophysiological maturation, requiring up to 6
Fig. 4 – Expression studies of genes related with functional maturation of DA neurons.

(A) TH expression during long-term differentiation. (Ai) Temporal TH mRNA expression profile determined by Q-RT-PCR. Data from each cell line was normalized to its own calibrator sample (value at day 0, set as value 1); for further details see the Materials and methods section, and the legend for Supplementary Fig. S1). Results are mean ± SEM (n=3) (**p<0.01, ***p<0.001 vs d0; ±p<0.01 vs previous day; t-test). (Aii) Bcl-XL effects on TH relative mRNA levels expressed as the ratio hVMI Bcl-XL/hVMI at each studied time. Results are mean ± SEM (n=3) (**p<0.01 indicates significantly from 1 (95% confidence interval), one sample t test).

(B) Temporal DAT, VMAT2, DRD2 and GIRK2 mRNA expression profile determined by Q-RT-PCR at d0, d12 and d30 of differentiation. Results are mean ± SEM (n=3) (**p<0.01, ***p<0.001 vs d0; ±p<0.05, ++p<0.01 vs previous day; t-test).

(C) Comparative expression level of genes related to dopaminergic system vs TH relative expression determined by ratio calculations (see Supp. Inf. Figs. 1 and 3 for more details). Results are mean ± SEM (n=3) (**p<0.01, ***p<0.001 indicate that the mean differs significantly from 1 (95% confidence interval), one sample t test); ++p<0.01 d12 vs d30 (t-test).

(D) Protein expression studies by immunoblots. The WBs show TH, VMAT2, DAT and GIRK2 protein levels in hVMI and hVMI Bcl-XL cells differentiated for 30 day. β-actin was used as loading control. (E) hVMI and hVMI Bcl-XL were analyzed by ICC at d30 of differentiation, detecting expression of TH, DAT, VMAT2 and DRD2 (red). These proteins colocalize with TH+ cells (green). Nuclei were counterstained using ToPro3 (blue) (scale bar=20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
weeks of differentiation, has also been reported for human telencephalic precursor-derived neurons in vitro [56].

Another functional property of interest is the ability of the differentiated cultures for DA uptake, mediated by functional DAT. Positive evidence for a functional DA transport not only adds on the functionality of the neurons, but also comes to validate the Q-RT-PCR data shown in previous sections (Fig. 4). First, we studied ASP transport and accumulation (Fig. 8A), what indicated a clear NMF-sensitive accumulation in primary cultures, and in control and Bcl-X, hVM1 differentiated cultures. Second, we studied the NMF-sensitive 6-OHDA-induced TH+ neuronal death (Fig. 8B) finding that 6-OHDA kills a substantial fraction of the TH+ neurons generated in these cultures, but not in the presence of NMF, meaning that it was induced by toxin accumulation via DAT-mediated transport (and not by unspecific 6-OHDA toxicity caused by auto-oxidation). And thirdly, we measured the actual transport of $[^{3}H]$-DA, finding no differences in the NMF-sensitive transport per TH+ cell in the three types of cultures (Fig. 8C). In conclusion, from the point of view of the presence and activity of a functional DAT, there are no differences between the DAN generated by a primary fetal neuronal culture and those generated by control or Bcl-X, hVM1 cells.

Discussion

When large numbers of identical neurons are required, as for drug testing or to complete a clinical trial, a continuous and stable source of genuine A9-DAN is needed. The present scenario is challenging since the capacity of human fetal VM precursor cultures to generate A9-DAN is controversial [8,28,29]. Human VM neurosphere show a limited expansion capacity, cease to persist until the midbrain/hindbrain boundary, maintaining of midbrain developmental genes (EN1, OTX2), neuroepithelial genes (SOX2), early midbrain and dopaminergic genes such as Pax6, En1, and generate neurons showing differential expression of A9-DAN genes (such as Girk2) (Fig. 1A). In the mouse embryo, Pax6 is expressed in the neuroepithelium of the FB and hindbrain [64]. In human stem cells Pax6 acts as a determinant of neuroectoderm fate [65-67]. Consistently, some expression was detected in hVM1 cells, but it was higher in hNS1 cells (Fig. 1A). En1, characteristic of the midbrain neuroepithelium, and TH, DAT and Girk2 expression were substantially higher in hVM1 cells, as compared to hNS1 ones. Otx2, involved in the positioning of the midbrain/hindbrain boundary, maintenance of precursor domains, and precursor proliferation and maturation [8,17,45,68] was also expressed in hVM1 cells (Supplementary information Fig. 1). Altogether, these data not only indicate the persistence of a developmental program and patterning preservation, but also identify the hVM1 cells as neural stem cells showing most features of FP VM precursors for A9-DAN generation in culture while retaining proper cell intrinsic information.

Regionalization and activation of developmental gene cascades in hVM1 precursor cells

The proposition that regionally specified precursors can be propagated long-term in culture implies that the cells must retain their patterning, which is not granted. Patterning preservation has been demonstrated in cortical, striatal and midbrain neurospheres [57,58,63], but there is only indirect evidence for FB or VM v-myc hNSC lines [34-36,40-42,64], and the cell lines have never been studied head-to-head. In the present work we first clarified that long-term cultured FB and VM hNSCs differentially express early neuroectodermal markers (Pax6, En1), and generate neurons showing differential expression of A9-DAN genes (such as Girk2) (Fig. 1A). En1, characteristic of the midbrain neuroepithelium, and TH, DAT and Girk2 expression were substantially higher in hVM1 cells, as compared to hNS1 ones. Otx2, involved in the positioning of the midbrain/hindbrain boundary, maintenance of precursor domains, and precursor proliferation and maturation [6,9,17,45,68] was also expressed in hVM1 cells (Supplementary information Fig. 1). Altogether, these data not only indicate the persistence of a developmental program and patterning preservation, but also identify the hVM1 cells as neural stem cells showing most features of FP VM precursors for A9-DAN generation in culture while retaining proper cell intrinsic information.

![Image 0x0 to 595x842]

Fig. 5 - Long term expression of dopaminergic transcription factors involved in maintenance and survival. (A) Relative mRNA expression level of EN1, LMX1b, NURR1 and PITX3, at d30 of differentiation. Results are mean ± SEM (n=3) (*p<0.05, **p<0.01 indicate a significant difference from value 1 (95% confidence interval), one sample t test). (B) Comparative expression level of dopaminergic transcription factors in hVM1 Bcl-X, vs hVM1 determined by ratio calculations at d30 of differentiation. Results are mean ± SEM (n=3) (*p<0.05, **p<0.01 indicate a significant difference from value 1 (95% confidence interval), one sample t test).
Fig. 6 - Morphometric analysis of dopaminergic neurons. Measurement of different parameters of TH+ cells in primary fetal (E12) mouse VM cultures, hVM1 and hVM1 Bcl-X_L cells after long-term differentiation. hVM1 and hVM1 Bcl-X_L were differentiated for 30 day, and VM control cultures were maintained 22 day in vitro. (A) ICC for TH (red) and nuclear stain (blue, ToPro3). Snapshots taken with NeuronJ plug-in show semi-automatically traced neurites in different colors (see legend) in four analyzed neurons per picture (scale bar=40 μm). (B) Cell body area was calculated by ImageJ and expressed as mean ± SEM (n≥10, ANOVA, post-hoc Tukey test). (C) Average number of each neurite type per TH+ cells. Results were expressed as mean ± SEM (n≥50), (p<0.05, **p<0.01 vs VM culture; +p<0.05 hVM1 vs hVM1 Bcl-X_L cells; Kruskal Wallis test). (D) Average length of each type of neurites present in TH+ cells expressed as mean ± SEM (n≥50), (p<0.05, **p<0.01 indicate significant differences with VM culture; Kruskal Wallis test). (E) Total arborization (neurite length). Results are expressed as mean ± SEM (n≥50), (p<0.05, ***p<0.001 vs dl2; +p<0.05, ++p<0.01, +++p<0.001 vs VM culture at each day; ANOVA, post-hoc Tukey test).
as LMX1A, LMX1B, NGN2, MASH1, PITX3, together with the expression of nestin, vimentin, and radial glia markers (such as 3CB2)). The expression of functional properties typical of A9-DA neurons in the progeny of hVM1 cells further substantiates this view (present work and [34–36]). Moreover, among calcium binding proteins, only calretinin is expressed in TH+ neurons, but not calbindin or parvalbumin typical of ventral tegmental areas DA neurons (E.G.Seiz unpublished). Regarding developmental stages, to consider the hVM1 cells as VM DA precursors is not in conflict with the gestational age of the fetus from which they were derived (10 weeks post conception (PC) [34]). This age is roughly equivalent to E14 in mice, a time when TH neurogenesis is considered over [69]. However, seminal studies indicate that, in humans, neurogenesis is protracted in time, peaking during PC weeks 6.5 to 8 and finishing by PC week 10–11, a stage where newborn TH+ neurons are still present in the ventricular zone and have not yet migrated to the mantle zone [70]. Furthermore, VM dopamine content in human embryos continuously increases well beyond PC week 10 [71].

When mitogens are withdrawn, the cells start to differentiate, and the percentage of nestin or Ki-67 positive cells quickly decreases to 20–30% by one-two weeks (Supplementary information Fig. 2), matching what has been described for FB-derived hNSC cells [42,72], and human cortical, striatal and VM neurosphere cultures [26,53]. Nestin+ cells only disappeared completely by d30, enforcing the view that hNSCs require long differentiation times in vitro to mature (present work and see also [26,40,63]). The forced expression of Bcl-X, did not alter the down-regulation of these markers (Supplementary information Fig. 2).

For neurogenesis to take place a set of proneural genes must be activated early during differentiation. Here we have studied MASH1, NGN2 and MSX1, essential for VM DA progenitors (NEUROD1 was studied in Ref. [34]). MASH1 cooperates in a permissive manner in the generation of VM DAn, both in rodents and humans [23,27,46,47]. MSX1 prevents alternative cell fates through the repression of Nkx6.1 and activates Ngn2 expression [6,24,43,45]. In the case of the human brain, there is no information in the literature about MSX1 expression, but for the lack of effect of its over-expression in the NGC-407 cell line of human VM precursors. However, in this cell line, endogenous levels of MSX1 were not studied [61]. NGN2 is essential for the conversion of Otx2+ precursors into Nurrl+ postmitotic DAn, and its ablation dramatically reduces VM DAn numbers [6,17,18,44,46,68]. For the case of MASH1 and NGN2 we have reproduced previous data [34,36] which, together with the data on MSX1 (Fig. 2), indicate that these proneural genes are expressed in hVM1 cells, following the expected time course, being MSX1 and NGN2 transiently activated. As discussed below, the forced expression of Bcl-X, profoundly alters the expression of MSX1 and NGN2.

**Activation of developmental pro-dopaminergic genes**

Regionally specified (SN1, OTX2) and neuron committed (MASH1, MSX1 and NGN2) precursors (Figs. 1 and 2) must activate genes required for the development of a correct A9 DA phenotype, at a time in the mouse when the cells exit the cell cycle [21,22]. Nurrl expression in the mouse cooperating with Pitx3 is critical for the acquisition of the correct DA phenotype (that requires co-expression of TH, AADC, VMAT2 and DAT) [17,22,73,74]. In relation to LMX1B, loss of function studies indicate that is required for the maintenance of VM DAn [75], and more recently it has been involved in patterning [12,18,76]. hVM1 cells activate Nurrl and LMX1B during the first week of differentiation (Fig. 3), in parallel to cell cycle exit (Supplementary information Fig. 2), and prior to the blow of TH expression, as temporally expected and normally occurs during mouse and human development. PITX3 expression,
However, did not activate, possibly explaining a lower-than-possible yield of DAn in control cells. The expression levels of LMX1B and NURR1, and most notably that of PITX3, are enhanced after forced Bcl-X<sub>L</sub> expression (Fig. 3B), what may be of critical importance to enhance DAn generation (see the Discussion section).

Maturation, function and maintenance of DAn

Consistent with our previous reports showing that DAn from hVM1 cells can synthesize and release DA after depolarization [34-36], here we show that the genes needed for DA synthesis (TH, AADC and GTPCH1) are in fact expressed during differentiation (Figs. 1, 3 and 4, S3). The electrophysiological data (showing active neurons) we present here for differentiated Bcl-X<sub>L</sub> hVM1 cells largely correspond to those previously reported for the control hVM1 cell line [40]. Though these neuronal electrophysiological properties seem to be relatively immature, it is possible that with longer differentiation times, or upon grafting, the cells may develop into more functionally mature neurons. This notion is partly supported by the observed tendency to an increase in membrane potential over time. Nevertheless, some of the properties exhibited by the cells (high input resistance, trains of action potentials, and long duration action potentials are typical of A9 SNpc DAn). In this context, Lepski et al. [78] have also reported that telencephalic human neural precursors require about six weeks of in vitro differentiation to complete their electrophysiological maturation.

In the present study we have also generated detailed data on the correct activation of genes essential for A9-DAn function (DAT, VMAT2, DRD2 and GHRK2, Fig. 4B), which gradually increased over the month of differentiation studied, again indicating a slow maturation. Furthermore, we studied DAT activity by three independent means, and consistently found that there is an active DA transport in the differentiated DAn (Fig. 7), not different from that in primary neurons. Morphologically, TH<sup>+</sup> neurons did not substantially differ from those in primary culture (Fig. 6). In summary, DAn generated from hVM1 precursors express all genes needed for an A9 type DAn to be functional, available data indicate a high degree of both morphological and functional maturation of the obtained DAn. Bcl-X<sub>L</sub> effects on maturation are marginal, as discussed later.

EN1, LMX1B, NURR1 and PITX3 are not only involved in development, but also in the survival/maintenance of functional A9-DAn ([50-54] reviewed in [6,15,24,49,77]). In control hVM1 cells the expression levels of these genes first increase during differentiation (but for PITX3), to later return to values close to basal levels (Fig. 3A and Fig. 5A). This may explain why the percentage of TH<sup>+</sup> neurons decreased from 12 to 11% (d7-d12), down to 8% (d30) (Fig. 1C). The lack of up-regulation of PITX3 may also contribute to this decline, since it is crucial for DAn maintenance [6,22,49]. In sum, these data would point to a compromised maintenance of the DAn in the long perspective, although their morphology does not differ from that of the TH<sup>+</sup>
cells in primary cultures (Fig. 6, S4) and they are still functional (Figs. 7 and 8, and Ref. [40]).

Integration of Bcl-X<sub>L</sub> effects

Bcl-X<sub>L</sub> has been described to exert multiple functions in hNSCs, by molecular and cellular mechanisms involving apoptosis, changes in gene expression, control of cell cycle duration, and expansion of intermediate progenitors [34,36] Bcl-X<sub>L</sub> has been shown to act in FB and VM hNSCs in a dose-response fashion, conclusion validated by interfering RNA studies (such as those in [78]). In the case of VM, Bcl-X<sub>L</sub> enhances the survival of A9-DAn, both in vitro and in vivo [35,36], and rescues the neurogenic potential of hVM1 cells lost with passaging [36]. Those studies focused on cell death counteraction (reduced LDH release, Annexin-V<sup>+</sup> or caspase-3<sup>+</sup> cells, DNA fragmentation). DA synthesis was also enhanced, as expected by the increased number of TH<sup>+</sup> cells generated. In the present study we have conducted a detailed long-term study of gene expression changes to explain further the Bcl-X<sub>L</sub>-induced enhanced generation of DAn. Present data indicate that Bcl-X<sub>L</sub> has a dramatic effect in MSX1 temporal expression, anticipating it, and increasing the net levels of MSX1 and NGN2, in comparison to control cells (Fig. 2, see also Ref. [36]). At later stages, NURR1 is also enhanced, but most importantly, PITX3 activation is rescued (Fig. 4). Altogether, these gene expression changes (plus a small, 3-fold increase in OTX2 expression, Supplementary information Fig. 1), are sufficient to explain the enhanced number of TH<sup>+</sup> neurons generated and TH mRNA levels at all times studied (Figs. 1, 3 and 4). Note that by d7, TH mRNA levels increase by 4-fold (Fig. 3B, 4Aii), whereas the number of TH<sup>+</sup> cells is only doubled (Fig. 1C). This, together with the increase in the expression of AADC and GTPCH1 (Fig. S2B), would suggest an enhanced dopaminergic phenotype. However, one cannot affirm that the cells are more mature in the presence of elevated levels of Bcl-X<sub>L</sub>. Long-term data on expression of function-related genes (Fig. 4) only indicate that the cells might mature faster, and that by d30 Bcl-X<sub>L</sub> prevents the loss of the DA phenotype that occurs in control cells (at least when the data are normalized to TH mRNA, Fig. 4C). In addition, electrophysiology (Fig. 7), DAT activity determinations (Fig. 8), and morphological data (Fig. 6) also indicate that Bcl-X<sub>L</sub> does not accelerate functional maturation in vitro.

Conclusion

In the present work we provide evidence demonstrating that VM hNSCs with the capacity to generate functional A9 DAn can be propagated in culture (at the expense of v-myc), while retaining their patterning. These precursors resume their developmental instructions during differentiation, offering a stable and reproducible source of functional DAn for basic developmental studies, but also applied research in the fields of drug screening and cell therapy. The forced expression of Bcl-X<sub>L</sub> results in an increased yield of DAn from VM hNSCs via cell death counteraction [36], but also enhancing neuron generation and maintenance (present work).

Acknowledgments

We would like to acknowledge the excellent technical assistance of Ignacio Tardieu de Chorro, Marta González Mella and Beatriz Moreno Moreno, Dr. Elisa García-Garcia (CBMSO) for fruitful discussions, Dr. Ricardo Ramos (Scientific Park, Madrid) for Q-RT-PCR determinations and Veronica Labrador (CBMSO) for confocal microscopy assistance. Dr. Isabel Liste was partially supported by the Ramón y Cajal programme. This work was supported by EU (NMP-SL-2008-214706 EXCELL), MCYT (SAF2004-03405), MICINN/MINECO (BI02007-66807, PLE2009-0101 and SAF2010-17167), Carlos III Institute of Health grant RETICS TerCel (RD06/0101/0009) and La Caixa Foundation (BM05-22-0). This work was also supported by an institutional grant from Foundation Ramón Areces to the Center of Molecular Biology Severo Ochoa.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jeyexc.2012.07.018.

REFERENCES


T. Kallur, V. Darsalia, O. Lindvall, Z. Kokaia, Human fetal cortical...