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Phenazopyridine induces and synchronizes neuronal differentiation of embryonic stem cells

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Abstract

Embryonic stem (ES) cells are powerful tools to understand mechanisms of neuronal differentiation and to engineer neurons for *in vitro* studies and cell therapy. We developed a screening approach to identify small organic molecules driving neuronal differentiation of ES cells. For this purpose, we used a lentivector carrying a dual luciferase reporter system to engineer an ES cell line which allowed us to screen for small organic molecules enhancing neuronal differentiation. One of them, phenazopyridine, was further analysed in human ES cells. Phenazopyridine: (*i*) enhanced neuronal differentiation, (*ii*) increased cell survival, (*iii*) decreased the amount of non-neuronal and undifferentiated cells and (*iv*) synchronized the cellular differentiation state. Phenazopyridine allowed the development of a differentiation protocol compatible with the generation of clinical grade neural precursors, which were able differentiate into different neuronal subtypes, astrocytes and oligodendrocytes. In summary, we describe a powerful approach to identify small molecules directing stem cell differentiation. This led to the establishment of a new application for an old drug and the development of a novel clinical grade protocol for neuronal differentiation of ES cells.

Keywords: embryonic stem cells/neuronal differentiation/phenazopyridine • Main topics: neuronal differentiation of embryonic stem cells • small molecule-driven differentiation

Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. Their ability to differentiate into any somatic cell type makes them ideally suited for the study of early developmental steps, but they also represent a promising source for future cell therapy. Although a heterogeneous mixture of different cell types derived from ES cells is easy to obtain in culture, their targeted differentiation towards a specific lineage remains challenging. Even more difficult is to obtain cell populations which are synchronized at a particular differentiation stage [1].

Methods to drive differentiation of ES cells include use of growth factors, coculture with stromal cells, and adhesion on specific extracellular matrix proteins (reviewed in [2]). These approaches are cost- and labour intensive, and often result in the generation of heterogeneous cell populations. Transgenic expression of transcription factors has also been used to drive ES cell differentiation [3, 4], but is hampered by the risk of malignant transformation upon integration of the transgene into the host cell genome. An alternative is the use of cell-permeant transcription factors, which has however been of limited efficacy so far [5].

A more recent concept is the use of small molecules to control ES cell differentiation [6–9]. Advantages of such an approach include binding to intracellular targets, profound modification of cell signalling and the possibility to be readily integrated into clinical grade protocols. Also, in contrast to bioactive proteins, the number of small organic molecules is huge and large size chemical libraries are available.

In the present study, we describe a technology that allows screening for molecules modulating neuronal differentiation of ES cells. This approach led to the discovery of neurogenic properties of phenazopyridine, a clinical grade small molecule. We show that phenazopyridine enhances neuronal differentiation of human ES cells and allows the generation of a monolayer of synchronized neural progenitors.

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Materials and methods

Reagents

Reagents and their sources were as follows: the pDONR221 vector (Invitrogen Corp., Carlsbad, CA, USA); the pGEM®-T Easy plasmid, the pRL-CMV Vector (Promega Corp., Madison, WI, USA) [10]. The pENTR eGFP, pENTR mRFP1, pENTR EF1-αS, pENTR Tα1 α-tubulin, $2K7_{bsd}$ vectors, have been described before [11]. The murine CGR8 ES cell line (European Collection of Cell Culture); the human H1 ES cell line (Wicell Research Institute, Inc., Madison, WI, USA); the human HS401 ES cell line (kindly provided by Outi Hovatta, Karolinska Institute, Stockholm, Sweden); the bone marrow stromal MS5 cell line (kindly provided by Katsuhiko Itoh [12]); cell culture media, foetal bovine serum, serum replacement, penicillin, streptomycin, N2 supplement, non-essential amino acids, sodium pyruvate, collagenase IV (Gibco, Paisley, Scotland); basic human fibroblast growth factor (Invitrogen Corp.); recombinant brain-derived neurotrophic factor (Sigma-Aldrich, St. Louis, MO, USA); Gateway® clonase enzymes (Invitrogen Corp.); Dual-Luciferase® Reporter Assay System (Promega); L-Polyornithine, human laminin (Sigma-Aldrich). Antibodies and dilutions were as follows: mouse anti-CNPase II (1:1000), rabbit anti-Musashi (1:500), rabbit anti-nestin (1:500), mouse anti-Vglut-1 (1:1000), mouse anti−GAD67 (1:2000), mouse anti-vimentin (1:200), rabbit anti-Sox1 (1:100), rabbit anti-tyrosine hydroxylase (1:1000) (Chemicon, Temecula, CA, USA), mouse anti-Pax6 (1:50) (Developmental studies Hybridoma bank), rabbit anti-glial fibrillary acidic protein (GFAP) (1:1000) (Dako, Glostrup, Denmark), mouse anti-βIII-tubulin (1:1000) (Sigma), rabbit anti-βIII-tubulin (1:3000) (Covance, Princeton, NJ, USA). The following fluorochrome-labelled secondary antibodies were used: AlexaFluor (555, 488 or 350)-labelled antibodies from goat or donkey against mouse, goat or rabbit (Invitrogen-Molecular Probes). Small organic molecules: NINDS custom collection II, phenazopyridine hydrochloride (Microsource Discovery, Inc., Gaylordsville, CT, USA).

Vector constructions

We previously described the construction of the $2K7_{GFP}$ [13]. To generate the $2K7_{EFSGFP,}$ the SV40 promoter sequence was replaced by the EF1-αS promoter sequence. To generate the $2K7_{EFSRluc,}$ the GFP coding sequence from $2K7_{EFSGFP}$ was replaced by the Renilla luciferase coding sequence from pRL-CMV. The construction of pENTR mRFP1, pENTR Tα1 α-tubulin and pENTR Fluc were described before [11, 13]. The resulting entry vectors were then recombined into $2K7_{EFSGFP}$ or $2K7_{EFSRluc}$ lentivectors using the Gateway® LR plus clonase enzyme mix.

Cell cultures and neuronal differentiation

CGR8 mouse ES cells and H1 human ES cells were cultured as described [11], and human HS401 ES cells were cultured on irradiated human foreskin fibroblasts. For the primary screening assay, CGR8 cells were seeded in 96-well plates at 10^3 cells per well in differentiation medium (BHK-21 medium supplemented with 20% foetal calf serum, L-glutamine, non-essential amino acids, sodium pyruvate, penicillin and streptomycin). Forty-eight hours later, the medium was removed and replaced by 330 μl of fresh differentiation medium. One microlitre of the drug library diluted in DMSO at 3.3 mM was added to each well to obtain a final drug concentration of 10 μM. Seventy-two hours later, cells were assayed for Firefly and Renilla luciferase activity.

To perform the secondary screening assay, neuronal differentiation of CGR8 cells was carried out in SR medium (DMEM high glucose supplemented with 15% KO serum [Gibco], non-essential amino acids, penicillin and streptomycin). Cells were plated at 10^5 cells per 10-cm cell culture dish and maintained in SR medium for 5 days with compounds found in the primary screen. Cells were subsequently dissociated and replated at 4×10^4 cells/cm^2 in N2 medium (DMEM high glucose, N2 supplement, 10 ng/ml basic fibroblast growth factor, penicillin and streptomycin) and cultured for four additional days without compound addition.

Two different protocols were used to induce neuronal differentiation of human ES cells. In the first protocol, human ES cells were mechanically dissociated into small aggregates and plated on laminin/polyornithine coated six-well plates. Cells were cultured 2 weeks in human SR medium (DMEM F-12 supplemented with 15% knockout serum [Gibco], non-essential amino acids, penicillin and streptomycin), followed by 4 weeks in N2 medium. Compounds were added throughout these first 6 weeks. Subsequently, cells were mechanically dissociated and replated on polyornithine/laminin-coated six-well plates. Throughout the text, this first differentiation protocol will be referred to as 'differentiation protocol 1'. The second differentiation protocol was derived from previously described differentiation conditions [14]. In the second differentiation protocol, undifferentiated human ES cells were mechanically dissociated into small aggregates and cultured in suspension on low-attachment six-well plates. For the first 4 days, they were cultured in neuronal induction medium (DMEM F-12, N2 supplement, penicillin and streptomycin), which was changed to simple neuronal proliferation medium (DMEM F-12, N2 supplement, 20 ng/ml bFGF, penicillin and streptomycin) for two additional days. Subsequently, aggregates were plated on polyornithine/laminin-coated six-well plates and maintained in simple neuronal proliferation medium for 7 days. Cells were then mechanically dissociated and replated in N2 medium at a density of 2×10^4 cells per cm^2 for two additional weeks. Compounds were added throughout these first 4 weeks. Cells were then replated at a density of 5000 cells per cm^2 in neuronal differentiation medium (Neurobasal medium, B-27 supplement, BDNF [10 ng/ml], penicillin and streptomycin). Throughout the text, this second differentiation protocol will be referred as to 'differentiation protocol 2'.

For three-dimensional neuronal differentiation, 5–10 spheres were generated as described in differentiation protocol 2 and plated on a pre-cut patch of hydrophilic polytetrafluoroethylene membrane (confetti, 6-mm diameter, 0.4 μm, BioCell-Interface, La Chaux-de-Fonds, Switzerland). Next, the membrane was placed in a Millicell®-CM (0.4 μm) Culture Plate Insert (Millipore, Invitrogen) on one ml of N2 medium. The medium was changed every 2–3 days. This method has been for tissue slices previously (Luc Stoppini) and has been adapted to generated engineered neural tissue from ES cells as recently described [15].

ES cell transductions

ES cell transductions were performed as previously described [11]. To generate the $CGR8_{EFSGFP}$Tα1mRFP1 cell line, ES cells were transduced with the $2K7_{EFSGFP}$Tα1mRFP1 lentivector and eGFP$^+$ cells were subsequently sorted by flow cytometry. To generate the $CGR8_{EFSRluc}$Tα1Fluc cell line, ES cells were transduced with the $2K7_{EFSRluc}$Tα1Fluc lentivector and subsequently grown as clones in 96-well plates. Four weeks later, cells were assayed for Renilla luciferase activity. Several clones were found to be positive and the clone with the highest activity was chosen to perform the primary screen.

Dual luciferase assays

CGR8 ES cells were lysed in 96-well plates according to the manufacturer's instructions. Luminescence measurements were performed on a Fluostar Optima (BMG Labtech GmbH, Hanns-Martin-Schleyer-Str. 10, D-77656 Offenburg/Germany).

Immunofluorescence microscopy

Immunofluorescence was carried out according to standard techniques. Briefly, mouse ES cells were grown on polyornithine-coated glass cover slips in six-well plates, and human ES cells were grown on plastic or glass cover slips coated with laminin/polyornithine in six-well plates. Cells were fixed with 2% paraformaldehyde for 30 min., washed with HBSS and permeabilized with 0.5% (v/v) Triton X-100 for 30 min. Cells were then exposed to primary antibodies overnight at 4°C. After two washes in HBSS containing 1% FBS (blocking buffer), cells were stained with secondary antibodies at RT for 1 hr (1:1000 dilution in blocking buffer). Cell nuclei were stained with 1 μg/ml 4'-6-diamidino-2-phenylindole (DAPI) for 10 min. Pictures were taken on an ImageXpress Micro (Molecular Devices, Sunnyvale, CA, USA) or a Zeiss axioplan microscope equipped for epifluorescence.

Immunostaining quantification

Immunostaining and nuclear staining quantifications were performed using the MetaXpress software (Molecular Devices). Total neurite outgrowth was quantified using the neurite outgrowth analysis module, and total cell numbers were quantified with the count nuclei analysis module.

Quantitative analysis of cells expressing fluorescent proteins

For the studies investigating the activity of the Tα1 α-tubulin and the EF1-αS promoter during neuronal differentiation of the $CGR8_{EFSGFP}T\alpha1mRFP1$ cell line, fluorescence intensity of eGFP and mRFP1 in a given cell was quantified using the Metamorph® software.

Real time PCR

Reactions were run on an ABI Prism 7900 HT detection system (Applied Biosystems, Rotkraz, Switzerland). ALAS and GusB were used as housekeeping genes. As these genes behaved similarly in all samples examined, data was normalized to ALAS level. Sequences of the primers are shown in Table S1.

Results

Primary screening of neuronal differentiation of ES cells

We first developed a method to screen for small molecules that affect neural lineage commitment of ES cells. For this purpose, we used the 2K7 lentivectors we recently described [11] to generate dual reporter mouse ES cell lines. We generated a cell line in which GFP is expressed under the control of the ubiquitous EF1-α short promoter and mRFP1 under the control of the neuron-specific Tα1 α-tubulin promoter (Fig. 1A). To validate this cell line, we differentiated it towards neurons using an established protocol based on coculture with the MS5 stromal cell line [16], and monitored changes of fluorescence in cells undergoing neuronal differentiation. We analysed cells by immunofluorescence on day 5 and investigated the correlation between the red/green fluorescence ratio and the staining for β_3-tubulin, revealed by a blue secondary antibody (Fig. 1B). We quantified the red/green fluorescence ratio in undifferentiated cells as well as in β_3-tubulin-negative and positive cells during neuronal differentiation (Fig. 1C). The correlation between the red/green ratio and β_3-tubulin staining was good, allowing us to monitor neuronal differentiation of ES cells. The use of fluorescent proteins allowed verifying the general validity of our approach. However, we suggested that the use of luminescent reporter genes could enhance the sensitivity of the system. We therefore constructed a second lentivector using luminescence rather than fluorescence reporters. For this purpose, we modified the above-described vector by replacing mRFP1 with firefly luciferase (Fluc) and GFP by renilla luciferase (Rluc) (Fig. 1D), and generated a cell line carrying this construct (see 'Materials and methods'). To validate this cell line, cells were cultured either on MS5 stromal cells to induce neuronal differentiation, or on mouse embryonic fibroblasts (MEF) which do not induce neuronal differentiation. We performed a time-course experiment in both conditions, and observed an up to 46-fold increase of Fluc/Rluc when ES cells were cultured on MS5 but not when cultured on MEF (Fig. 1E). Therefore, we decided to use this cell line to perform the primary screening assay. It will be referred as to $CGR8_{dual\ luc}$ throughout the text.

We next used $CGR8_{dual\ luc}$ to screen a small molecule library containing compounds approved by the food and drug administration (FDA). The advantages of such a library include the fact that the compounds are proven to be bioactive and may readily be used under clinical grade conditions. A total of 1040 compounds were screened, among which 975 compounds gave analysable results and 65 compounds resulted in the absence of any luciferase signal, probably due to cellular toxicity. Figure 1F shows values of the Fluc/Rluc ratio for 975 compounds, normalized to the mean Fluc/Rluc ratio found in control-treated cells.

For further studies, we selected 32 compounds with high Fluc/Rluc ratio. We then performed dose–response analysis of Fluc/Rluc ratio in the same conditions as used for the primary screen, using concentrations ranging from 100 nM to 100 μM (data not shown). For each compound whose activity was confirmed, the best concentration was selected to further investigate its activity in a neuronal differentiation protocol of mouse ES cells (see 'Materials and methods'). We quantified the amounts of neurons by calculating total neurite outgrowth identified by β_3-tubulin immunoreactivity using an automated imaging system (see 'Materials and methods'). One compound, phenazopyridine, resulted in an increase of total neurite outgrowth as compared to

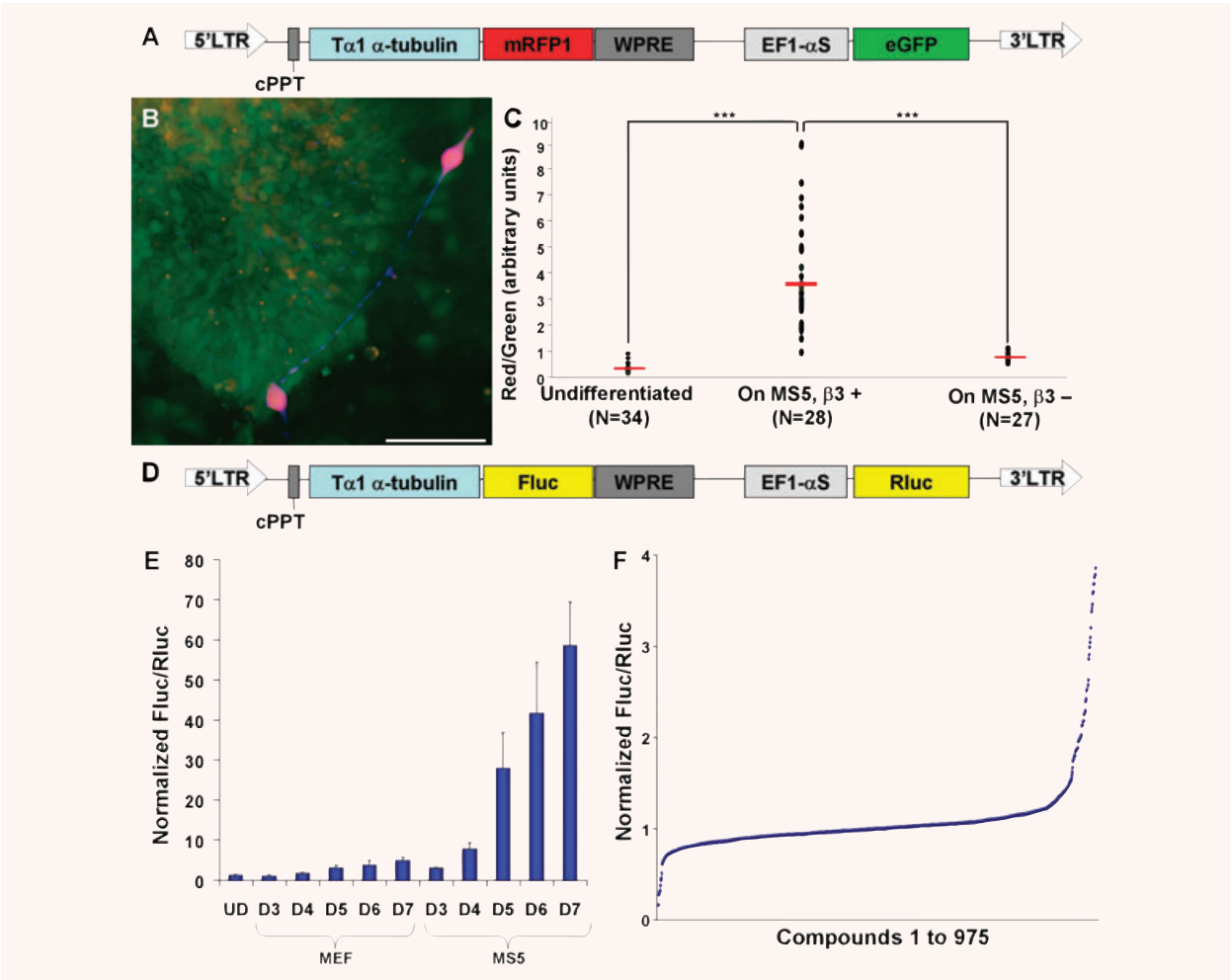

Fig. 1 Primary screen on mouse ES cells. (**A**)–(**C**): CGR8 mouse ES cells were transduced with a lentivector expressing mRFP1 under the control of the Tα1 α-tubulin neuron-specific promoter (Tα1) and GFP under the control of the ubiquitous EF1-α short promoter (EF1-αS) (**A**). cPPT: central polypurine tract. WPRE: Woodchuck hepatitis virus posttranscriptional regulatory element. (**B**) Cells undergoing neuronal differentiation were immunostained against β3-tubulin (blue). Scale bar: 100 μm. The ratio between red and green fluorescence was quantified in undifferentiated cells as well as β3-tubulin-positive and β3-tubulin-negative cells undergoing neuronal differentiation (**C**). Red bars indicate mean values. (**D**) Lentiviral construct used for the primary screening assay. Firefly luciferase (Fluc) expression is controlled by the Tα1 α-tubulin promoter, and Renilla luciferase (Rluc) expression is controlled by the EF1-α short promoter. (**E**) Mouse ES cells were transduced with the construct described in (**D**); the ratio between Firefly luciferase and Renilla luciferase activities (Fluc/Rluc) was measured in undifferentiated cells (ES) and in cells induced towards neuronal differentiation (MS5) or not (MEF) at different time-points. Values were normalized on those obtained with MEFs at day 3. (**F**) Results of the primary screening assay, shown as Fluc/Rluc ratio values of $CGR8_{dual\ luc}$ treated with each compound of the small molecule library, normalized to values obtained with DMSO alone. ***: $P < 0.001$. Error bars: standard error of the mean.

control cells (data not shown) and its activity was further investigated on human ES cell differentiation.

Phenazopyridine enhances neuronal differentiation of human ES cells

We next investigated the effects of phenazopyridine on neuronal differentiation of H1 ES cells, in a system with relatively low basal neuronal differentiation ('differentiation protocol 1', described in 'Materials and methods'). Human ES cells were treated either with DMSO alone or phenazopyridine for the first 6 weeks of differentiation, and cells were subsequently immunostained for neuronal markers at different time-points. After 8 weeks of differentiation, the DMSO-treated cell population was highly heterogeneous as assessed by immunostaining for β3-tubulin, nestin, vimentin and GFAP (Fig. 2A and B). In contrast, phenazopyridine-treated cells were more homogeneous, most cells being positive for nestin,

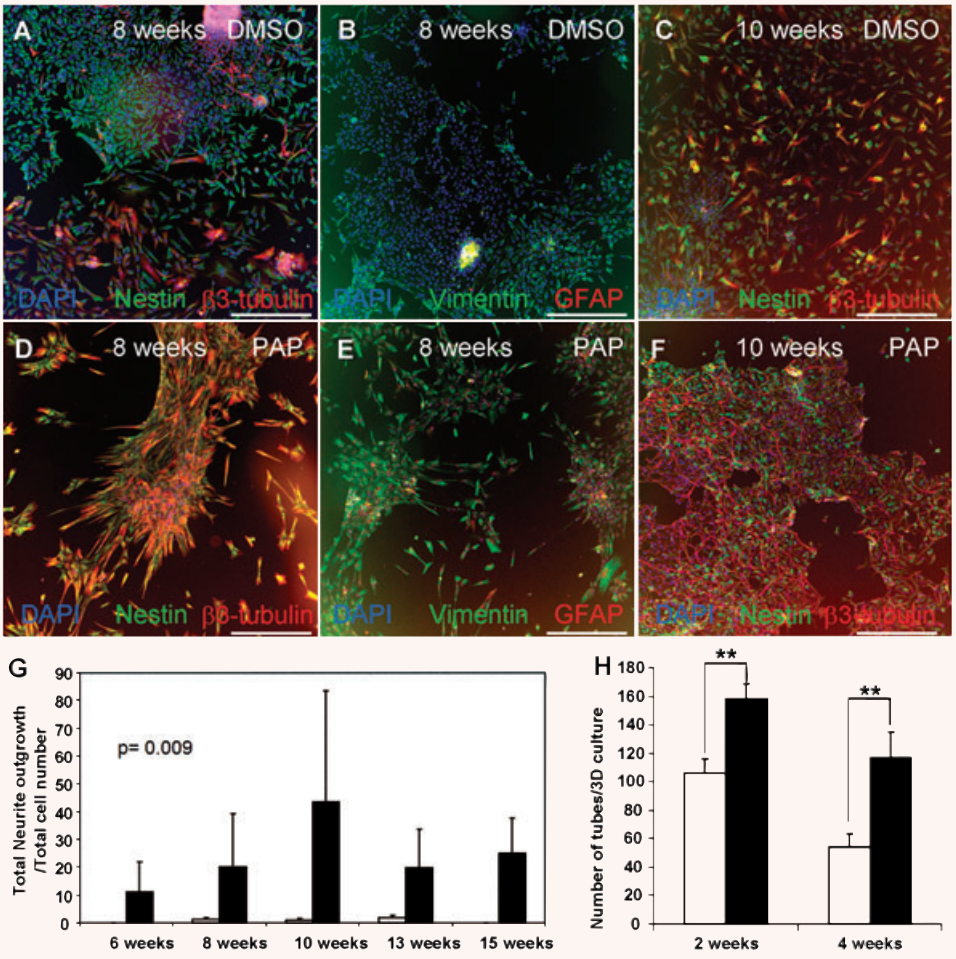

Fig. 2 Phenazopyridine enhances neuronal differentiation of human ES cells. Human ES cells were cultured for 6 weeks with phenazopyridine (PAP) at 3 μM or DMSO only, and subsequently replated without further treatment. Cells were immunostained for neuronal markers at different time-points. (**A**)–(**C**): DMSO-treated cells. (**D**)–(**F**): phenazopyridine-treated cells. (**G**) quantification of neurite outgrowth divided by total cell number at different time-points. Blue: control-treated cells; purple: phenazopyridine-treated cells. Scale bars: 100 μm. (**H**) quantification of neural tube number per three-dimensional human ES cell neuronal differentiation culture after 2 weeks and 4 weeks of differentiation. White: control-treated cells; black: phenazopyridine-treated cells. **: $P < 0.01$. Error bars: standard error of the mean.

β_3-tubulin and vimentin (Fig. 2D and E), with occasional neuronal networks (data not shown). After 10 weeks, further neuronal networks formed in phenazopyridine-treated cells (Fig. 2F), but not in DMSO-treated cells (Fig. 2C). To quantify neuronal differentiation, we chose to measure total neurite outgrowth and divided it by total cell number at different time-points in three independent experiments (Fig. 2G). Efficiency of differentiation of phenazopyridine-treated cells into mature neurons was variable, but always superior to DMSO-treated cells, as confirmed by Kruskal–Wallis analysis of ranks statistical analysis ($P = 0.045$).

Next, we tested the activity of phenazopyridine on neuronal differentiation in a three-dimensional culture system [15]. At two different time-points (Fig. 2H), we observed a significant increase in the total number of neural tubes when phenazopyridine was added to the culture medium. We conclude that phenazopyridine enhances neuronal differentiation of human ES cells.

Phenazopyridine permits the generation of homogeneous and synchronous populations of neuronal precursors

We next investigated the effects of phenazopyridine (3 μM) using a differentiation protocol in which all components can be replaced

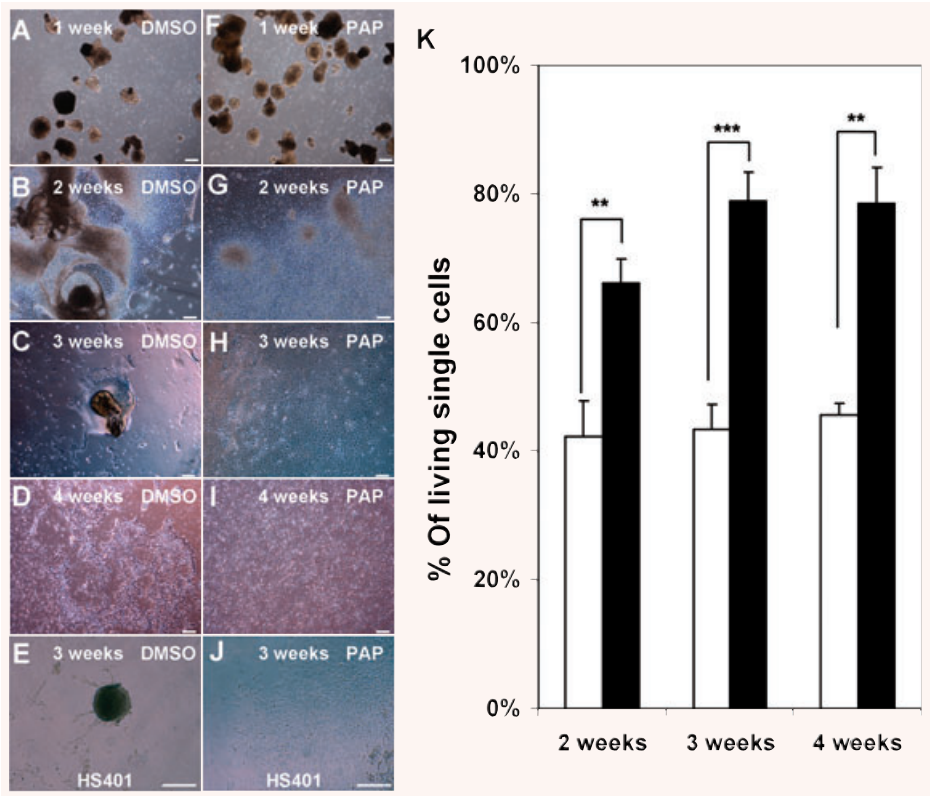

Fig. 3 Effect of phenazopyridine in the second neuronal differentiation protocol of human ES cells. (**A**)–(**J**): phase contrast pictures of control (**A**–**E**) and phenazopyridine (**F**–**J**) treated cells (3 μM) at different time-points. (**A**)–(**D**) and (**F**)–(**I**): H1 human ES cells. (**E**) and (**J**): HS401 ES cells. (**K**): percentage of living single cells by trypan blue exclusion at different time-points in control (white bars) or phenazopyridine-treated cells (black bars). PAP: phenazopyridine. Scale bars: 500 μm. **: $P < 0.01$; ***: $P < 0.001$. Error bars: standard error of the mean.

by clinical grade equivalents (differentiation protocol 2, described in 'Materials and methods'). Figure 3 shows representative phase contrast pictures of human ES cells at different stages of differentiation. DMSO-treated and phenazopyridine-treated spheres were morphologically indistinct after 1 week of differentiation (Fig. 3A and F). At 2 weeks of differentiation, control spheres remained relatively compact (Fig. 3B), whereas phenazopyridine-treated spheres developed bigger areas of monolayer at their periphery (Fig. 3G). Interestingly, many mitotic figures and $PCNA^+$ cells could be found in this area of phenazopyridine-treated cells, whereas only very few were present in DMSO-treated cells (data not shown). After mechanical passaging, cells remained as aggregates in DMSO-treated cells (Fig. 3C and D) while phenazopyridine-treated cells (Fig. 3H and I) could easily be dissociated into single cells. These observations were also confirmed in the HS401 cell line (Fig. 3E and J). Interestingly, there was a marked difference between the percentages of living single cells in control cells compared to phenazopyridine-treated cells as assessed by trypan blue staining at different time-points throughout the procedure (Fig. 3K). This suggests that phenazopyridine favours cell growth in monolayers and promotes survival of dissociated cells.

To investigate the phenotype of control and phenazopyridine-treated cells, we performed immunostainings for several neuronal markers after 4 weeks of differentiation in three independent experiments. Control-treated cells were mainly present as clusters (Fig. 4A), which were often containing typical rosette areas positive for nestin and β_3-tubulin (Fig. 4B), but there were also clusters negative for both markers (Fig. 4C). Musashi, Pax6, Sox1, vimentin and GFAP were distributed heterogeneously (Fig. 4D–G). In contrast, phenazopyridine-treated cells were mainly present as isolated cells, with only very few small clusters. Virtually all cells were nestin (Fig. 4I and J), Sox1 (Fig. 4M) and vimentin-positive (Fig. 4O), expressed β_3-tubulin (Fig. 4J) and Pax6 (Fig. 4M) at low levels, and were negative for musashi (Fig. 4L). Cells therefore displayed a homogeneous distribution of neuronal markers, at the exception of few strongly β_3-tubulin-positive (Fig. 4J), and some $GFAP^+$ cells (Fig. 4N). To quantify the synchronization of the cell population induced by phenazopyridine, we counted the number of cells negative for nestin and β_3-tubulin (non-neural cells), positive for nestin with low levels of β_3-tubulin (early neuronal precursors), positive for both markers at high levels (young neurons), or expressing β_3-tubulin only (differentiated neurons) (Fig. 4H and 4P). In DMSO-treated cells, all expression patterns were well represented (Fig. 4H), reflecting a highly heterogeneous cell population. In contrast, approximately 98% of phenazopyridine-treated cells were nestin-positive with low levels of β_3-tubulin, and no non-neural cells or differentiated neurons were observed. We therefore conclude that phenazopyridine treatment resulted in the generation of a homogeneous neuronal precursor cell population.

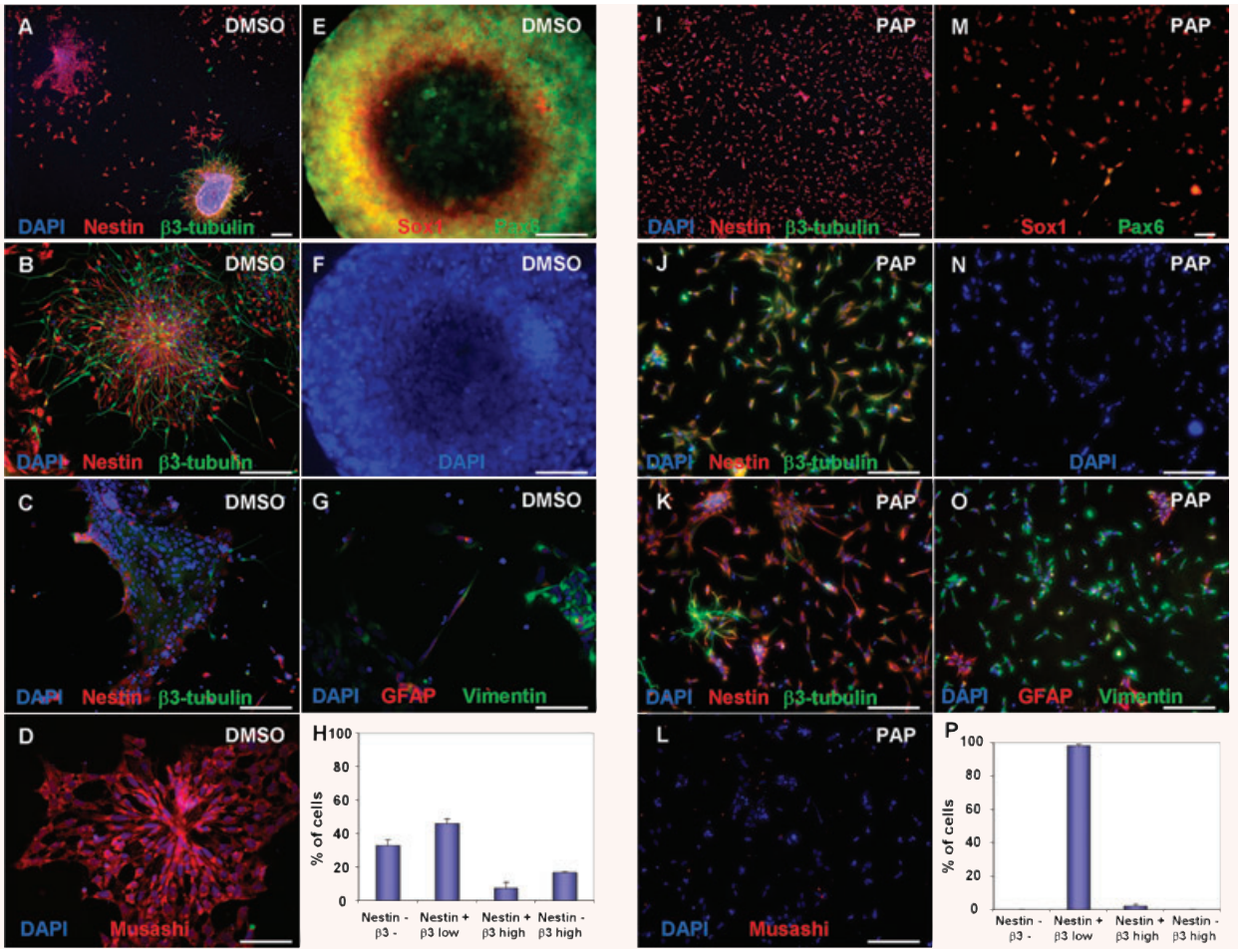

Fig. 4 Phenazopyridine treatment allows the generation of a homogenous monolayer of synchronized neuronal precursors. Human ES cells were cultured for 4 weeks using the differentiation protocol 2 (see 'Materials and methods'), treated with DMSO alone or phenazopyridine (3 μM). Cells were subsequently immunostained for markers of neuronal differentiation and for alkaline phosphatase, marking undifferentiated ES cells. (**A**)–(**G**): DMSO-treated cells. I-O: phenazopyridine-treated cells (PAP). (**F**) and (**M**) show DAPI staining corresponding to the fields shown in E and L, respectively. Scale bars: 100 μm. (**H**) and (**P**): quantification of cells expressing different markers in DMSO-treated cells (**H**) and phenazopyridine-treated cells (**P**). Error bars: standard error of the mean.

Phenazopyridine accelerates emergence of early neuronal markers and decreases markers of undifferentiated and non-neural cells

We next investigated the kinetics of emergence of neural and non-neural markers in DMSO and 3 μM phenazopyridine-treated H1 ES cells by real time PCR. After 2 weeks of differentiation, we observed an up-regulation of markers of early neuronal differentiation (Pax6, nestin), intermediate (β_3-tubulin, vimentin) and late differentiation (β_3-tubulin, Map2) in phenazopyridine-treated cells as compared to control cells (Fig. 5A). Markers for forebrain (Mash1), ventral hindbrain (Nkx2.2, HoxB4, Olig2), were also up-regulated, whereas Pax7 (marking the dorsal neural tube) and Pax2 (marking midbrain) were similar to control-treated cells. However, we did not see significant differences in the expression of the same markers at 4 weeks of differentiation (data not shown); the latter observation fits well with the observed induction of synchronized differentiation by phenazopyridine (Fig. 4). We then investigated expression of non-neural markers. Unexpectedly, after 2 weeks of differentiation, Oct-4 (marking undifferentiated ES cells) and brachyury (marking early mesoderm) were increased in phenazopyridine-treated cells, and α-fetoprotein (AFP, marking primitive endoderm) did not vary between both conditions (Fig. 5B). We reasoned that the persistence of these markers could be temporary and therefore investigated their expression after 4 weeks

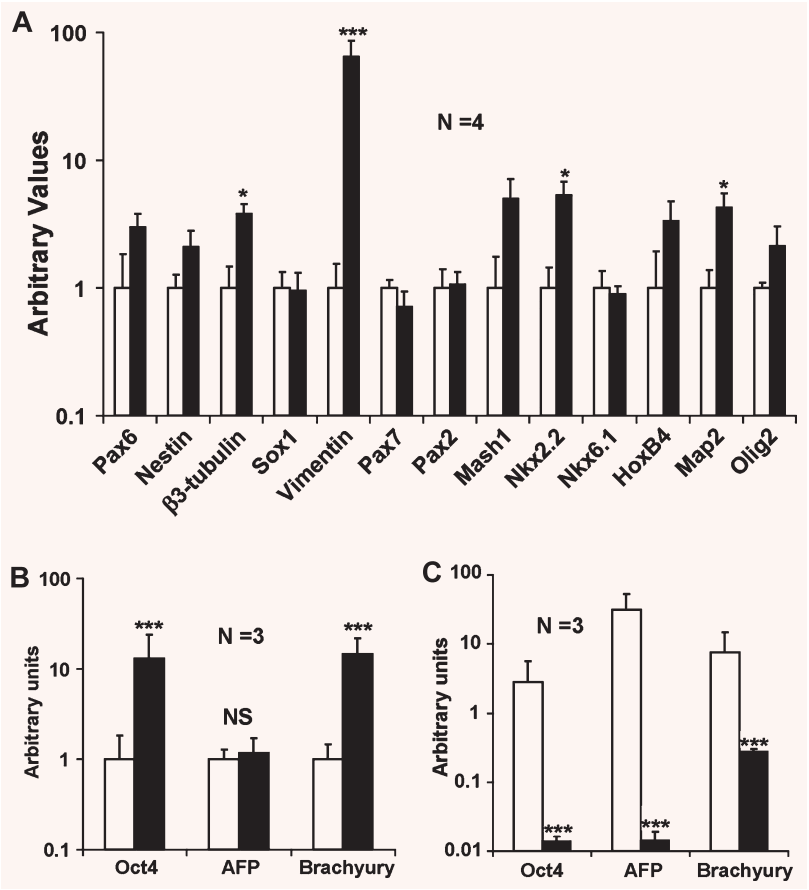

Fig. 5 Real Time PCR analysis of phenazopyridine-treated ES cells. Human ES cells were differentiated into neuronal precursors using the second differentiation protocol with DMSO or phenazopyridine (3 μM), and real time PCR was performed. (**A**) Neural marker expression after 2 weeks of differentiation. (**B**) and (**C**): Non-neural marker expression after 2 (**B**) or 4 weeks of differentiation (**C**). Data were normalized to values obtained with DMSO-treated cells at 2 weeks of differentiation. White bars: DMSO-treated cells. Black bars: phenazopyridine-treated cells. NS: no statistical differences. *: $P < 0.05$; ***: $P < 0.001$. Error bars: standard error of the mean.

of differentiation. Indeed, all three markers were markedly down-regulated in phenazopyridine-treated cells (>1000-fold for Oct4 and α-fetoprotein, and >10-fold for brachyury; Fig. 5C). These results suggest that (*i*) phenazopyridine accelerates the emergence of neural markers and that (*ii*) 4 weeks of treatment with phenazopyridine decreases the amount of undifferentiated and non-neural cells.

Phenazopyridine-treated cells can differentiate into all three neural lineages

To investigate the potential of neuronal precursors generated through phenazopyridine treatment, we devised a two step differentiation protocol. Cells were first treated with 3 μM phenazopyridine for 4 weeks and subsequently replated in the absence of phenazopyridine. For the second step, we cultured cells at low density (5×10^3 cells/cm^2) on polyornithine/laminin-coated support in neuronal differentiation medium and investigated their phenotype at different time-points in three independent experiments. We analysed several markers of neuronal differentiation as well as alkaline phosphatase, which is considered to be one of the best markers of undifferentiated ES cells [17]. In control-treated cells after 1 week of differentiation, we observed a heterogeneous cell population: neuronal networks in some regions (Fig. 6A$_1$) juxtaposed to areas containing tightly packed neuronal precursors (Fig. 6A$_2$), and very few isolated cells positive for Sox1 and occasionally for Pax6 (Fig. 6A$_3$). After 4 weeks of differentiation, control-treated cells still contained cells at variable stages of differentiation. Well-developed neuronal networks (Fig. 6C$_1$), alkaline phosphatase-positive cell areas (Fig. 6C$_2$) and Sox1 and Pax6$^+$ neuronal precursors (Fig. 6C$_3$) coexisted in the same culture. In contrast, after 1 week, the phenazopyridine-treated cells developed into a relatively homogenous monolayer of neural precursors and early neurons with short neurites (Fig. 6A$_{4–6}$). We analysed subtypes of neurons in the phenazopyridine-treated cells and observed glutamatergic (Fig. 6B$_1$), GABAergic (Fig. 6B$_2$) and – more rarely – TH$^+$ neurons (Fig. 6B$_3$). We did not observe the presence of astrocytes or oligodendrocytes precursors, as assessed by GFAP and CNPase immunostaining, respectively. After 4 weeks, we also observed slow-growing cell clusters homogeneously positive for nestin, β$_3$-tubulin, Sox1 and vimentin (Fig. 6C$_{4–7}$), together with more mature neurons, astrocytes and oligodendrocyte progenitors (Fig. 6D$_{1–2}$). The majority of cells were belonging to the neuronal

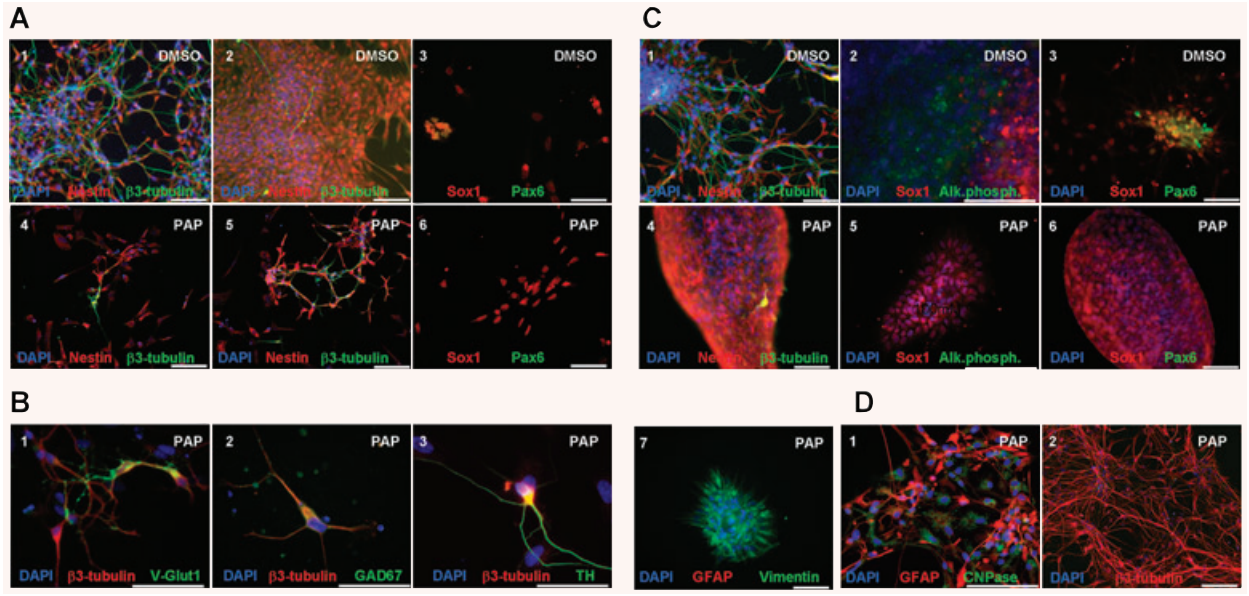

Fig. 6 Neuronal precursors generated from phenazopyridine-treated cells generate different neuronal subtypes, astrocytes and oligodendrocytes. Human ES cells were differentiated towards neuronal precursors for 4 weeks with DMSO or phenazopyridine (PAP) at 3 μM, and were subsequently replated at low density in neuronal differentiation medium. Cells were then immunostained for neuronal markers and alkaline phosphatase (marking undifferentiated ES cells) after 1 week (**A**) and 4 weeks of differentiation (**B**). Scale bars: $A_{1–6}$: 100 μm; $B_{1–3}$: 20 μm; (**C**) and (**D**): 100 μm.

lineage. However, the proportion of the different cell types was variable. Importantly, in phenazopyridine-treated cells, we never detected alkaline phosphatase-positive cells (Fig. $6C_5$).

Discussion

In the present study, we describe a dual reporter screening approach to discover small molecules modulating ES cell differentiation. The screening of a FDA-approved drug library allowed us to discover the neurogenic potential of phenazopyridine. This molecule not only enhanced and synchronized neuronal differentiation of ES cells, but also decreased the amount of non-neuronal and undifferentiated cells. Phenazopyridine allowed us to develop a novel differentiation protocol of human ES cells using only media formulations which are compatible with clinical grade.

Over the past years, small organic molecules have been of increasing interest in stem cell biology as tools to direct cell fate [6–9, 18–20]. So far, such approaches have been applied to mouse ES cells and molecules which maintain cells in an undifferentiated state [8, 20] or enhance their differentiation [6, 9, 20] have been described. One study demonstrated that the GSK-3β inhibitor TWS119 enhanced neuronal differentiation of mouse ES cells [6]. In our hands, this compound was not applicable to human ES cells (decreased cell growth and only small effect on neuronal differentiation; data not shown). In this study we have screened a collection of FDA-approved compounds. The advantages of using compounds derived from such a collection include their proven bioactivity, low toxicity and compatibility with future clinical applications. A key aspect of our screening method is the use of a double promoter/reporter system that allows ratio measurements. In contrast to studies using single promoter–reporter modules [6, 9], our system allows the activity of the neuronal promoter to be normalized and therefore allows the extent of neuronal differentiation and the size of the cell population analysed to be discriminated. The use of the 2K7 lentivector system [11] was instrumental for the construction of the cell lines, as it can carry two independent expression modules. It therefore allows the same copy numbers of the neuronal and the ubiquitously expressed reporter to be obtained. As demonstrated by our results, this leads to a reliable and sensitive primary screen. We therefore think that this approach is likely to have a wider application in ES cell research.

We used mouse rather than human ES cells for the primary screen and confirmed the results subsequently in human ES cells. The choice of mouse ES cells for the primary screen was dictated by the long time required for human ES cell differentiation. And, even if occasionally compounds active in the mouse system might not work in human cells (TWS119, see above), the striking effects of phenazopyridine in human ES cells justifies the approach. Phenazopyridine is an old molecule which has been widely used for symptomatic pain relief caused by irritation of the lower

urinary tract mucosa [21]. In the United States, it is still available as an over-the-counter drug, however the molecular mechanism of action of this common drug remains to date unknown. The molecular structure of phenazopyridine can give us some hints about its possible targets. First, it shares important structural similarities with some non-competitive metabotropic glutamate receptor 5 (mGluR5) antagonists such as SIB-1757 and SIB-1893. It was recently reported that treatment of mouse ES cells with the non-competitive mGluR5 antagonist MPEP enhances their differentiation towards neurons [22]. It remains to be seen whether phenazopyridine acts through this pathway.

One of the striking properties of phenazopyridine is its capacity to synchronize ES cell differentiation. Our results suggest that phenazopyridine provides a powerful exogenous cue that might be involved in this synchronization. However, the fact that in the presence of phenazopyridine, differentiating ES cells are capable of growing as a monolayer rather than as self-organizing cell clusters with hierarchical internal organization (Fig. 3) might also participate in this effect. The ability to obtain homogenous cell populations is of fundamental importance both for the study of defined differentiation stages *in vitro* and for cell therapy applications. One study proposed a cell sorting approach to allow synchronous neuronal precursor populations to be obtained [1]. However, such strategies remain tedious. Here we show that the simple addition of phenazopyridine to the differentiation medium resulted in the synchronization of cell differentiation, generating a homogenous population of neuronal progenitors virtually devoid of non-neural cells. Thus, our method is an important step towards the engineering of high quality human neuronal cell populations derived from ES cells.

Classical tools for ES cell differentiation include growth factors, matrix proteins and, coculture with stromal cells. The results shown here provide strong evidence that small organic molecules should be added to this list. The chemical space is of enormous size and it is likely that for many biological processes, small molecular agonists or antagonists exist. So far, the efficient use of the chemical variety in stem cell research was limited because of low throughput procedures. Thus, innovative assays allowing searching chemical libraries for active substances are the key to take advantage of this promising interface between the chemical and the biological world. Our study not only shows such an innovative assay, but also yields one most interesting compound that directs the cell fate of differentiating ES cells.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Primer used for real time PCR.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1582-4934.2009.00660.x
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